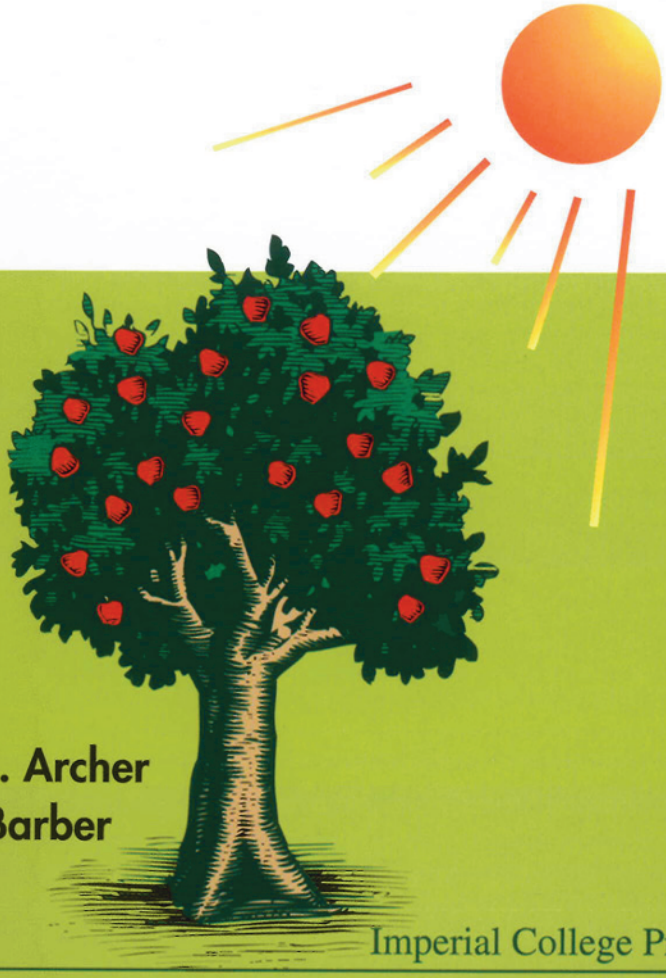
Series on Photoconversion of Solar Energy — Vol. 2

MOLECULAR TO GLOBAL PHOTOSYNTHESIS



Editors

Mary D. Archer
James Barber

Imperial College Press

MOLECULAR TO GLOBAL PHOTOSYNTHESIS

SERIES ON PHOTOCONVERSION OF SOLAR ENERGY

Series Editor: Mary D. Archer *(Cambridge, UK)*

Vol. 1: Clean Electricity from Photovoltaics
eds. Mary D. Archer & Robert Hill

Vol. 2: Molecular to Global Photosynthesis
eds. Mary D. Archer & Jim Barber

Forthcoming

Photochemical and Photoelectrochemical Approaches to Solar Energy Conversion
eds. Mary D. Archer & Arthur J. Nozik

From Solar Photons to Electrons and Molecules
by Mary D. Archer

Series on Photoconversion of Solar Energy — Vol. 2

MOLECULAR TO GLOBAL PHOTOSYNTHESIS

Editors

Mary D. Archer
Imperial College, UK

James Barber
Imperial College, UK

ICP Imperial College Press

Published by

Imperial College Press
57 Shelton Street
Covent Garden
London WC2H 9HE

Distributed by

World Scientific Publishing Co. Pte. Ltd.
5 Toh Tuck Link, Singapore 596224
USA office: Suite 202, 1060 Main Street, River Edge, NJ 07661
UK office: 57 Shelton Street, Covent Garden, London WC2H 9HE

Library of Congress Cataloging-in-Publication Data
Archer, Mary D.
Molecular to global photosynthesis / editors, Mary D. Archer, James Barber.
p. cm. - (Series on photoconversion of solar energy; v.2)
Includes bibliographical references and index.
ISBN 1-86094-256-3
1. Photosynthesis. 2. Energy crops. I. Archer, Mary D. II. Barber, J. (James), 1940-
III. Title. IV. Series.

QK882 .M84 2004
662'.8--dc22 2004044011

British Library Cataloguing-in-Publication Data
A catalogue record for this book is available from the British Library.

Desk Editor: Tan Rok Ting
Artist: Lionel Seow

This book is printed on acid-free paper.

Printed in Singapore by Mainland Press

This volume is dedicated

to

George Porter

The Rt Hon The Lord Porter of Luddenham OM FRS

Nobel Laureate

1920 – 2002

a fine man and a great scientist

This page intentionally left blank

CONTENTS

ABOUT THE AUTHORS

Mary Archer read chemistry at Oxford University and took her PhD from Imperial College, London, in 1968. From 1968 to 1972, she did post-doctoral work in electrochemistry with Dr John Albery at Oxford, and she then spent four years at The Royal Institution in London, working with Lord Porter (then Sir George Porter) on photoelectrochemical methods of solar energy conversion. She taught chemistry at Cambridge University, 1976–86. From 1991 to 1999, she was a Visiting Professor in the Department of Biochemistry at Imperial College, London, and from 1999 to 2002, she held a Visiting Professorship at ICCEPT (Imperial College Centre for Energy Policy and Technology). She is President of the UK Solar Energy Society and the National Energy Foundation, a Companion of the Institute of Energy and a member of the Royal Society's Energy Policy Advisory Group. She was awarded the Melchett Medal of the Energy Institute in 2002.

Jim Barber works on the molecular processes of photosynthesis as the Ernst Chain Professor of Biochemistry at Imperial College, London. After graduating as a chemist from the University of Wales, he gained his PhD in biophysics from the University of East Anglia. Following a post-doctoral year in Holland, he joined the staff at Imperial College as a lecturer in 1968. He was Dean of the Royal College of Science, 1988–89, and head of the Biochemistry Department of Imperial College, 1989–99. He continues as Director of the Centre for Photomolecular Sciences at Imperial College. Much of his research has focused on the reactions and proteins involved in the photochemically-driven oxidation of water. He was elected to the European Academy (Academia Europaea) in 1989 and he was awarded the Flintoff Medal of the Royal Society of Chemistry in 2002.

Vladimir Boichenko is a senior researcher and project leader at the Institute of Soil Science and Photosynthesis of the Russian Academy of Sciences. He graduated from Voronezh State University in 1971, and received his doctor's degree from Moscow State University in 1980. His scientific interests centre on the functional organisation of the photosynthetic apparatus and mechanisms of oxygen and hydrogen photoproduction. He has published about sixty papers in this field, collaborating extensively with colleagues in Hungary and Germany.

Janet Bornman is Research Director of the Department of Plant Biology at the Research Centre Flakkebjerg, Slagelse, Denmark, which forms part of the Danish Institute of Agricultural Sciences (DIAS). She is interested in the interplay of stress factors in plants and the mechanisms behind acclimation and longer-term adaptation with regard to plant quality and performance. Her areas of research include the potential interactive effects of

several environmental factors on plant crops, such as ultraviolet radiation, high visible irradiance, drought and temperature, as well as the measurement of internal light microenvironments within plant material using fibre optics. She is a member of the Environmental Effects Panel of the United Nations Environment Programme (UNEP) that is concerned with depletion of the ozone layer and global climate changes. She is currently President of the European Society for Photobiology, and Scientific Editor of the journal *Physiologia Plantarum*.

Tony Bridgwater is a Professor of Chemical Engineering at Aston University in Birmingham, specialising in thermal conversion of biomass for production of fuels and chemicals. He obtained his first degree in Chemical Engineering from UMIST, followed his PhD and DSc from Aston. Most of his professional career has been spent at Aston University, where he leads the Bio-Energy Research Group and heads the Chemical Engineering and Applied Chemistry Department. His current interests are focussed on the development of both the technologies for fast pyrolysis of biomass and the fuel and chemical products that can be derived from the liquids. He leads the biomass pyrolysis network PyNe sponsored by the EC and IEA Bioenergy.

Mike Bullard is the Managing Director of Bio-Renewables Ltd, a company established in 2002 in order to develop energy crops commercially in the UK. After gaining his doctorate from the University of York, and a brief period working at the Department of Agricultural and Environmental Science at the University of Newcastle, he spent his career at ADAS' Arthur Rickwood Research Station in Cambridgeshire, UK, most recently leading the novel crops programme, until leaving to establish the new company. His current research interests are the optimisation of yield and quality in *Miscanthus spp.* and *Salix spp.*, the development of small-scale electricity generation units fuelled by biomass, and the development of next-generation technologies such as bio-hydrogen.

Rebecca Carr is a forestry and land use consultant at the Edinburgh Centre for Carbon Management (ECCM). She gained her degree in Ecological Science from the University of Edinburgh, during which she spent a year at Queens University, Canada and took part in a community project in the Philippines. Her thesis looked at restoration of floodplain woodlands with the Borders Forest Trust. Since joining ECCM, she has worked on assessment of the carbon-offset potential of planting schemes throughout the UK. Currently she is providing technical advice on carbon sequestration to the Scottish Forest Alliance and working on a European project looking at synergies between carbon verification and forest certification processes.

Zvy Dubinsky graduated in 1978 from the Department of Life Sciences at Bar Ilan University, Israel, where he is a member of the faculty. His research interests include the energy conversion efficiencies of algal communities, the remote sensing of phytoplankton dynamics, the biochemistry of algal pigments, photoadaptation of the photosynthetic apparatus, combined systems for sewage treatment and algal protein production, and the potential of microalgae as sources of fine chemicals and pharmaceuticals. He is currently working on projects with Rockefeller University, New York (photoacoustic determination of phytoplankton biomass and the rate of photosynthesis), Brookhaven National Laboratory (biological oceanography) and Germany, Egypt, Jordan and the Palestinian Authority (marine science and coral conservation in the Red Sea).

Gerry Edwards is Professor of Botany in the School of Biological Sciences, Washington State University. His area of expertise is C_4 photosynthesis, with particular emphasis on environmental stress. He was awarded his doctorate from the University of California, Riverside, in 1969. Subsequently, he worked at the University of Georgia as a National Institutes of Health postdoctoral fellow, and then moved to the Horticulture Department, University of Wisconsin, Madison, rising to the rank of Professor. In 1981, he relocated to the Botany Department, Washington State University, serving as its chairman until 1986. Currently, besides being Professor of Botany, he is also a Fellow of the Institute of Biological Chemistry and Adjunct Professor of Horticulture and Landscape Architecture. Over the course of his career, Dr. Edwards has received both a Guggenheim Fellowship and a Fulbright Fellowship. His current research projects include several collaborations, with topics ranging from effects of UV-B radiation, to bioenergetics of C_4 photosynthesis, to source-sink relationships in rice, to studies on the diversity of C_4 photosynthesis in the family Chenopodiaceae.

Paul Falkowski is a Professor in the Institute of Marine and Coastal Sciences and Department of Geology at Rutgers University, New Jersey. He obtained his BSc and MA from the City College of New York, and a PhD in Biology from the University of British Columbia. His research interests include biophysics, photosynthesis, photobiology, molecular evolution, signal transduction, apoptosis, biogeochemical cycles, and symbiosis. He is a John Simon Guggenheim Fellow, a Fellow of the American Geophysical Union, a Cecil and Ida Green Distinguished Professor, and the recipient of the Huntsman Medal and Hutchinson Award. He has authored or co-authored over 190 papers in peer-reviewed journals and books, and has co-invented and patented a fluorosensing system which is capable of measuring phytoplankton photosynthetic rates non-destructively and in real time. Falkowski lives in Princeton, New Jersey with his wife and two daughters.

Richard Geider is a Professor in the Department of Biological Sciences at the University of Essex. After gaining his doctorate in Biological Oceanography from Dalhousie University, Canada in 1984, he held post-doctoral positions at the University of Dundee, Scotland and at the University of Birmingham, England, before taking up a faculty position at the University of Delaware, USA, in 1988. He became a Fellow of the Marine Biological Association of the United Kingdom in 1995, and moved to his current post at the University of Essex in 1999. His current research includes laboratory, field and theoretical studies of the factors that limit primary productivity in the sea.

Doris Godde studied biology and chemistry at Ruhr University, Bochum,Germany. Her PhD work in the Department of Plant Biochemistry with AchimTrebst was concerned with solar energy conversion and photosynthetic hydrogen production. In 1982 she went to Dartmouth College, USA for a year to work on a bacterial menaquinone-cytochrome c oxidoreductase with Bernhard Trumpower. After the birth of her two sons she worked on a joint project of the Departments of Plant Ultrastructure and Experimental Physics at Ruhr University on element distribution in plants. In 1989 she returned to the Department of Plant Biochemistry, where she worked on the regulation of photosynthesis under stress conditions. In 1995 she received her Habilitation from the Faculty of Biology and is now Privat-Dozentin.

Elias Greenbaum is an Oak Ridge National Laboratory Corporate Fellow and Adjunct Professor of Biotechnology at The University of Tennessee. He is a fellow of the American Physical Society and the American Association for the Advancement of Science. His main areas of research are the physico-chemical mechanisms of photosynthesis and their application to the production of renewable fuel and chemicals. He is a recipient of the US Department of Energy's Biological and Chemical Technologies Research Program Award.

Alfred Holzwarth has worked at the Max-Planck Institute for Radiation Chemistry in Mülheim, Germany since 1977, and has also held a Visiting Professorship at Heinrich Heine University, Düsseldorf since 1993. His research interests centre round the study of structure/function relationships and dynamics in pigment–protein complexes and the design of artificial photosynthetic units based on self-organised photoactive nanodevices with energy and electron transfer competence. He obtained his Diploma in Chemistry from the Eidgenossische Hochschule in Zürich in 1974, his PhD in Physical Chemistry from the ETH Zürich in 1977 and his Habilitation from Phillips University, Marburg in 1987.

Winfried Leibl graduated in Physics from the University of Regensburg in his home country, Germany. He then decided on a research career in the field of Biophysics and completed his PhD at the University of Osnabrück in 1989. A post-doctoral fellowship from the Deutsche Forschungsgemeinschaft brought him to the Bioenergetics Section at CEA Saclay, France, where he set up time-resolved photovoltage measurements in the picosecond and nanosecond time range. He is currently a research scientist at the institute. His main research interests are structure-function relationships in bioenergetics, and the kinetics of electron and proton transfer in photosynthetic reaction centres.

Kyriakos Maniatis is a Principal Administrator with the Directorate General for Energy and Transport of the European Commission, where he is responsible for the sector of Energy from Biomass and Waste in the unit for New & Renewable Energy Sources. After obtaining his PhD from Aston University, Birmingham, he worked as an Adjunct Assistant Professor at the Vrije Universiteit, Brussels and as a consultant for various companies, research institutes and international organisations before joining the European Commission. His main expertise is in thermochemical conversion of biomass and waste. In addition to the management of about 60 research contracts on biomass and waste on behalf of DG TREN, he is also involved in policy development on waste and renewable energy sources. During 1999–2001, he served as Vice-Chairman of the International Energy Agency's Executive Committee for Bioenergy.

Rosa Martínez obtained her PhD at the University of Valencia, Spain in 1973, and is currently Professor of Ecology in the Department of Water Science and Technology at the University of Cantabria, Spain. Her research interests include plankton production and respiration, Antarctic plankton biochemistry and ambient-driven gene expression in phytoplankton. She has participated in several national and international oceanographic cruises. Presently, she is working on a European Union project with partners from Sweden, France, Norway and Germany, dealing with harmful dinoflagellate blooms, and a Spanish project on toxic diatoms.

Paul Mathis received a degree in agricultural sciences in Paris, followed by a PhD in physical chemistry on transient states in carotenoids and post-doctoral training in Berkeley. In Saclay, he set up several instruments for flash absorption spectroscopy and used them to study functional properties of photosynthetic reaction centres. His present interest lies in electron transfer in purple photosynthetic bacteria. He is the head of the Bioenergetics Section of the French Atomic Energy Commission in Saclay, a former President of the French Photobiology Society and past-President of the International Society for Photosynthesis Research.

Denis Murphy is a well-known researcher in plant molecular and cell biology, particularly in the area of seed development and lipid metabolism. He has published over 100 research papers, written numerous reviews and edited a book on biotechnology. He has also been involved in a variety of public events relating to agbiotech, ranging from media interviews to debates in schools and universities. He received his PhD in plant biochemistry from York University, UK in 1977, and was then awarded a Fulbright Scholarship at the University of California, where he spent three years working on lipid biochemistry. He subsequently did post-doctoral fellowships at the Sheffield and Munster Universities and the Australian National University, Canberra. In 1985, he was appointed New Blood Lecturer at the University of Durham, where he founded the Oilseeds Research Group. In 1990, he accepted the post of Head of the newly established Brassica and Oilseeds Research Department at the John Innes Centre, UK, where he spent ten years as a senior manager as well as continuing an active personal research programme. In 1998, he was awarded an Honorary Professorship by the University of East Anglia in recognition of his services to education and research, and he currently has a long-term Visiting Fellowship at UEA. In 2001, he was appointed to his present post at the University of Glamorgan.

John Raven is Boyd Baxter Professor of Biology at the University of Dundee. He has broad interests in resource acquisition by photosynthetic organisms, and is especially interested in aquatic phototrophs. After gaining his BA and PhD at the University of Cambridge, UK, he stayed in Cambridge as a University Demonstrator and a Fellow of St John's College before moving to the University of Dundee in 1971, becoming a Professor in 1980. Professor Raven has made contributions to the understanding of inorganic carbon acquisition and assimilation by marine and freshwater phototrophs, the costs and benefits of the diversity of light-harvesting systems in algae, and the implications of variations in light harvesting, inorganic carbon acquisition mechanisms and the inorganic nitrogen source for the trace metal requirements of aquatic phototrophs.

Michael Seibert is Principal Scientist at the National Renewable Energy Laboratory and a Research Professor of Biology at the University of Denver and the Colorado School of Mines. He is a Fellow of the American Association for the Advancement of Sciences and a 1999 Glenn Awardee of the American Chemical Society. His research interests encompass primary processes of photosynthesis including charge separation and water oxidation, regulation of hydrogenase function and biotechnology of algal hydrogen production. He has published over 150 papers in these areas and holds six patents.

Richard Tipper is the Director of Carbon Asset Management for Greenergy Carbon Partners and the Edinburgh Centre for Carbon Management. Over the past seven years, he has worked on the development of systems to facilitate the generation and management of climate change mitigation projects in the land use sector, particularly in developing countries. From 1996 to 2001, he was the leader of a project funded by the UK government's Department for International Development to develop a model for carbon sequestration by forestry that could deliver benefits to rural communities in Latin America. He was lead author on the IPCC Special Report on Land Use Change and Forestry and has also worked as a consultant to the IEA, OECD and DETR and to a number of private companies on issues relating to the quantification and economics of carbon assets.

David Alan Walker is Emeritus Professor of Photosynthesis at the University of Sheffield, and was formerly Director of the Robert Hill Institute at that university. He is probably best known for isolating chloroplasts with intact limiting envelopes that would support carbon dioxide-dependent oxygen evolution as rapidly as the parent leaf. Work with these organelles led him to propose the existence of specific permeases able to support direct obligatory exchange between external (cytosolic) orthophosphate and internal (stromal) sugar phosphates.

This page intentionally left blank

PREFACE

In the midst of all this dwells the Sun ... Sitting upon the royal throne, he rules all the family of planets which turn about him ... we find in this arrangement an admirable world harmony.

Nicholas Copernicus, *On the Revolutions of the Heavenly Spheres,* Book I, Ch. 10.

The Sun has been shining for some four and a half billion years, and is expected to do so for as long again. It is the Earth's only truly sustainable source of energy. Photosynthesis, the process by which the energy of sunlight absorbed in the chlorophyll pigments of green plants fixes atmospheric carbon dioxide (CO_2) to carbohydrates, supplies us directly or indirectly with all our food. The oxygen discarded by plants as part of this process replenishes the atmosphere with the oxygen humans and animals need for survival.

Photosynthesis in past eras laid down the fossil fuels—oil, coal and gas—which today supply the major portion of the world's energy. These are being rapidly and irreversibly consumed, and we currently contemplate global resource depletion of oil and gas within a few decades. Only renewable sources of energy can fill this looming gap sustainably. Present-day photosynthesis already supplies about 14% of the world's energy, mainly in the form of fuelwood, and photosynthesis could in future be harnessed to provide a greater fraction of our energy needs. The end product of photosynthesis, the organic matter called biomass, is a rich store of energy, which can be burned to liberate heat or converted by a number of chemical and biological means to biofuels such as methane and ethanol. In the course of this, the CO_2 fixed in the biomass by photosynthesis is liberated to the atmosphere once again. Thus the use of sustainably grown biomass and energy crops as energy sources is CO_2-neutral and avoids increasing the burden of atmospheric CO_2.

One does not need to subscribe to Jim Lovelock's image of the Earth as the living organism Gaia to recognise that life has adapted to the way things naturally are, and the way things naturally are has adapted to life. What is new and unnatural about life today is the global influence that we exert on the atmosphere and biosphere. The burning of fossil fuels is causing atmospheric levels of CO_2 to rise steadily, and damaging consequences will be avoided only if the world moves to low-carbon or carbon-neutral sources of energy.

As the Prince of Wales remarked in a lecture in Cambridge some years ago,

> "The strategic threats posed by global environment and development problems are the most complex, interwoven and potentially devastating of all the challenges to our security ... Scientists ... do not fully understand the consequences of our many-faceted assault on the interwoven fabric of atmosphere, water, land and life in all its biological diversity. Things could turn out to be worse than the current scientific best guess. In military matters, policy has long been based on the dictum that we should be prepared for the worst case. Why should it be so different when the security is that of the planet and our long-term future?"

This book is about photosynthesis in all its biological diversity—how it works at a molecular level, how plants store its products, and how we may increase the amount of carbon fixed in the biosphere and the use of biomass as an energy source to improve the security of our planet.

We thank first our authors, who have provided such readable accounts of their various fields and who have remained (mostly) patient through the elephantine gestation of this book. We are also grateful to those who have worked on various parts of this book, in particular Alexandra Anghel, Jeffrey Archer, Lynn Barber, Barrie Clark and Stuart Honan, and to Gabriella Frescura, Laurent Chaminade, Joy Quek and Yugarani Thanabalasingam of Imperial College Press. Before he retired, Professor Jim Bolton wrote the earliest version of some parts of Chapter 1.

Finally, we remember with gratitude and respect George Porter, our friend and mentor at the Royal Institution and Imperial College. His early recognition that better understanding of the mechanisms of photosynthesis would lead to improved design of *in vitro* methods of solar energy conversion was the wellspring of much of his later research. It was he who, as Chairman of the Scientific Advisory Board of Imperial College Press, inspired this book, and the book series on the photoconversion of solar energy of which this book constitutes the second volume.

Mary Archer
Jim Barber

Imperial College, London
October 2003

CHAPTER 1

PHOTOSYNTHESIS AND PHOTOCONVERSION

MARY D. ARCHER
Centre for Energy Policy and Technology,
Imperial College London SW7 2AZ, UK
mda12@cam.ac.uk
and
JAMES BARBER
Wolfson Laboratories, Department of Biological Sciences, South Kensington Campus,
Imperial College London SW7 2AZ, UK
j.barber@imperial.ac.uk

Nature set herself the task of capturing the light flooding toward the earth and of storing this, the most elusive of all forces, by converting it into an immobile force ... the plant world constitutes a reservoir in which the fleeting sun rays are fixed and ingeniously stored for future use, a providential measure to which the very existence of the human race is inescapably bound.

Julius Robert Mayer, *The Organic Motion in its Relation to Metabolism*, 1845.

1.1 Introduction

The word photosynthesis means 'building up by light', and the process is the building up, by plants, algae and certain bacteria under the action of sunlight, of organic compounds (mainly carbohydrates) from two very simple inorganic molecules, water (H_2O) and carbon dioxide (CO_2). Put another way, photosynthesis is the light-driven reduction of atmospheric carbon dioxide by water to energy-rich organic compounds. But this reductionist, chemist's view gives little hint of the central role of photosynthesis in sustaining life on Earth. Photosynthesis is the primary engine of the biosphere, essential to life since it is almost the sole process by which the chemical energy to maintain living organisms is made. It provides all our food, either directly in the form of green plants or indirectly in the form of animals that eat green plants or other animals that have eaten green plants. The only living organisms not sustained directly or indirectly by photosynthesis are the *chemoautotrophs*, primitive bacteria that harness the energy of inorganic compounds such as H_2S to obtain the metabolic energy they need to grow and replicate, and the organisms that feed off them. Humans and other animals are *heterotrophs*—they cannot synthesise their own organic

compounds from inorganic sources, but must ingest them as food. Photosynthetic organisms are *photoautotrophs*—able to harness solar energy to fix CO_2 (that is, store it in solid form as products of photosynthesis). Modern photosynthetic organisms come in a wide range of shapes and sizes, ranging from the 1–10 μm-sized photosynthetic bacteria and small, nonvascular mosses to giant sequoia trees that can reach more than 100 m in height.

1.1.1 Photosynthesis as the creator of fossil fuels and biomass

Photosynthesis in past geological eras has provided the dominant contribution to today's energy supplies: the fossil fuels (oil, coal and natural gas) on which we currently so heavily depend were laid down by the decay of plant matter and marine organisms in the Carboniferous Period between 345 and 280 million years ago. Photosynthesis also provides an often-underrated contribution to world energy resources today by creating *biomass*, the organic matter associated with living or recently living organisms. Traditional plant and animal biomass—mainly fuelwood and animal dung—are important sources of non-commercial energy, currently providing about 14% of the world's total final energy consumption and nearly twice that in the developing world, as shown in Table 1.1. Traditional plant biomass is, however, often gathered and burned unsustainably, leading to deforestation, soil degradation and net CO_2 emissions. Only better land management techniques throughout the developing world can prevent this.

Table 1.1 Supply and consumption of biomass energy in 1998

Mtoe	OECD	Non-OECD	World
TPES [a]	5097.0	4518.2	9615.2
of which biomass [c]	169.1	893.3	1062.4
% biomass	3.3	19.8	11.1
TFC [b]	3467.0	3178.8	6645.7
of which biomass [c]	99.6	842.9	942.5
% biomass	2.9	26.5	14.2

[a] TPES = Total Primary Energy Consumption; [b] TFC = Total Final Consumption; [c] Biomass is here defined as combustible renewables and (organic) wastes. All figures are in Mtoe (million tonnes of oil equivalent). TPES and TFC differ mainly because of the inefficiency of electricity generation from burning fossil fuels. Source: IEA World Energy Outlook 2000 (*www.iea.org/statist/keyworld*).

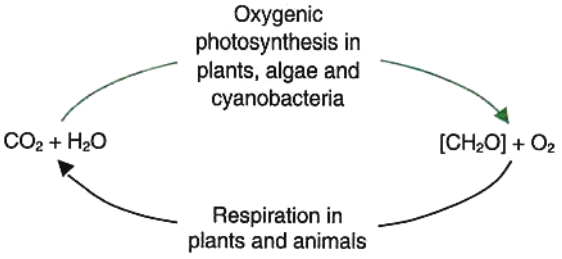


Figure 1.1 The photosynthetic respiratory cycle. Adapted from Hall and Rao (1999).

'New' biomass in the form of *energy crops* (crops grown specifically for energy production) have only a small commercial presence today, but could make an important contribution to future, more sustainable energy supplies. Indeed, many experts see biomass as providing *the* dominant renewable energy resource in the post-fossil fuel era. Production of biomass and energy crops represents one of the few ways of turning solar energy—a fluctuating, intermittent and dilute source of energy—into a compact, storable chemical form that can provide energy on demand. Energy crops can simply be dried and burned to provide heat or generate electricity. More value is added, though at a cost that is likely to be uneconomic at times of low fossil fuel prices, by converting biomass or energy crops into biofuels by biological, thermal or chemical means such as fermentation, pyrolysis and gasification.

1.1.2 Photosynthesis and the modern atmosphere

Photosynthesis in algae and higher plants provides a further vital service to man in that it is the sole replenisher of oxygen in the atmosphere. Indeed, it was the advent of oxygenic photosynthesis (this is oxygen-evolving photosynthesis as carried out by green plants, discussed in Section 1.2.1) that changed the atmosphere of the Earth from its primitive, reducing state containing virtually no free oxygen to today's breathable air. During oxygenic photosynthesis, photosynthetic organisms absorb carbon dioxide (CO_2), fix it as carbohydrates (of empirical formula $[CH_2O]$) and discard oxygen (O_2) to the atmosphere, as shown in Fig. 1.1. During respiration, animals and plants (as well as algae) do the opposite—take in oxygen, 'burn' stored carbohydrates by the process of metabolism, hence obtaining the energy needed to sustain life, and discard carbon dioxide to the atmosphere. Animals and plants thus live in symbiosis, but not symmetrically so. If animal life were suddenly to cease, forests would reclaim agricultural land and weeds the cities, but photosynthesis would

continue to cycle oxygen and carbon dioxide through the atmosphere, missing little more from the kingdom of Animalia than pollinating insects.

If photosynthesis were suddenly to cease, it would be altogether another matter, because animal life could not survive for long without fresh supplies of oxygen. Some 105 net GtC[1] is fixed by photosynthesis on land and in the oceans each year, and this releases about 260 Gt of oxygen into the atmosphere. The atmosphere contains 1.2×10^6 Gt oxygen, so the cycling time of oxygen through the biosphere is ~4600 years. If photosynthesis were to down tools, the atmosphere would return to its primordial reducing state on this timescale as plants and animals respired and expired. Humans would start to suffer from hypoxia as the partial pressure of oxygen in the atmosphere fell from its current level of 21 kPa to anything much below 15 kPa. This would hardly be our most urgent problem since we would run short of food in a matter of months. Even if we did not (say we made food from fossil fuels or biomass), the oxidative decay of dead trees and plants and organic carbon in soils would release the ~2500 Gt of organic carbon currently sequestered in the biosphere into the atmosphere as CO_2 on a timescale of decades. Much of this would eventually dissolve in the oceans but the atmospheric concentration of CO_2 would be temporarily quadrupled and the global warming experiment would be fast-forwarded.

1.1.3 Fluxes and sinks of photosynthetic carbon

The carbon cycle, described by David Schimel in Chapter 11, is the flow of carbon in various chemical forms through the Earth's atmosphere, oceans, biosphere and lithosphere. It involves chemical (geological) processes such as the formation of carbonate rock, physical processes such as the dissolution of atmospheric carbon dioxide in surface waters, mechanical processes such as the transport of dissolved carbon dioxide to the deep ocean—and the biological processes of photosynthesis and respiration. Compared with the other processes, the biological cycle is fast: although the amounts of carbon stored in rocks and the deep ocean are much greater than those in biomass or the atmosphere (see Fig. 11.1), the amount of carbon taken up annually by photosynthesis and released back to the atmosphere by respiration is 1,000 times greater than the annual flow of carbon through the geological cycle.

---

[1] 1 GtC = 1 gigatonne of carbon = 10^9 tonnes of carbon, a convenient unit in which to express carbon masses in different carbon compounds on a common basis. Another unit that is often used is the numerically equivalent PgC: 1 PgC = 1 petagramme of carbon = 10^{15} grammes of carbon = 1 GtC.

Over geological time, the flow of carbon through the carbon cycle has remained essentially responsive to climatic conditions, as they in turn have been determined by changes in the Earth's orbit, volcanic action and continental drift. The concentration of CO_2 in the atmosphere has always changed in response to these natural changes in Earth's climate. But now the boot is on the other foot. Anthropogenic (man-made) CO_2 emissions, coming mainly from fossil fuel burning, cement production and changes in land use, now constitute some 4% of total CO_2 emissions to the atmosphere. The balance of the carbon cycle is a fine one and some of the steps in it are slower than others. In particular, the rate at which we are adding CO_2 to the atmosphere is faster than natural uptake processes can adapt to take it out again. The concentration of CO_2 in the atmosphere is rising by about 0.4% per year as a result mainly of fossil fuel burning and land use change, and global warming is upon us. The UN's Intergovernmental Panel on Climate Change (IPCC) estimates that greenhouse gas emissions have already led to an increase in global mean surface temperature of about 0.6 C, and that this is likely to increase by a further 1.4–5.8 C over the next 100 years (Climate Change, 2001).

Photosynthesis removes CO_2 from the atmosphere and stores it as fixed carbon for the lifetime of the photosynthetic organism. The subsequent decay of dead photosynthetic biomass returns the temporarily fixed carbon to the atmosphere as CO_2 (except in the case of marine photosynthetic organisms, which may be exported to the deep ocean). The mass of carbon fixed annually by photosynthesis is termed primary production. *Gross primary production* (GPP), also known as assimilation, is the gross amount of carbon fixed per year. Perhaps surprisingly, GPP in the oceans (~103 GtC per year) is nearly as great as that on land (~120 GtC per year).[2] This is because phytoplankton, despite containing only about 5 GtC, or 0.2% of total photosynthetic biomass, turn over much faster than land plants.[3] Falkowski, Geider and Raven discuss the evolution and role of aquatic photosynthesis in Chapter 6.

Photosynthetic organisms consume some of their fixed carbon by autotrophic respiration, and *net primary production* (NPP) is what remains after this has occurred. On land, NPP is ~60 GtC per year, and in the oceans it is ~45 GtC per year. NPP is thus about half GPP, both on land and in the oceans. The remaining photosynthetic biomass sooner or later dies and returns its carbon as CO_2 to the atmosphere through decay, fire or conversion to compounds resistant to decomposition, so the total amount of photosynthetically fixed carbon varies only slowly over time.

---

[2] All GPP and NPP figures come from Chapter 6 of this book or from Climate Change (2001).

[3] People contain even less fixed carbon. Although there are currently about 6 billion of us, even the obese are mostly made of water, and we collectively store only ~0.1 GtC.

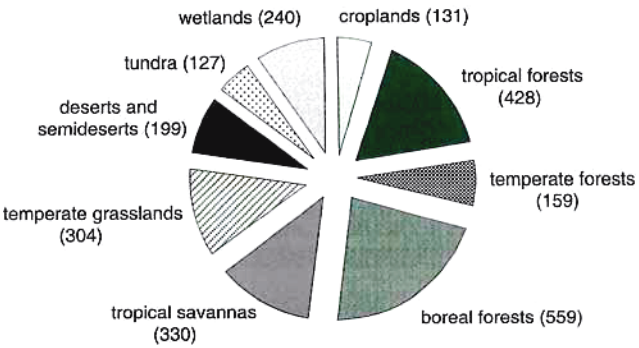


Figure 1.2 Global carbon stocks in different terrestrial systems (figures in brackets are GtC). Total terrestrial fixed carbon = 2471 GtC. Source: Royal Society (2001).

Figure 1.2 shows the distribution of land stocks of carbon between different vegetation types. The longer-lived the photosynthetic organism, the more carbon it tends to amass in its lifetime. Of the ~2500 GtC stored in terrestrial biomass, nearly all is contained in long-lived higher land plants—a term that includes trees—and their associated soils. Tropical, temperate and boreal trees contain nearly half the total, in the forests that cover about one-quarter of the land area of Earth. The forest soils of temperate forests store much more carbon than do tropical moist forests, but tropical forests grow much faster because the fall and regrowth of leaves occur continuously throughout the year and the forest is always active.

Planting and growing more trees could make a useful, though one-off, contribution to arresting the increase of atmospheric CO_2 levels by fixing more carbon in wood and soils. Richard Tipper and Rebecca Carr discuss these possibilities, and how they might be encouraged and validated, in Chapter 12. It is also possible, though currently judged unlikely in the long term, that the feedback from increased levels of CO_2 in the atmosphere could 'fertilise' photosynthesis, *i.e.* increase its net rate; David Schimel discusses this in Chapter 11.

1.1.4 Oxygenic and anoxygenic photosynthesis

Photosynthesis is a highly complex, multistep process. Only the first step, a photochemical electron transfer reaction, is driven by light. All the subsequent reactions are thermodynamically spontaneous and can therefore in principle take place in the dark, but do so rapidly under physiological conditions only because they are facilitated by sophisticated enzyme catalysts within the photosynthetic organism. Several chapters

in this book are devoted to the intricate architecture of the photosynthetic apparatus and the different organisms that carry out photosynthesis, and here we shall provide no more than a brief introduction.

Complex though the mechanisms of photosynthesis are, the overall chemical reactions are quite simple and fall into two categories: *oxygenic photosynthesis* is photosynthesis in which oxygen is produced, and *anoxygenic photosynthesis* is a simpler type of photosynthesis in which oxygen is not produced. Oxygenic photosynthesis occurs in plants, algae, oxyphotobacteria (including cyanobacteria) and anoxygenic photosynthesis occurs in green and purple photosynthetic bacteria.

The overall chemical reaction of oxygenic photosynthesis is the sunlight-driven, chlorophyll-sensitised transformation of water and atmospheric carbon dioxide to make energy-storing carbohydrates, which have the empirical formula $[CH_2O]$, and oxygen as a by-product.

$$CO_2 + 2H_2O \xrightarrow[\text{chlorophylls}]{\text{sunlight}} [CH_2O] + O_2 + H_2O \qquad (1.1)$$

This crucially important reaction, also shown in Fig. 1.1, produces essentially all of the Earth's biomass, and this book is primarily concerned with its mechanism and consequences.

The photosynthetic apparatus of plants and algae is located in organelles called chloroplasts. In leaves of higher plants they are mostly found in the palisade layer of cells under the waxy sun-facing surface of the leaves. Each cell contains up to 100 chloroplasts. These are disk-like bodies 5–10 μm in diameter containing a colourless supporting matrix known as the stroma in which double-layer lipid membranes about 50–70 Å across provides support for the pigment-proteins involved in the capture of light energy. These membranes are known as thylakoids and, as can be seen in Fig.1.3, in chloroplasts of plants they are partly stacked in dense regions known as grana and partly more loosely arranged in the stroma as unstacked lamellae. The intrathylakoid region between the thylakoid double membranes is known as the lumen. In the chloroplasts of algae, the thylakoid membranes do not tend to have such an extensive separation of stacked and unstacked regions. In the case of red algae and cyanobacteria, there is no differentiation into stacked and unstacked regions.

Anoxygenic photosynthesis occurs in purple and green sulphur photosynthetic bacteria. Here the overall reaction does not involve the oxidation of water to oxygen. Instead, these anoxygenic organisms obtain the reducing equivalents they need to fix CO_2 from a range of electron sources such as H_2S and organic acids, which we denote H_2A. Bacteriochlorophylls are the light-absorbing pigments in these organisms, and the overall process can be written

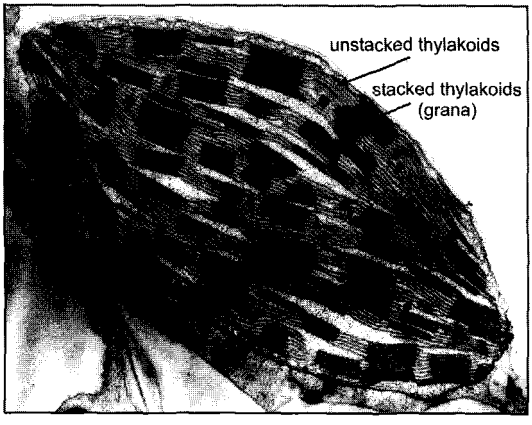


Figure 1.3 Cross section of a higher plant chloroplast (from a mesophyll cell of *Zea mays*) as seen by electron microscopy (fixed with gluteraldehyde and stained with Os and Pb) showing the stacked regions (grana) and unstacked regions of the thylakoid membrane. The dark spots are plastoglobuli (little droplets of plastoquinone). Magnification × 10,000.

$$CO_2 + 2H_2A \xrightarrow[\text{bacterio-chlorophylls}]{\text{sunlight}} [CH_2O] + 2A + H_2O \qquad (1.2)$$

All modern anoxygenic photosynthetic bacteria are oxygen-intolerant prokaryotic[4] organisms that thrive only in reducing conditions, for example in the anaerobic environment of mud, soil and stagnant ponds where their particular electron donor H_2A is available. Depending on the nature of H_2A, reaction 1.2 may be exergonic or mildly endergonic.

Photosynthetic bacteria play an important ecological role, but in terms of biomass creation they are insignificant. In scientific terms, however, they have been very important experimental systems. Indeed, as explained in Chapter 3 by Leibl and Mathis, they have provided valuable clues to the structure of the more complex photosynthetic apparatus of green plants. There are probably many thousands of different species of photosynthetic bacteria yet to be discovered, but to date detailed studies have focussed on just a few: the purple photosynthetic bacteria *Rhodopseudomonas viridis*, *Rhodobacter sphaeroides*, *Rhodobacter capsulatus*, *Rhodospirillum rubrum* and *Rhodopseudomonas acidophila* and the related green, non-sulphur bacterium *Chloroflexus aurantiacus*; and the green sulphur bacteria *Chlorobium limicola*, *Prostherochloris aestuarii* and the related *Heliobacillus mobilis*.

[4] Prokaryotes have simple cell structures with no cell nucleus.

The light-absorbing pigments and cofactors[5] in a photosynthetic organism are arranged in protein structures whose architecture depends on the type of organism, generically known as *photosynthetic units*. We discuss these structures in Section 1.3. For now, we note that each photosynthetic unit has two principal components: a *reaction centre* (RC), where the early, light-driven events of photosynthesis take place, and a *light-harvesting* (LH) antenna system, which contains most of the photosynthetically active pigments. In plants and green algae, the principal photosynthetic pigments are the chlorophylls (Chl *a* and *b*). Other types of algae (*e.g.* brown algae) can contain Chl *c* and Chl *d* as well as Chl *a*. Red algae and cyanobacteria, have only Chl *a* but also contain pigments known as phycobilins. In anoxygenic photosynthetic bacteria, the main pigments are the bacteriochlorophylls *a* and *b* (BChl), which are doubly-reduced chlorophylls that absorb at longer wavelengths than Chl *a* or Chl *b* so are more suited to aquatic organisms. All photosynthetic organisms contain carotenoids, of which there are many different types. They are involved in light harvesting but also play an important role in photoprotection. Figure 1.4 shows the structures of the main photosynthetic pigments.

The LH systems absorb the photosynthetically active radiation (PAR) of incoming sunlight and channel it to the RCs. These are of two types, known as Type I and Type II, that seem to have a common evolutionary origin. However, they differ in the nature of their cofactors and thermodynamic properties. Photosynthetic bacteria fall into two classes, distinguished by their RC type: green sulphur bacteria and heliobacteria have Type I RCs, while purple photosynthetic bacteria and filamentous green, non-sulphur photosynthetic bacteria have Type II RCs. Cyanobacteria, algae and higher plants have one RC of each type, known as Photosystem I (PSI) and Photosystem II (PSII), which act in series to drive oxygenic photosynthesis.[6]

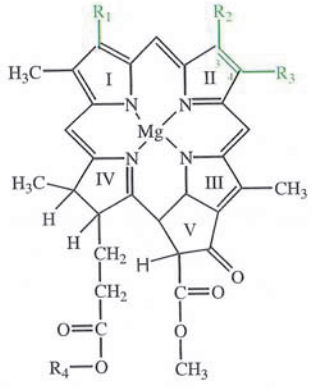
Within the reaction centre lie two closely spaced Chl or BChl molecules usually known as the *special pair*,[7] and given the symbol P (for pigment) followed by a number. This is the wavelength in nanometres where maximum bleaching is observed during photosynthesis, and lies close to the absorption maximum of the particular chlorophyll dimer. Because of the dimeric or quasi-dimeric character of P, its absorption maximum lies to longer wavelengths than that of the monomeric antenna pigment molecules. This significant shift to the red makes P an efficient trap for the excitation energy from the many surrounding LH antenna pigment molecules.

---

[5] Cofactors are the redox-active molecules involved in the electron-transfer reactions of photosynthesis.

[6] PSI and PSII have evolved from the symbiotic linkage of one Type I and one Type II photosynthetic bacterium, but now differ from their ancestors in molecular detail.

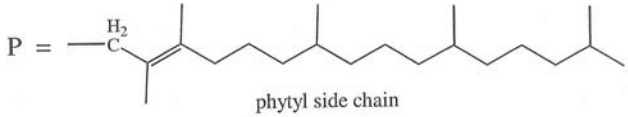
[7] Photosystem II of green-plant photosynthesis provides an exception, discussed below.



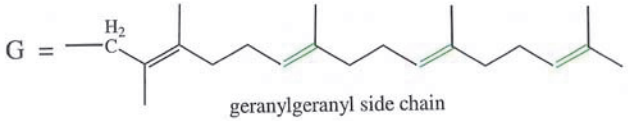
chlorophyll

	R_1	R_2	R_3	R_4
chlorophyll *a*	$-\overset{H}{C}=CH_2$	$-CH_3$	$-\overset{H_2}{C}-CH_3$	P
chlorophyll *b*	$-\overset{H}{C}=CH_2$	$-\overset{O}{\overset{\|\|}{C}}-H$	$-\overset{H_2}{C}-CH_3$	P
bacteriochlorophyll *c*	$-\overset{O}{\overset{\|\|}{C}}-CH_3$	$-CH_3{}^a$	$-\overset{H_2}{C}-CH_3{}^a$	P or G
bacteriochlorophyll *b*	$-\overset{O}{\overset{\|\|}{C}}-CH_3$	$-CH_3{}^a$	$=\overset{H}{C}-CH_3{}^a$	P

[a] no double bond between positions C3 and C4

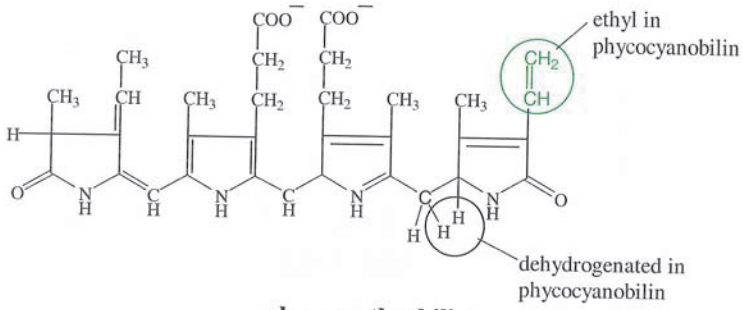


phytyl side chain

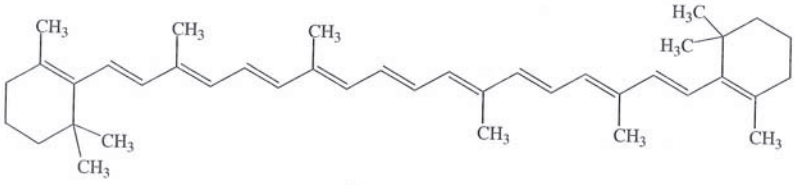


geranylgeranyl side chain

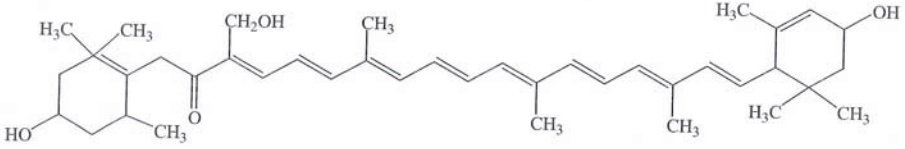
Figure 1.4a Molecular structures of the chlorophylls.



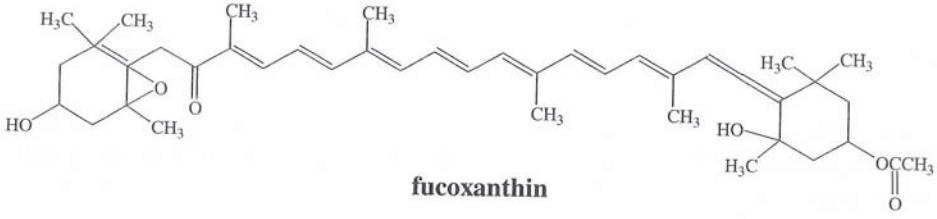
phycoerythrobilin



β-carotene



lutein



fucoxanthin

Figure 1.4b Molecular structures of phycoerythrobilin, phycocyanobilin and the common carotenoids structures β–carotene, lutein and fucoxanthin.

Figure 1.5 shows a highly schematic energy-level diagram of the photosynthetic units and overall photosynthetic reactions in anoxygenic (bacterial) and oxygenic (green-plant) photosynthesis. Photosynthetic bacteria possess *either* Type I *or* Type II RCs, and Fig. 1.5a shows P870, the Type II RC of purple photosynthetic bacteria, as an example. When P870 receives excitation from its LH system, the electronically excited state P870* is formed. Like all 'special pairs' of (bacterio)chlorophylls in photosynthesis, P870* acts as a reducing agent, and transfers an electron to a primary electron acceptor and thence to a series of secondary acceptors that take the electron outside the RC. In this way a chain of dark (thermal) events are initiated culminating in the reduction of CO_2 to carbohydrate. Meanwhile, the 'hole' (positive charge) on $P870^+$ is transferred to a nearby primary acceptor, and thence to secondary acceptors in a chain of events that culminates in the oxidation of H_2A. The bacterial RC is thus regenerated in an active state, ready to receive the next excitation from its LH system. A minimum of four absorbed photons is required to reduce one molecule of CO_2 although in many photosynthetic bacteria, cyclic electron flow also occurs which generates metabolic energy (ATP) without fixing CO_2.

In cyanobacteria, algae and higher plants, the Type I and Type II reaction centres PSI and PSII act in series, so that two photons now drive each electron through the electron-transfer chain, and a minimum of eight photons is required to reduce each CO_2 molecule. Excitation of P700, the 'special pair' of Chl *a* molecules in PSI, generates a strong reductant, P700*, capable of reducing CO_2, while the Chls of P680 in PSII generate an unusually strong oxidant, $P680^+$, capable of oxidising water to oxygen. The overall electron-transfer sequence is completed by the transfer of an electron from P680* to $P700^+$ (see Fig 1.5b).

1.2 Evolution and progress of ideas

1.2.1 Evolution of photosynthetic organisms

Life on Earth may have established itself as long as four billion years ago, a mere half a billion years after the Earth itself was formed, in the simple form of short replicating RNA strands adsorbed on pyrites (Maynard Smith and Szathmáry, 1999). How complex organisms evolved from such a primitive form of organisation is only now becoming clearer from study of the gene sequences in the RNA of the Bacteria and Archea (Woese, 2000). The common chemoautotrophic ancestor of these early kingdoms of organisms probably appeared in the waters that then covered the Earth in sunless locations where highly reducing substances such as FeS, H_2 or H_2S were

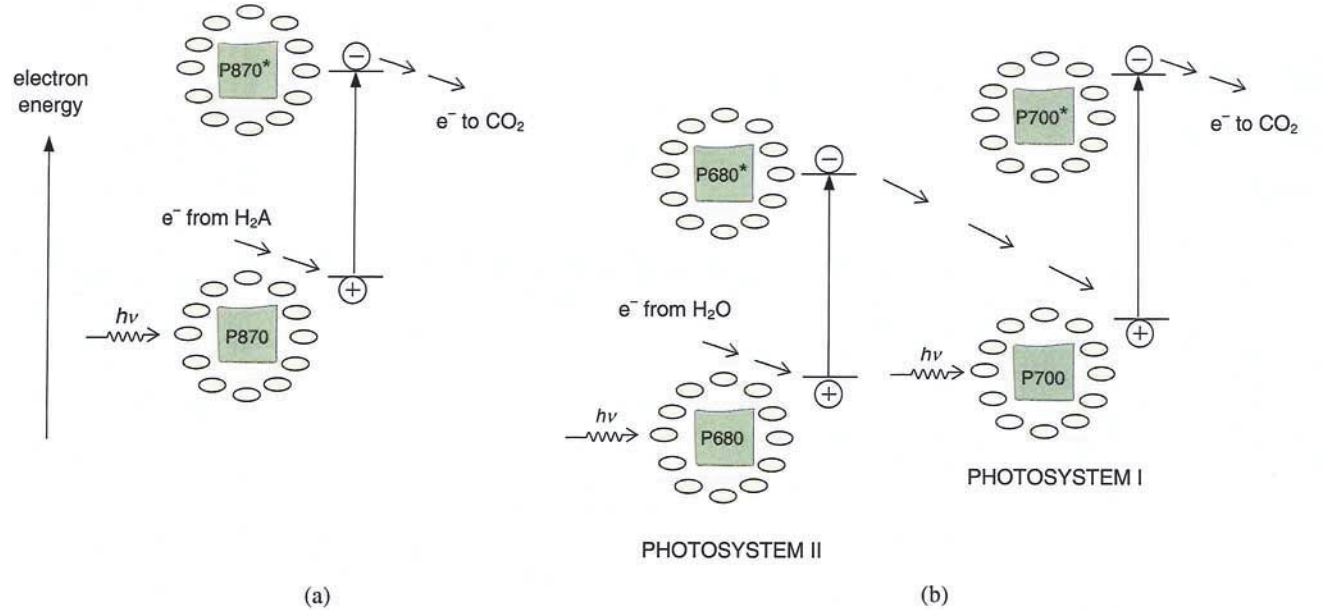


Figure 1.5 Schematic representation of (a) anoxygenic (bacterial) photosynthesis, where the energy of one photon absorbed by the pigment special pair, here shown as P870, drives the transfer of each electron through the entire process, leading to the reduction of CO_2 and the oxidation of H_2A; (b) oxygenic photosynthesis, carried out by cyanobacteria, algae and higher plants, where two linked photosystems PSII and PSI containing pigments P680 and P700 respectively act in series in the Z-scheme of photosynthesis, so called because the scheme as conventionally drawn looks like a Z on its side. Here the energy of two absorbed photons is used to drive each electron through the entire process, leading to the reduction of CO_2 and the oxidation of H_2O. The arrows show the direction of electron transfer, the reaction centres are shown as green squares and the surrounding light-harvesting systems as paler green ellipses.

available to permit fixation of dissolved CO_2 by exergonic (having negative ΔG) reactions such as

$$4FeS + 4H^+ + CO_2 \rightarrow 4Fe^{3+} + 4S^{2-} + [CH_2O] + H_2O \qquad (1.3)$$

Chemoautotrophs feeding on such an energy-rich diet had no need of light in order to grow, maintain themselves and replicate (albeit even the simplest of organisms must possess wonderfully subtle enzymes to facilitate the successive electron transfers and other reactions involved in these vital processes). One may therefore ask why photosynthesis ever evolved. One attractive hypothesis is that it originally arose as a protection mechanism: the atmosphere of the early Earth contained almost no free oxygen, so there was then no ozone layer to screen the delicate components of living organisms from the damaging hard UV wavelengths in sunlight. A pigmented organism could survive in shallow, sunlit water where an unpigmented one could not. The protective action of a chlorin pigment such as a bacteriochlorophyll would inevitably involve formation of its electronically excited state by the absorption of light. From there it is not a great leap, in imagination at least, to the excited state of the pigment acting as the donor in the first electron-transfer step of photosynthesis—and thus to the appearance of photosynthetic bacteria.

Photosynthetic bacteria are confined to the locations where both sunlight and their particular electron source is available. About 2.8 billion years ago, the evolutionary pressure to use less strongly reducing (and therefore more abundant) sources of electrons appears to have culminated in the symbiotic linkage of members of the two classes of photosynthetic bacteria (containing either Type I or Type II RCs) so that their two photosystems acted in series. Thus appeared the cyanobacteria (formerly called the blue-green algae), the earliest class of extant photosynthetic organism able to carry out oxygenic photosynthesis.

Cyanobacteria fall into a general class of oxygen-producing prokaryotic organisms called oxyphotobacteria. They are found throughout oceans, seas and lakes, and contribute significantly to the global oxygen/carbon cycle. They are also found on land, where they often have symbiotic relationships with eukaryotic organisms such as lichens. As oxygenic organisms, cyanobacteria contain PSI and PSII derived from the bacterial Type I and Type II reaction centres. Often called blue-green algae, the 'cyano/blue' colouration that gives cyanobacteria their name is due to the presence of light-harvesting phycobiliproteins, typically phycocyanin and allophycocyanin. It is not known exactly how many different species of cyanobacteria exist but it must be many tens of thousands. They can be either single cell or multicellular filamentous organisms. Experimentally favoured representatives are different strains of *Synechococcus* and *Synechocystis*, *Nostoc flagelliforme* and *Cyanidium calderium*.

Closely related to cyanobacteria are the green oxyphotobacteria often called prochlorophytes. These prokaryotic oxygen-evolving organisms do not contain phycobilins, but chlorophyll *b* instead. For this reason they were once considered to represent the type of organism that could have been the precursor to the modern chlorophyll *a*/chlorophyll *b*-containing chloroplast of higher plants and green algae (Lewin, 1976). However, gene sequencing (La Roche *et al.*, 1996) has thrown some doubt on this hypothesis. Representative species of this class of oxyphotobacteria are *Prochloron didemni*, *Prochlorothrix hollandica* and *Prochlorococcus marinus*. The latter is a picoplankton distributed extensively throughout the world's oceans and is thought to be the most abundant photosynthetic organism on our planet (Partensky *et al.*, 1999). Many cyanobacteria, such as *Spirulina*, can fix atmospheric nitrogen and are a source of protein-rich food in some parts of the world.

Cyanobacteria appeared early in evolution, as described above, and evidently spread widely throughout the sunlit surface waters of Earth. At first, the oxygen they evolved was used up in oxidising the then-abundant Fe^{2+} and S^{2-} in the oceans and lithosphere. Once this great 'rust event' was complete, about 2.2 billion years ago (see Fig. 6.2), the level of oxygen in the atmosphere rapidly rose to its present day level.

Non-photosynthetic eukaryotes[8] evolved about 1.6 billion years ago, opening the way to the appearance of *algae*, created by the endosymbiosis of a cyanobacterium by a non-photosynthetic organism. Algae are oxygenic eukaryotic organisms that are mainly aquatic, although there are some groups that can live on moist soil, tree trunks and rocks. Like cyanobacteria, some algae can have symbiotic associations with lichens and thus are not solely restricted to sunlit surface waters. Together with oxyphotobacteria, algae can often be found at considerable depths in the ocean even below the photic (sunlit) zone. Algal colonies give corals their colour and attach to rock on shallow seabeds. They exhibit considerable diversity of form, size and hue, as suits their ecological niche. Green, red and brown algae take their names from their dominant antenna pigments—chlorophylls, phycobilins and fucoxanthins, respectively. As sunlight passes through water, the longer wavelengths are absorbed first, so green algae (absorbing in the red) are found nearest the surface, while red algae and cyanobacteria (absorbing in the blue) are found at greater depths. The presence of chlorophyll *b* in *Prochlorococcus* means that this highly abundant green, marine oxyphotobacterium can also flourish at considerable depths.

Algae fix carbon to produce a range of substances. Green algae convert CO_2 mainly to starch, while other algae store oil globules, and brown and red seaweeds form sugar alcohols, polysaccharides and gummy substances such as agar and algin.

[8] Eukaryotes have complex cells that contain a nucleus and other organised internal structures.

Although the red tides and algal blooms that sometimes appear in nutrient-rich waters can be toxic and unsightly, many useful products come from algae, as Rosa Martínez and Zvy Dubinsky describe in Chapter 7.

By the beginning of the Cambrian Period 540 million years ago, the high oxygen content of the atmosphere and oceans allowed the evolution of a great variety of marine invertebrate animals deriving their energy from respiration. Air-breathing land animals appeared about 400 million years ago. 350 million years ago, the higher land plants (descended from green algae, and sharing with them the pigments chlorophyll *a* and *b*) at last proliferated, leading the way to the Carboniferous Period (345–225 million years before present), during which Earth's deposits of fossil fuel were laid down by the large-scale growth and decay of vegetation.

Like oxyphotobacteria and algae, higher plants contain both Type I and Type II reaction centres, in the form of PSI and PSII respectively. The modern plant kingdom comprises over 300,000 known species. The carbon fixed by plants is largely in the form of carbohydrates (energy-storage compounds such as glucose, sucrose and starch and plant structural materials such as cellulose and lignin). As in other types of photosynthetic organisms, the pantheon of other organic molecules needed in smaller quantities to maintain cellular activity, growth and reproduction are synthesised by elegant biochemical pathways that occur in the dark, but ultimately depend on the metabolic energy derived from photosynthesis.

1.2.2 Landmarks in photosynthesis research

Modern photosynthesis research dates from 1771 when Joseph Priestley discovered that plants can grow in air 'injured by the burning of candles' and restore the air so that a candle can burn again. He thus showed that oxygen gas is a product of the photosynthesis reaction. In 1779, the Dutch physician Jan Ingenhousz confirmed Priestley's findings and also discovered that plants can improve air only when illuminated, thus showing the importance of sunlight in the reaction. He also showed that only the green parts of the plant could carry out this process. In 1788, the Swiss pastor Jean Senebier noted that 'fixed air' or carbon dioxide was a necessary ingredient for the growth of plants. In 1804, another Swiss scholar, Nicolas Theodore de Saussure, demonstrated that water was consumed in the reaction. In 1817, two French chemists, Pelletier and Caventou, isolated the green pigment of leaves and called it chlorophyll.[9]

[9] Greek: *chloros*, green; *phyllon*, leaf.

It was not until 1845 that the German physician, Julius Robert Mayer, first realised the importance of photosynthesis as an energy-storage reaction storing sunlight as chemical energy. The stoichiometry of photosynthesis was established in 1864 by the work of the French plant physiologist T. B. Boussingault, who showed that the mole ratio of oxygen evolved to carbon dioxide consumed is close to unity. Finally, also in 1864, the German botanist, Julius Sachs, demonstrated that carbohydrates are produced in photosynthesis via the now famous starch–iodide experiment on a half-darkened leaf. In 1880, Engelmann determined that chlorophylls are the key pigments of green algae, by using oxygen-seeking motile bacteria to demonstrate that oxygen was evolved only by the chloroplast in the algae, and that the red and blue portions of the spectrum (absorbed strongly by chlorophyll) were the most active in generating oxygen.

The concepts of the antenna pigment system and the photosynthetic unit were developed by Emerson and Arnold (1932), who measured O_2 evolution from algae subjected to short flashes of light, and concluded that one O_2 molecule could be evolved per ~2400 chlorophyll molecules. Later, Gaffron and Wohl (1936) suggested that absorbed quanta are transferred from one chlorophyll molecule to another until they can be trapped at a reaction centre.

The Dutch microbiologist C. B. van Niel (1935) hypothesised that the oxygen evolved in green-plant photosynthesis comes from H_2O (rather than from CO_2, as had been previously thought). Ruben *et al.* (1941) later confirmed this hypothesis by the use of ^{18}O-labelled water. Another important development was the isolation by Hill (1937) of chloroplasts from leaves. He showed that these isolated chloroplasts were able to evolve oxygen when supplied with an artificial electron acceptor such as ferricyanide, but were not able to reduce CO_2, thus proving that the oxygen evolution and CO_2 reduction reactions are physically separable. In the same year, Pirson (1937) established the requirement for manganese in oxygenic photosynthesis. Hill and Bendall (1960) first proposed the coupling of the two photosystems in the Z–scheme.

The detailed mechanism of CO_2 fixation was elucidated by Melvin Calvin and his co-workers between 1947 and 1950 (Bassham and Calvin, 1957), using the ^{14}C radioactive tracer technique to detect the chronological history of the pathway of carbon in C_3 plants from its initial fixation into phosphoglyceric acid to the 'ultimate' product D-glucose. For this work Calvin received the Nobel Prize for Chemistry in 1961. Several years later, Hatch and Slack (1966) discovered the C_4 pathway for carbon fixation, in which CO_2 is initially converted to C_4 dicarboxylic acids (malic, aspartic and oxaloacetic) before entering the C_3 pathway. Photophosphorylation (the production of ATP in the light) was discovered in the chloroplasts of algae and green plants by Arnon *et al.* (1954), and in the chromatophores of photosynthetic bacteria

by Frenkel (1954). Vishniac and Ochoa (1951), Arnon (1951) and Tolmach (1951) independently discovered the photochemical reduction of $NADP^+$ by chloroplasts. A number of useful chloroplast-driven reduction reactions other than CO_2 fixation are thermodynamically possible. By far the most interest has centred on the photobiological production of hydrogen from water. Gaffron and Rubin (1942) were the first to observe that certain algae, containing the enzyme hydrogenase, adapted in certain conditions to evolve hydrogen. Arnon *et al.* (1961) observed hydrogen evolution from isolated chloroplasts coupled to hydrogenase in the presence of artificial electron donors. Benemann *et al.* (1973) demonstrated hydrogen evolution from isolated chloroplasts plus hydrogenase in a system where water was clearly the electron donor.

Enormous advances in our knowledge of the mechanisms of photosynthesis have been made since these pioneering studies by workers such as Witt, Duysens, Kok, Jagendorf, Clayton, Feher, Sauer, Joliot, Babcock and many others (see Ke, 2001). However, prior to the 1980s, the structures of reaction centres and other important photosynthetic components could only be inferred indirectly from spectroscopic and kinetic studies. Then in 1982, Hartmut Michel succeeded in crystallising the reaction centre protein of the purple photosynthetic bacterium *Rhodopseudomonas viridis* (Michel, 1982). X-ray crystallographic determination of the structure followed (Deisenhofer *et al.*, 1984, 1985). This seminal achievement, for which Johann Deisenhofer, Robert Huber and Hartmut Michel shared the 1988 Nobel Prize in Chemistry, revealed for the first time the exact locations of the redox active cofactors involved in the earliest steps of photosynthesis and the arrangement of the bacteriochlorophyll special pair. Since then, several reaction centre proteins and light-harvesting complexes have been crystallised and their structures determined.

1.3 The 'blue print' of the photosynthetic apparatus

Our present-day understanding of the light-driven processes of photosynthesis has reached a high level, as detailed by Leibl and Mathis in Chapter 3, although there remain some unanswered questions. The dark, carbon fixation processes are also very well understood, as described by Edwards and Walker in Chapter 4. In this section, we shall summarise the main components of photosynthetic reaction centres and their structures and functions, and then briefly sketch in the dark chemistry that results in the fixation of atmospheric CO_2 as carbohydrates.

1.3.1 Reaction centres

All types of photosynthetic systems are constructed around an exquisitely designed basic blueprint. All contain a reaction centre (RC) protein complex in which the conversion of light energy to electrochemical potential occurs. This energy conversion process involves the movement of electrical charge in the form of an electron across a membrane, generating an electrical gradient as well as a chemical potential gradient in the form of 'redox' energy, as indicated in Fig. 1.6. As stated earlier, the primary electron donor—the pigment—P is always a chlorophyll or bacteriochlorophyll, while the primary electron acceptor A can be either (bacterio)chlorophyll (as in Type I RCs) or (bacterio)pheophytin (as in Type II RCs). Typically the generation of the primary radical pair[10] P^+A^- occurs within a few picoseconds at very high quantum efficiencies.

The subsequent reduction of P^+ and oxidation of A^- occur on a slower time scale. In both Type I and Type II RCs, A^- is initially re-oxidised by a quinone molecule (Q). In Type I reaction centres, this quinone is phylloquinone, which typically has a low midpoint redox potential[11] of about –0.6 V and is strongly bound to the RC proteins. In Type II RCs, the quinone electron acceptor (called Q_A) is also tightly bound but has a higher midpoint potential of about –0.1 V. The identity of this Q_A quinone depends on the organism: it is plastoquinone in plants and algae, and ubiquinone or menaquinone in purple photosynthetic bacteria. The transfer of electrons from A^- to Q occurs on a timescale of ~200 ps, resulting in the charge transfer state P^+AQ^-.

It is the next step in the reductive electron flow that clearly distinguishes Type I and Type II reaction centres. In Type I centres, the electron is passed to an iron–sulphur centre (given the symbol F_X) which is contained within the reaction centre protein, as shown in Fig. 3.8. From F_X, the electron proceeds to two further iron–sulphur centres (F_A and F_B) and ultimately to ferredoxin, which as a water-soluble protein, transfers the reducing equivalent away from the membrane. In contrast, Type II reaction centres transfer the electron on Q_A^- to a second quinone, Q_B (as shown in Fig. 3.1). When Q_B receives a second electron from the next photochemical turnover, it is protonated to form a quinol, which diffuses away from the reaction-centre protein into the lipid matrix of the membrane. In plants and algae, Q_B is a plastoquinone, while in purple photosynthetic bacteria it is a ubiquinone. These secondary electron transfer events leading to the ejection of reducing equivalents from the reaction centre occur on a timescale stretching from microseconds to milliseconds.

---

[10] The radical pair P^+A^- is sometimes written $P^{\bullet+}A^{\bullet-}$ to emphasise the presence of unpaired electrons.

[11] See Section 3.1.1 for an explanation of midpoint potentials.

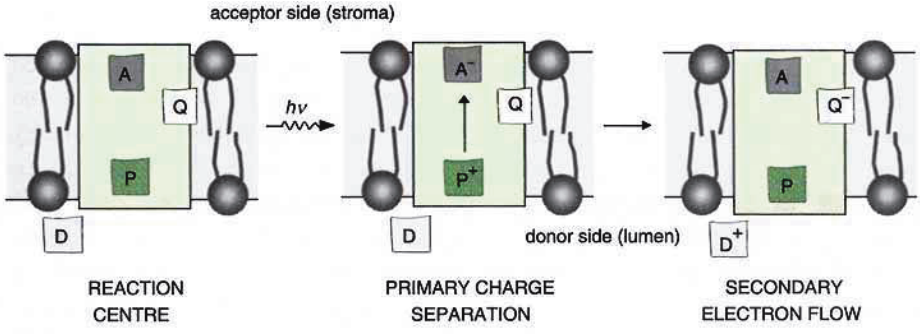


Figure 1.6 Diagrammatic representation of a photosynthetic reaction centre embedded in a bilayer liquid membrane. The absorption of a quantum of light ($h\nu$) brings about charge separation across the membrane from a chlorophyll pigment P to a primary acceptor A, followed by secondary electron flows to a quinone Q and from a donor D.

Meanwhile, the reduction of P^+ by the electron donor D occurs on the nanosecond to millisecond time scale, depending on conditions. The nature of the electron donor also depends on the particular system. Cytochromes are usually the donors in both Type I and Type II RCs of photosynthetic bacteria, while in plants and algae, water is the electron donor to PSII and plastocyanin or cytochrome c_6 is the electron donor to PSI. In those photosynthetic organisms that evolve O_2 (plants, algae and cyanobacteria), the Type I (PSI) and Type II (PSII) reaction centres are coupled as shown schematically in Fig. 1.5b and in more detail in Fig. 1.7, so as to use two photons to drive each electron through the system, providing sufficient energy to oxidise water and reduce CO_2. In all cases, the fundamental principle is that energy storage is accomplished by rapidly separating the initial oxidants and reductants of the primary charge separation so as to avoid wasteful recombination reactions.

1.3.2 Light-harvesting systems

As Alfred Holzwarth explains in detail in the next chapter, photosynthetic organisms have evolved light-harvesting (LH) antenna systems that service photosynthetic reaction centres so that they can operate efficiently under relatively low light intensities. The nature of these LH systems varies considerably according to the type of organism, but all function to intercept light and transfer the excitation energy rapidly to the reaction centre. The process is efficient, so the overall transfer rate must be faster than the singlet lifetimes of the pigments, which are typically in the nanosecond time domain. In fact, overall transfer times of energy migration from the

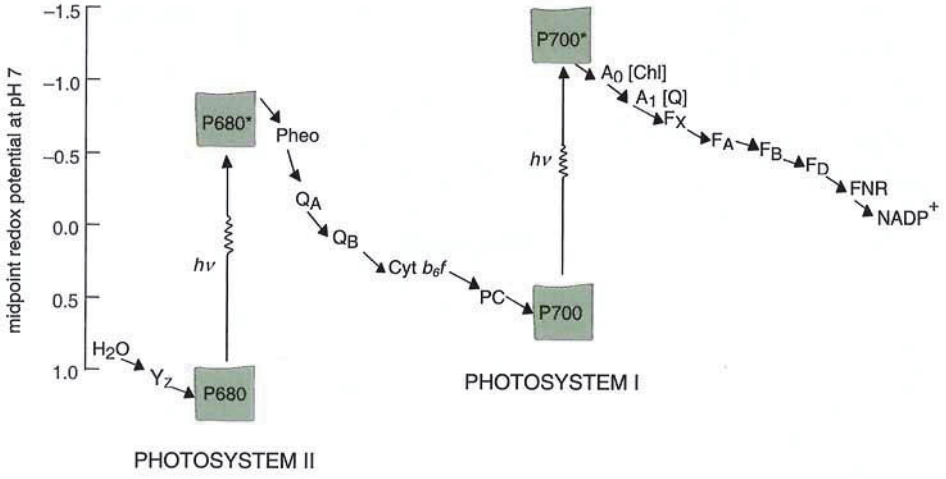


Figure 1.7 The Z-scheme for electron transfer in oxygenic photosynthesis. Y_Z = tyrosine; P680 = primary electron donor of PSII composed of chlorophyll (Chl); Pheo = pheophytin; Q_A and Q_B = plastoquinone; Cyt b_6f = cytochrome b_6f complex, consisting of an Fe–S Rieske centre, cytochrome *f* (Cyt *f*), cytochrome *b* low- and high-potential forms (Cyt b_{LP} and Cyt b_{HP}), plastoquinone binding sites, Q_i and Q_o; PC = plastocyanin; P700 = primary electron Chl donor of PSI; A_0 = Chl; A_1[Q] = phylloquinone; F_X, F_A and F_B = Fe–S centres, F_D = ferredoxin; FNR = ferredoxin NADP reductase; $NADP^+$ = oxidised nicotinamide adenine dinucleotide phosphate.

LH system to the RC are in the sub-nanosecond time domain, and in most cases transfer seems to occur by the Förster resonance mechanism. This requires good overlap between the absorption and emission spectra of the pigments, location of each pair of energy donor and acceptor pigment molecules to be close (typically within 10–15 Å centre-to-centre) and with appropriate orientations. To achieve these properties, the pigment molecules are bound to a protein scaffold and these pigment-proteins associate with the RC. The number of light-harvesting pigment molecules servicing an RC varies according to the type of organism and the growth conditions, from 50 (in some purple photosynthetic bacteria) to many thousands (as in the case of the chlorosome of green sulphur bacteria). In plants and algae, the number is around 250 pigment molecules per reaction centre.

The LH system and RC together comprise the photosynthetic unit. In the case of higher plants and green algae, the pigments bound to LH proteins are chlorophyll *a*, chlorophyll *b* and carotenoids. In addition to chlorophyll *a* and carotenoids, red algae contain the phycobilin pigments that covalently bind to protein to form the phycobilisomes, large macromolecular structures that attach to the outer (stromal) surface of the photosynthetic membrane. Cryptomonads also contain phycobilins but they do not associate to form phycobilisomes and are located on the other side of the membrane.

Like red algae, brown algae, dinoflagellates and diatoms do not contain chlorophyll *b*, but differ again in that they contain chlorophyll *c* as an LH pigment as well as chlorophyll *a* and carotenoids. Cyanobacteria also do not contain chlorophyll *b*, but like red algae they contain phycobiliproteins that assemble into phycobilisomes. However, as mentioned in Section 1.2, there are related prokaryotic organisms (oxyphotobacteria) known as prochlorophytes that do not contain phycobilins but instead have an LH system composed of chlorophyll *a* and chlorophyll *b*. In contrast, the purple and green sulphur bacteria contain different forms of bacteriochlorophyll and carotenoids.

The photosynthetic unit is a marvellously tuned sunlight-gathering apparatus. The different spectral properties of the wide range of LH pigments, coupled with fine-tuning of the IR spectra by interactions with the proteins to which they bind, allow photosynthetic organisms to absorb at all the wavelengths available in the solar spectrum at the Earth's surface (350–1000 nm).

1.3.3 Photosynthetic membranes

The reaction centres of purple and green sulphur bacteria are localised in membranes, often called chromatophore membranes, which lie close to or include the outer cell membrane. In purple photosynthetic bacteria, the LH proteins are also intrinsic to the chromatophore membrane. However, in green bacteria the very large LH chlorosome, packed with many thousands of molecules of bacteriochlorophyll, is stacked into rod-like structures attached to the cytoplasmic side of the photosynthetic membrane, which does not invaginate as it does in purple bacteria (Fig. 1.8).

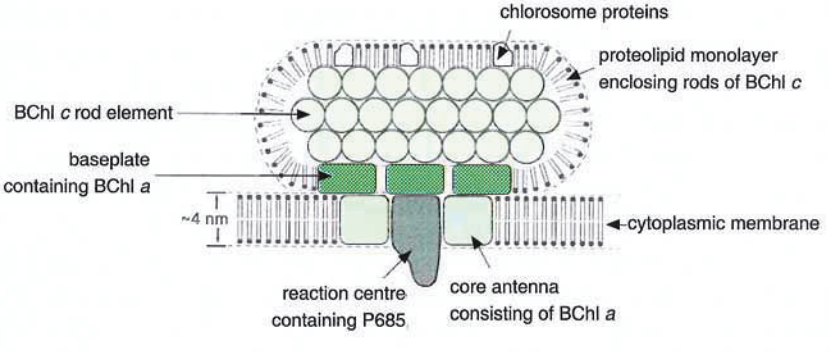


Figure 1.8 Model of the chlorosome in *Chloroflexus aurantiacus* (modified from Ke (2001)).

In oxygenic photosynthetic organisms, the photosynthetic apparatus involved in light reactions is embedded in the specialised thylakoid membrane (see Fig.1.3). In cyanobacteria, the thylakoid membranes tend to form concentric rings within the cytoplasm and are characterised by the presence of the large LH phycobilisomes attached to their surfaces, which induces a considerable spacing between them. In the green oxyphotobacteria (prochlorophytes), the same concentric rings are present but the membranes lie more closely together because of the absence of bulky phycobilisomes. The presence of phycobilisomes in the chloroplast of red algae leads to a thylakoid membrane organisation reminiscent of cyanobacteria. In striking contrast, the thylakoid membranes of higher plant chloroplasts, and to a lesser extent those of green algae, are arranged in stacked (grana) and unstacked regions (see Fig.1.3). The granal thylakoids are highly enriched in PSII, while PSI is found in the unstacked regions. However, this extreme lateral separation does not seem to occur in the thylakoid membranes of cyanobacteria and many forms of algae and therefore cannot be an absolute requirement for oxygenic photosynthesis to occur.

1.3.4 Energetics of electron-transfer processes in reaction centres

Before discussing the structural and functional properties of the reaction centres of different types of photosynthetic organisms, it is necessary to appreciate their specific electron-transfer pathways in terms of redox potentials. Figure 3.2 compares the Type I and Type II reaction centres of anoxygenic and oxygenic organisms. In purple photosynthetic bacteria (specifically *R. sphaeroides*), the primary donor is called P870, because the long wavelength absorption peak of its special pair of bacteriochlorophylls is at 870 nm. Similar notation is used for other primary donors *e.g.* P840 (green sulphur bacteria), P870 (green non-sulphur bacteria), P700 (PSI) and P680 (PSII). However, as hinted in Section 1.1.4, P680 differs from the other primary electron donors in that it seems not to be a special pair (Barber and Archer, 2001).

As we noted in Section 1.3.1, when excitation arrives at the RC from the LH system, primary charge separation occurs and this is followed by secondary electron flow to a terminal electron acceptor, ferredoxin (Fd) in the case of green sulphur bacteria and PSI (Type I RCs), or quinone (Q_B), in the case of purple bacteria and PSII (Type II RCs). In the case of anoxygenic bacteria, some of the reducing potential is used to convert NAD^+ to the NADH needed for CO_2 fixation and some is utilised in cyclic electron flow, whereby the reductant indirectly reduces the oxidised primary donor. This cyclic electron flow involves the cytochrome *bc* complex, which is also embedded in the chromatophore membrane and which couples the electron flow to

the vectorial movement of protons across the membrane, as shown in Fig. 3.6. The resulting pH and electrical gradients are then used to drive the conversion of ADP to ATP in accordance with the chemiosmotic mechanism of Peter Mitchell (1966), a contribution for which he received the Nobel Prize for Chemistry in 1978.

As already mentioned and shown diagrammatically in Figs. 1.4, 1.7 and 3.2, Photosystem I and Photosystem II work together in oxygenic photosynthetic organisms to oxidise water and reduce ferredoxin. PSII functions as the water–plastoquinone oxidoreductase while PSI is a plastocyanin–ferredoxin oxidoreductase. The redox coupling between the two reaction centres is accomplished by a cytochrome *bc* complex rather like that found in anaerobic photosynthetic bacteria but called, for historical reasons, the cytochrome b_6f complex. An important feature of this scheme is that two photons are used to drive one electron from water to ferredoxin. The cytochrome b_6f complex acts as a plastoquinol–plastocyanin oxidoreductase and, like its counter part in photosynthetic bacteria, facilitates the maintenance of the electrochemical potential gradient of protons across the thylakoid membrane needed to convert ADP to ATP. In oxygenic photosynthesis, the reduced ferredoxin is used to convert $NADP^+$ to NADPH, which together with ATP is required to convert CO_2 to carbohydrate.

Green sulphur bacteria also use reduced ferredoxin in the same way as PSI except that they, like purple bacteria, use non-phosphorylated nicotinamide adenine dinucleotide (NAD^+) rather than $NADP^+$. The similarity in the redox properties and electron transport pathways of the Type I and Type II RCs is evident in Fig. 3.2 except for the important fact that $P680^+$ is a much stronger oxidant (with a midpoint potential of ~1 V) than $P700^+$, $P840^+$ and $P870^+$ (~0.4 V). This is because $P680^+$ must be sufficiently oxidising to remove electrons from water, which is a very stable molecule and difficult to oxidise compared with the substrates oxidised by other reaction centres. This oxidation reaction involves a cluster of 4 Mn atoms and the transfer of electrons and protons from the substrate water molecules is facilitated by a redox-active tyrosine, named Y_Z, positioned between the $(Mn)_4$-cluster and P680 (see Fig.1.7). As the production of dioxygen from water is a four-electron process

$$2H_2O \rightarrow O_2 + 4e^- + 4H^+ \qquad (1.4)$$

and a dioxygen molecule is produced at a single PSII reaction centre, the Mn cluster must accumulate four oxidising equivalents. This is why the evolution of O_2 oscillates with a period of four when oxygenic organisms are subjected to single turnover flashes of light, as discovered by Pierre Joliot and colleagues in 1969. This discovery caused Kok *et al.* (1970) to propose the S-state cycle, whereby the absorption of four successive photons drives the series of reactions

$$S_0 \xrightarrow{h\nu} S_1 \xrightarrow{h\nu} S_2 \xrightarrow{h\nu} S_3 \xrightarrow{h\nu} S_4 \qquad (1.5)$$

When S_4 is formed, dioxygen is released and the cycle resets itself to the S_0-state. Although the precise chemical mechanism of the S-state cycle is unknown, it is generally believed that the two water substrate molecules bind at the S_0-state and that H^+ and electrons are extracted before arriving at the S_4-state. The late Jerry Babcock and colleagues (Tommos and Babcock, 2000) have suggested an attractive 'hydrogen-atom abstraction' hypothesis for the water oxidation mechanism.

Not surprisingly, the high redox potential of $P680^+$ and the possibility of forming reactive oxygen species during the water-splitting reaction give rise to oxidative damage of the PSII RC. This manifests itself as rapid degradation and regular replacement of protein, as Godde and Bornman describe in Chapter 5. Plants and other oxygenic organisms have evolved a range of protective strategies that reduce the frequency of photoinduced PSII damage and allow the repair process to cope under normal conditions. The effect of this intrinsic and detrimental property of PSII is, however, observed when organisms are exposed to environmental stress, when the rate of repair does not match the rate of damage and photoinhibition occurs. When this happens, the efficiency of photosynthesis and biomass/crop productivity decline.

1.3.5 Reaction centre structures

Figure 1.9 shows how PSI and PSII are functionally coupled with cyt b_6f and ATP synthase in the thylakoid membrane. We now know for certain from x-ray crystallographic studies that all reaction centres are characterised by a pseudo-2 fold symmetry axis that relates the cofactors and the proteins that bind them. In Type II RCs, this symmetry gives rise to a redox-active branch and an inactive branch, as shown for PSII in Fig. 1.9. Despite intense studies on the purple bacterial RC it is still not clear how Type II centres are able to differentiate their active and inactive branches. However, this property has distinct advantages when the terminal acceptor (*i.e.* Q_B) requires two electrons to be fully reduced. In Type I reaction centres, where a single, centrally located iron–sulphur centre F_X is the electron acceptor (as for PSI in Fig. 1.9), it seems possible that primary charge separation occurs with similar probability up either branch; this must be so in green sulphur bacterial RCs, which are homodimeric while in the case of PSI the situation is less clear.

The two protein subunits that constitute the RCs of PSI, PSII and purple bacteria are not identical, as in the case of green sulphur bacteria, but form a heterodimer. In purple bacteria, the two subunits are called L and M, while in PSII the closely related

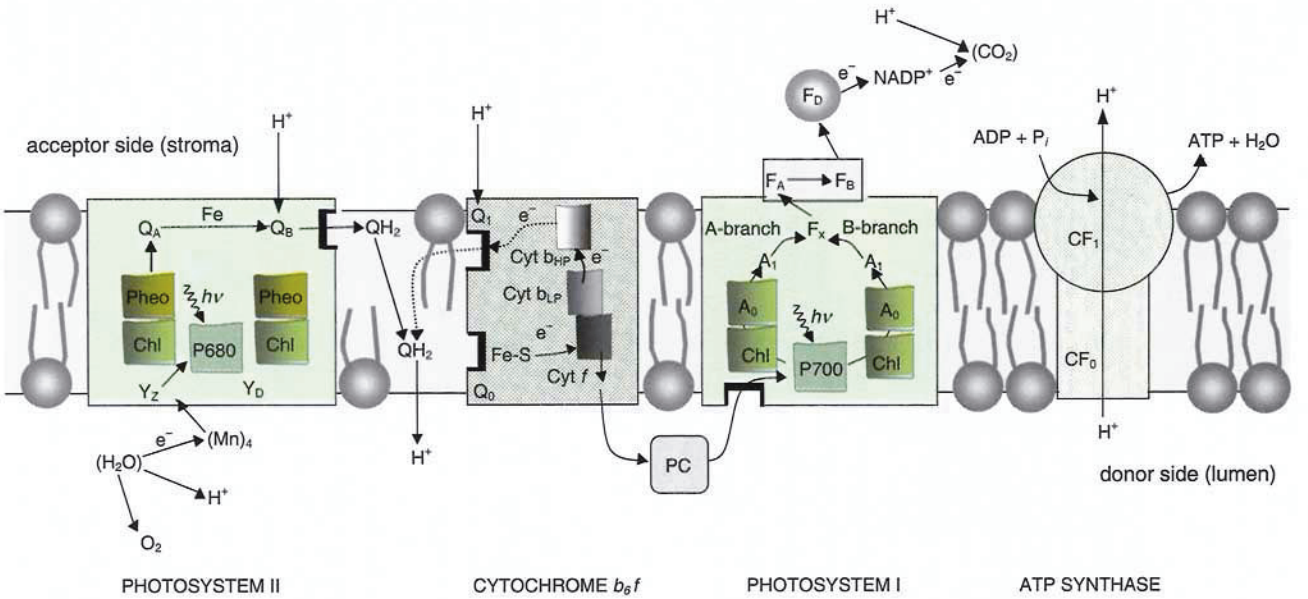


Figure 1.9 Schematic diagram of the electron–proton transport chain of oxygenic photosynthesis in the thylakoid membrane, showing how Photosystem I (PSI) and Photosystem II (PSII) work together to use absorbed light to oxidise water and reduce $NADP^+$, in an alternative representation to the Z–scheme shown in Fig. 1.7. The diagram also shows how the proton gradient generated by the vectorial flow of electrons across the membrane is used to convert ADP to ATP at the ATP synthase complex (CF_0CF_1). In both PSI and PSII, the redox-active cofactors are arranged around a pseudo-two-fold axis. In PSII, primary charge separation and subsequent electron flow occurs along one branch of the reaction centre. However, in the case of PSI, it is likely that electron flow occurs up both branches as shown. Electron flow through the cytochrome b_6f complex also involves a cyclic process known as the Q cycle. The symbols used for the various redox cofactors are defined in the legend of Fig. 1.7 except for Y_D = symmetrically related tyrosine to Y_Z but not directly involved in water oxidation, and QH_2 = reduced plastoquinone (plastoquinol), which acts as a mobile electron/proton carrier from PSII to the cytochrome b_6f complex.

subunits are known as the D1 and D2 proteins (see Figs. 3.3 and 3.10). All four proteins show considerable homologies, and all have five transmembrane helices related to each other in their reaction centres by the same pseudo-2 fold axis that relates the cofactors.

The two proteins that make up Type I reaction centres, PsaA and PsaB, are also arranged around the pseudo-2 fold axis that relates the cofactors (see Fig. 3.8), but in this case they have eleven transmembrane helices. Interestingly the five transmembrane helices at the C-terminal ends of these Type I RC proteins are arranged in a similar, but not identical, manner as in Type II RCs (Rhee *et al.*, 1998; Schubert *et al.*, 1998).

The structural details briefly described above have emerged from X-ray crystallographic studies which began with the elucidation of the structure of a Type II RC isolated from the purple bacterium *R. viridis* in the 1980s by Deisenhofer, Huber, Michel and colleagues (Deisenhofer *et al.*, 1984, 1985) and have recently advanced to the determination of the structure of PSI at 2.5 Å (Jordan *et al.*, 2001) and PSII at resolutions ranging from 3.8 Å to 3.5 Å (Zouni *et al.*, 2001; Kamiya and Shen, 2003; Ferreira *et al.*, 2004). As discussed in detail in Chapter 3, these studies have given a structural basis for the interpretation of data obtained by a variety of spectroscopic techniques and for developing general theories of electron transfer in proteins. Moreover, the determination of the structures of the cytochrome *bc* (Iwata *et al.*, 1998; Zhang *et al.*, 1998) and ATP synthase (Abrahams *et al.*, 1994) complexes of the respiratory membranes allows realistic structural extrapolations to the corresponding complexes of photosynthesis.

1.3.6 The dark reactions of photosynthesis

The sequences of reactions by which CO_2 is reduced to carbohydrate are sometimes referred to as the 'dark' reactions of photosynthesis because CO_2 can be fixed in the dark by a leaf or photosynthetic organism if the appropriate reagents are available. The dark reactions take place separately from the light-driven reactions in the stroma or cytoplasm, as indicated in Fig. 1.10. Electrons from the light-driven process in the thylakoid membrane reduce either nicotinamide adenine dinucleotide (NAD^+) or its phosphorylated form ($NADP^+$), and the reduced forms NADH or NADPH provide the reducing power for CO_2 fixation, with the help of some additional free energy in the form of adenosine triphosphate (ATP) generated by photosynthetic phosphorylation. There are several mechanisms of CO_2 reduction, characteristic of different photosynthetic species. The reductive pentose cycle or C_3 cycle (so called because the

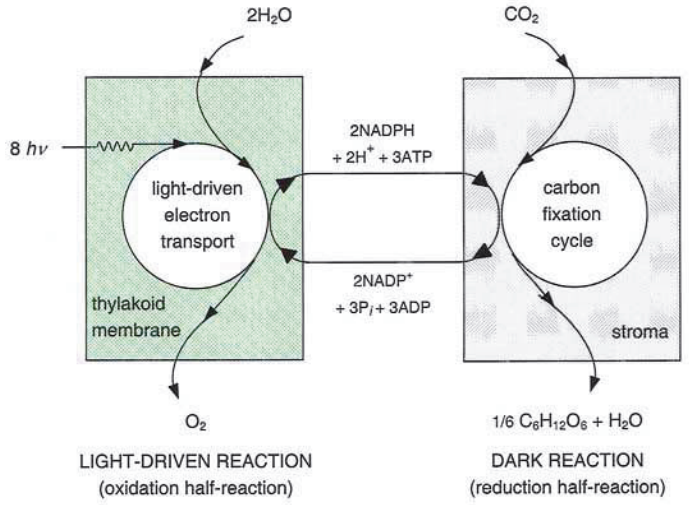


Figure 1.10 The light and dark reactions of oxygenic photosynthesis. The light-driven production of oxygen occurs in reaction centres embedded in the thylakoid membrane. The electrons from this process reduce NADP to NADPH, and also enable the production of ATP from ADP and inorganic phosphate (P_i). The dark carbon fixation cycle occurring in the stroma is driven by NADPH and ATP.

first 'stable' product of CO_2 reduction is a three-carbon compound) is the commonest mechanism, operating in algae and most plants. Some plants, especially those indigenous to hot climates, such as corn (maize) and sugar cane, operate the C_4 cycle. Edwards and Walker discuss these and other carbon fixation cycles such as the CAM cycle in Chapter 4; Blankenship (2002) provides a full account.

1.4 Energy-storage efficiency of photosynthesis

Photosynthesis is the only natural process able to store a significant amount of solar energy as chemical energy in biomass: terrestrial plants, particularly trees, are the main repositories. However, nature has not entered photosynthesis for any energy efficiency awards—the imperative for any photosynthetic organism is replication, not the accretion of biomass. Nonetheless, if the energy-storage process were not adequately efficient, it would not serve this primary purpose.

In our context, the energy-storage efficiency of photosynthesis is of course of great interest. It determines the flux of energy into the biosphere, the land area required to produce a given number of food calories, and the biomass yield from a

given area of an energy crop plantation. In this section, we first look at the structures of the carbohydrates that are the main energy-storage compounds of photosynthesis, and then the maximum gross and net efficiencies permitted by the characteristics of the photosynthesis reaction, and finally the energy-storage efficiencies actually achieved in the wild and in cultivated crops.

1.4.1 Carbohydrates

The immediate end product of photosynthesis is the monosaccharide D-glucose. Fig. 1.11a shows its structure in its usual cyclic form. Both plants and animals break down this simple sugar to obtain energy via the metabolic process known as glycolysis. The end product of this depends on the nature of the organism and whether oxygen is present. In green plants in normal, aerobic conditions, glycolysis proceeds (via the citric acid cycle) to form the fully oxidised products CO_2 and H_2O, and the energy released by this process, when coupled with respiratory electron flow, drives the synthesis of 36 molecules of ATP (adenosine triphosphate), the energy-carrying molecule that is found in the cells of all living organisms.

Glucose that is not immediately required by a photosynthetic organism is polymerised, to provide both the oligosaccharides often associated with lipids and proteins and the polysaccharides that constitute the main structural materials and nutritional reservoirs of plants. Cellulose (Fig. 1.11b) is the most abundant structural material, constituting about half the cell-wall material of wood and higher plants, and accounting for over half of all the fixed carbon in the biosphere. It is a linear polymer of up to 15,000 D-glucose residues held by hydrogen bonds in a rigid assembly of great strength. Glycogen (Fig. 1.11c), the chief food reserve of photosynthetic bacteria and animals, is a branched polymer of D-glucose residues. Starch, which is the main food reserve of plants as well as a major nutrient for herbivorous animals, is a mixture of the two polysaccharides α-amylose (an isomer of cellulose) and amylopectin (similar to, but more highly branched than, glycogen).

1.4.2 Gross efficiency ignoring respiration

Green plants contain carbon in a reduced state, mainly as carbohydrates. The chemical energy stored in these compounds is released when they are metabolised in the living plant, or when the plant biomass is burned or otherwise oxidised to CO_2 and H_2O. Since plant biomass is mainly comprised of D-glucose polymers, the energy

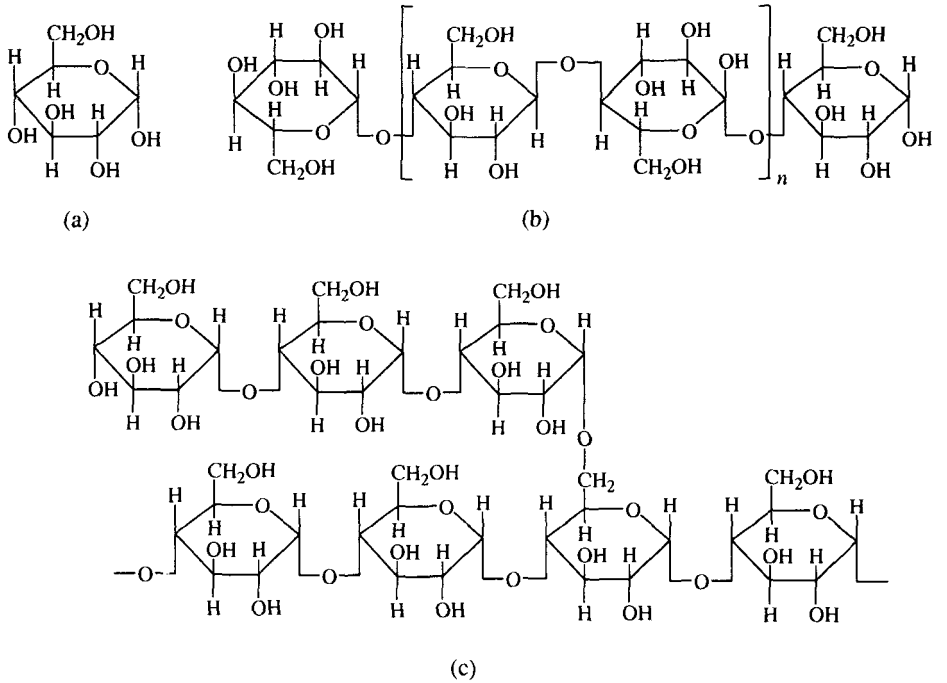


Figure 1.11 Structures of the energy-storing carbohydrates of photosynthesis: (a) D-glucose, in its common cyclic form α-D-glucopyranose; (b) the linear polymer of D-glucose residues that is the primary structure of cellulose; (c) glycogen, a highly branched polymer of D-glucose residues.

released when biomass is oxidised to CO_2 and H_2O is roughly equal to the enthalpy of synthesis of D-glucose from CO_2 and H_2O. Under normal atmospheric conditions, we can write this reaction as

$$CO_2\ (g, 0.00036\ atm) + H_2O\ (liq.) \rightarrow \frac{1}{6} C_6H_{12}O_6\ (s) + O_2\ (g, 0.21\ atm) \qquad (1.6)$$

where $C_6H_{12}O_6$ is D-glucose. For this reaction, ΔH = 467 kJ and ΔG = 496 kJ at 298 K; the standard values are ΔH° = 467 kJ and ΔG° = 480 kJ (Bolton, 1979).
Neglecting for the moment the inevitable loss of some biomass in a living plant by the process of respiration, we can define the photosynthetic energy (enthalpy) storage efficiency η_{PS} of a green plant, acting as a photoconverter of solar energy, as

$$\eta_{PS} = \frac{\text{rate of enthalpy storage (W m}^{-2}\text{) in the plant as D-glucose}}{\text{solar irradiance (W m}^{-2}\text{) incident on the plant}} \qquad (1.7)$$

To calculate η_{PS} for sunlight of a given spectral distribution, Schneider (1973) and Bolton (1979) rewrote eq. 1.7 as

$$\eta_{PS} = \frac{\int_{\lambda_{min}}^{\lambda_{max}} \frac{j_\lambda^S \alpha_\lambda \phi_\lambda}{N_A} \Delta H \, d\lambda}{\int_0^\infty E_\lambda^S \, d\lambda} \tag{1.8}$$

where N_A is the Avogrado constant and, for the wavelength band λ to $\lambda + d\lambda$, j_λ^S is the incident solar spectral photon flux (photons $m^{-2}\,s^{-1}\,nm^{-1}$), E_λ^S (W $m^{-2}\,nm^{-1}$) is the incident solar spectral irradiance α_λ is the spectral absorptivity (fraction of light absorbed by the plant), ϕ_λ is the quantum yield for the production of O_2 or consumption of CO_2, ΔH is the enthalpy of the photosynthesis reaction, and λ_{min} and λ_{max} are the minimum and maximum wavelengths that effect the reaction,.

Using experimental data at 10 nm intervals for α_λ, j_λ^S, E_λ^S and ϕ_λ, Bolton (1979) calculated η_{PS} from eq. 1.8 and obtained a value of (9.2 ± 0.8)%. This is the upper bound on the gross efficiency of energy storage in a healthy growing leaf, ignoring respiration. In using experimental values of α_λ and ϕ_λ, it takes account of the dip in α_λ values in the green and ϕ_λ values towards the red. If ϕ_λ were to maintain the 'ideal' value of 0.125 (or 8 photons per O_2 molecule evolved) from 360 nm to 700 nm and then drop off as the experimental values from 700 nm to 720 nm, the gross efficiency would rise even higher, to 13.3%.

1.4.3 Net efficiency allowing for respiration

The calculated gross energy-storage efficiency of ~9% of a green plant can never be achieved in real life because all photosynthetic organisms must constantly consume a portion of their stored energy in the process of respiration to obtain the energy to stay alive. Respiration effectively reverses oxygenic photosynthesis, and so reduces the energy-storage efficiency to a net value below the gross value.

There are two types of respiration—dark respiration and photorespiration, the latter occurring only in the light. The rate of dark respiration in green leaves lies in the range 0.5–4.0 mg CO_2 dm^{-2} hr^{-1} at 25 C (Zelitch, 1971). This has only a small effect on the efficiency in bright sunlight, subtracting perhaps ~0.2% from the gross efficiency. Photorespiration, on the other hand, is responsible for a much more serious loss of fixed carbon. In plants using the normal C_3 carbon fixation cycle, photorespiration occurs at such a rate that some 30% (at 25 C) to 40% (at 35 C) of the gross yield of photosynthesis is lost. Plants using the C_4 cycle lose rather less.

The combination of dark respiration and photorespiration reduces the calculated maximum net efficiency of photosynthesis to a value between 5.3% at 35 C and 6.2% at 25 C (Bolton, 1979). This agrees well with other estimates: 5.3% (Bassham, 1976); 5.5% (Hall, 1977); 5% (Boardman and Larkum, 1975). This is the expected instantaneous maximum efficiency for a healthy leaf growing in optimal conditions. There are a number of factors, explored in the next section, that reduce time-average values well below this, although short-term values can approach 5%.

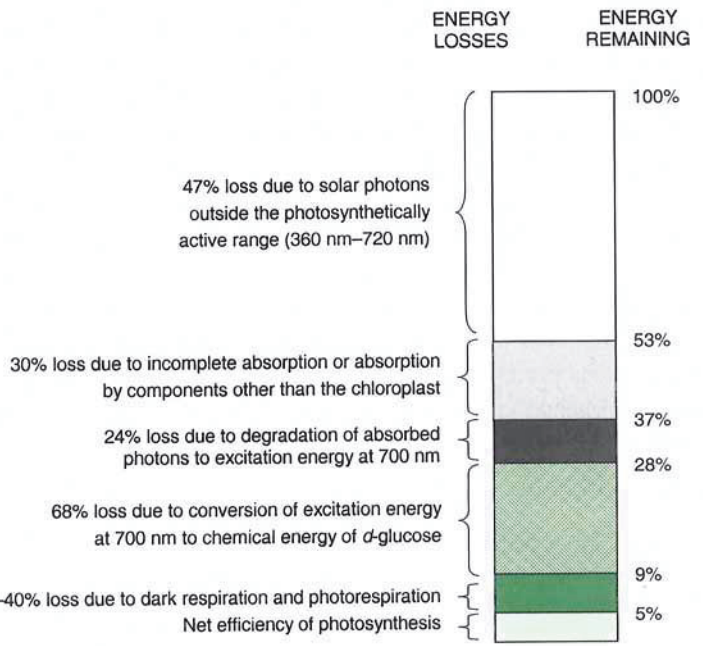


Figure 1.12 Energy losses in photosynthesis.

Figure 1.12 shows in another way how the upper bound of ~5% on the energy-storage efficiency of a green plant comes about. Nearly half (47%) of the solar energy incident on a plant is lost because it lies outside the photosynthetically active range of 400–700 nm. A further 16% is lost by incomplete absorption of PAR (Photosynthetically Active Radiation) or by its absorption by components other than the chloroplast. A further 9% is lost by thermalisation—the degradation to heat of the 'excess' energy of absorbed photons of wavelength below 700 nm, that is, energy above 1.77 eV, which is the threshold or 'bandgap' energy U_g of P700. A further substantial loss of 19% arises because the synthesis of D-glucose stores only the

fraction ($\Delta H / 8U_g$) of the energy of eight thermalised P700* states. That leaves only ~5% of the incident solar energy to be stored as chemical energy. This is still a formidable value; the instantaneous energy-storage capability of a green leaf in the Sun leaves most artificial molecular photoconverters of solar energy in the shade.

1.4.4 Efficiencies achieved in wild and cultivated crops

The time-average energy-storage efficiency of green-plant photosynthesis is much lower than the instantaneous maximum values of ~5% calculated in the previous section, for a number of obvious reasons. For example, the growing season is limited, the plant canopy does not intercept all incident sunlight, light levels may be too low or too high for maximum photosynthetic efficiency, and plant growth may be inhibited by factors such as water or thermal stress.

The global energy-storage efficiency of photosynthesis can be calculated from knowledge of net primary production (NPP), the mass of carbon fixed annually by photosynthesis. Global NPP is ~100 GtC yr^{-1}, about half on land and half in the oceans (see Section 6.5). The molar free energy of the photosynthesis reaction (eq. 1.6) is 496 kJ mol^{-1}, which corresponds to a specific energy-storage capacity of 41.3 kJ g^{-1} fixed carbon. Global NPP of 100 GtC yr^{-1} thus corresponds to ~4×10^{21} J of chemical energy stored in photosynthetic biomass per year.

The total solar energy received at the Earth's surface is 2.75×10^{24} J yr^{-1}. Thus the net efficiency of photosynthesis averaged over Earth's surface (land and oceans) is ~0.15%. About half of the incoming solar energy is in the PAR range of 360–720 nm, so the energy efficiency of PAR utilisation is ~0.3%. Modest as these efficiencies are, the amount of energy stored annually by photosynthesis is about ten times greater than current world energy consumption.

Agriculture—the cultivation of plants for food—arose in the Fertile Crescent about 10,500 years ago. Traditional methods of plant breeding by the selection and crossing of species with favourable characteristics (for example, hardiness and plant size) have hugely improved food crop yields since then. Energy-storage efficiencies of 0.5–1.0% on an annual basis are typical in modern food crops, and short-term yields can be as high as ~4%. C_4 plants, with their modified CO_2 fixation pathway, have considerably higher efficiencies than C_3 plants, especially in tropical and sub-tropical areas where their growth rate is less likely to saturate under high light levels. In future, global warming may extend the geographic range of some crops and trees to higher latitudes, and increased CO_2 levels may increase growth rates.

It is not widely appreciated that traditional plant-breeding techniques were effectively an imprecise form of genetic engineering: plants that have favourable characteristics and are therefore selected for breeding have favourable genotypes, which are therefore selectively replicated in future generations of plants. About half of past improvements in yields of rice, wheat and maize is due to genetic inputs. Recently, plant-breeding methods have been extended by two new 'genetic' techniques: tissue culture, which allows the crossing of favourable genotypes at cellular level to form new cultivars, and genetic modification, which involves the incorporation of individual genes directly into plant genomes. Despite the current outcry about GMOs (genetically modified organisms), a significant part of future improvement is likely to come from transgenically improved plants. Denis Murphy describes the enormous potential of 'agbiotech'—the application of genetic techniques to improve food and non-food crop traits and yields in Chapter 13.

1.5 Energy and chemicals from biomass

Biomass was one of humanity's earliest energy sources, and it remains an important resource today and for the future. Traditional plant biomass, particularly fuelwood, currently supplies a significant part of the energy needs of the developing world. Used and regrown sustainably, such energy sources could become an important component in a future CO_2-neutral energy economy. In the USA, biomass provides nearly 4% of final energy consumption. In the EU, biomass provided 7% of total primary production in 1997. Methane produced by landfill wastes is used to generate electricity as a matter of good practice in many countries.

Plant biomass resources include wood and wood wastes, agricultural crops and their residues, and aquatic plants and algae. Energy can be derived from biomass in three main ways:

- by direct combustion to provide heat and light as such, or to raise steam and hence generate electricity;
- by gasification to provide 'biogas', a combustible gas mixture predominantly consisting of H_2, CO and CO_2, that can be used for heating or electricity generation or converted to useful chemicals such as methanol;
- by fast pyrolysis at temperatures around 480–550 C, giving a high yield of 'bio-oil', an espresso-coffee-like liquid that can substitute for conventional fuel oil in transport and static applications.

Wood is substantially the largest source of biomass energy and indeed of renewable energy, providing more than twice the contribution of hydroelectricity worldwide. Energy crops (herbaceous plants and forest plantations grown specifically for their energy content) and biofuels[12] (solid, liquid or gaseous fuels made from biomass) are established or emerging features in several parts of the world. At the moment, biofuels and electricity made from biomass are expensive compared with conventional alternatives, and their use generally needs to be stimulated by subsidy or regulation. Future cost improvements should come from volume production, the development of more efficient chemical processes and the production of value-added chemicals.

The chemical composition of dry biomass varies somewhat with species but is roughly 75% carbohydrates or sugars and 25% lignin, with the empirical formula $C_3H_4O_2$. The main carbohydrates are cellulose—a polymer of glucose and the single most abundant product of photosynthesis—and hemicellulose. Because of their oxygen content, biomass and biofuels have a much lower calorific value (~18 GJ tonne^{-1} @100% dry matter) than conventional fuel oils (42–44 GJ tonne^{-1}). Biomass generally has low (<0.1%) sulphur content. Its nitrogen content depends on the protein content, which should be kept low to minimise the emission of NO_x on combustion.

Wood and straw are widely used as fuels. However, most other plant products are not suitable to be directly burned because they are too wet. Even fresh wood contains a considerable amount of water (30–50% by weight) and is best dried before use. Charcoal, produced by slow pyrolysis of wood in limited air, is used as a domestic and industrial fuel. In Brazil, the world's largest charcoal producer and consumer, charcoal is used in heavy industries such as pig-iron, steel making and cement manufacture.

Mike Bullard discusses energy crops in Chapter 9. These are (generally perennial) species that grow rapidly and can be harvested for their biomass annually or every few years. In northern Europe, the most promising energy crops are perceived to be coppiced willow or poplar, often referred to as short rotation coppice (SRC) or arable energy coppice (AEC). Other energy crops include conventional arable crops, novel annual crops such as sweet sorghum and perennial species such as elephant grass (*Miscanthus*). Although a native of Asia and Africa, *Miscanthus* grows well in temperate climates. Its C_4 photosynthetic pathway permits biomass yields of up to 55 t ha^{-1} in the UK (compare wheat, for example, with a theoretical maximum UK

[12] The terms biomass and biofuels are sometimes used interchangeably, but we distinguish here between the undifferentiated plant mass and well-defined chemical products derived from it.

yield of 33 t ha^{-1}). Field trials of such energy crops have been underway for some years in Europe and the USA. In developed countries, the economic viability of energy crops is generally marginal, or even poor at times of low energy prices. However, the growth of energy crops on idle or set-aside farmland could represent a major future opportunity if robust supply chains and markets can be established and costs held down.

Tony Bridgwater and Kyriakos Maniatis discuss biofuels in Chapter 10. The most widely used biofuel is ethanol, made by fermentation of starch crops. Brazil and USA have pioneered large-scale ethanol fuel programmes. Nearly 1.5 billion gallons of ethanol are produced from corn annually in the USA to blend with gasoline and reduce air pollution. Brazil began its Pro-Alcohol programme, making ethanol from sugar cane, to counter the oil price hikes of the 1970s and its dependency on imported oil. It is by far the world's major producer of cane alcohol, producing 10–15 million cubic metres annually and with significant export opportunities now opening up.

Biodiesel is another significant biofuel. Chemically, this consists of a range of fatty acid alkyl esters. It can be made from any vegetable oil, produced from any oil-bearing crop or microalga, by esterification with ethanol or methanol. The properties of biodiesel are quite similar to those of conventional diesel oil, enabling it to be used neat or in blends in conventional diesel engines. Rape methyl ester (RME) produced from oilseed rape is the main form of biodiesel in Europe and Canada. The USA produced about 5 billion gallons of biodiesel in 2000 from recycled cooking oils and soy oil.

Hydrogen, an important energy vector of the future, can be produced biologically under certain growth conditions by a range of photosynthetic microorganisms that contain either the hydrogenase enzyme or the closely related nitrogenase enzyme. Some strains of cyanobacteria have developed intracellular protective mechanisms that enable them to do this in air. Photosynthetic bacteria and algae have not developed protective systems, and can produce H_2 under anaerobic but not aerobic conditions. Historically, these cultures have been easily poisoned and short-lived, but recent cyanobacterial cultures are encouragingly robust and other tricks are being used to enhance H_2 evolution from green algae. Boichenko, Greenbaum and Seibert discuss progress in Chapter 8.

Many other chemicals and products can be derived from biomass. Cellulose fibres from wood are used to make paper and textiles, natural pharmaceuticals can be extracted from many plants. In Chapter 7, Rosa Martinez and Zvi Dubinsky discuss the range of products that can be derived from algae.

Statistics on the use and trends in use of biomass for energy generation are needed in energy and CO_2 emissions modelling, particularly where developing countries are

moving from traditional biomass use to fossil fuel use. Unfortunately, reliable data do not generally exist. Traditional biomass (fuelwood and crop and animal residues) is either collected by the user or traded in highly informal markets. The volume of these transactions is not metered and can only be roughly estimated from spot consumer surveys. Statistics on commercial use of biomass in forestry, paper and sugar industries and large-scale CHP and power generation are better.

Table 1.1 showed what are probably still the most reliable statistics on global and regional biomass use, derived from two workshops held by the IEA in 1997 and 1998. According to this survey, biomass accounts for ~11% of world total primary energy supply (TPES) and ~14% of world total final consumption (TFC). Nearly all of this consumption is in non-OECD (developing) countries, where biomass comprises ~20% of TPES and ~27% of TFC. (Most biomass is simply burned in such countries, rather than being converted to derived biofuels or used to generate electricity, so the proportion of biomass in final energy use is higher than in primary supply.)

The main sources of national biomass data are the UN's Food & Agriculture Organisation (*www.fao.org*) and Energy Statistics Division and, for Asia, the Center for Energy-Environment Research and Development (*www.ceerd.ait.ac.th/*). The FAO (*www.fao.org/waicent/faoinfo/forestry/energy/*) has made considerable efforts to gather statistics on forest woodfuel, through its series of publications *Wood Energy Today for Tomorrow* (FAO, 1997–1999). Other useful sources of information are the latest biennial survey of the World Energy Council (WEC, 2001), the world biomass scenarios of Hoogwijk *et al.* (2001) and the final paper by that most indefatigable of champions of biomass, the late Professor David Hall of King's College, London (Hall *et al.*, 2000).

References

Abrahams J. P., Leslie A. G. W., Lutter R. and Walker J. E. (1994), 'Structure at 2.8 Å resolution of F_1–ATPase from bovine heart mitochondria', *Nature* **370**, 621–628.

Andersson B., Salter H. A. and Barber J. (1996), *Molecular Genetics of Photosynthesis*, Oxford University Press, Oxford.

Arnon D. I. (1951), 'Extracellular photosynthesis reactions', *Nature* **167**, 1008–1010.

Arnon D. I., Mitsui A. and Paneque A. (1961), 'Photoproduction of hydrogen gas coupled with photosynthetic phosphorylation', *Science* **134**, 1425.

Arnon D. I., Whatley F. R. and Allen M. B. (1954), 'Photosynthesis by isolated chloroplasts. II. Photosynthetic phosphorylation, the conversion of light into phosphate-bond energy', *J. Am. Chem. Soc.* **76**, 6324–6329.

Barber J. and Archer M. D. (2001), 'P680, the primary electron donor of Photosystem II', *J. Photochem. Photobiol.* **A142**, 97–106.

Bassham J. A. and Calvin M. (1957), *The Path of Carbon in Photosynthesis*, Prentice-Hall, Englewood Cliffs, N.J.

Bassham J. A. (1976), 'Mechanism and efficiency of photosynthesis in green plants', *Symp. Clean Fuels from Biomass, Sewage, Urban Refuse and Agricultural Wastes*, Orlando, Florida, Institute of Gas Technology, Chicago, pp. 205–208.

Benemann J. R., Berenson J. A., Kaplan N. D. and Kamen M. D. (1973), 'Hydrogen evolution by a chloroplast–ferredoxin–hydrogenase system', *Proc. Nat. Acad. Sci. USA* **70**, 2317–2320.

Blankenship R. E. (2002), *Molecular Mechanisms of Photosynthesis,* Blackwell Science Ltd., Oxford.

Boardman N. K. and Larkum A. W. D. (1975), 'Energy exchange in the living world', in Messel H. and Butler S. T., eds., *Solar Energy*, Pergamon Press, Oxford, pp. 125–144.

Bolton J. R. (1979), 'Solar energy conversion in photosynthesis—features relevant to artificial systems for the photochemical conversion of solar energy', in King J. B., Hautala R. R. and Kutal C. R., eds., *The Chemical Conversion and Storage of Solar Energy*, Humana Press, Clifton, N.J., pp. 31–50.

Climate Change (2001): The Scientific Basis, Houghton J. T. *et al.*, eds., Cambridge University Press, Cambridge.

Deisenhofer J., Epp O., Miki K., Huber R. and Michel H. (1984), 'X-ray structure analysis of a membrane and protein complex. Electron density maps at 3Å resolution and a model of the chromophores of the photosynthetic reaction centre from *Rhodopseudomonas viridis*', *J. Mol. Biol.* **80**, 385–398.

Deisenhofer J., Epp O., Miki K., Huber R. and Michel H. (1985), 'Structure of the protein subunits in the photosynthetic reaction centre of *Rhodopseudomonas viridis* at 3Å resolution', *Nature* **318**, 618–624.

Deisenhofer J.and Norris J. R. (1993 and 1997), eds., *The Photosynthetic Reaction Centre*, Vol. 1 (1993) and Vol. 2 (1997), Academic Press, New York.

Emerson R. and Arnold W. (1932), 'A separation of the reactions in photosynthesis by means of intermittent light', *J. Gen. Physiol.* **15**, 391–420.

Falkowski P. G. and Raven J. (1997), *Aquatic Photosynthesis*, Blackwell Science, Oxford.

FAO (1997–1999), *The Role of Wood Energy in Europe and the OECD*, van den Broek R., March 1997; *The Role of Wood Energy in Asia*, Lefevre T., November 1997; *The Role of Wood Energy in Africa*, Amous S., May 1999.

Ferreira K. N., Iverson T., Maghlaoui K., Barber J. and Iwata S. (2004), 'Architecture of the photosynthetic oxygen-evolving center', *Sciencexpress*, 5 February 2004/ *Science* 1093087.

Frenkel A. W. (1954), 'Light-induced photophosphorylation by cell-free preparations of photosynthetic bacteria', *J. Am. Chem. Soc.* **76**, 5568–5569.

Gaffron H. and Wohl K. (1936), 'Zur Theorie der Assimilation', *Naturwissenschaften* **24**, 81–90, 103–107.

Gaffron H. and Rubin J. (1942), 'Fermentation and photochemical production of hydrogen in algae', *J. Gen. Physiol.* **26**, 219–240.

Hall D. O. (1977), 'Will photosynthesis solve the energy problem?', in Bolton J. R., ed., *Solar Power and Fuels*, Academic Press, New York, pp. 27–52.

Hall D. O. and Rao K. K. (1999, 6th. ed.), *Photosynthesis*, Cambridge University Press, Cambridge.

Hall D. O., House J. I. and Scrase I. (2000), 'Overview of biomass energy', Ch. 1 in *Industrial Uses of Biomass Energy*, Rosillo-Calle F., Bajay S. and Rothman H., eds., Taylor & Francis, London, pp. 1–26.

Hatch M. D. and Slack C. R. (1966), 'Photosynthesis by sugar cane leaves: a new carboxylation reaction and the pathway of sugar formation', *Biochem. J.* **101**, 103–111.

Hill R. (1937), 'Oxygen evolved by isolated chloroplasts', *Nature* **139**, 881–882.

Hill R. and Bendall F. (1960), 'Function of the two cytochrome components of chloroplasts: a working hypothesis', *Nature* **186**, 136–137.

Hoogwijk M., van dem Broek R., Berndes G. and Faaij A. (2001), 'A review of assessments on the future contribution of biomass energy', *Proc. 1st. World Conf. on Biomass for Energy and Industry*, James & James, Vol. 1, pp. 296–299.

Iwata S., Lee J. W., Okada K., Lee J. K., Iwata M., Rasmussen B., Link T. A., Ramaswamy S. and Jap B. K. (1998), 'Complete structure of the 11-subunit bovine mitochrondrial cytochrome *bc*(1) complex', *Science* **281**, 64–71.

Joliot P., Barbieri G. and Charbaud R. (1969), 'Un nouveau modele des centres photochimique du systeme II', *Photochem. Photobiol.* **10**, 309–329.

Jordan P., Fromme P., Witt H. T., Klukas O., Saenger W. and Krauss N. (2001), 'Three-dimensional structure of cyanobacterial photosystem I at 2.5 Å resolution', *Nature* **411**, 909–917.

Kamiya N. and Shen J. R. (2003), 'Crystal structure of oxygen-evolving photosystem II from *Thermosynechococcus vulcanus* at 3.7 Å resolution', *Proc. Nat. Acad. Sci. USA* **100**, 989–102.

Ke B. (2001), Photosynthesis: Photobiochemistry and Photobiophysics, Kluwer Academic Press, New York.

Kok B., Forbush B. and McGloin M. (1970), 'Cooperation of charges in photosynthetic O_2 evolution. 1. A linear four step mechanism', *Photochem. Photobiol.* **11**, 457–475.

La Roche J., van der Staay G. W. M., Partensky F., Ducret A., Aabersold R., Li R., Golden S. S., Hiller R. G., Wrench P. M., Larkum A. W. D. and Green B. R. (1996), Independent evolution of the prochlorophyte and green plant chlorophyll *a/b* light-harvesting proteins', *Proc. Nat. Acad. Sci. USA* **93**, 15244–15248.

Lewin R. A. (1976), 'Prochlorophyta as a proposed new division of algae,' *Nature* **261**, 697–698.

Maynard Smith J. and Szathm◁ry E. (1999), *The Origins of Life*, Oxford University Press, Oxford.

Michel H. (1982), 'Three-dimensional crystals of a membrane–protein complex. The photosynthetic reaction centre from *Rhodopseudomonas viridis*', *J. Mol. Biol.* **158**, 567–572.

Mitchell P. (1966), 'Chemiosmotic coupling in oxidative and photosynthetic phosphorylation', *Biol. Rev.* **41**, 445–502.

Partensky F, Hess W. R. and Vaulot D. (1999), '*Prochlorococcus*, a marine photosynthetic prokaryote of global significance', *Microbiol. Mol. Biol. Rev.* **63**, 106–127.

Pirson A. (1937), 'A study of the nutrition and metabolism of *Fontinalis Chlorella*', *Z. Bot.* **31**, 193–267.

Rhee K.-H., Morris E. P., Barber J. and Kuehlbrandt W. (1998), 'Three dimensional structure of the PSII reaction centre at 8 Å', *Nature* **396**, 282–286.

Royal Society (2001), 'The role of land carbon in mitigating global climate change', The Royal Society (*www.royalsoc.ac.uk*).

Ruben S., Randall M., Kamen M. D. and Hyde J. L. (1941), 'Heavy oxygen (^{18}O) as tracer in the study of photosynthesis', *J. Am. Chem. Soc.* **63**, 877–878.

Schneider T. R. (1973), 'Efficiency of photosynthesis as a solar energy converter', *Energy Conversion* **13**, 77–85.

Schubert W. D., Klukas O., Saenger W., Witt H. T., Fromme P. and Krauss N. (1998), 'A common ancestor for oxygenic and anoxygenic photosynthetic systems: a comparison based on the structural model of photosystem I', *J. Mol. Biol.* **280**, 297–314

Tolmach L. J. (1951), 'Effect of triphosphopyridine nucleotide upon oxygen evolution and carbon dioxide fixation by illuminated chloroplasts', *Nature* **167**, 946–948.

Tommos C. and Babcock G. T. (2000), 'Proton and hydrogen currents in photosynthetic water oxidation', *Biochim. Biophys. Acta* **1458**, 199–219.

van Niel C. B. (1935), 'Photosynthesis of bacteria', in *Cold Spring Harbor Symp. Quant. Biol.* **3**, 138.

Vishniac W. and Ochoa S. (1951), 'Photochemical reduction of pyridine nucleotides by spinach grana and coupled carbon dioxide fixation', *Nature* **167**, 768–769.

WEC (2001), *Survey of Energy Resources*, World Energy Council (*www.worldenergy.org*).

Woese C. R. (2000), 'Interpreting the universal phylogenetic tree', *Proc. Nat. Acad. Sci. USA* **97**, 8392–8396.

Zelitch I. (1971), *Photosynthesis, Photorespiration and Plant Productivity*, Academic Press, New York.

Zhang Z. L., Huang L. S., Shulmeister V. M., Chi Y. I., Kim K. K., Hung L. W., Crofts A. R., Berry E. A. and Kim S. H. (1998), 'Electron transfer by domain movement in cytochrome bc_1', *Nature* **392**, 677–684.

Zouni A., Witt H. T., Kern J., Fromme P., Krauss N., Saenger W. and Orth P. (2001), 'Crystal structure of photosystem II from *Synechococcus elongatus* at 3.8 Å resolution', *Nature* **409**, 739–743.

This page intentionally left blank

CHAPTER 2

LIGHT ABSORPTION AND HARVESTING

ALFRED R. HOLZWARTH
Max-Planck-Institut für Bioanorganische Chemie [1]
D-45470 Mülheim a.d. Ruhr, Germany
Holzwarth@mpi-muelheim.mpg.de

A model should be as simple as possible—but not simpler.
Albert Einstein

2.1 Introduction

Photosynthesis is the complex series of processes that enable the conversion of solar energy into chemically stored energy. The absorption of light takes place in photosynthetic antennae systems composed of supramolecular arrays of pigments, and is followed by the ultrafast migration of electronic excitation energy through the antenna arrays to reaction centres (RCs), where energy storage occurs by electron transfer across the photosynthetic membrane, as outlined in Chapter 1. This chapter describes the molecular components and structures of light-harvesting systems, and the mechanisms by which energy transfer occurs. The emphasis will mainly be on the antennae systems of oxygen-evolving organisms (higher plants, algae and cyanobacteria), and the antennae of purple and other photosynthetic bacteria will be described only briefly. In recent years, the detailed structures of the light-harvesting systems and reaction centres of several organisms have been determined, some to atomic resolution. This chapter discusses our current understanding of the structural, dynamic and other features that allow efficient light harvesting in photosynthesis.

2.1.1 The photosynthetic unit

Before delving into the details of specific light-harvesting systems in photosynthetic organisms, we will first consider the general concept of a photosynthetic unit (PSU). As Fig. 2.1 shows, a PSU consists of an array of light-absorbing chromophores

[1] Previously called the Max-Planck-Institut für Strahlenchemie.

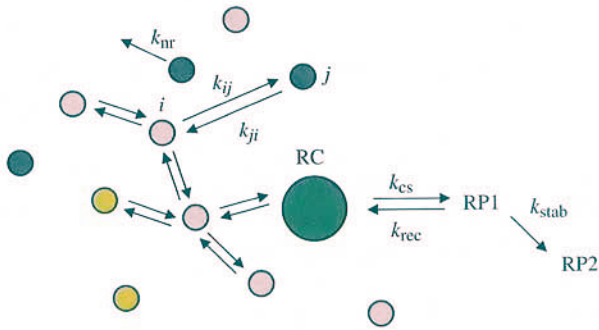


Figure 2.1 General model of a photosynthetic unit (PSU) consisting of a reaction centre (RC), antenna pigments (small circles coloured differently to indicate different spectral properties), and two radical pairs (RP1 and RP2). The rate constant of energy transfer between pigments i and j are k_{ij} and k_{ji}, k_{nr} is the rate constant for nonradiative loss of the excited states. In the reaction centre, k_{cs}, k_{rec}, and k_{stab} are the rate constants for charge separation, recombination and stabilisation, respectively.

arranged around a reaction centre. The antenna chromophores may be arranged regularly or irregularly in space, as we shall see later when discussing specific systems. There exist two essential requirements for the functioning of an antenna system. These are: (1) the distances between the chromophores must be short enough in order to allow sufficient electronic interaction for fast, efficient transfer of energy to occur between individual chromophores; and (2) the ordering of their excited-state energies must be such that there is sufficient energetic overlap between adjacent chromophores for energy transfer to occur at physiological temperatures. While there exist additional limitations for an efficient antenna system, the presently known structures tell us that these essential requirements can be satisfied by a very wide range of spatial arrangements of pigments, pigment compositions *etc*. As we shall see when we discuss specific antennae systems, typical interchromophore distances are in the range 7–15 Å, and the number of antenna chromophores in a photosynthetic antenna unit ranges from a minimum of 32 in the LHI antenna of purple photosynthetic bacteria to several hundred per reaction centre in higher plants and algae, and possibly even higher in green photosynthetic bacteria.

2.1.2 *Why are antenna systems necessary?*

One may well ask why photosynthetic antenna systems are necessary for the functioning of photosynthesis. After all, the reaction centres themselves contain several chromophores that absorb visible light, and they are in principle fully capable

of performing the processes of light absorption and charge separation by themselves. However, all photosynthetic organisms have developed photosynthetic antennae, thereby dramatically increasing the effective absorption cross-section of their RCs. The reason is that the complex machinery of an RC would not be able to work at an optimal rate and yield under typical sunlight conditions (we are thinking here of higher plants located at Earth's surface: conditions for most photosynthetic bacteria would be even worse). A bare RC would have a light-limited turnover rate of 1–10 min^{-1}, but it is capable of operating at turnover rates of up to a few hundreds per second. Thus an RC without its antenna would use only a small fraction of its photosynthetic capacity. Moreover, the limitation of the absorption cross-section would further reduce turnover rates by additional quantum losses in the intermediate charge-separated states, which have a finite lifetime before recombining to the ground state. This can only be prevented if a second photon, leading to another turnover, is absorbed within a short time. Thus an increase in the light-limited turnover rate by a factor of at least several hundred as compared with the bare RCs would be optimal. This has been achieved by the development of light-harvesting antennae that increase the effective absorption cross-section of the RC by factors of up to several hundred. Moreover, antennae systems coupled to RCs can harvest a larger bandwidth of the solar spectrum by combining pigments that absorb at different energies.

One might conclude that very large antenna sizes would be the most desirable. However, there are several limitations on maximal antenna size, because it is essentially limited by the relative rates of energy migration through the antenna to the RC and the rate of charge separation in the RC. The larger the antenna system, the longer the average time for the arrival of excitation energy at the RC (this is often called the 'first passage time' of the antenna), and thus the larger the energy lost through competing processes in the antenna such as fluorescence and radiationless decays. Furthermore, larger antennae (other than the so-called diffusion-limited PSUs) are typically associated with longer average times for charge separation, which also increases the probability that loss processes will occur.

Let us consider some basics in order to gain insight into the principles. We can distinguish two extreme cases of antenna kinetics: (1) The so-called *energy-transfer-limited case*, where the overall rate-limiting step is energy transfer through the antenna to the RC. In this case, the intrinsic electron-transfer step in the RC is very fast (faster than the first passage time); and (2) the so-called *trap-limited case*, where energy transfer through the antenna is faster than charge separation in the RC. This leads to quasi-equilibrium between excited antenna and excited RC chromophores, such that energy migrates back and forth between antenna and RC several times before charge separation occurs.

For case (1), the average time τ_{ET} for energy transfer from a regular lattice of identical chromophores to the RC can be estimated as

$$\tau_{ET} = N \ln (N\tau_{ss}) \tag{2.1}$$

where N is the number of antenna chromophores (the antenna size), and τ_{ss} the time of a single pairwise energy-transfer step. For a typical τ_{ss} of ~100 fs, the overall transfer time is ~50–100 ps for antenna sizes N of 200–300 and antenna pigments with excited-state lifetimes of ~1–3 ns. Under these conditions the yield of the photosynthetic process is limited to a maximum of about 90%; the rest of the excitation energy would be dissipated in the antenna as heat and would be lost. However, the yield is well above 90% in most photosynthetic systems. Thus for the transfer-limited case the maximal antenna size is expected to be ~200 chromophores per RC, or somewhat higher in the case of energetically heterogeneous antennae.

A similar limitation on antenna size arises, albeit for different reasons, in the other extreme case, case (2), that of trap-limited kinetics. In this case, energy transfer is assumed to be very fast (although there are limits on the maximal rates), but the excitation migrates through the system from pigment to pigment in a random, hopping process. If we assume that the excited-state energy of the RC is identical to that of the antenna pigments, the probability of the excitation being located on the RC becomes smaller as the antenna size increases for statistical reasons. This reduces the effective rate k_{cs} of charge separation in the system, according to

$$k_{cs} = k_{int}/N \tag{2.2}$$

where k_{int} is the intrinsic rate of charge separation for the RC alone and N is the number of pigment molecules. The total quantum yield of charge separation ϕ_{cs} is then given by

$$\phi_{cs} = k_{cs}/(k_{rad} + k_{nr} + k_{cs}) \tag{2.3}$$

Again using typical values for the deactivation processes in the antenna, it becomes clear that k_{cs} must be at least 2 ns^{-1}, corresponding to an overall charge separation lifetime of about 500 ps, in order to achieve a quantum yield of more than 90%. This again limits the maximal antenna size to about 200 chromophores if severe losses in overall quantum yield are to be avoided. For heterogeneous antenna systems with chromophore energies higher than the RC energy, the maximal antenna sizes are somewhat higher.

The numbers resulting from these simple considerations agree very well with actual antenna sizes of higher plants, which range from about 100 up to a maximum of 250–300 chromophores per RC. However, in organisms which contain highly heterogeneous antenna systems, such as green photosynthetic bacteria, the number of chromophores per RC can increase to several thousands, because excitation is funnelled to the RC rather than encountering it by random hops.

2.2 Theoretical aspects of energy transfer in photosynthetic antennae

2.2.1 Förster energy transfer

The light energy that is absorbed in a particular antenna pigment molecule must be transported non-radiatively over relatively large distances of the order of hundreds Ångstroms from the location of initial absorption to the RC. As we have already noted, this energy transfer must be very rapid in order to compete with intramolecular deactivation of excited states by processes such as internal conversion and intersystem crossing, which occur in the time range of a few ns. Several mechanisms that in principle allow energy migration through the antenna complexes on this timescale exist. The best known of these is the so-called very weak dipole interaction mechanism, better known as the Förster mechanism (Förster, 1965; Förster, 1959) (see Pearlstein, 1982a and 1982b for early reviews on photosynthetic energy transfer). The Förster mechanism is also known as the 'hopping process' of excitation energy transfer, since an excited state migrates through the system in a type of random walk process, albeit one which may be directionally biased depending on the amount of energetic funnelling built into the system architecture. The Förster mechanism is the most abundant mechanism of energy transfer in photosynthesis, being relevant in all photosynthetic systems, except at the shortest time scales where other mechanisms (see below) may apply.

The basis for the Förster mechanism is the coupling energy between the transition dipoles of the donor and acceptor molecules and an energetic restriction that is dealt with by the so-called overlap integral in the Förster equation. The advantage of the Förster formulation of energy-transfer rates is that it is directly based on easily accessible experimental quantities such as the absorption coefficient ε_A of the donor, the emission spectrum I_F of the acceptor, the distance R between them, and the relative orientations of the donor and acceptor as reflected in the so-called orientation factor κ^2 of the transition dipole moments of the donor and acceptor. The rate constant k_{ET} of a single energy-transfer step is given by

$$k_{ET} = \frac{\kappa^2}{R^6 n^4} \frac{\phi_F}{\tau_F} \int_0^\infty \frac{\varepsilon_A(\nu) \cdot I_F(\nu)}{\nu^3} \, d\nu \tag{2.4}$$

where n is the refractive index of the medium, ϕ_F the fluorescence yield, τ_F the lifetime of the donor molecule without energy transfer, and ν the frequency. The part of the equation under the integral is the spectral overlap factor. Due to the inverse R^6 dependence of the energy-transfer rate constant, the Förster mechanism allows energy transfer over relatively large distances, well up to 100 Å and beyond, depending on the molecular transition dipoles involved. However, for the fast transfer required in photosynthetic antenna, where single-step transfer times should typically be shorter than 1 ps, the typical maximum allowed distances R for Chl pairs are up to 15–20 Å, depending somewhat on orientation.

The Förster mechanism assumes that both the donor and the acceptor molecules are in thermal equilibrium with the environment and that the interaction energy between the dipoles is very small compared with the energy of a typical molecular vibration. This means that the coupling does not influence the absorption spectra of the involved pigments in any appreciable fashion as compared with the uncoupled system. The mechanism allows electronic singlet-to-singlet, and also triplet-to-singlet, energy transfer. Transfer from a singlet to a triplet state is, however, not possible because the overlap integral is negligible (the ground-state-to-triplet absorption probability lying close to zero).

The kinetics of energy migration in the case of Förster transfer in the very weak coupling limit[2] are described by the master equation

$$\frac{d p_i}{d t} = k_i^{nr} p_i + \sum_{i \neq j; j=1}^{n} (p_j k_{ji} - p_i k_{ij}) - \delta_{i,RC} k_{RC} p_{RC} \tag{2.5}$$

where p_i is the probability of the excitation being located on pigment i, k^{nr} is the rate of loss processes other than energy transfer, k_{ij} is the Förster rate of energy hopping from pigment i to pigment j, k_{RC} is the rate of charge separation at the RC and p_{RC} the probability of the RC being excited. Using this equation and the known Förster rate constants, the overall energy-transfer dynamics in a complex antenna system can in principle be fully calculated. However, the problem with using eq. 2.5 is the uncertainties in the Förster rate constants, which depend critically on the often-

[2] This case is also referred to as the 'incoherent limit', as compared to the 'coherent limit', meaning exciton coupling.

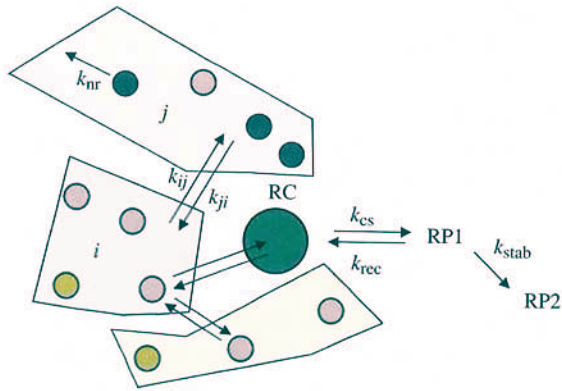


Figure 2.2 Compartment model of a photosynthetic antenna/RC complex (compare with Fig. 2.1). The individual pigments (circles) are now grouped into three different antenna compartments or pools, indicated by the coloured boxes surrounding the pigments. The reaction centre also forms a compartment of its own. The rate constants k_{ij} and k_{ji} now no longer denote the rate constants of energy transfer between different individual pigments, but between the different compartments i and j.

unknown spectral properties of the individual pigments and on the distances and relative orientations of the pigments.

Due to the very large number of antenna chromophores comprising a particular antenna system, it is often not practical or possible to take into account all the individual chromophores. Rather, groups of pigments or chromophores with similar spectroscopic and kinetic properties are lumped together in a pseudo-pigment complex, called a 'compartment' when interpreting antenna energy-transfer spectra and kinetics. Such a 'compartment model', representing the general photosynthetic unit shown in Fig. 2.1, is given in Fig. 2.2. The 11 chromophores present in the antenna are lumped together into three different antenna compartments, which would differ in their spectra and kinetics. Very often such a compartment can be identified with a particular biochemical subunit of the antenna, although this is not necessarily always the case.

2.2.2 *Coherent exciton motion*

At the opposite end of the scale of pigment interactions is the strong-coupling or so-called exciton coupling mechanism, which also occurs through the Coulomb interaction of pigments. In this case the coupling energy is sufficiently strong, typically greater than the vibrational quantum energy $h\nu$, that it influences the shape

of the absorption spectrum of the pigments involved, replacing the excited states of the individual pigments by a new set of excited states characteristic of the coupled system. Excitation energy is no longer located on a single pigment, but is delocalised over the ensemble of pigments involved in the excitonic coupling.

In the simplest case of an excitonically coupled dimer made up from a pair of identical molecules, the new states are obtained by solving the Schrödinger equation for the dimer

$$(H_1 + H_2 + V_{12})\Psi = E\Psi \tag{2.6}$$

where $H_{1,2}$ are the Hamilton operators for the two pigments, V_{12} is the coupling energy, E the excited-state energy of the coupled system and Ψ the excitonic wavefunction. Solution of eq. 2.6 gives the energies and wavefunctions of the excitonically coupled system. This leads to the two excitonic excited states energies

$$E_{1,2} = \varepsilon_1 \pm V_{1,2} + D \tag{2.7}$$

where D is the (ground-state) energy shift due to the change in environment for each pigment (going from the gas phase into the solution environment) and ε_1 is the excited-state transition energy of the uncoupled pigment. Thus excitonic coupling leads to two new excited states separated by an energy $2V_{12}$. The result is a modification of the absorption spectrum of the dimer as compared to the monomers. The dipole moments $d_{1,2}$ of the two new delocalised exciton states are oriented perpendicularly to each other and depend on the orientations of the transition moments of the monomers according to

$$d_{1,2} = d_0(1 \pm \cos\theta) \tag{2.8}$$

where d_0 is the dipole strength of the uncoupled monomer and θ the angle between the transition-dipole moments of the two monomers, thus conserving the total dipole strength of the combined monomers. Where more than two molecules are involved in the excitonic coupling, the relevant equations can be solved either analytically when symmetry prevails, or numerically in the general case (Pearlstein, 1982b and 1982c).

While the exciton coupling determines the shape of the absorption, linear dichroism and CD spectra (Pearlstein, 1982b and 1982c), the exciton states do not live for very long at room temperature since the phase relationship of the electronic wavefunctions between the two molecules making up the coupled dimer are disturbed by thermal motions. Typically, dephasing occurs within 1 ps or less. This dephasing leads to a localisation of the excited-state energy on one of the monomers. From then on, Förster-type hopping transfer of energy between the two molecules may occur.

However, during the lifetime of the excitonic states, the energy-transport dynamics are controlled by entirely different equations than for the Förster hopping mechanism, which is particularly important for larger assemblies of excitonically coupled systems. (For a modern in-depth treatment of the exciton concept in photosynthetic systems, see van Amerongen *et al.*, 2000). It turns out that the major part of the energy transfer processes in photosynthetic systems can be quite well described using the Förster mechanism, if appropriate adaptations are made for the calculation of coupling strengths, spectral properties *etc.* (Yang and Fleming, 2002; Konermann *et al.*, 1997). A rigorous excitonic description only seems necessary for larger assemblies such as the LHI or LHII complexes of purple photosynthetic bacteria, the FMO antenna complex (discussed in Section 2.4.7) and the supramolecular pigment aggregates in the chlorosomes of green sulphur bacteria (Prokhorenko *et al.*, 2000).

2.3 General principles of organisation of light-harvesting antennae

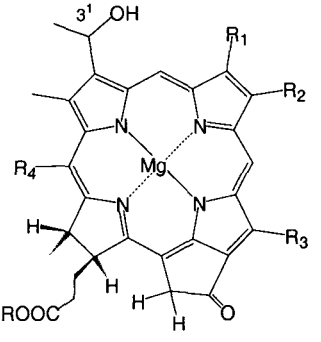
2.3.1 Chlorophylls and carotenoids

The most important functional parts of a photosynthetic antenna are the chromophores. All photosynthetic organisms use either chlorophylls (mainly Chl *a* and Chl *b*, but also a few other Chls) or bacteriochlorophylls (*e.g.* BChl *a* and BChl *b*) as pigments. The chromophore moiety of the Chls and BChls are magnesium-containing cyclical tetrapyrroles, the so-called chlorins and bacteriochlorins. Figure 1.4*a* shows the structures of the most common Chls and BChls. All Chls also contain a long ester side chain, which help in anchoring them in their surrounding protein by hydrophobic interactions. In addition all photosynthetic antennae contain carotenoids, which play a dual function as energy donors and photoprotective pigments (for an overview, see Smith, 1991).

Because the pigments in antenna systems must be held at a certain range of optimal distances and orientations to each other and to the RCs, all known antenna systems (with one notable exception—the chlorosome structures discussed below) are pigment–protein complexes. The proteins both hold the pigments in a defined position and provide the possibility of fine-tuning their absorption properties by specific pigment–protein interactions. All Chls and BChls, as well as the carotenoids, are attached non-covalently to the proteins by way of either weak ligand–metal interactions of the central Mg of the Chls to amino acid side chains (often histidine), or by other non-covalent interactions such as hydrogen bonding, $\pi\pi$–interactions or hydrophobic interactions with their long ester side chains (Smith, 1991).

A few families of photosynthetic organisms make use of additional chromophores and/or other interaction principles in their antennae. A notable, and indeed in all respects exotic, antenna system is employed by the green sulphur bacteria and green gliding bacteria in their chlorosomal antennae. As briefly explained in Chapter 1 and shown in Fig. 1.8, these are extramembranous antennae, located on the inner surface of the cytoplasma membrane, which do not contain proteins in their central part. Instead these chlorosomal antennae consist of huge (several tens to hundred thousands of Chls each) pigment aggregates formed by self-organisation of the special bacteriochlorophylls BChl *c*, *d*, *e*. As Fig. 2.3 shows, these special BChls of green bacteria are characterised by their 3^1-OH groups. These are not found in any of the usual Chls or BChls and they enable the molecules to interact closely with each other. Actually these 'bacteriochlorophylls'—as they are traditionally called—are chlorophylls, rather than true bacteriochlorophylls in terms of their electronic structures (Smith, 1991).

Another class of photosynthetic pigments is found in the phycobiliproteins of cyanobacteria, red algae and cryptomonads. These are open-chain tetrapyrroles which are covalently bound to their apoproteins, typically via S–cysteine linkages (Stanier, 1974; Wildman and Bowen, 1974; Gantt, 1975; Zuber, 1978; Scheer, 1981; Holzwarth, 1986; Holzwarth, 1991; Schaffner *et al.*, 1991; MacColl and Guard-Friar, 1987).



BChl *c*: R_4 = Me; R_1 = Me; R_2 = Et; R_3 = Me
BChl *d*: R_4 = H; R_1 = Me; R_2 = Me, Et, Bu; R_3 = Me, Et
BChl *e*: R_4 = H; R_1 = CHO; R_2 = Me, Et, Bu; R_3 = Me, Et
R = farnesyl and other C-16 and C-18 alcohols

Figure 2.3 Structure of the chlorosomal bacteriochlorophyll pigments BChl *c*, *d* and *e*, which differ in the nature of the side-chain substituents R_1 to R_4 and also in the nature of the esterifying alcohol R. Note the 3^1-OH group, which does not appear in any other Chls or BChls, and the lack of the 17^2 carboxyl group next to the carbonyl group.

2.4 Structural and functional basis for light absorption and harvesting

In plants, green algae, cyanobacteria and a few other oxygen-evolving photosynthetic organisms, the primary steps of photosynthesis occur in two membrane-bound protein supercomplexes, Photosystem I (PSI) and Photosystem II (PSII), introduced in Chapter 1. Recently the structures of several of the antenna systems of oxygenic photosynthetic organisms have been determined to various degrees, some to atomic resolution. The best characterised of these complexes is the PSI antenna/RC complex of the cyanobacterium *Synechococcus elongatus*. The structures of the PSII core/RC complex and most of the peripheral light-harvesting complexes have so far only been determined to significantly lower resolution.

2.4.1 Photosystem I

The PSI antenna/RC complex appears as a native trimeric unit in the membrane of cyanobacteria, in contrast to the monomeric PSI complex occurring in higher plant species. Despite substantial differences in the number of total polypeptides and differences in structural details, including the different macroorganisation of PSI in the different organisms, there probably exist many similarities in the structure of their core antenna, as judged on the basis of the sequence homology. No detailed structure is as yet available from a higher-plant PSI complex, although a 4.5 Å X-ray structure of LHI–PSI is in press at the time of writing; see also Kargul *et al.* (2003).

The PSI complex of *S. elongatus* consists of 12 protein subunits and contains 96 Chls (95 Chl *a* and one Chl *a*′[3], which is located in the 'special pair' of the RC), 22 carotenoids (mostly *β*-carotene), two phylloquinones and the [4Fe–4S] centres involved in electron transfer as electron acceptors.

Figure 2.4 (p. 83) shows the structure of Photosystem I (only one monomer of the trimeric structure is shown for clarity) to a resolution of 2.5 Å (Fromme and Witt, 1998; Fromme *et al.*, 2001; Jordan *et al.*, 2001) at near atomic resolution. These studies have for the first time revealed the orientations of all the chlorin ring systems, enabling more rigorous theoretical calculations of energy-transfer properties based on the distances and transition-dipole orientations of the Chls. A salient feature of the PSI complex is that a large number of the antenna pigments (89 Chls), as well as the 6 RC Chls, are bound to the same two polypeptides, namely the highly homologous *psa*A and *psa*B units forming the core of the structure. These two polypeptides are

[3] This differs from normal Chl *a* by its inverse stereochemistry at the 13^2-C position.

related by a pseudo-C_2 axis located at the centre of the PSI monomer. The organic cofactors of the electron-transfer chain of RCs are arranged in two branches along this pseudo-C_2 axis.

Figure 2.5 (p. 84) shows the arrangement of the pigments in one monomer of the Photosystem I trimer. Viewed from above (upper diagram), the antenna Chls form an elliptically distorted, cylindrical ring structure around the six central RC Chls. Except for the two 'linking Chls', which may possibly connect energetically to the antennae and the RC pigments, the distance between the nearest antenna pigments and the RC pigments is relatively large (>18 Å). The antenna Chls have centre-to-centre distances of 7–16 Å, with a maximum in the distance distribution around 10 Å, well within the range allowing ultrafast energy transfer. The side view of the pigment arrangement (lower diagram of Fig. 2.5) shows that in the greater part of the PSI antenna, except close to the RC, the Chls are arranged in two layers located near the two membrane surfaces, with large distances between the two. Thus energy transfer is expected to occur preferentially within the layers, while layer–layer transfer will occur only near the RC. Thus a large part of the PSI antenna is quasi-two-dimensional with respect to the arrangement of the Chls.

There are three regions in the antenna structure (highlighted in red in Fig. 2.5) where close stacking of two or three Chls occurs. These arrangements should lead to a substantial excitonic coupling, conferring special spectroscopic properties on these Chls. In all probability, these stacking regions contain the so-called special 'red Chls' that are present in all PSI complexes to various extents. This term denominates those groups of Chls that absorb beyond 700 nm, above the absorption maximum of the RC, which is located around 700 nm. These 'red Chls' are particularly prominent in cyanobacterial PSI and are believed to play a special limiting role in the overall energy-transfer process to the special pair.

Energy transfer in PSI core complexes of cyanobacteria and green algae has been extensively studied, both experimentally and theoretically, but no general agreement has so far been reached as to the rates of the various energy-transfer steps and rate-limiting processes. Most experimental studies have been performed on the core complexes of either cyanobacteria or green algae and higher plants, while relatively few detailed studies are available for intact higher-plant PSI complexes that also carry the light-harvesting I complexes (LHCI) of the outer antenna. These studies have been interpreted either qualitatively or in more detail in terms of so-called *compartment models*. In these simpler models, energy and electron-transfer processes between groups of pigments are analysed, rather than those between each pair of pigments in each complex.

Figure 2.6 summarises various compartment models for the energy-transfer processes in the PSI core. These models differ in the relative rates of energy transfer from the core antenna to the RC and the effective charge-separation rates. Figure 2.6*a* shows the so-called trap-limited scheme, where the energy transfer from the core antenna to the RC is much faster than the charge-separation lifetime. This model has been adopted by several groups (for reviews see Karapetyan *et al.*, 1999 and Melkozernov, 2001). A contrasting model is the so-called transfer-to-trap limited model, which is primarily based on data from cyanobacterial core complexes (for reviews, see Gobets and van Grondelle, 2001 and Gobets *et al.*, 2001). A common theme in these models, shown in Figs. 2.6*b–d*, is the overall transfer time from the core antenna pigments to the RC, which is the rate-limiting step, having a lifetime of ~20 ps. The models differ, however, in the charge-separation lifetimes within the RC, with variations from 1 ps to 10 ps. Individual energy-transfer steps between groups of pigments are very rapid, in the range of 100–200 fs, leading to a very fast sub-picosecond energy equilibration within the core antenna. A complication in the kinetics arises from the presence of the special 'red pigments' in PSI of cyanobacteria, which generally slow down energy transfer to the RC if they are located in the antenna.

Figure 2.7 gives the absorption spectra of some PSI and PSII preparations, showing the much larger red tail in the absorption of the PSI as compared with the PSII particles. The tail is particularly pronounced for *Spirulina platensis* PSI because of the extreme content of red pigments. The various cyanobacterial PSI complexes are believed to contain different numbers of red pigment molecules, ranging from about 2 in *Synechocystis* to an extreme of about 6–8 in *Spirulina platensis*. They also differ in their spectral signatures, ranging from 708 nm absorption (Chl708) in *Synechocystis*, through 718 nm in *Synechococcus elongatus*, to 735 nm in *Spirulina*. The latter seems to contain the longest-wavelength absorbing Chls of any PSI complex, giving rise to 760 nm fluorescence at low temperature (Karapetyan *et al.*, 1999). The red pigments are believed to exchange energy with the core antenna in about 5–10 ps (Gobets and van Grondelle, 2001). This relatively slow energy exchange slows down the overall energy-transfer rate to the RC and severely complicates the observed kinetics. The time constant of the overall energy-trapping process (as measured by the kinetics of formation of the charge-separated states) in cyanobacterial core complexes at room temperature ranges from about 23 ps for *Synechocystis*, a species with minimal red-pigment content, to about 35 ps in *S. elongatus* with an intermediate amount of red pigments, to a maximum of about 50 ps in *Spirulina platensis*. This slowing down in the overall trapping rate has been ascribed to the effects of the red pigments (Gobets *et al.*, 2001).

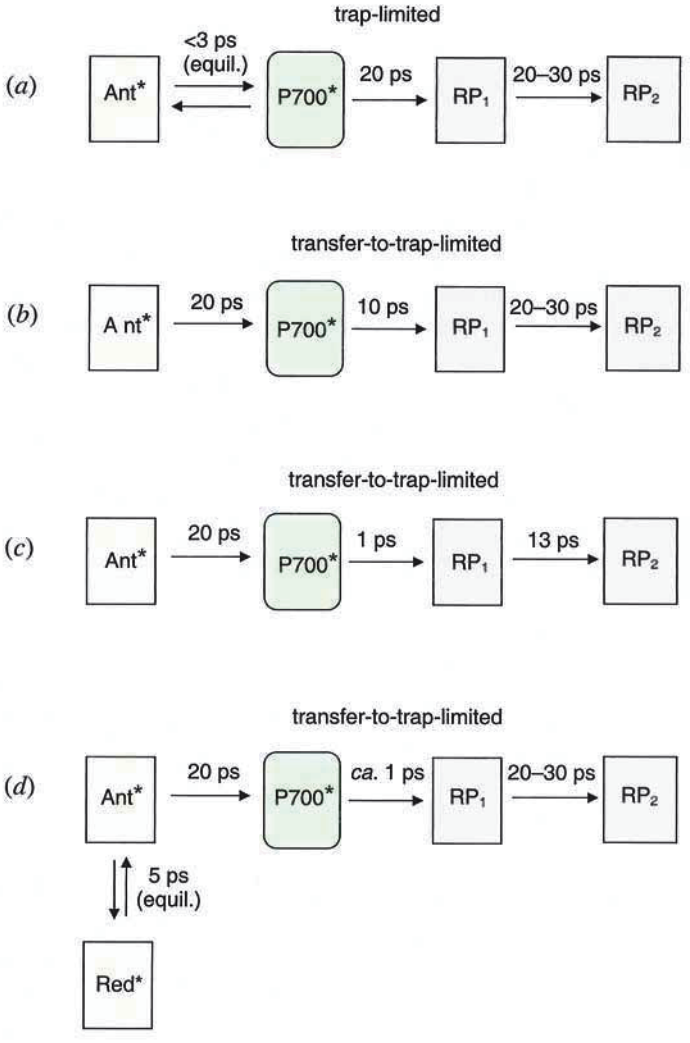


Figure 2.6 Selected compartment models of PSI energy and electron transfer processes applying to cyanobacterial and green algae core PSI complexes. Ant refers to the core antenna, P700 to the equilibrated reaction centre, and RP_1 and RP_2 refer to the first and second radical pair states. (a) trap-limited model, *(Melkozernov, 2001, and Müller et al., 2003)*; *(b) transfer-to-trap-limited model (Savikhin et al., 2000)*; (c) modified transfer-to-trap-limited model with faster charge separation (Savikhin *et al.*, 2001); (d) transfer-to-trap-limited model with additional red antenna pool; this model applies primarily to cyanobacterial PSI (Gobets and van Grondelle, 2001); Source: Müller *et al.* (2003).

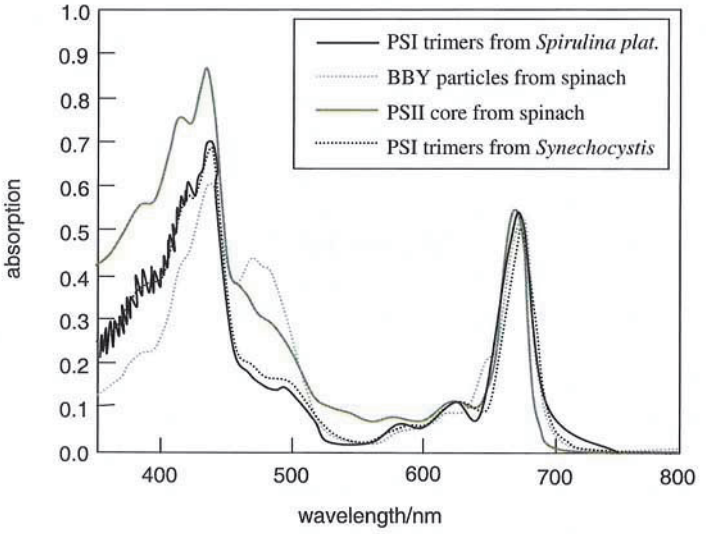


Figure 2.7 Absorption spectra of various PSI and PSII unit preparations.

A problem for all these models at present is that until recently no detailed spectra of the intermediate species involved in the kinetics have been resolved. Based on such data, Müller *et al.* (2003) have recently proposed the new model shown in Fig. 2.8. This does not so far take into account the effects of the very long-wavelength red pigments, but is limited to PSI particles with low red pigment content, such as the green algae *Chlamydomonas reinhardtii* and the cyanobacterium *Synechocystis* (Müller *et al.*, 2003). It is essentially a trap-limited model where energy equilibration within the core is subpicosecond, the transfer between the core antenna and the RC is very rapid (at most a few ps), and the effective charge-separation step is still fast (about 6–9 ps) but significantly slower than the energy equilibration between the core antenna and RC. Energy equilibration within the RC itself is also ultrafast, typically about 200 fs. In this model, the intrinsic charge-separation step in the RC is estimated to occur on a sub-picosecond time scale. This model is the first time to provide consistent spectra of the various intermediates, along with a description of the dynamics of the core antenna processes of PSI. Eventually it will have to be extended to include the effects of the more extreme red pigments.

These data imply that PSI is the fastest RC known, featuring an intrinsic initial charge-separation step taking about 0.5–0.8 ps, which is a factor of five faster than bacterial RCs, with their charge-separation time of about 3 ps. Despite disagreements in the literature about the relative rates of antenna/RC equilibration and charge

separation, there is general agreement that energy equilibration within the PSI core, barring the slower transfer to/from the small number of red pigments, is very fast, typically in the time range of 200–600 fs (Melkozernov *et al.*, 2000; Kennis *et al.*, 2001; Gibasiewicz *et al.*, 2001; Müller *et al.*, 2003). Such fast equilibration is also in agreement with the detailed structure-based modelling studies mentioned above.

We have already noted that *S. elongatus* contains several close-lying groups of Chls that could be assigned to the special red Chl pigments. This is borne out by data showing that these red pigments derive their bathochromic shift from excitonic coupling rather than a special protein environment. Several groups have tried to model the spectral and kinetic properties of the *S. elongatus* PSI in detail, based on crystallographic information (Beddard, 1998; Byrdin *et al.*, 2002; Sener *et al.*, 2002; Damjanovic *et al.*, 2002). However, even a very precise high-resolution structure does not provide the exact spectroscopic properties of a specific Chl in a protein complex. This energetic position is determined by the detailed interaction of the pigments with the environment, which can be obtained only by a full quantum-mechanical calculation based on an exact structure. PSI is particularly variable in this respect: the core antenna contains only Chl *a*, but the absorption maxima of the various Chls range from about 640 nm up to 735 nm, indicating a wide range of pigment environments. Much of this range is due to pigment–protein interactions, but pigment–pigment interactions such as charge-transfer and excitonic interaction also have an effect. Thus theoretical descriptions usually treat the spectral properties of the individual pigments as a fitting parameter ((Byrdin *et al.*, 2002; Sener *et al.*, 2002).

One attempt has been made (for PSI) to calculate the energetic locations of the pigments quantum-mechanically, taking into account the interaction of each specific Chl with its environment (Damjanovic *et al.*, 2002). At present, the conclusions of these studies, based on the information available for the PSI complex of *S. elongatus*, vary substantially. More work is needed to arrive at final conclusions about the specific location of the red pigments as well as many other details of the spectral and kinetic properties of PSI of *S. elongatus*. However, irrespective of the outcome of such calculations, it can already be stated that the exact details of the pigment arrangement in the antenna and the distribution of spectral forms across the antenna do not have any decisive influence on the overall kinetics, because of the quasi-statistical averaging of pigment properties that occurs in such a large antenna array. Thus the functioning of the PSI antenna system seems to be fairly robust against even relatively drastic changes in spectral distribution and other properties. By contrast, the particular spectral and kinetic properties of the reaction centre, as well as its electron-transfer rate, seem to be much more decisive for the overall trapping kinetics and the total yield of charge separation. This is not surprising since these parameters directly

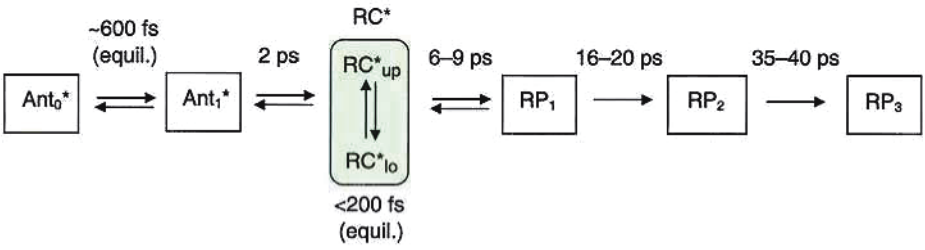


Figure 2.8 Improved compartment model for PSI based on measurements of the energy transfer and electron-transfer processes in the PSI core particles of *Chlamydomonas reinhardtii*, showing two antenna pools (Ant_0 and Ant_1), which equilibrate energy rapidly (within about 2 ps) with the RC. The overall trapping by charge separation occurs in 6–9 ps. Also depicted is the ultrafast energy equilibration between exciton states in the RC. RC^*_{lo} and RC^*_{up} are the lower and upper exciton states of the reaction centre, respectively. Source: Müller *et al.* (2003).

influence the key steps in the energy equilibration between antenna and RC and the overall energy flow toward the RC. This can be understood in more detail from the scheme in Fig. 2.8. Photosynthetic organisms have probably used these parameters to fine-tune PSI function in different organisms.

The PSI core antenna of higher plants and green algae binds various amounts of LHCI light-harvesting complexes, whose structures may be similar to those of LHCII (discussed below) but are not known in any structural detail. These peripheral antenna complexes in higher plants also contain red pigments (Melkozernov, 2001). The average energy of the Chls in the LHCI complexes is above 700 nm, *i.e.* above the RC absorption maximum. The overall energy equilibration within the PSI antenna systems of higher plants seems however to be very rapid, with lifetimes from a few ps to about 12 ps, depending on the amount and type of peripheral antenna complexes present. The overall trapping times in the intact complex range from ~50 ps to ~100 ps at room temperature: see Melkozernov (2001) for a detailed review. Much longer lifetimes may be observed at low temperatures because of trapping on long-wavelength pigments located far from the RCs. Thus the overall trapping times in PSI of higher plants are generally slower than in cyanobacterial PSI by a factor of 2–5. However, the overall trapping times are still fast, allowing a high yield of >90% for charge separation. The slower trapping in higher-plant PSI is mainly a consequence of the larger number of pigment molecules per RC but partly due to the distant location of red pigments in the peripheral antenna. Thus in the PSI complexes of higher plants with associated peripheral light-harvesting complexes, there may be a mixed situation, with trap-limited kinetics in the core, but diffusion-limited kinetics in at least part of the peripheral LHC complexes (Jennings *et al.*, 1998; Croce *et al.*, 2000).

The contribution of red pigments to the kinetics of the cyanobacterial core complexes could also perhaps be described using such a mixed model.

Some structural details of the arrangement of the peripheral antenna complexes in PSI of higher plants and green algae have recently been obtained by electron microscopy (Boekema *et al.*, 2001b; Germano *et al.*, 2002; Kargul *et al.*, 2003). It was found that the LHCI complex binds on one side of the PSI core only. There are, however, substantial differences in the size and arrangement of the outer antenna complexes between different organisms. Figure 2.9 (p. 84) shows the arrangements of the various PSI complexes in the green algae *Chlamydomonas reinhardtii*, spinach and the cyanobacterial trimeric PSI core. In contrast to PSII (discussed below), there does not seem to be high symmetry in the antenna PSI complexes of green algae and higher plants.

As regards the carotenoids, about 60 of the antenna chlorin heads in the PSI structure of *Synechococcus* are in van der Waals contact with the 22 carotenoids (Jordan *et al.*, 2001), which should provide excellent conditions for light harvesting through the carotenoids as well as photoprotection of the antenna by Chl triplet quenching. Little experimental information is available at present on carotenoid-to-Chl energy transfer in the core antenna of PSI as compared with PSII. However, one can conclude from simple fluorescence excitation spectra that the carotenoids contribute significantly (typically above 50% yield, but there are exceptions: see below) to the light-harvesting function in the wavelength range 450–650 nm where the Chls do not absorb well.

A very interesting case of regulation involving a major restructuring of the PSI antenna has recently been found in cyanobacteria (Bibby *et al.*, 2001a; Boekema *et al.*, 2001a) and *Prochlorococcus* (Bibby *et al.*, 2001b), the most abundant photosynthetic organism in the oceans. Iron deficiency, which is often the limiting factor for growth of photosynthetic organisms in aquatic ecosystems, leads to the induction of additional proteins around the PSI core such as IsiA in cyanobacteria and a similar protein in *Prochlorococcus*. IsiA has been implicated in chlorophyll storage, energy absorption and protection against excessive light. However, it has now been shown that a PSI–IsiA supercomplex is abundant under conditions of iron limitation. Electron microscopy has revealed that this supercomplex consists of trimeric PSI surrounded by a giant closed ring of 18 IsiA proteins binding about 180 chlorophyll molecules (Bibby *et al.*, 2001a and 2001b; Boekema *et al.*, 2001a; Nield *et al.*, 2003). Figure 2.10 (p. 85) shows the structure of this giant ring.

Energy transfer within this supercomplex has been recently studied by time-resolved absorption and emission spectroscopy (Melkozernov *et al.*, 2003), showing that the ring is energetically tightly coupled to the core, and energy equilibration

within the ring occurs on a sub-picosecond time scale. One should realise that this time constant probably does not reflect energy transfer through the whole giant ring, but simply energy equilibration between directly neighbouring subunits. Energy transfer along the outer ring cannot be resolved by time-resolved spectroscopy since it occurs among spectrally identical subunits. Energy transfer from the outer ring to the nearest Chl molecules in the central core occurs with a time constant of ~1.7 ps, while overall energy transfer from the ring to the core takes ~10 ps.

2.4.2 *Photosystem II core antenna complex*

In oxygenic photosynthesis, Photosystem II is the antenna/RC supercomplex that carries out light-induced charge separation across the membrane by doubly reducing plastoquinone on the acceptor side, and the water-splitting reactions in the oxygen-evolving complex attached to the donor side (Sauer, 1979; Barber and Santini *et al.*, 1994; Kühlbrandt, 1999). The intermediate-resolution X-ray structures shown in Fig. 2.11 (p. 86) at 3.8 Å resolution have recently been reported for the core PSII complex of the cyanobacterium *S. elongatus*; this occurs in its native form as a homodimer in the membrane (Zouni *et al.*, 2001; Kamiya and Shen, 2003).

The monomeric unit of the PSII core complex is made up of at least 21 protein subunits, 18 of them located within the photosynthetic membrane. In contrast to the PSI supercomplex, the RC pigments and the antenna pigments of the PSII core complex are bound to different protein subunits. The RC pigments, 4 Chls and 2 pheophytins are located on the D1 (*psb*A) and D2 (*psb*D) subunits (Fig. 2.12, p. 87). These RC subunits are highly homologous to the L and M subunits of the purple bacterial RC complex and to the cofactor positioning.

The subunits carrying the antenna pigments are CP43 (PsbC) and CP47 (PsbB), which are arranged around a local pseudo-C2 axis in the monomer. Each has 6 transmembrane helices arranged as a pseudo-trimer. This structure is very similar to the six amino-terminal transmembrane-helices of PsaA and PsaB, the large core subunits in the PSI RC, suggesting that the two types of reaction centre had a common ancestor.

According to the X-ray structures, CP43 and CP47 bind 12 and 14 Chl *a* molecules respectively. As in the PSI RC complex, the Chl pigments are arranged in two layers close to the two membrane surfaces, most of them presumably being bound via histidine ligands to the protein (Fig. 2.13, p. 88). The range of centre-to-centre distances for the Chls is 8.5–13 Å, within the range expected for rapid energy transfer. Compared with the purple bacterial RC, the D1 and D2 subunits each carry

one additional Chl, Chl z_{D1} and Chl z_{D2}, respectively. These Chls, despite being bound to the RC subunits, are relatively far distant (about 30 Å) from the RC pigments. They may act partly as linker Chls in energy transfer to the core RC. The nearest antenna Chls of the CP43 and CP47 subunits are somewhat closer, at a distance of 20 Å, but this is still a relatively large distance for energy transfer to the RC.

The energy-transfer steps have been studied in isolated CP43 and CP47 complexes, and overall energy trapping in the intact core complex. In the isolated complexes, energy equilibration is very fast, mostly in the time range of 0.2–0.4 ps, with some slower 2–3 ps contributions (de Weerd *et al.*, 2002a and 2002b). The fast transfer steps, which have mainly been assigned to equilibration in the Chl layer near the stromal side of the membrane, imply average single-step transfer times of ~100 fs between pairs of Chls. Such fast rates are expected from the relatively close packing of the Chls, and are in reasonable agreement with theoretical calculations (de Weerd *et al.*, 2002b). The slower transfer step has been assigned to transfer from the lumenal to the stromal layer of Chls, and to the transfer to the lowest-energy excitonic state in each system at low temperature. This final transfer step may however be substantially faster at room temperature. De Weerd *et al.* (2002b) have concluded that the rates of energy transfer within the isolated CP43 and CP47 complexes are fast enough not to limit the overall trapping of excitation energy in Photosystem II.

The initial trapping of energy by charge separation in the RC of PSII core complexes occurs in about 40 ps in cyanobacterial complexes of *S. elongatus* (Schatz *et al.*, 1987 and 1988) and increases up to ~200–250 ps for the large PSII antenna/RC particles of higher plants (Holzwarth and Roelofs, 1992). From a comparison of the trapping lifetimes and rates with open and closed RCs, it had been concluded before the X-ray structure determination that kinetics in the PSII core are mostly trap-limited (Schatz *et al.*, 1987 and 1988). Since the antenna and the RC are essentially isoenergetic at room temperature, the 40 ps lifetime and the 32 pigments per RC would, according to the simple picture of trapping presented in Section 2.1.2, imply a primary charge-separation rate of about $(1.3–1.5\ \text{ps})^{-1}$ within a trap-limited model.

However, recent modelling based on the structural data has led to widely deviating conclusions (Vasiliev *et al.*, 2001 and 2002). According to these workers, the fastest transfer steps from the antenna Chls into the RC are slower than 5 ps because of the large antenna-to-RC distances, which may be necessary for avoiding oxidation of the antenna Chls by oxidised P680. They thus advocated a transfer-to-trap-limited model for energy transfer in the PSII core complex, arguing that electron transfer in the RC is much faster than transfer from the antenna to the RC.

Many assumptions about unknown spectral properties are involved in such modelling, and as yet it is not clear whether the transfer-to-trap-limited model is

correct since there are several unexplained contradictions with other data. For example, it is well known that the fluorescence yield and lifetime of PSII critically depend on whether the RC is open or closed (see, for example, Holzwarth and Roelofs, 1992). This cannot easily be explained within a transfer-to-trap-limited model, but it is easily understood within an essentially trap-limited model (Holzwarth and Roelofs, 1992): if one assumes at the extreme a situation where energy equilibration between antenna and RC occurs at about the same rate as charge separation in the RC, one would still have an essentially trap-limited model which would at the same time be consistent with the RC redox-state sensitivity of the fluorescence lifetime and the antenna size dependence of the PSII lifetime. We thus believe that the models of Vassiliev *et al.* (2002) and Dekker and van Grondelle (2000) are too extreme, and further work is necessary finally to clarify the transfer steps in the PSII core.

A recent detailed study of the dependence of trapping kinetics on antenna size in various PSII particles also came to the conclusion that indeed there is a shallow equilibrium established in PSII prior to charge separation (Barter *et al.*, 2001), consistent with the early model and conclusions of Schatz *et al.* (1988) and Holzwarth and Roelofs (1992). Barter *et al.* (2001) concluded that a shallow equilibrium between the antenna and reaction centre in Photosystem II would facilitate regulation via, for example, non-photochemical quenching, and went on to propose that Photosystem II is optimised for regulation rather than for efficiency. However, the efficiency of PSII in the absence of quenching is very high (usually better than 92%), which implies that regulation capabilities and high efficiency are not in any way mutually exclusive.

In conclusion, at present it seems likely that Photosystem II kinetics are essentially trap-limited rather than transfer-to-trap-limited.

2.4.3 Peripheral LHCII complex of PSII and minor light-harvesting complexes

The Photosystem II core antenna/reaction centre complex of higher plants and green algae is very similar to that of cyanobacteria, described above (Barber, 2003). However, higher plants and green algae employ additional peripheral light-harvesting complexes to form a variety of supramolecular antenna structures of various sizes and compositions. The major components of these peripheral complexes are the light-harvesting complex LHCII and the highly homologous minor complexes CP24, CP26 and CP29. As yet, only the structure of LHCII has been determined to an intermediate resolution of 3.4 Å using cryo-electron microscopy on two-dimensional crystals (Kühlbrandt, 1984 and 1994; Kühlbrandt *et al.*, 1994). Unfortunately, this resolution

did not permit unequivocal assignment of the positions and orientations of the pigments, but complementary information about the structure has recently been obtained using other methods.

The LHCII complex, which occurs as a native trimer, consists of a single polypeptide that forms three membrane-spanning α-helices. It binds 7 Chl *a* and 5 Chl *b* pigments and contains 3 carotenoid tight-binding sites. Two of the carotenoid sites, located in the centre of the structure, contain luteins arranged in a crossed structure. These two luteins are essential for the stability of the complex. An additional carotenoid site, preferentially containing neoxanthin but able also to accommodate other carotenoids, lies in a peripheral position.

The Chl pigments are arranged in two layers near the membrane surface, as in the core complexes. The LHCII complex is one of the most abundant proteins on Earth, binding about 50% of all the chlorophyll in higher plants. The Chl–Chl distances range from 8–11 Å, again in an expected optimal range for efficient, rapid energy transfer. Seven of the chlorin head groups in the centre of the structure are at essentially van der Waals distance from the carotenoids, enabling rapid energy transfer from carotenoids to Chls. At the same time, this arrangement is favourable to the photoprotection function of the carotenoids, allowing for Chl triplet quenching.

Unfortunately the structural resolution of Kühlbrandt's studies did not allow determination of the phytyl ester side chains of the Chls and thus did not provide the exact orientation of the Chls, nor could Chl *a* and Chl *b* be distinguished. More recent biochemical work, combining point mutations, overexpression of the protein in *E. coli*, and *in-vitro* pigment reconstitution of the whole complex from a mixture of the overexpressed denatured protein has provided a large variety of artificial complexes with modified pigment composition (Paulsen and Hobe, 1992; Paulsen *et al.*, 1993; Giuffra *et al.*, 1995 and 1997; Bassi *et al.*, 1999). These, in combination with polarisation spectroscopy, have yielded a fairly detailed picture of both the Chl assignments and their orientations in the complex. Thus, despite some remaining uncertainties, it has been possible to obtain highly valuable information beyond the electron microscopic structure. This is a prime example of how molecular biology, structural methods and spectroscopy can be used together to solve problems that could not have been solved previously by any of these disciplines alone.

Besides providing all the structural details, this work has further shown, quite surprisingly, that several of the Chl binding sites in LHCII are not specific: they can bind either Chl *a* or Chl *b*, which substantially complicates the assignment and later functional analysis. Using the original site notation of Kühlbrandt and Wang (1991), the non-selective sites are a3/b3 and b5/b6, irrespective of whether Chl *a* or Chl *b* is actually bound to a particular position (Giuffra *et al.*, 1997; Sandona *et al.*, 1998;

Bassi *et al.*, 1999). The same sites, namely a3/b3 and a6/a7/b6, are also involved in forming some closely-spaced pigment clusters which are prime candidates for exciton coupling and may give rise to special optical properties of these Chls.

The minor LHCII complexes CP24, CP26 and CP29 are highly homologous with the major LHCII complex (Bassi *et al.*, 1987, 1993 and 1997). They bind fewer Chls and only two carotenoids in the two central sites. All structural details and the pigment composition of these complexes have been obtained by a combination of biochemical, molecular genetics and spectroscopic work. The modelling has been based on homology with the major LHCII complex. Clearly the ability to reconstitute these complexes *in vitro* to form the polypeptides and pigment mixtures has been crucial for this work. The reconstituted complexes do seem to have basically the same spectroscopic properties as the native ones.

Native CP29 binds 6 Chl *a*, 2 Chl *b* and two carotenoids, as indicated in Fig. 2.14 (p. 89, righthand side) (Giuffra *et al.*, 1996). Again, four of the Chl binding sites, the equivalent ones to those in LHCII, are non-specific in their binding, as indicated by the colouring of these Chls in Fig. 2.14. The carotenoid site L1 binds lutein, as in LHCII, but the site L2 binds either violaxanthin or neoxanthin with equal probability. However, the protein is relatively stable when only the L1 site is occupied by a lutein, a situation that can be obtained when reconstituting without neoxanthin or violaxanthin in the mixture. The two other minor complexes, CP24 and CP26, are highly homologous and bind pigments with a similar composition to CP29.

Numerous studies of the energy-transfer dynamics in the isolated LHCII and the minor complexes have been performed both at room temperature and at low temperatures (Connelly *et al.*, 1997b; Cinque *et al.*, 2000; van Amerongen and van Grondelle, 2001; Novoderezhkin *et al.*, 2003; Croce *et al.*, 2003a). As would be expected from the rather irregular arrangement of Chls, the transfer times between pairs of Chls are dispersive. At low temperatures, Chl *b* to Chl *a* transfer in the major LHCII complex occurs with roughly three lifetimes, of about 300 fs (40%), 600 fs (40%) and 4–9 ps (20%). At room temperature, the energy-transfer lifetimes are all somewhat faster: 175 fs, 625 fs and 5 ps, and there is a tendency for higher contributions to the overall yield of energy transfer by the faster components. The largest exciton coupling strengths between Chl *a*/*b* pairs in the LHCII complex are about 120 cm^{-1}, which is smaller than their energetic separation of up to 450 cm^{-1}. Thus the contribution of excitonic coupling to the spectral and dynamic properties of Chl *a*/*b* pairs is limited, and the pigments behave as monomers for most transfer steps. It is therefore likely that energy transfer occurs by the Förster mechanism. Cinque *et al.* (2000) also found this from their detailed calculations on the CP29 complex.

In-depth studies of Chl *a/a* transfer have been performed by Visser *et al.* (1996) at low temperature, since the transfer steps cannot be well resolved at room temperature due to strong spectral overlap. According to these studies, Chl *a/a* transfer is also highly heterogeneous, occurring with lifetimes of ~400 fs, 2 ps and 15 ps, reflecting the large variation in distances and orientations of the pigments. At room temperature, these transfer lifetimes should be shorter, owing to better spectral overlap in the Förster mechanism and to the contribution of back-transfer steps. In conclusion, energy equilibration within a trimeric LHCII complex at room temperature should be mostly complete within a few picoseconds, with some minor contribution of a component of lifetime up to ~10 ps. These lifetimes are in reasonable agreement with expectations from Förster transfer calculations (Visser *et al.*, 1996; Cinque *et al.*, 2000).

A problem for an exact calculation of the energy-transfer processes in LHCII is the site heterogeneity, in addition to the incompletely solved problem of pigment assignment and orientation. Novoderezhkin *et al.* (2003) have recently performed a rather involved exciton level calculation using Redfield theory, varying all these structural factors. A large body of experimental data, including femtosecond transient absorption and kinetics and linear dichroism spectra, could be explained fairly well although some significant deviations between the experimental and calculated spectra remained. In essence, the calculations confirmed the lifetimes of energy transfer between groups of pigments as discussed above, and are consistent with the assumption that, for the most part, the kinetics can be explained by non-excitonic hopping processes.

A very detailed model of the energy-transfer steps in CP29, including the transfers from the carotenoids to the Chls, has recently been worked out by Croce *et al.* (2003a and 2003b). In this model, shown in Fig. 2.15, the rates of individual transfer steps have been assigned to specific carotenoid/Chl and Chl/Chl pairs. This was possible because of the relative simplicity of the CP29 complex as compared with the LHCII complex, and also because several reconstituted mutant complexes were available for study, which allowed the contributions by specific Chls to be assigned. As far as the Chl/Chl transfers are concerned, the lifetimes are in a similar range as in LHCII (ranging from ~150 fs to a few ps), as was expected from the high similarity of the two complexes. However, a much clearer picture of single-step transfer processes between pigment pairs could be obtained in this case. In summary, the internal transfer steps in the peripheral LHCII antenna complexes, including the minor ones, are not in any way rate-limiting for overall energy transfer in the PSII reaction centres of higher plants.

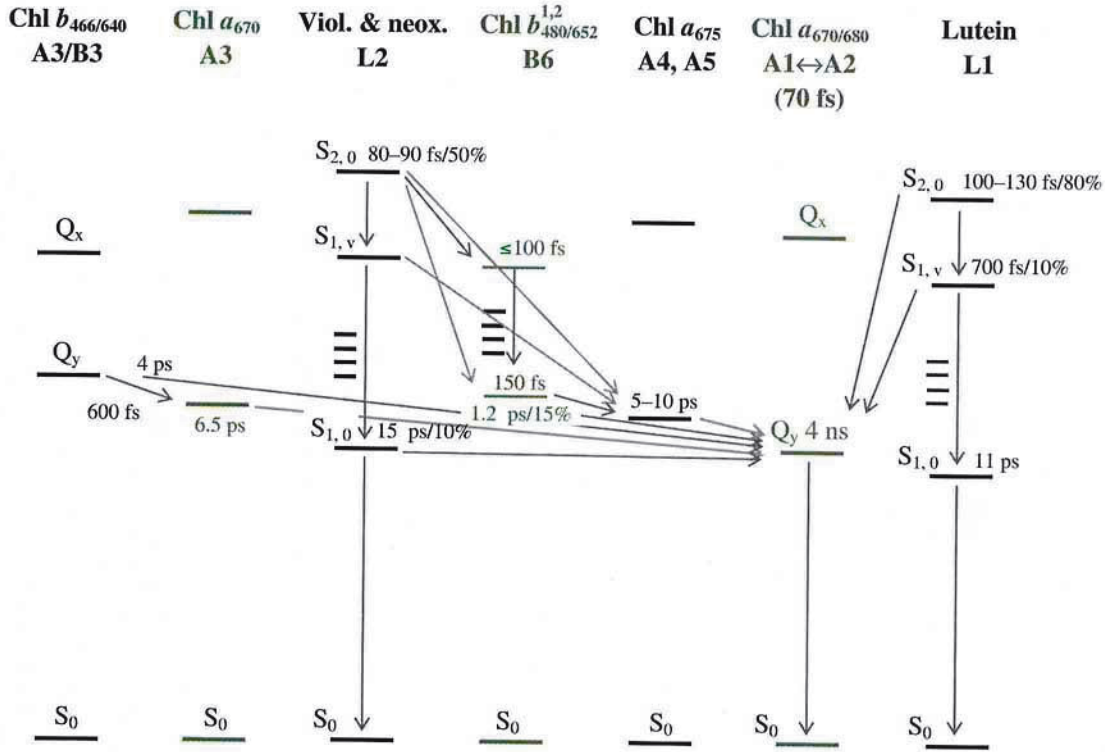


Figure 2.15 Schematic presentation of the major energy-transfer pathways in the CP29 complex, including the carotenoid-to-Chl transfers. The spectral assignment of the pigments involved is given at the top. In the second row are shown the corresponding binding sites of the Chls in the complex, using the nomenclature for the LHCII complex (see Fig. 2.14). The arrows show the major energy-transfer pathways and the numbers assigned to the horizontal lines, which represent the various excited states, give the lifetimes of each state and the overall yield (in %) that each contributes to the energy transfer. Source: modified from Croce *et al.* (2003b).

2.4.4 *The role of carotenoids in PSII*

Both the core complex and the peripheral antenna complexes of PSII, like all other antenna systems, contain a substantial amount of carotenoids. These pigments play multiple roles. The first is to enhance the light-harvesting function by absorbing in wavelength ranges where chlorophylls do not absorb and transferring their energy to them. While the transfer efficiency of carotenoids varies greatly, in some cases this ranges up to 85%. The second role of carotenoids in Photosystem II is a protective function against the detrimental effects of Chl triplet states in the system, which are likely to develop in all photosynthetic antenna, as well as in reaction centres. Carotenoids are able to quench the triplets directly as well as deactivating the resulting aggressive oxygen species, such as singlet oxygen, which may be created via the Chl triplets (Groot *et al.*, 1995; Peterman *et al.*, 1997). The latter function is particularly important in PSII reaction centres since those are particularly prone to damage by oxygen species. Scheme 2.9 below shows the multiple functions performed by carotenoids in photosynthetic units.

Light harvesting

$$^1\text{Car} + h\nu \rightarrow {}^1\text{Car}^*$$
$$^1\text{Car}^* + {}^1\text{Chl} \rightarrow {}^1\text{Car} + {}^1\text{Chl}^*$$

Chl triplet and singlet oxygen formation

$$^1\text{Chl} + h\nu \rightarrow {}^1\text{Chl}^*$$
$$^1\text{Chl}^* \rightarrow {}^3\text{Chl}^*$$
$$^3\text{Chl}^* + {}^3\text{O}_2 \rightarrow {}^1\text{Chl} + {}^1\text{O}_2^*$$

Chlorophyll triplet quenching

$$^3\text{Chl}^* + {}^1\text{Car} \rightarrow {}^1\text{Chl} + {}^3\text{Car}^*$$
$$^3\text{Car}^* \rightarrow {}^3\text{Car} + \text{heat}$$

Singlet oxygen scavenging

$$^1\text{O}_2^* + {}^1\text{Car} \rightarrow {}^3\text{O}_2 + {}^3\text{Car}^*$$
$$^3\text{Car}^* \rightarrow {}^1\text{Car} + \text{heat} \qquad (2.9)$$

The lowest excited singlet state in carotenoids is a forbidden state that is barely visible in the absorption spectra (Christensen, 1999). The strongly blue-absorbing absorption characteristic of the carotenoids comes from excitation to the S_2 state. It is

this state that is involved in light harvesting. Numerous studies have shown that, despite the very short lifetime (only about 100–200 fs) of the S_2 state, it contributes a major part to the energy-transfer yield to the Chls in many antenna complexes. The large transition-dipole moment of the S_2 state favours a Förster energy-transfer mechanism. However, in parallel with direct energy transfer to the Chls from the carotenoid S_2 state, there occurs very rapid internal conversion to the carotenoid S_1 state. Since the S_1 state has a much longer lifetime, in the 10–20 ps range, it can also transfer its energy to nearby Chls. For a long time, it was believed that the mechanism for energy transfer from the S_1 state could not be Förster transfer because of the very unfavourable overlap factor arising from the low transition moment of the S_1 carotenoid state. It was assumed that energy transfer should instead occur by the so-called Dexter exchange mechanism, which involves electron exchange between the donor and acceptor molecules and thus would demand van der Waals contact to allow overlap of the electron orbital clouds (Naqvi, 1980).

However, more recent experiments and calculations on a variety of antenna complexes have shown that the Dexter mechanism is probably not involved in energy transfer from carotenoids to Chls or BChls. The problem is located in the extended spatial dimension of the carotenoids compared with the rather short distances to the neighbouring Chls. This means that the transition dipole of the carotenoid cannot be treated as a point dipole, as is usual in Förster transfer calculations. Instead, the Coulomb couplings must be calculated in small-volume elements and summed, as in the so-called transition density cube (TDC) method (Damjanovic *et al.*, 1999; Hsu *et al.*, 2001; Scholes *et al.*, 2001). Such calculations indicate that carotenoid S_1 states can indeed transfer energy efficiently to nearby Chls by way of a Coulomb (Förster transfer) mechanism. Numerous experimental data that support this idea are now available. By having two pathways of energy transfer from the carotenoid to the Chls, via S_2 and via S_1 the overall efficiency of transfer can be improved.

In higher-plant LHC complexes, the predominant pathway is usually the S_2 pathway, but significant amounts of energy are also transferred via the S_1 pathway, sometimes up to 20–30% of the total depending on the specific carotenoid site and species (Connelly *et al.*, 1997a; Croce *et al.*, 2001 and 2003b; Gradinaru *et al.*, 2000). In addition, there is evidence from both higher-plant LHC complexes (Croce *et al.*, 2003b; Walla *et al.*, 2002 and 2000b) and bacterial antenna complexes (Krueger *et al.*, 1999; Walla *et al.*, 2000a; Hsu *et al.*, 2001; Billsten *et al.*, 2002; Polivka *et al.*, 2002) that energy transfer from carotenoids to Chls also occurs from vibrationally hot S_1 states. In fact, the yields of transfer from the hot states are usually higher than from the vibrationally relaxed state. In many cases the yield of the latter is close to zero (see, for example, the case of CP29 in Fig. 2.15.). This phenomenon is not completely

understood at present, but it may be related to the better spectral overlap of the vibrational hot states with the energy-accepting states in the Chls. The overall yield of carotenoid-to-Chl transfer in the family of LHCII complexes ranges from 30% up to 80%, depending on the specific carotenoid and pigment complex.

In addition to the two functions described above, carotenoids in at least some of the antenna complexes of PSII perform a third function, which involves the xanthophylls violaxanthin, antheraxanthin and zeaxanthin, whose structures and spectra are shown in Fig. 2.16. Together these participate in the xanthophyll cycle, which brings about so-called qE-quenching as a protective mechanism against the high light intensities that are potentially damaging to PSII (Havaux and Niyogi, 1999; Bassi and Caffarri, 2000). In this mechanism, antheraxanthin, but more efficiently zeaxanthin, are formed from violaxanthin in response to over-reduction of the plastoquinone pool. As a consequence, the two related carotenoids are able to quench the Chl excited singlet states of the antenna (Frank *et al.*, 2000; Josue and Frank, 2002). This function is believed to be mainly located in the minor antenna complexes of PSII, but may also occur in the major LHCII complex. Zeaxanthin has now been shown (Frank *et al.*, 2000; Josue and Frank, 2002) to have a lower S_1 energy than the Chls, contradicting the earlier work of Polivka *et al.* (1999). This enables downhill energy quenching of excited Chl states.

The exact mechanism and location of zeaxanthin quenching in PSII is still a matter of debate. The only case where the quenching effect of zeaxanthin has so far been demonstrated is in a reconstituted CP29 complex containing zeaxanthin (Crimi *et al.*, 2001). The quenching rate in this complex is relatively slow, in the nanosecond range. Nevertheless, this rate should be quite sufficient to explain the quenching yields observed *in vivo*, assuming the quenching occurs in parallel at several sites in the antenna complex. Recent data show that the presence of a special small Chl binding protein, PsbS, may be a requirement for the non-photochemical quenching in the qE-process *in vivo* (Li *et al.*, 2000), but this observation still does not provide a mechanistic explanation for the quenching process.

The PSII core complex contains β-carotene as the only carotenoid, as does the PSII RC. It has been shown that the quantum yield of energy transfer from β-carotene to Chl in the core complex is very poor, only about 35%. Energy transfer occurs almost exclusively via the carotenoid S_2 state, with some small contribution from vibrationally hot S_1 states, while the vibrationally relaxed S_1 state does not contribute to the energy transfer at all (de Weerd *et al.*, 2003). Transfer from the S_2 state occurs with a lifetime of 80 fs, while the hot S_1 state transfers some energy with lifetimes in the range 0.4–1.0 ps.

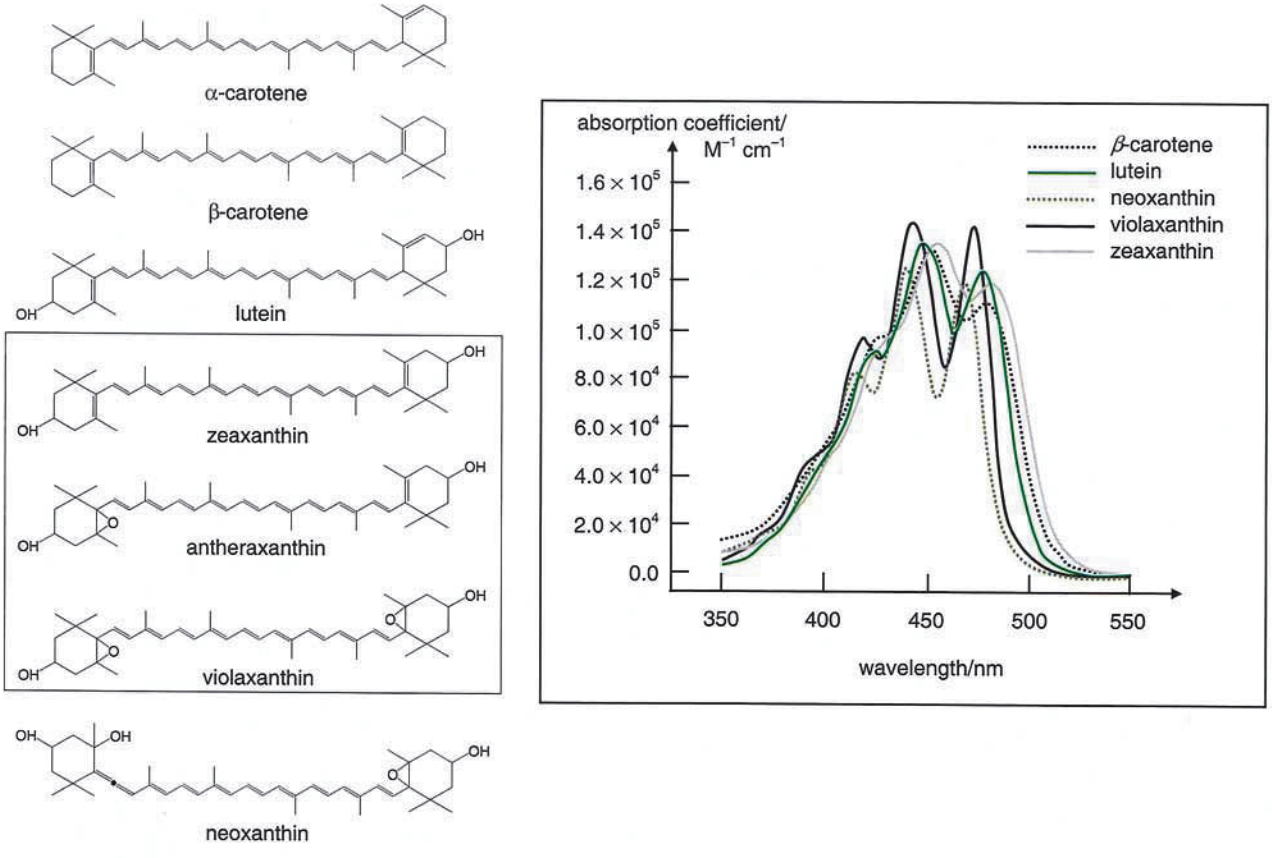


Figure 2.16 Left: structures of carotenoids contained in oxygen-evolving organisms. Right: the corresponding absorption spectra in acetone. Source: Bassi and Caffarri (2000).

2.4.5 *Supraorganisation of light-harvesting systems in Photosystem II*

The PSII complex of higher plants *in vivo* consists of supercomplexes of varying size, formed by a combination of a dimeric PSII core complex, very similar to that of cyanobacteria, and variable amounts of peripheral light-harvesting complexes. The structures of these supercomplexes have been studied by electron microscopy (Barber *et al.*, 1998; Boekema *et al.*, 1999; Dekker *et al.*, 1999; Nield *et al.*, 2000a,b; Hankamer *et al.*, 2001).

The basic unit of such a PSII supercomplex is formed by the D1/D2 reaction centre proteins and the CP43/CP47 core antenna proteins, the *cyt* b_{559} unit, and a number of low molecular weight single transmembrane-spanning proteins. This basic complex forms a homodimer. The next higher level of organisation in the supercomplex involves the addition of the peripheral complexes CP26/CP29, which are located next to the CP47/CP43 complexes. This unit adds two trimeric LHCII complexes to form the basic LHCII–PSII supercomplex, as shown in the centre of Fig. 2.17 (p. 90). This contains about 100 Chls per PSII RC, corresponding to a total of 200 Chls per dimeric complex (Hankamer *et al.*, 1997). Electron microscopy has shown that even larger supramolecular PSII antenna complexes are formed by adding further LHCII complexes at the periphery. This variability of the antenna size and composition allows for a wide range of total cross section and other properties of the PSII complex in response to cellular and environmental conditions such as varying light intensities and growth conditions.

It would be helpful to understand how energy migrates through an intact PSII supercomplex, but despite many time-resolved studies of overall energy trapping, carried out both *in vivo* and with intact isolated PSII complexes of higher plants and green algae, no detailed insight has been gained. The total trapping of excitation energy in higher plants occurs with lifetimes of about 150–250 ps, depending on the antenna size of the particular system; for a review, see Holzwarth and Roelofs (1992); Roelofs *et al.* (1992), and for a more recent study of the trapping rate dependence on antenna size, see Barter *et al.* (2001). The complexity of the PSII supercomplex and the strong spectral overlaps between the various antenna complexes are a major limiting factor in gaining detailed insight from time-resolved spectroscopic studies. The overall trapping seems to occur via a trap-limited mechanism, but the details of possible rate-limiting steps in the antenna supercomplex are not known. With the availability of more structural information, it may be possible to perform extended theoretical kinetic modelling, but so far this has not to our knowledge been done.

2.4.6 *Purple photosynthetic bacterial antennae systems*

In the photosynthetic membranes of purple bacteria, the RCs are surrounded by a single so-called LHI supramolecular antenna system, which is also called the 'B875 complex' due to its BChl *a* absorption maximum in *Rb. sphaeroides*, one of the most widely studied organisms in this field. Variations in some spectral details occur between various organisms, however. The RC LHI complex in *Rb. sphaeroides* is usually surrounded by a varying amount of the ring-forming LHII complexes, such as the B800–850 complex, so called because of its double-banded absorption spectrum (the absorption maxima again depend on the organism). The best-studied systems where either high-resolution X-ray structures or high-quality structural models are available are the antenna complexes of the purple photosynthetic bacteria *Rps. acidophila* and *Rs. Molischianum*. Unfortunately the detailed spectroscopic investigations, many carried out before the X-ray structure determination, were performed on complexes isolated from other organisms. The earliest high-resolution structures were those of the bacterial RCs of *Rps. viridis* (Deisenhofer *et al.*, 1984) and *Rb. sphaeroides* (Allen *et al.*, 1987). *Rps. viridis* contains BChl *b* and thus has a very red-shifted absorption (960 nm trap) as compared to the ~870 nm trap of most of the BChl *a*-containing reaction centres. Likewise, the BChl *a*-containing antenna systems of purple photosynthetic bacteria absorb at shorter wavelengths than the BChl *b*-containing antennae. Thus in *Rps. viridis*, the antenna absorbs at wavelengths above 1000 nm. No structural details are known for the latter although one may assume from the existing sequence homologies that the structures are similar to those of the BChl *a*-containing species. The antenna structures have so far been determined to atomic resolution for the LHII complexes of two purple bacterial species, *Rps. acidophila* (Freer *et al.*, 1996) and *Rs. molischianum* (Koepke *et al.*, 1996).

Bacterial light-harvesting complexes have a common organisation: they are all composed of a basic monomeric unit which itself is a heterodimer, made up of two short polypeptides, each forming a transmembrane helix. These two polypeptides non-covalently bind two Chls (in LHI) or three BChls (in LHII). Each heterodimer also binds one carotenoid molecule. Large ring-like oligomeric structures are formed by aggregation. For the LHII complex, two different ring structures containing nine (*Rps. acidophila*: Freer *et al.*, 1996) or eight (*Rs. molischianum*: Koepke *et al.*, 1996) monomers are known. The BChls are grouped in two layers, located close to the two membrane surfaces. One layer contains the 800 nm absorption BChl pigments, which are arranged with their head groups more or less parallel to the membrane plane. The other layer contains the 850 nm absorbing pigments. These are arranged with their head groups nearly perpendicular to the membrane plane and nearly parallel to each

other (at an angle of 167° in the *Rs. molischianum* complex). Fig. 2.18 (p. 91) shows the arrangement of the chromophores in the two layers. The BChls in the B800-containing layer are well separated from each other, with centre-to-centre distances of about 22 Å. In contrast, the pigments in the B850 ring are much more closely packed with partial $\pi\pi$-stacking. The distances are not exactly equal, the inter-dimer BChl–BChl distance being somewhat shorter (8.9 Å) than the intra-dimer BChl–BChl distance (9.2 Å) in *Rs. molischianum.* Short interchromophore distances below 10 Å give rise to substantial excitonic coupling in the B850 layer, which is the main cause (besides some possible direct protein–pigment contributions) of the greatly red-shifted absorption maximum of the B850 layer as compared with the monomeric B800 pigments, since all the three BChl forms are chemically identical.

Figure 2.18 shows the nonameric ring structure of the LHII complex of *Rps. acidophila.* Because of the high BChl content, the polypeptide width in the B850 layer is much larger than in the B800 layer. Figure 2.18 also indicates that BChl pigment–pigment interaction in this complex contributes significantly to the overall stability of the complex, presumably through hydrophobic interaction and $\pi\pi$-stacking.

No high-resolution structure is available for the LHI complex. However, Hu and Schulten (1998) have developed the detailed structural model shown in Fig. 2.19 (p. 92) based on a low-resolution electron micrograph (Karrasch *et al.*, 1995) and the homology to the LHII complex. The LHI complex forms a hexadecameric structure of heterodimers, each containing two BChls in an arrangement similar to the B850 pigment layer of the LHII complex. Thus there are 32 BChls arranged in a ring structure and the absorption due to excitonic coupling between the BChl pairs is at 875 nm (B875 complex). The centre-to-centre distances are 9.2 Å and 9.3 Å for the inter-dimer and intra-dimer pairs, respectively, very similar to the B850 ring in LHII (Fig. 2.19). Thus the LHI complex is essentially an enlarged LHII ring without the B800 pigment layer. The RC of purple photosynthetic bacteria fits tightly into the hole of the LHI complex, as modelled by Schulten and co-workers (Hu *et al.*, 1998; Hu and Schulten, 1998). The LHI/RC complex, together with a variable amount of LHII complexes surrounding the LHI complex, form the PSU of purple bacteria, as shown in Fig. 2.20 (p. 93). The LHII/LHI ratio is highly variable, depending on the species, growth conditions, light quality and intensity *etc.* Some purple photosynthetic bacteria also form another complex, LHIII, under certain conditions, which is similar to the LHII complex in structure but absorbs at 800 and 820 nm, allowing a larger variability for optimal light adaptation.

Many experimental studies have been performed to characterise the energy-transfer steps in detail, both within the isolated antenna complexes LHI and LHII and

the whole PSU of purple photosynthetic bacteria. Figure 2.21 (p. 93) shows the results of these studies, together with the conclusion of a theoretical attempt to calculate each transfer step from structural and spectroscopic information. Schulten and co-workers (Hu *et al.*, 2002) have developed the most detailed theoretical model of all these energy-transfer steps. The experimentally determined energy-transfer steps between pairs of chromophores and complexes are shown without parentheses and are compared with the theoretically calculated ones, based on exciton theory and Förster theory and taking into account every pigment in the PSU (theoretical values shown in parentheses). The agreement is reasonably good, but not perfect, indicating that our understanding of the spectroscopic properties and structure–function relationship of these complexes is now fairly good. The data summarised in Fig. 2.21 are actually the result of several decades of studies by a large number of research groups, often working together in a truly interdisciplinary fashion.

The PSU of purple photosynthetic bacteria essentially forms a shallow energetic funnel. On the periphery are the LHII B800–850 pigment complexes. The fastest energy-transfer steps occur in the B850 layer of LHII and among the analogous B875 pigments in LHI. The pairwise single-transfer steps in these rings are extremely fast, taking only ~150 fs, due to efficient excitonic coupling. The B800 pigments are too far apart from each other for rapid energy transfer to occur within the B800 ring. Instead, the B800 pigments transfer their energy with a 0.7 ps lifetime to the B850 pigments in the same LHII subunit. Owing to the large distance of the LHI pigments from the RC pigments, the slowest and overall rate-limiting step in the PSU is energy transfer from the LHI pigments to the RC (~25 ps, with a somewhat faster back transfer time of ~8 ps). The inter-complex transfer steps are also relatively slow, about 10 ps between LHII complexes and about 3 ps from LHII to LHI, due to the better spectral overlap and energetic downhill process of the latter. The whole PSU of purple bacteria represents a remarkable self-organised molecular machinery to perform energy transfer to the RC with high yield of above 90% involving hundreds of antenna BChls. The energy-transfer rates towards the RC and away from the RC back to the antenna are quite comparable, so there exists only a mild bias for forward transfer. An important driving force for the overall efficient forward reaction is charge separation in the RC, which is the mechanism that traps excitation irreversibly.

The picture discussed above for light harvesting in purple photosynthetic bacteria refers mostly to the widely studied antennae and RC systems of *Rb. sphaeroides* and related BChl *a*-containing organisms. The principal structures and mechanisms should also be valid for BChl *b*-containing organisms, although much less detailed information is available for them, with the exception of their RC structure (Deisenhofer *et al.*, 1984 and 1985).

2.4.7 *Non-protein containing antenna systems of green bacteria (chlorosomes)*

The general structural principle of photosynthetic antennae is that of a protein-bound pigment array, with pigment bound either non-covalently, as in Chls, BChls and carotenoids, or covalently, as in phycobiliproteins. The proteins in antenna systems have several roles: primarily they serve to maintain the chromophores at suitable distances and in favourable orientations for energy transfer, separated enough to avoid self-quenching, which may occur if pigment molecules interact directly. However, the proteins also have the subtler role of fine-tuning the spectroscopic properties of the pigments to create a more efficient, broadly-absorbing antenna system. This can be achieved in various ways: by providing charges in various distinct positions near the chromophores, by controlling the conformation of chromophores, by protonation, hydrogen bonding, $\pi\pi$-interaction with aromatic amino acids and other means (Gudowska-Nowak *et al.*, 1990). One prime example is the PSI antenna system of cyanobacteria, where the absorption of the chemically identical chromophore, Chl *a*, is tuned by pigment–protein and pigment–pigment interactions from an absorption maximum of about 650 nm up to 730 nm (Holzwarth *et al.*, 1998; Palsson *et al.*, 1998; Karapetyan, 1998; Karapetyan *et al.*, 1999; Gobets and van Grondelle, 2001).

Thus for many good reasons, the notion that photosynthetic antenna pigments necessarily have to be pigment–protein complexes developed into a kind of dogma. It therefore came as a big surprise when it was found that no proteins are involved in the organisation of the chlorin pigments in the outer antenna system—the so-called chlorosomes—of a particular group of photosynthetic organisms, the green sulphur bacteria and the green gliding bacteria (*Chloroflexaceae*) (Staehelin *et al.*, 1978; Schmidt, 1980; Schmidt *et al.*, 1980; Griebenow and Holzwarth, 1989; Griebenow and Holzwarth, 1990; Holzwarth *et al.*, 1990).

Figure 2.22 (p. 94) shows a schematic chlorosomal antenna and how it is attached to the membrane. The interior of the chlorosome contains a pure self-organised pigment aggregate made up of tens of thousands of special chlorophyll molecules, the so-called Bchls *c*, *d* or *e*. These differ from other chlorophylls or bacteriochlorophylls mainly by the presence of a 3^1-OH group, which plays a key role in the self-organisation. The hydroxyl group in the 3^1 position ligates the central Mg of a second neighbouring BChl. This in turn activates the OH group to become a very strong hydrogen-bond donor, facilitating a strong hydrogen bond with the 13^1 carbonyl group of a third BChl. This structural arrangement, comprised of three BChls, is shown in Fig. 2.23 (p. 95, lower left). On the right-hand side are shown higher aggregates, forming the rod structures in chlorosomes (see below). While representing the decisive structural element of the self-organised supramolecular pigment

aggregate of chlorosomes (Balaban *et al.*, 1995b; van Rossum *et al.*, 2001), this building block of three neighbouring BChls is not yet sufficient to determine the overall structure of the chlorosomal pigment aggregates. Important additional roles are played by the type and chirality of the side chains on the chlorin rings (Chiefari *et al.*, 1995; Balaban *et al.*, 1995a, 1997 and 2000), the type of the ester side chains, and the environment of the aggregates. Overall, the pigment aggregates form stacks of chlorins that in turn are bound together by hydrogen-bonding networks. The sheets formed in this way then organise themselves into single- or double-tube rod structures, depending on the organism, as shown in Fig. 2.23 (p. 95, lower right). Single-tube rods with diameters of about 5.4 Å are formed by *Chloroflexaceae* while *Chlorobiaceae* form double-tube rods of ~10 Å diameter (Staehelin *et al.*, 1978 and 1980; van Rossum *et al.*, 2001).

At present, no crystal structure is available for the pigment arrangement in a chlorosomal aggregate. It is doubtful whether chlorosomes can be crystallised at all, since they vary substantially in size and shape. However, a combination of many spectroscopic data characterising the pigment interactions (Olson, 1980; Olson *et al.*, 1985; van Dorssen *et al.*, 1986; Brune *et al.*, 1987b and 1988; Blankenship *et al.*, 1988; Olson and Pedersen, 1988 and 1990; Griebenow and Holzwarth, 1989 and 1990; van Mourik *et al.*, 1990; Fages *et al.*, 1990; Miller *et al.*, 1993; Griebenow *et al.*, 1991; Hildebrandt *et al.*, 1991; Uehara *et al.*, 1991; Holzwarth *et al.*, 1990 and 1992; Chiefari *et al.*, 1995; Balaban *et al.*, 1995a and 1997), of molecular modelling (Holzwarth *et al.*, 1990 and 1992; Holzwarth and Schaffner, 1994; Möltgen *et al.*, 2002), and more recently of solid-state NMR structure information (Nozawa *et al.*, 1994; Balaban *et al.*, 1995b; Boender *et al.*, 1995; Mizoguchi *et al.*, 1998; van Rossum *et al.*, 1998 and 2001) has led to a refined structure that more or less provides the information that would be obtained from an X-ray structure, if one is obtained.

The nomenclature used for BChls present in the chlorosomes is basically incorrect since all of them are essentially Chls in chemical terms. As monomers in solution, they show an absorption spectrum and maximum very similar to those of Chl *a*. However, the strong exciton interaction introduced by the stacking of the chlorins red-shifts the absorption maximum of the chlorosomal aggregates to wavelengths of around 720–740 nm, as described above. Incidentally, this wavelength is also characteristic of the Chl *a*–water aggregates that have been known for a long time (Worcester *et al.*, 1986 and 1989; Katz *et al.*, 1991). Not surprisingly, the structural arrangement of the chlorins in the chlorophyllide *a* water crystal and in Chl *a* water aggregates, which also absorb around 740 nm, bear many similarities to the arrangement of the chlorins in the chlorosomal aggregates (Strouse, 1974; Kratky and Dunitz, 1977; Boender *et al.*, 1995; van Rossum *et al.*, 2002). The major difference

between the chlorophyll water aggregates and the chlorosomal aggregates is the absence of water in the latter, since the structural role of the water is now played by the internal 3^1 hydroxyl group, and the fact that the sheet structures in the chlorophyllide *a* crystals and the Chl *a* aggregates are planar and not rolled up into tubular rod structures, as in the chlorosomes. However, the latter effect might simply be due to conformational restrictions arising from the more demanding requirements of hydrogen bonding to the internal hydroxyl group and the effect of the large side chains in the chlorin rings of the chlorosomal BChls.

One of the striking features of chlorosomes is the presence of an outer monolayer of lipids, a very unusual arrangement in biological systems. The chlorosome rods present the ester chains to the outside and interact with each other in this way. Thus a lipid monolayer as an envelope of the chlorosomal rod arrangement provides a favourable interaction to the outside aqueous phase, which fully explains this unusual structural element. For reviews of chlorosomes, see Blankenship *et al.* (1988 and 1995), Olson *et al.* (1990) and Olson (1998).

The optical properties of chlorosomal aggregates are mainly determined by the strong excitonic coupling between the chlorin rings in the aggregates, which are located at essentially van der Waals distance from each other, with a plane–plane chlorin distance of ~4.5–5 Å. This corresponds to a Mg–Mg distance of ~6.7 Å in the chlorin stack due to the tilted arrangement of the chlorin rings (Prokhorenko *et al.*, 200 and 2003). Figure 2.24 shows the spectroscopic properties of the BChls, in

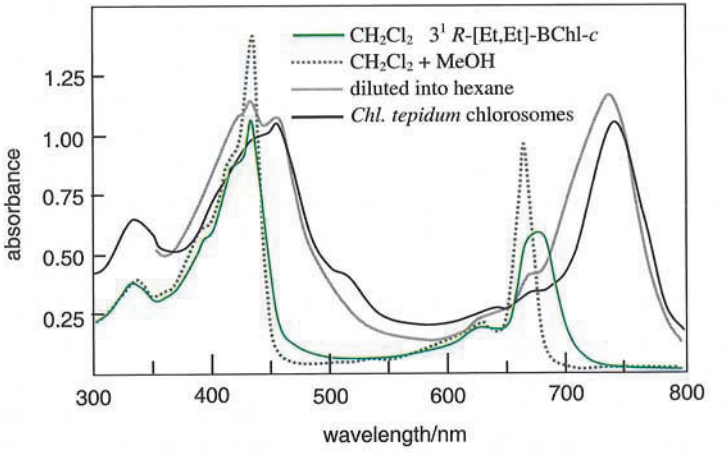


Figure 2.24 Absorption spectra showing the effects of aggregation of BChls in the chlorosomal antennae. The figure shows the monomer absorption of BChl *c* in methanol and the dimer absorption in dichloromethane. The absorption of aggregated BChl structures are shown for intact chlorosomes and for artificial supramolecular aggregates of BChl *c* in hexane.

particular the red shift in the spectra *vs.* monomeric BChls, resulting from the aggregation. This figure compares the absorption spectrum of intact chlorosomes of *C. tepidum* (black) with the spectra of the monomeric BChl, partially aggregated BChl in dichloromethane and pure BChl aggregates in hexane.

There is little difference spectroscopically between single- and double-tube rods (*cf.* Fig. 2.25, p. 96) occurring in different species of green bacteria, since the optical properties are primarily determined by the strong intra-tubular interactions, and the inter-tube interactions only slightly modify the excitonic structure. The close excitonic coupling gives rise to highly delocalised exciton states. Recent data suggest that delocalisation (coherence) extends for a substantial time (many picoseconds) over domains of one thousand or more chlorins, even at room temperature (Prokhorenko *et al.*, 2000, 2002a and 2003). This exceeds the delocalisation lengths in other photosynthetic antennae, such as the LHI or LHII complexes of purple photosynthetic bacteria, by two orders of magnitude. Consequently time-resolved spectroscopy shows a wide dispersion in antenna relaxation lifetimes, ranging from femtoseconds to many picoseconds, corresponding to relaxation between different exciton states in the delocalised chlorosomal antenna structure (Savikhin *et al.*, 1998; Prokhorenko *et al.*, 2000 and 2002a). While the CD spectra of chlorosomes are primarily determined by the excitonic interactions, it has for a long time been a puzzle that the CD signatures differ strongly, sometimes even changing their signs, in chlorosomes of the same organism and with presumably the same or very similar structure (van Mourik *et al.*, 1990; Griebenow *et al.*, 1991). It has recently been shown that this behaviour is explained by different rod lengths of chlorosomes, being introduced either in response to external factors during isolation or in particular growth conditions (Didraga *et al.*, 2002; Prokhorenko *et al.*, 2003).

At their base, chlorosomes form a so-called baseplate containing BChl *a* (Staehelin *et al.*, 1980). This is made up of a regular small pigment–protein complex of unknown structure (Montano *et al.*, 2001). The function of this baseplate is to form the structural and energetic link to the antenna and reaction centres in the cytoplasmic membrane. In green sulphur bacteria, there exists an additional pigment–protein complex, the so-called FMO complex, located between the chlorosomes and the membrane, whereas in *Chloroflexaceae* the chlorosomes sit directly on the membrane (Staehelin *et al.*, 1980), as indicated in Figs. 1.8 and 2.23, without the intervening FMO complex.

Energy transfer through the tens of thousands of chlorins in the complete chlorosome occurs extremely rapidly. While full understanding of the dynamics is still lacking, it is known that energy trapping from the chlorosomes into the membrane antenna of *Chloroflexaceae* takes about 20–40 ps (Griebenow *et al.*, 1990;

Alden *et al.*, 1992; Holzwarth *et al.*, 1992; Müller *et al.*, 1993; Savikhin *et al.*, 1995 and 1996), while energy transfer to the baseplate and the FMO complex in the *Chlorobiaceae*, which form larger chlorosomes, takes rather longer, about 60–100 ps, depending on the organism, exact preparation and redox conditions (Brune *et al.*, 1987a; Causgrove *et al.*, 1990; Alden *et al.*, 1992; Causgrove *et al.*, 1992; Sakuragi *et al.*, 1998; Steensgaard *et al.*, 1999 and 2000; van Walree *et al.*, 1999; Prokhorenko *et al.*, 2000; Psencik *et al.*, 2003).

This very rapid energy transfer out of the chlorosomal antenna into neighbouring antenna complexes makes chlorosomes the most efficient antenna systems in photosynthesis. This high efficiency probably arose because the evolutionary pressure on the development of chlorosomal antenna systems in green bacteria was very high: these organisms usually live under extremely low light conditions at considerable depths in ponds, hot springs, lakes or the sea, where light has been filtered out by many other photosynthetic organisms at shallower depths. This might in turn explain the unusual pigment arrangement of chlorosomes, since more conventional antenna structures would hardly be able to provide such a huge absorption cross-section without severe loss of energy-transfer efficiency. Thus in the chlorosomal antenna, the excitonic interactions that are present to varying degrees in most antenna systems play a decisive role. Only the special properties created by the strong excitonic interactions and the large delocalisation lengths allow this huge antenna system to function. Indeed, chlorosomal antennae are capable of harvesting essentially all photons impinging on the green bacterial cell and transporting them to the reaction centres.

2.4.8 *The FMO complex*

We have already noted that green sulphur bacteria contain a special pigment–protein complex, the FMO protein, which functions as the energy relay between the chlorosomes and the reaction-centre complex in the membrane. The FMO complex is a water-soluble pigment–protein complex containing seven BChl *a* molecules per monomer (Fig. 2.26, p. 97). The protein forms a barrel structure mostly made up of β-sheets and the BChl molecules are arranged in a non-symmetric fashion. The protein forms a trimeric structure containing 21 BChl *a* molecules in its stable native form. Although their structures are very similar, there are slight spectroscopic differences between the FMO complexes of different organisms. The FMO protein of *Prosthecochloris aestuarii* was the first antenna system to have its structure determined to high resolution by X-ray crystallography (Fenna and Matthews, 1975 and 1980).

More recently, the resolution has been improved and the structure of the FMO complex from another species, *Chlorobium tepidum*, has been determined to high resolution as well (Li *et al.*, 1997).

Energy transfer through the FMO complex is actually represented by the dynamics of the relaxation between the different excitonic states, much more so than in any other antenna complex, with the notable exception of chlorosomes. It occurs very rapidly, in times of sub-picoseconds to a few picoseconds, showing the expected strong temperature dependence for excitonic band relaxation. Relaxation to the lowest-energy exciton state is somewhat slower, taking about 10 ps (Louwe and Aartsma, 1997; Vulto *et al.*, 1997 and 1999; Prokhorenko *et al.*, 2002b). Van Amerongen *et al.* (2000) discuss the spectroscopic properties and exciton dynamics of the FMO complex in great detail in their book on photosynthetic excitons.

Many attempts have been made to understand the spectroscopic properties of the FMO complex in terms of its structure; the availability of a high-resolution structure has made this a long-standing challenge. While it fairly quickly became clear that excitonic interactions play a decisive role in the FMO complex, as they do in chlorosomes, it turned out to be difficult to relate structure and spectroscopy rigorously. Early attempts (Pearlstein and Hemenger, 1978; Pearlstein, 1991) to explain the optical properties of FMO complexes on the basis of excitonic coupling were unsatisfactory, partly because of insufficient theory and partly because the experimental data were imprecise. More recently, several attempts have been made, taking into account new spectroscopic data, and these have finally led to a fairly good understanding of the spectroscopic properties in terms of the structure (Louwe *et al.*, 1997; Vulto *et al.*, 1998 and 1999; Wendling *et al.*, 2000 and 2002). Absorption, CD, linear dichroism and transient absorption data are now explained within a unified exciton picture. This work, performed on a structurally well-known and highly defined small antenna complex, represents a challenge to the biophysics of antenna complexes in general. It is the final goal to relate structure and spectroscopy so as to understand in detail the function of the photosynthetic pigment complexes employed in light harvesting.

2.5 Concluding remarks

The study of the structural and functional properties of antenna and antenna/reaction centre units over the last few decades has shown that efficient light harvesting can be achieved in many different ways. The early contention that photosynthetic antenna systems and photosynthetic units are fully optimised for energy transfer to the

reaction centre has turned out to be wrong. In reality, a large range of relative distributions and orientations of pigment arrays are able to fulfil the same function efficiently. While purple bacterial systems typically have highly symmetric antenna systems, which may be mainly determined by the necessity for efficient protein–protein interaction in multimer protein assemblies composed of small polypeptides, the higher plant and cyanobacterial antenna by contrast feature very irregular and unsymmetrical pigment arrangements. Some of the most extensive calculations to date, carried out for PSI core complexes and the PSUs of purple photosynthetic bacteria, furthermore show that energy-transfer functions are quite tolerant of fairly large modifications in the pigment arrangement and distribution of the component complexes. Certain restrictions as to average distances and orientations do apply, however, in order to achieve an acceptable minimal rate of energy transfer, as dictated by the parameters controlling the Förster mechanism. Such tolerance is probably needed because photosynthetic organisms have to meet many challenges in addition to efficient light harvesting. Under certain conditions, there may in fact be too much light available, which could be damaging to the organism as a whole or at least to its photosynthetic apparatus. In this case, efficient regulation mechanisms must be available to the photosynthetic apparatus, down-regulating the efficiency of light capture and utilisation. This can only be achieved by substantial variations in the composition and arrangement of the antenna systems. Thus tolerance to a large range of arrangements is a necessary requirement for the survival of photosynthetic organisms, and they have developed a variety of adaptation mechanisms to meet that challenge.

Despite many open questions, such as the details of energy transfer from the core antennae to the reaction centres of higher plants and cyanobacteria, the main principles of light harvesting by antenna systems are today reasonably well understood and many details are known. The new challenge comes from the availability of high-resolution structures of individual complexes or even intact PSU units or supercomplexes on the one hand, and our ability to acquire ever more detailed kinetic and spectral information on the other. What we would like to achieve in the coming years is a full understanding of the function and spectral properties of light-harvesting complexes based on a detailed knowledge of their structure. This will involve the full quantum-mechanical and kinetic simulation of the processes occurring within and between the complexes. If measured by this goal, our understanding of the properties of most antenna complexes is still in its early stages, but we are making rapid progress using the powerful experimental and theoretical techniques that have been developed in recent years.

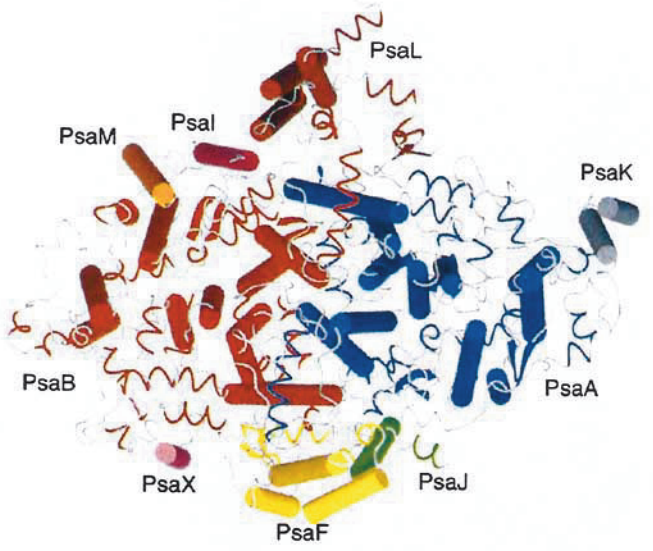


Figure 2.4 Overview structure of a monomer of the overall trimeric PSI structure, showing the location of the transmembrane helices and the subunit composition. View perpendicular to the photosynthetic membrane. Source: Fromme *et al.* (2001).

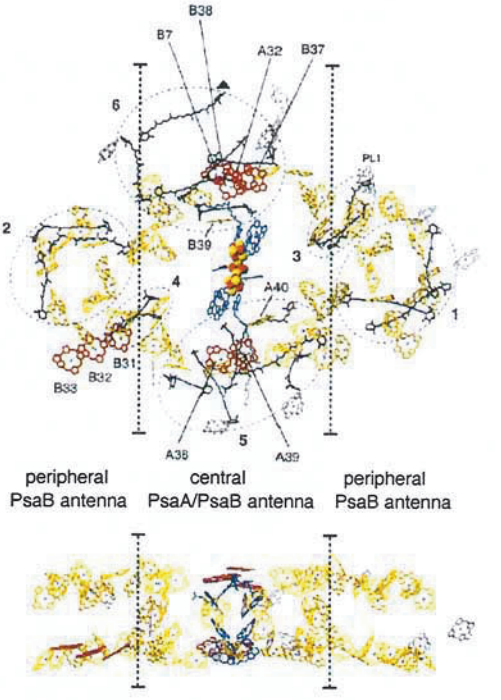


Figure 2.5 Pigment arrangement in the structure of PSI of *S. elongatus* (only one monomer of the trimeric structure is shown). Top: view perpendicular to the membrane plane. Bottom: view parallel to the membrane plane. The Chl clusters that are thought to form the excitonically coupled red pigment aggregates are shown in red. The RC pigments in the centre are shown in blue. Source: Fromme *et al.* (2001).

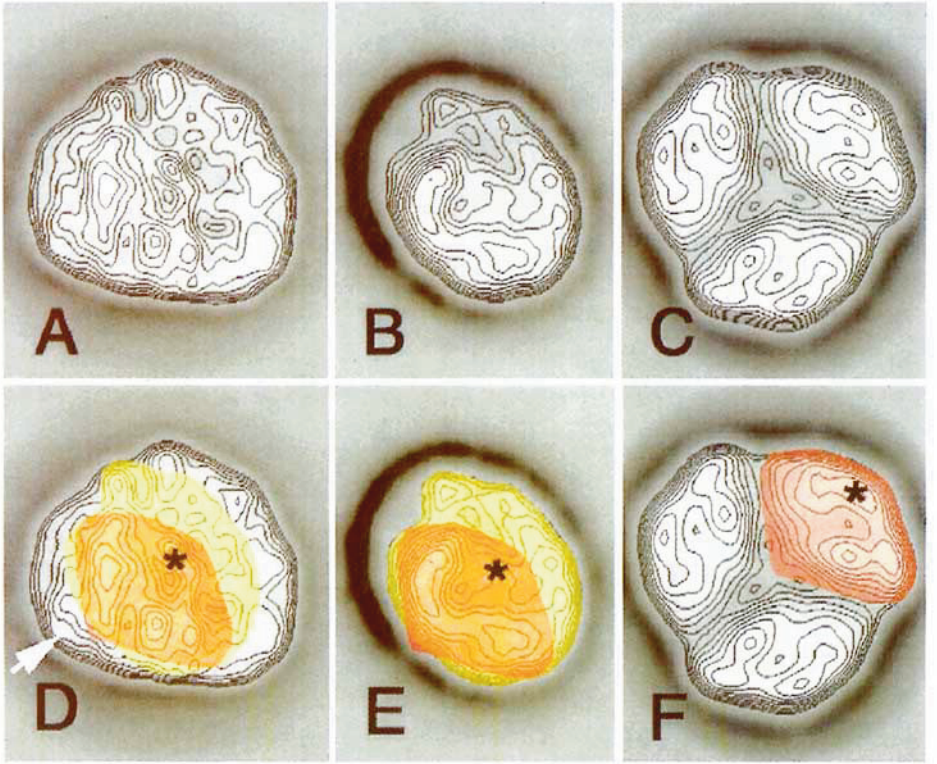


Figure 2.9 Electron micrographs of the structure of the PSI complexes of *Chlamydomonas reinhardtii* (A), spinach (B), and *Synechococcus* trimers (C). The bottom row shows the same structures, but superimposed are the monomeric structures of the *Synechococcus* monomer (red) and the LHCI part of the spinach PSI complex (yellow) in D and E. In part F, the monomeric contribution to the trimeric structure of *Synechococcus* PSI is marked in red. Source: Germano *et al.* (2002).

Figure 2.10 Giant ring around the PSI core in *Synechocystis* PCC 6803

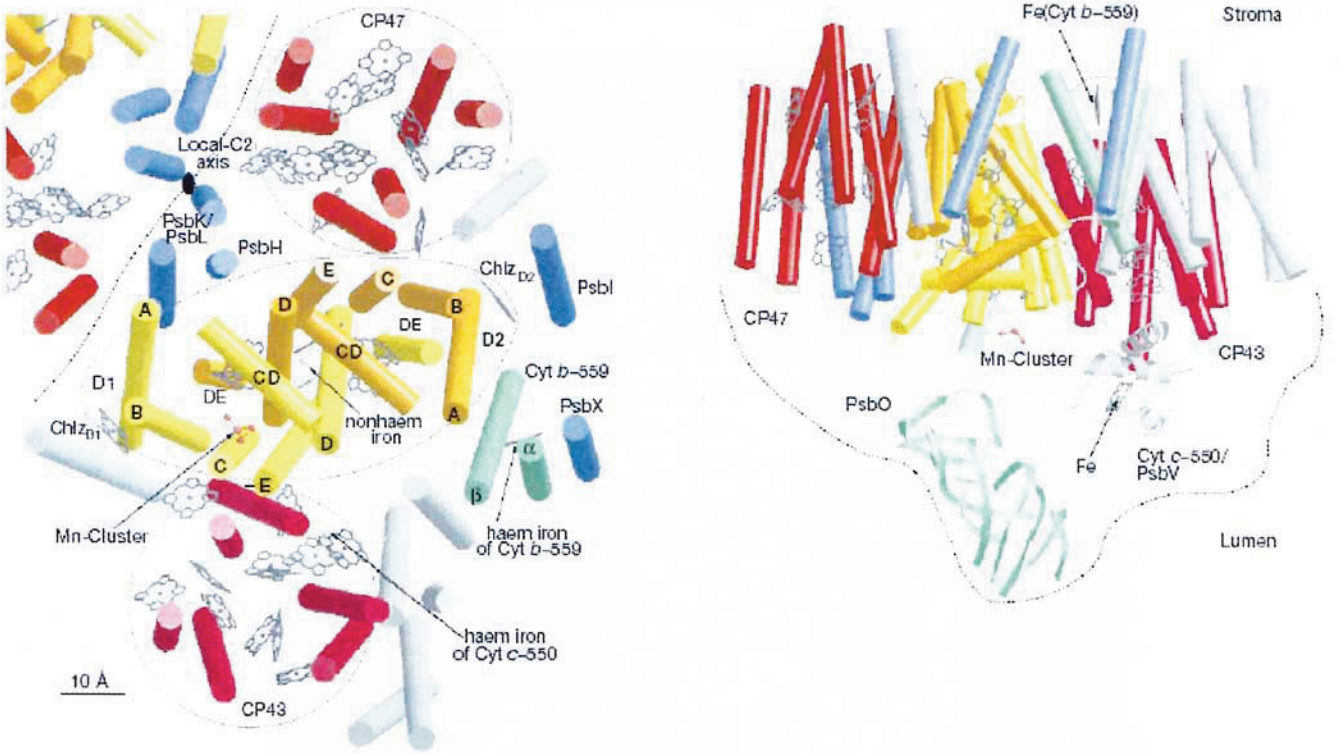


Figure 2.11 Structure of the cyanobacterial PSII core complex of *S. elongatus*, showing the transmembrane helices and the subunit composition (coloured). For clarity, only one monomer of the dimeric structure is shown. Left: view perpendicular to the membrane plane. Right: view parallel to the membrane plane. The polypeptides of the oxygen-evolving complex on the lumenal side are shown as ribbons. Source: Zouni *et al.* (2001).

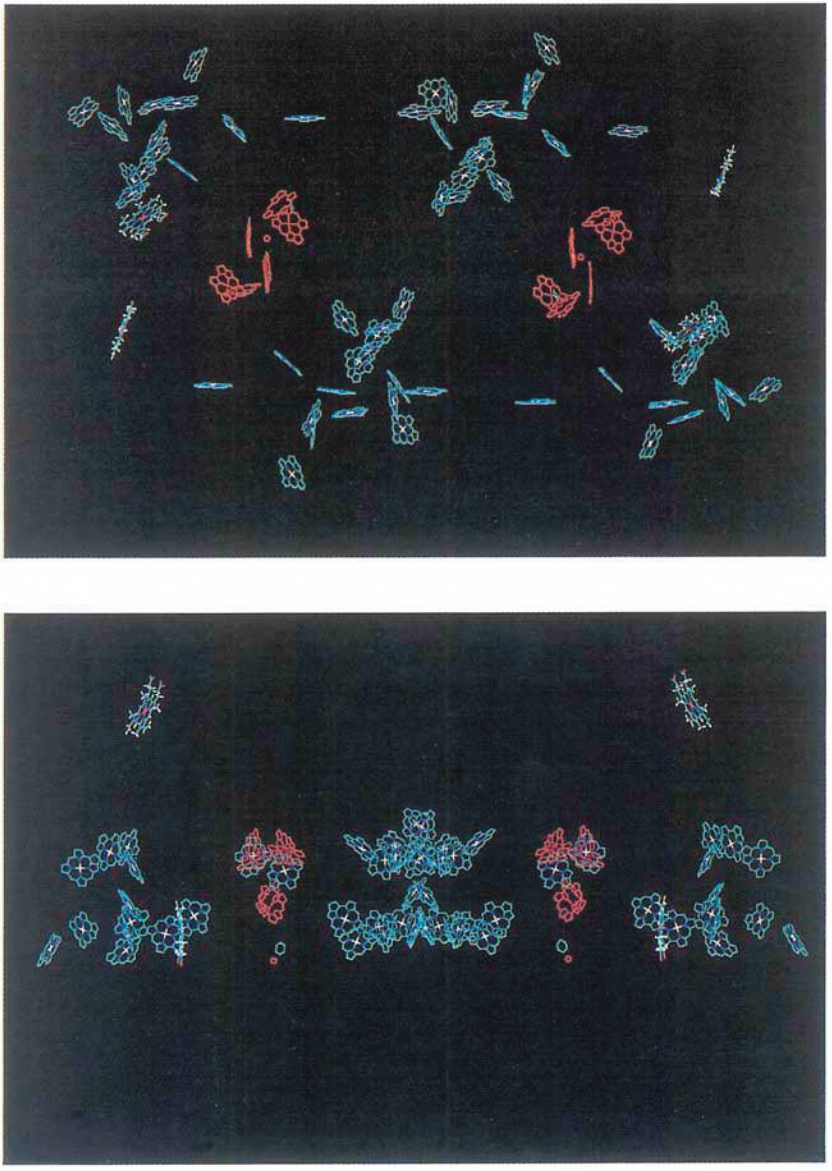


Figure 2.12 Pigment arrangement in the PSII core of *S. elongatus*. The antenna Chls are shown in blue and the RC pigments in red. The full dimer structure is shown from above (upper box) and from the side (lower box). The two pigments located at the top of the lower box are the haeme groups of the *cyt* b_{559} units. Source: Zouni *et al.* (2001).

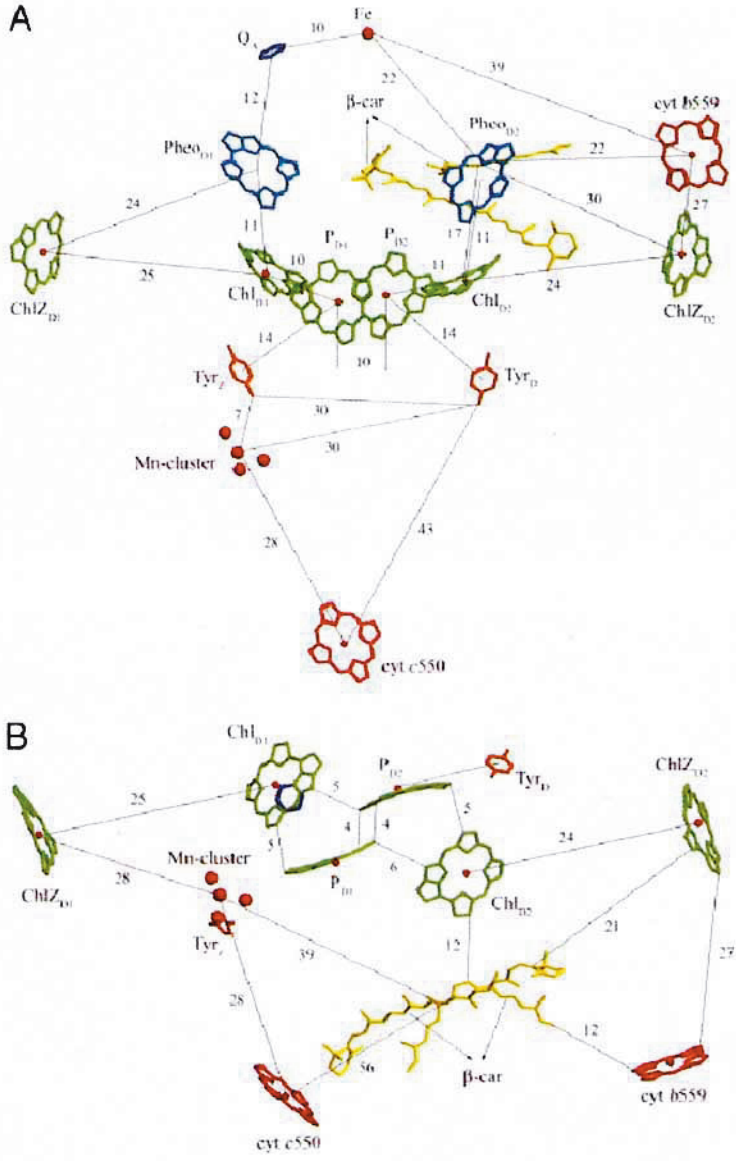


Figure 2.13 Pigment arrangement around the reaction centre of the PSII complex, showing distances and orientations of the RC pigments and the nearest antenna Chls. Also shown are the two β-carotene molecules in the structure. Source: Kamiya and Shen (2003). A: view in membrane plane; B: view perpendicular to membrane plane.

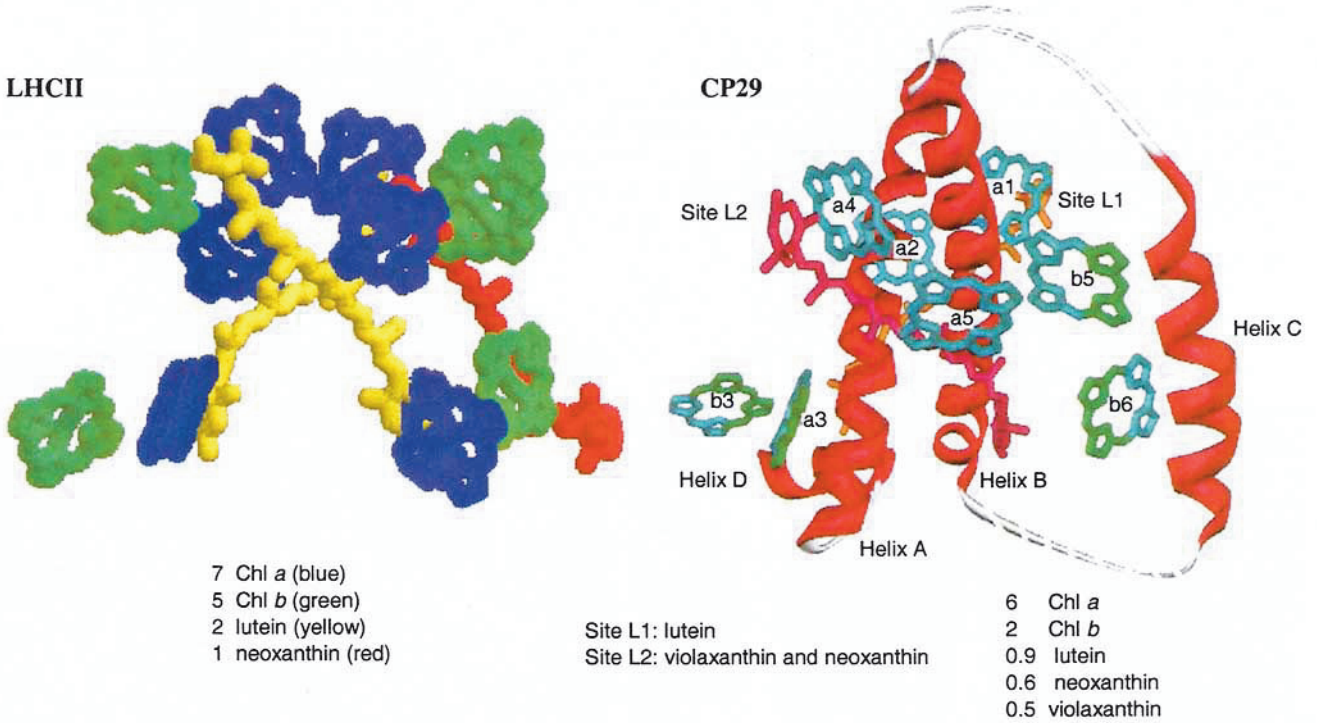


Figure 2.14 Pigment arrangement in the LHCII complex (left) and the minor CP29 complex (right). Left: Proposed Chl *b* molecules are shown in green, Chl *a* molecules in blue, the two central lutein carotenoids in yellow, and the third carotenoid (neoxanthin) binding site in red. The latter was not resolved in the original structure determination (Kühlbrandt and Wang, 1991) and was established by molecular modelling by Bassi and coworkers. Adapted from Bassi and Caffarri (2000).

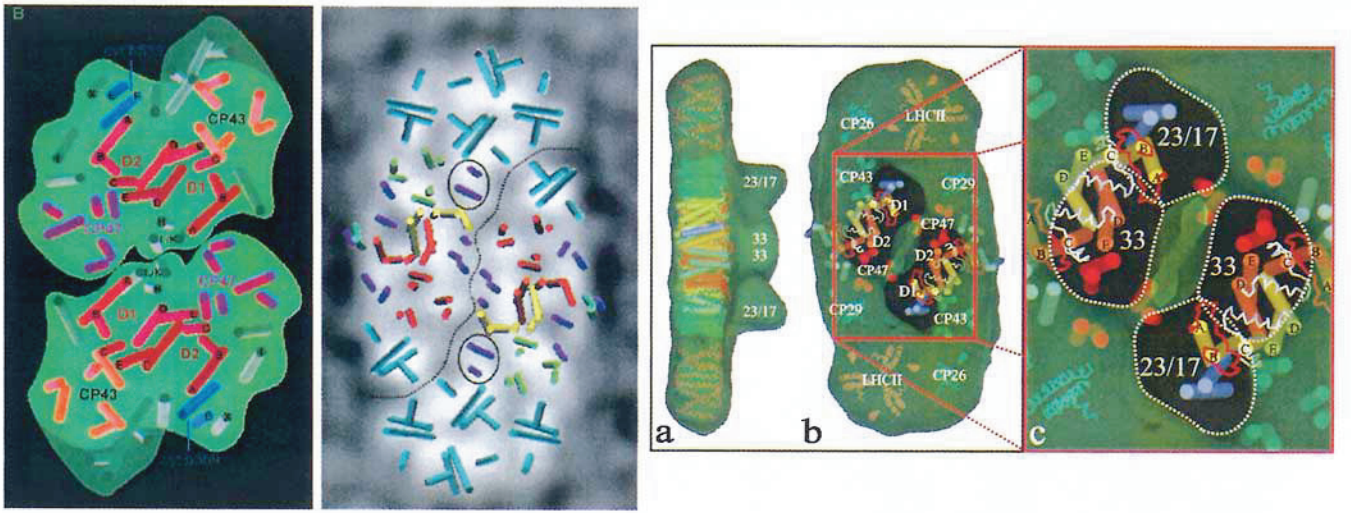


Figure 2.17 Left: low-resolution structure of the Photosystem II core dimer of higher plants as determined by electron microscopy, showing the helical parts of the polypeptides of the CP43, CP37, D1, D2 and *cyt-b*$_{559}$ polypeptides. Centre: structure of the core/light-harvesting complex of PSII from higher plants, showing the helices of the PSII core (central region coloured) surrounded by the LHII trimer antenna complexes and the minor antenna (blue). Source: Hankamer *et al.* (2001). Right: (a) side and (b) top schematic views of the PSII supercomplex of higher plants, showing the arrangement of the various pigment complexes. Source: Nield *et al.* (2000a).

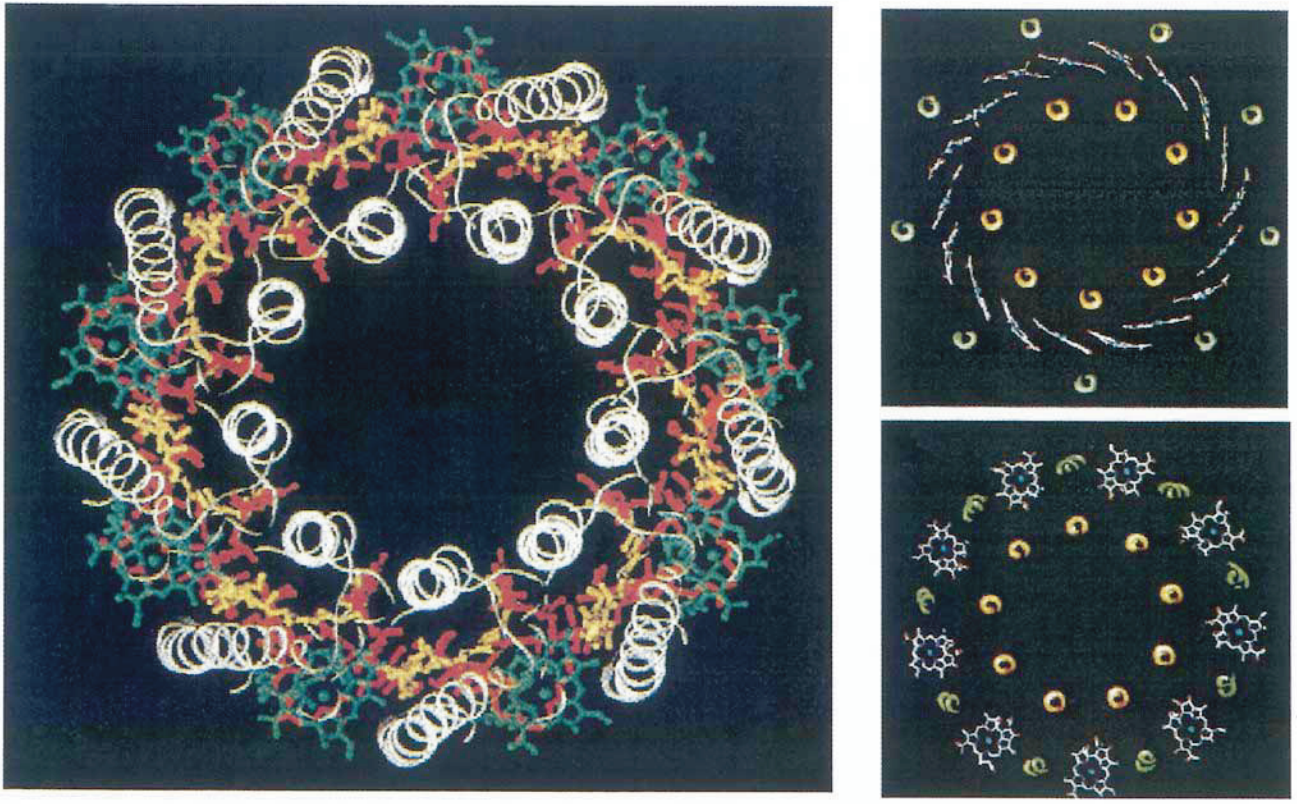


Figure 2.18 Structure of the nonameric LHII antenna complex of the purple photosynthetic bacterium *Rps. acidophila*. Left: structure of the transmembrane helices of the α- and β-polypeptides (white) with the BChls B800 (green) and B850 (red) and the carotenoids (yellow). Right: sections through the structure, showing the region around the pigment layers of the B850-containing pigments (top) and the B800-containing layer (bottom). The circles symbolise the helices of the polypeptides. Source: McDermott *et al.* (1995).

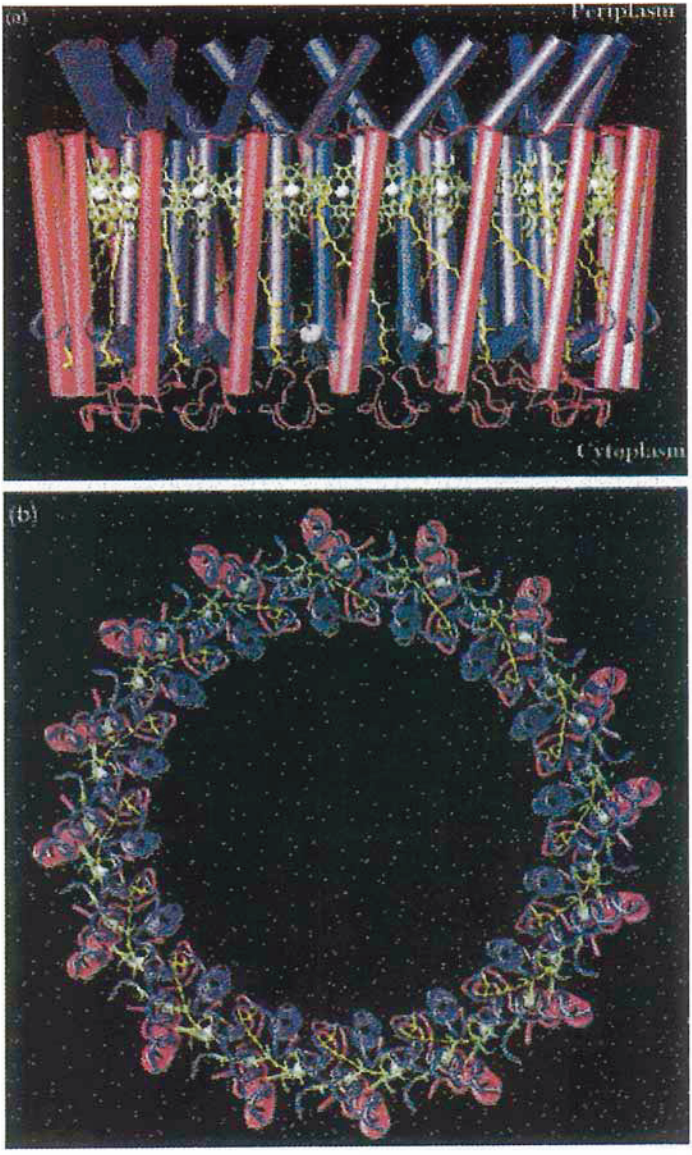


Figure 2.19 Model of the bacterial LHI antenna complex modelled after the structure of the LHII complex (Fig. 2.17) and the low-resolution electron micrograph of LHI. Top: side view showing the transmembrane helices of the α- and β-polypeptides and the B875 BChl pigment layer near the periplasmic membrane surface. Bottom: top view of the same complex, showing the 16-fold symmetric ring structure. Source: Hu and Schulten (1998).

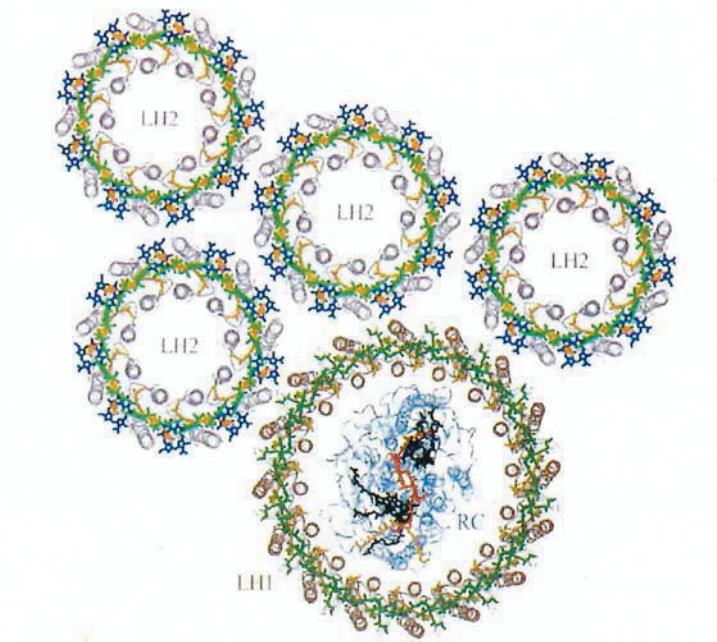


Figure 2.20 Arrangement of the LHI/RC complex and the surrounding LHII complexes in the photosynthetic unit (PSU) of purple photosynthetic bacteria antennae. The B800 pigments are shown in blue, the B850 pigments in light green, and the B875 pigments of the LHI complex in dark green. Adapted from Hu *et al.* (1997).

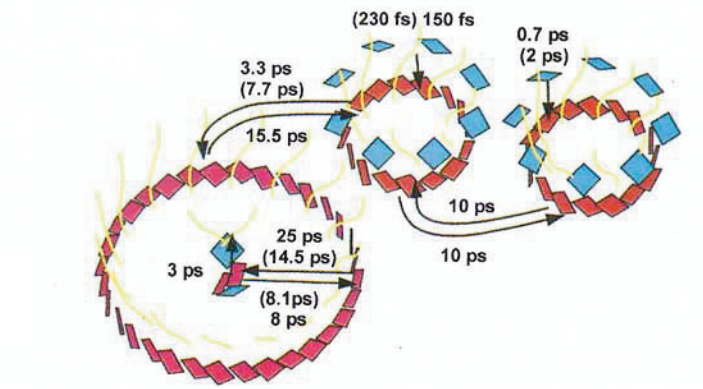


Figure 2.21 Energy-transfer pathways in the supercomplex of the PSU of purple photosynthetic bacteria. The energy-transfer lifetimes between pigments and complexes are indicated by numbered arrows. Source: Hu *et al.* (2002).

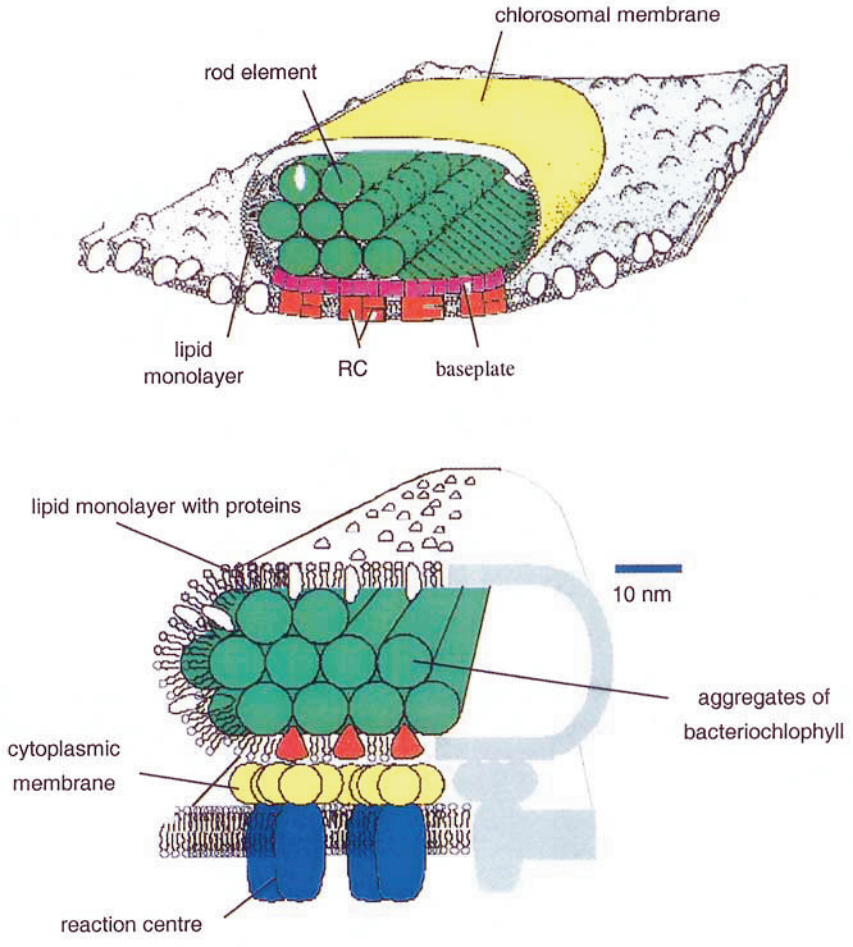


Figure 2.22 Schematic diagrams of the supramolecular chlorosomal antenna complexes and their attachment to the membrane. Top: chlorosome of the green gliding bacterium *C. aurantiacus*. The green rod elements are the protein-free BChl *c* pigment aggregates, which form an extended supramolecular structure containing several thousand BChls each (rod diameter 5.4 nm). The yellow surface denotes the lipid monolayer surrounding the chlorosome body. The pink structures are the BChl *a*–protein complexes that form the so-called baseplate of the chlorosome. The LH1/RC complex in the cytoplasmic membrane (shown in grey) is depicted in red. Bottom: chlorosome structure of the green sulphur bacteria: Protein-free pigment aggregates (green) are arranged in rod structures (10 nm diameter). The chlorosome is surrounded by a lipid monolayer containing small polypeptides. Baseplate proteins (red) and PSI–FeS-type reaction centre complexes are shown in blue. The chlorosomes in green sulphur bacteria are attached to the cytoplasmic membrane via the FMO complex (yellow).

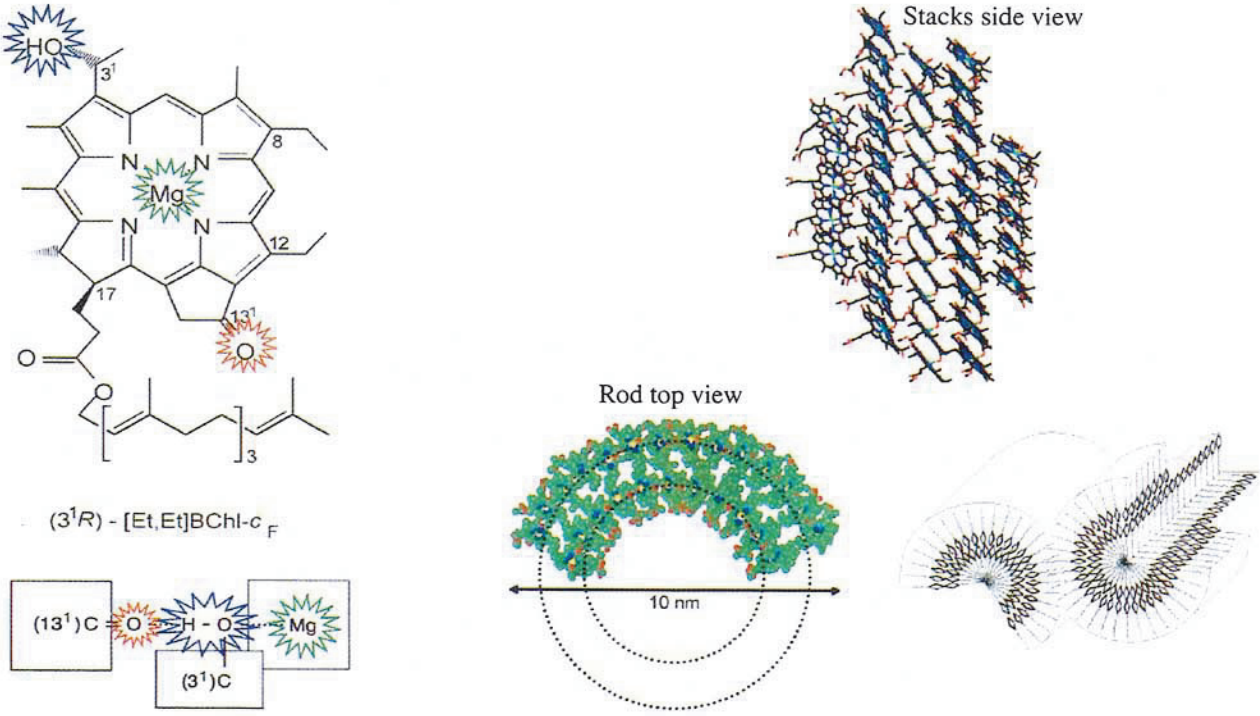


Figure 2.23 Top left: structure of BChl *c*, indicating with asterisks the relevant binding sites for supramolecular BChl/BChl aggregation. Lower left: schematic view of the binding of 3 BChl monomers to a supramolecular aggregate. Top right: 5 stacks of supramolecular BChl aggregates in side view. This structure represents part of a chlorosome rod element. Lower right: structure of a bilayer rod of the green sulphur bacterium *Chlorobium* as space-filling models with inner and outer rod elements seen along the rod axis, and a schematic view of two bilayer rods in side view (the right-hand rod is cut open to show the inner parts). Structures based on solid-state NMR data and molecular modelling. Sources: Holzwarth and Schaffner (1994) and van Rossum *et al.* (2001).

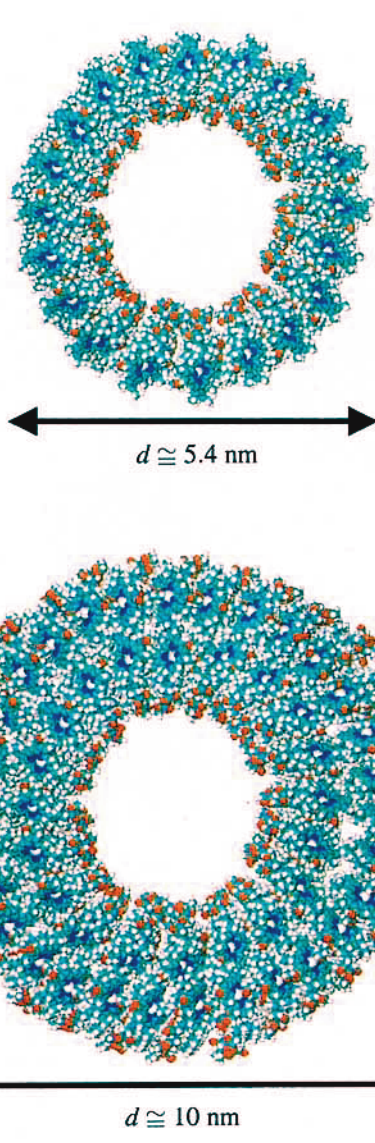


Figure 2.25 Space-filling models of the single layer rod structures of *C. aurantiacus* (top, 4 nm diameter) and the bilayer rod structure of *Chlorobium* (bottom, 10 nm diameter), shown as slices of the rods viewed along the rod axis.

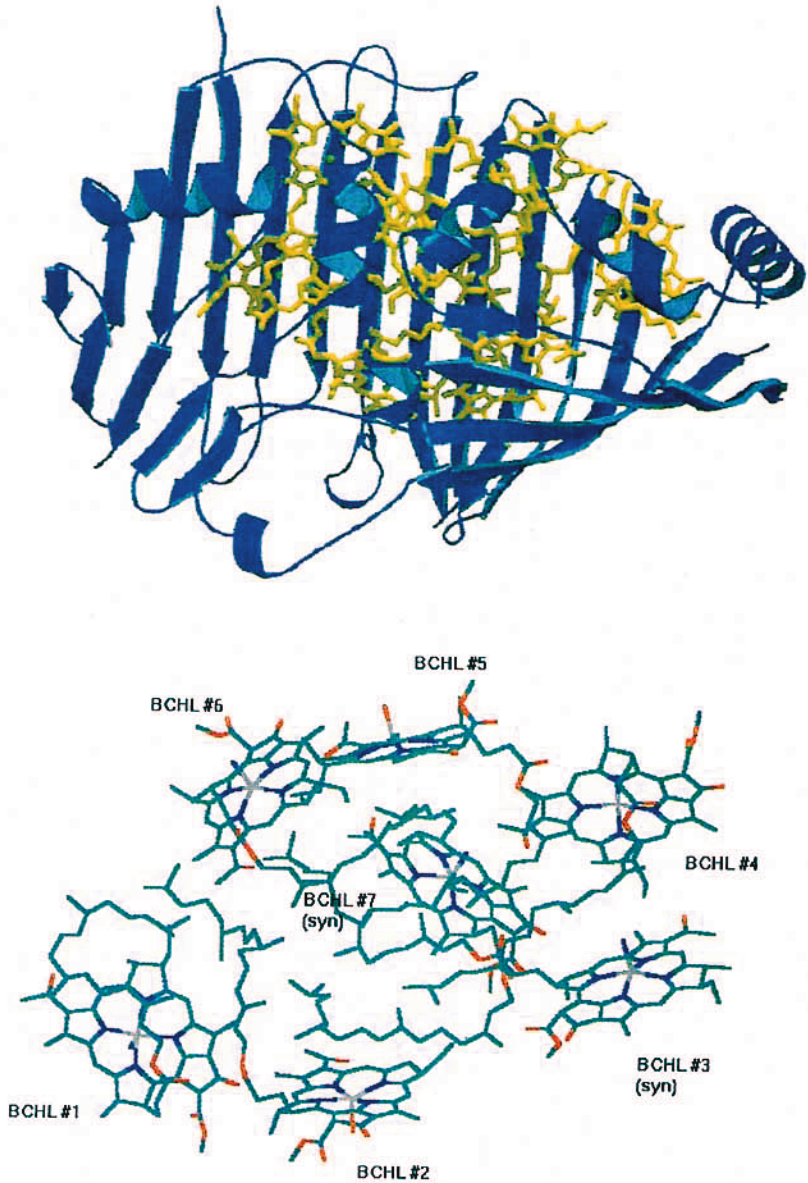


Figure 2.26 Structure of the FMO antenna complex of *Prosthecochloris aestuarii*, showing the polypeptides with the BChl *a* pigments (top, taken from Cogdell and Lindsay, 2000) and the pigment arrangement alone (bottom), indicating also the numbering of the 7 BChl *a* molecules in the structure. Drawn according to the Brookhaven database structure of the FMO complex.

References

Alden R. G., Lin S. H. and Blankenship R. E. (1992), 'Theory of spectroscopy and energy transfer of oligomeric pigments in chlorosome antennas of green photosynthetic bacteria', *J. Luminesc.* **51**, 51–66.

Allen J. P., Feher G., Yeates T. O., Komiya H. and Rees D. C. (1987), 'Structure of the reaction center from *Rhodobacter sphaeroides* R-26. The protein subunits', *Proc. Nat. Acad. Sci. USA* **84**, 6162–6166.

Balaban T. S., Holzwarth A. R. and Schaffner K. (1995a), 'Circular dichroism study on the diastereoselective self-assembly of bacteriochlorophyll c_s', *J. Mol. Struct.* **349**, 183–186.

Balaban T. S., Holzwarth A. R., Schaffner K., Boender G. J. and de Groot H. J. M. (1995b), 'CP-MAS ^{13}C-NMR dipolar correlation spectroscopy of ^{13}C enriched chlorosomes and isolated bacteriochlorophyll *c* aggregates of *Chlorobium tepidum:* the self-organization of pigments is the main structural feature of chlorosomes', *Biochem.* **34**, 15259–15266.

Balaban T. S., Tamiaki H., Holzwarth A. R. and Schaffner K. (1997), 'Self-assembly of methyl zinc (3^1R)- and (3^1S)- bacteriopheophorbides', *J. Phys. Chem. B* **101**, 3424–3431.

Balaban T. S., Leitich J., Holzwarth A. R. and Schaffner K. (2000), 'Autocatalyzed self-assembly of (3^1R)-[Et,Et]bacteriochlorophyll c_F in nonpolar solvents. Analysis of the kinetics', *J. Phys. Chem. B* **104**, 1362–1372.

Barber J., Rhee K. H., Morris E., Hankamer B., Nield J., Boekema E. and Kühlbrandt W. (1998), 'Towards the elucidation of the structure of Photosystem II at high resolution', *Biophys. J.* **74**, A328.

Barber J. and Kühlbrandt W. (1999), 'Photosystem II', *Curr. Opinion Struct. Biol.* **9**, 469–475.

Barber J. (2003), 'Photosystem II: the engine of life', *Quart. Rev. Biophys.* **36**, 71–89.

Barter L. M. C., Bianchietti M., Jeans C., Schilstra M. J., Hankamer B., Diner B. A., Barber J., Durrant J. R. and Klug D. R. (2001), 'Relationship between excitation energy transfer, trapping, and antenna size in Photosystem II', *Biochem.* **40**, 4026–4034.

Bassi R., Hoyer-Hansen G., Barbato R., Giacometti G. M. and Simpson D. J. (1987), 'Chlorophyll proteins of the Photosystem II antenna system', *J. Biol. Chem.* **262**, 13333–13341.

Bassi R., Pineau B., Dainese P. and Marquardt J. (1993), 'Carotenoid-binding proteins of Photosystem II', *Eur. J. Biochem.* **212**, 297–303.

Bassi R., Sandona D. and Croce R. (1997), 'Novel aspects of chlorophyll *a*/*b*-binding proteins', *Physiol. Plant.* **100**, 769–779.

Bassi R., Croce R., Cugini D. and Sandona D. (1999), 'Mutational analysis of a higher plant antenna protein provides identification of chromophores bound into multiple sites', *Proc. Nat. Acad. Sci. USA* **96**, 10056–10061.

Bassi R. and Caffarri S. (2000), 'LHC proteins and the regulation of photosynthetic light-harvesting function by xanthophylls', *Photosynth. Res.* **64**, 243–256.

Beddard G. S. (1998), 'Excitations and excitons in Photosystem I', *Phil. Trans. R. Soc. Lond. A* **356**, 421–448.

Bibby T. S., Nield J. and Barber J. (2001a), 'Three-dimensional model and characterization of the iron stress-induced CP43 Photosystem I supercomplex isolated from the cyanobacterium *Synechocystis* PCC 6803', *J. Biol. Chem.* **276**, 43246–43252.

Bibby T. S., Nield J., Partensky F. and Barber J. (2001b), 'Oxyphotobacteria—antenna ring around Photosystem I', *Nature* **413**, 590.

Billsten H. H., Herek J. L., Garcia-Asua G., Hashoj L., Polivka T., Hunter C. N. and Sundström V. (2002), 'Dynamics of energy transfer from lycopene to bacteriochlorophyll in genetically-modified LH2 complexes of *Rhodobacter sphaeroides*', *Biochem.* **41**, 4127–4136.

Blankenship R. E., Brune D. C. and Wittmershaus B. P. (1988), 'Chlorosome antennas in green photosynthetic bacteria', in *Light-Energy Transduction in Photosynthesis: Higher Plants and Bacterial Models,* Stevens S. E. and Bryant D. A., eds., American Society of Plant Physiologists, Rockville MD., 32–46.

Blankenship R. E., Olson J. M. and Miller M. (1995), 'Antenna complexes from green photosynthetic bacteria', in *Anoxygenic Photosynthetic Bacteria*, Blankenship R. E., Madigan M. T. and Bauer C. E., eds., Kluwer, Dordrecht, pp. 399–435.

Boekema E. J., van Roon H., van Breemen J. F. L. and Dekker J. P. (1999), 'Supramolecular organization of Photosystem II and its light-harvesting antenna in partially solubilized Photosystem II membranes', *Eur. J. Biochem.* **266**, 444–452.

Boekema E. J., Hifney A., Yakushevska A. E., Piotrowski M., Keegstra W., Berry S., Michel K.-P., Pistorius E. K. and Kruip J. (2001a), 'A giant chlorophyll–protein complex induced by iron deficiency in cyanobacteria', *Nature* **412**, 745–748.

Boekema E. J., Jensen P. E., Schlodder E., van Breemen J. F. L., van Roon H., Scheller H. V. and Dekker J. P. (2001b), 'Green-plant Photosystem I binds light-harvesting complex I on one side of the complex', *Biochem.* **40**, 1029–1036.

Boender G. J., Balaban T. S., Holzwarth A. R., Schaffner K., Raap J., Prytulla S., Oschkinat H. and de Groot H. J. M. (1995), 'Comparison of the stacking of chlorophylls in chlorosomes versus aggregates of bacteriochlorophyll *c* and chlorophyll *a* using 2-D MAS NMR spectroscopy', in *Photosynthesis: From Light*

to Biosphere, Vol. I. *Proc. Xth. Int. Photosynthesis Congress*, Montpellier, Mathis P., ed., Kluwer Academic Publishers, Dordrecht, pp. 347–50.

Brune D. C., King G. H., Infosino A., Steiner T., Thewalt M. L. W. and Blankenship R. E. (1987a), 'Antenna organization in green photosynthetic bacteria. 2. Excitation transfer in detached and membrane-bound chlorosomes from *Chloroflexus aurantiacus*', *Biochem.* **26**, 8652–8658.

Brune D. C., Nozawa T. and Blankenship R. E. (1987b), 'Antenna organization in green photosynthetic bacteria. 1. Oligomeric bacteriochlorophyll *c* as a model for the 740 nm absorbing bacteriochlorophyll *c* in *Chloroflexus aurantiacus* chlorosomes', *Biochem.* **26**, 8644–8652.

Brune D. C., King G. H. and Blankenship R. E. (1988), 'Interactions between bacteriochlorophyll *c* molecules in oligomers and in chlorosomes of green photosynthetic bacteria', in *Photosynthetic Light-Harvesting Systems*, Scheer H. and Schneider S., eds., de Gruyter, Berlin, pp. 141–51.

Byrdin M., Jordan P., Krauss N., Fromme P., Stehlik D. and Schlodder E. (2002), 'Light harvesting in Photosystem I: Modeling based on the 2.5Å structure of Photosystem I from *Synechococcus elongatus*', *Biophys. J.* **83**, 433–457.

Causgrove T. P., Brune D. C. and Blankenship R. E. (1992), 'Förster energy transfer in chlorosomes of green photosynthetic bacteria', *J. Photochem. Photobiol. B* **15**, 171–179.

Causgrove T. P., Brune D. C., Wang J., Wittmershaus B. P. and Blankenship R. E. (1990), 'Energy transfer kinetics in whole cells and isolated chlorosomes of green photosynthetic bacteria', *Photosynth. Res.* **26**, 39–48.

Chiefari J., Griebenow K., Fages F., Griebenow N., Balaban T. S., Holzwarth A. R. and Schaffner K. (1995), 'Models for the pigment organization in the chlorosomes of photosynthetic bacteria: diastereoselective control of *in-vitro* bacteriochlorophyll c_s aggregation', *J. Phys. Chem.* **99**, 1357–1365.

Christensen R. L. (1999), 'The electronic states of carotenoids', in *The Photochemistry of Carotenoids,* Frank H. A., Young A. J., Britton G. and Cogdell R. J., eds., Kluwer Academic Publishers, Dordrecht, pp. 137–59.

Cinque G., Croce R., Holzwarth A. R. and Bassi R. (2000), 'Energy transfer among CP29 chlorophylls: calculated Förster rates and experimental transient absorption at room temperature', *Biophys. J.* **79**, 1706–1717.

Cogdell R. J. and Lindsay J. G. (2000), 'Tansley Review No. 109—The structure of photosynthetic complexes in bacteria and plants: an illustration of the importance of protein structure to the future development of plant science', *New Phytol.* **145**, 167–196.

Connelly J. P., Müller M. G., Bassi R., Croce R. and Holzwarth A. R. (1997a), 'Femtosecond transient absorption study of carotenoid to chlorophyll energy transfer in the light-harvesting complex II of Photosystem II', *Biochem.* **36**, 281–287.

Connelly J. P., Müller M. G., Hucke M., Gatzen G., Mullineaux C. W., Ruban A. V., Horton P. and Holzwarth A. R. (1997b), 'Ultrafast spectroscopy of trimeric light-harvesting complex II from higher plants', *J. Phys. Chem. B* **101**, 1902–1909.

Crimi M., Dorra D., Bösinger C. S., Giuffra E., Holzwarth A. R. and Bassi R. (2001), 'Time-resolved fluorescence analysis of the recombinant Photosystem II antenna complex CP29. Effects of zeaxanthin, pH and phosphorylation', *Eur. J. Biochem.* **268**, 260–267.

Croce R., Dorra D., Holzwarth A. R. and Jennings R. C. (2000), 'Fluorescence decay and spectral evolution in intact Photosystem I of higher plants', *Biochem.* **39**, 6341–6348.

Croce R., Müller M. G., Bassi R., and Holzwarth A. R. (2001), 'Carotenoid-to-chlorophyll energy transfer in recombinant major light-harvesting complex (LHCII) of higher plants. I. Femtosecond transient absorption measurements', *Biophys. J.* **80**, 901–915.

Croce R., Müller M. G., Bassi R. and Holzwarth A. R. (2003a), 'Chlorophyll *b* to chlorophyll *a* energy transfer kinetics in the CP29 antenna complex: a comparative femtosecond absorption study between native and reconstituted proteins', *Biophys. J.* **84**, 2508–2516.

Croce R., Müller M. G., Caffarri S., Bassi R. and Holzwarth A. R. (2003b), 'Energy transfer pathways in the minor antenna complex CP29 of Photosystem II: a femtosecond study of carotenoid-to-chlorophyll transfer on mutant and WT complexes', *Biophys. J.* **84**, 2517–2532.

Damjanovic A., Ritz T. and Schulten K. (1999), 'Energy transfer between carotenoids and bacteriochlorophylls in light-harvesting complex II of purple bacteria', *Phys. Rev. E* **59**, 3293–3311.

Damjanovic A., Vaswani H., Fromme P. and Fleming G. R. (2002), 'Chlorophyll excitations in Photosystem I of *Synechococcus elongatus*', *J. Phys. Chem. B* **106**, 10251–10262.

de Weerd F. L., Palacios M. A., Andrizhiyevskaya E. G., Dekker J. P. and van Grondelle R. (2002a), 'Identifying the lowest electronic states of the chlorophylls in the CP47 core antenna protein of Photosystem II', *Biochem.* **41**, 15224–15233.

de Weerd F. L., van Stokkum I. H. M., van Amerongen H., Dekker J. P. and van Grondelle R. (2002b), 'Pathways for energy transfer in the core light-harvesting complexes CP43 and CP47 of Photosystem II', *Biophys. J.* **82**, 1586–1597.

de Weerd F. L., Dekker J. P. and van Grondelle R. (2003), 'Dynamics of β-carotene-to-chlorophyll singlet energy transfer in the core of Photosystem II', *J. Phys. Chem. B* **107** 6214–6220.

Deisenhofer J., Epp O., Miki K., Huber R. and Michel H. (1984), 'X-ray structure analysis of a membrane–protein complex. Electron density map at 3Å resolution and a model of the chromophores of the photosynthetic reaction centre from *Rhodopseudomonas viridis*', *J. Mol. Biol.* **180**, 385–398.

Deisenhofer J., Epp O., Miki K., Huber R. and Michel H. (1985), 'Structure of the protein subunits in the photosynthetic reaction centre of *Rhodopseudomonas viridis* at 3Å resolution, *Nature* **318**, 618–624.

Dekker J. P., van Roon H. and Boekema E. J. (1999), 'Heptameric association of light-harvesting complex II trimers in partially solubilized Photosystem II membranes', *FEBS Lett.* **449**, 211–214.

Dekker J. P. and van Grondelle R. (2000), 'Primary charge separation in Photosystem II', *Photosynth. Res.* **63**, 195–208.

Didraga C., Klugkist J. A. and Knoester J. (2002), 'Optical properties of helical cylindrical molecular aggregates: the homogeneous limit', *J. Phys. Chem. B* **106**, 11474–11486.

Fages F., Griebenow N., Griebenow K., Holzwarth A. R. and Schaffner K. (1990), 'Characterization of light-harvesting pigments of *Chloroflexus aurantiacus*. Two new chlorophylls: oleyl (octadec-9-enyl) and cetyl (hexadecanyl) bacteriochlorophyllides-*c*', *J. Chem. Soc. Perkin Trans. I* **1990**, 2791–2797.

Fenna R. E. and Matthews B. W. (1975), 'Chlorophyll arrangement in a bacteriochlorophyll protein from *Chlorobium limicola*', *Nature* **258**, 573–577.

Förster T. (1959), 'Energy transfer with special reference to biological systems. 10th Spiers Memorial Lecture: transfer mechanisms of electronic excitation', *Faraday Discuss. Chem. Soc.* **27**, 7–17.

Förster T. (1965), 1. 'Delocalized excitation and excitation transfer', in *Modern Quantum Chemistry,* Part III, *Action of Light on Organic Crystals*, Sinanoglu O., ed., Academic Press, New York, pp. 93–137.

Frank H. A., Bautista J. A., Josue J. S. and Young A. J. (2000), 'Mechanism of non-photochemical quenching in green plants: energies of the lowest excited singlet states of violaxanthin and zeaxanthin', *Biochem.* **39**, 2831–2837.

Freer A., Prince S., Sauer K., Papiz M., Hawthornthwaite-Lawless A. M., McDermott G., Cogdell R. and Isaacs N. W. (1996), 'Pigment–pigment interactions and energy transfer in the antenna complex of the photosynthetic bacterium *Rhodopseudomonas acidophila*', *Structure* **4**, 449–462.

Fromme P. and Witt H. T. (1998), 'Improved isolation and crystallization of Photosystem I for structural analysis', *Biochim. Biophys. Acta* **1365**, 175–184.

Fromme P., Jordan P. and Krauβ N. (2001), 'Structure of Photosystem I review', *Biochim. Biophys. Acta* **1507**, 5–31.

Gantt E. (1975), 'Phycobilisomes: light-harvesting pigment–protein complexes', *Bioscience* **25**, 781–788.

Germano M., Yakushevska A. E., Keegstra W., van Gorkom H. J., Dekker J. P. and Boekema E. J. (2002), Supramolecular organization of Photosystem I and light-harvesting complex I in *Chlamydomonas reinhardtii*', *FEBS Lett.* **525**, 121–125.

Gibasiewicz K., Ramesh V. M., Melkozernov A. N., Lin S., Woodbury N. W., Blankenship R. E. and Webber A. N. (2001), 'Excitation dynamics in the core antenna of PSI from *Chlamydomonas reinhardtii* CC 2696 at room temperature', *J. Phys. Chem. B* **105**, 11498–11506.

Giuffra E., Cugini D., Pagano A., Croce R., Sandona D. and Bassi R. (1995), '*In vitro* reconstitution and pigment-binding properties of recombinant CP29 and CP24', in *Photosynthesis: From Light to Biosphere*', Vol. I, Mathis P., ed., Kluwer Academic Publishers, Dordrecht, pp. 271–4.

Giuffra E., Cugini D., Croce R. and Bassi R. (1996), 'Reconstitution and pigment-binding properties of recombinant CP29', *Eur. J. Biochem.* **238**, 112–120.

Giuffra E., Zucchelli G., Sandona D., Croce R., Cugini D., Garlaschi F. M., Bassi R. and Jennings R. C. (1997), 'Analysis of some optical properties of a native and reconstituted Photosystem II antenna complex, CP29: pigment-binding sites can be occupied by chlorophyll *a* or chlorophyll *b* and determine spectral forms', *Biochem.* **36**, 12984–12993.

Gobets B. and van Grondelle R. (2001), 'Energy transfer and trapping in Photosystem I review', *Biochim. Biophys. Acta* **1507**, 80–99.

Gobets B., van Stokkum I. H. M., Rogner M., Kruip J., Schlodder E., Karapetyan N. V., Dekker J. P. and van Grondelle R. (2001), 'Time-resolved fluorescence emission measurements of Photosystem I particles of various cyanobacteria: a unified compartmental model', *Biophys. J.* **81**, 407–424.

Gradinaru C. C., van Stokkum I. H. M., Pascal A. A., van Grondelle R. and van Amerongen H. (2000), 'Identifying the pathways of energy transfer between carotenoids and chlorophylls in LHCII and CP29. A multicolor, femtosecond pump–probe study', *J. Phys. Chem. B* **104**, 9330–9342.

Griebenow K. and Holzwarth A. R. (1989), 'Pigment organization and energy transfer in green bacteria. 1. Isolation of native chlorosomes free of bound bacteriochlorophyll *a* from *Chloroflexus aurantiacus* by gel-electrophoretic filtration (GEF)', *Biochim. Biophys. Acta* **973**, 235–240.

Griebenow K. and Holzwarth A. R. (1990), 'Biochemical evidence for chromophore–chromophore interactions as the main organizational principle in chlorosomes of *Chloroflexus aurantiacus*', in *Mol. Biol. of Membrane-Bound Complexes in Phototrophic Bacteria,* Drews G. and Dawes E. A., eds., Plenum Press, New York, pp. 375–381.

Griebenow K., Müller M. G. and Holzwarth A. R. (1990), 'Picosecond energy-transfer kinetics between different pigment pools in chlorosomes from the green bacterium *Chloroflexus aurantiacus*', in *Molecular Biology of Membrane-Bound Complexes in Phototrophic Bacteria,* Drews G. and Dawes E. A., eds., Plenum Press, New York, pp. 383–387.

Griebenow K., Holzwarth A. R., van Mourik F. and van Grondelle R. (1991), 'Pigment organization and energy transfer in green bacteria. 2. Circular and linear dichroism spectra of protein-containing and protein-free chlorosomes isolated from *Chloroflexus aurantiacus* strain OK–70–fl*', *Biochim. Biophys. Acta* **1058**, 194–202.

Groot M.-L., Peterman E. J., van Stokkum I. H. M, Dekker J. P. and van Grondelle R. (1995), 'Triplet and fluorescing states of the CP47 antenna complex of Photosystem II studied as a function of temperature', *Biophys. J.* **68**, 281–290.

Gudowska-Nowak E., Newton M. D. and Fajer J. (1990), 'Conformational and environmental effects on bacteriochlorophyll optical spectra: correlations of calculated spectra with structural results', *J. Phys. Chem.* **94**, 5795–5801.

Hankamer B., Nield J., Zheleva D., Boekema E., Jansson S. and Barber J. (1997), 'Isolation and biochemical characterisation of monomeric and dimeric Photosystem II complexes from spinach and their relevance to the organisation of Photosystem II *in vivo*', *Eur. J. Biochem.* **243**, 422–429.

Hankamer B., Morris E. P. and Barber J. (1999), 'Revealing the structure of the oxygen-evolving core dimer of Photosystem II by cryoelectron crystallography', *Nature Struct. Biol.* **6**, 560–564.

Hankamer B., Morris E., Nield J., Gerle C. and Barber J. (2001), 'Three-dimensional structure of the Photosystem II core dimer of higher plants determined by electron microscopy', *J. Struct. Biol.* **135**, 262–269. Havaux M. and Niyogi K. K. (1999), 'The violaxanthin cycle protects plants from photooxidative damage by more than one mechanism', *Proc. Nat. Acad. Sci. USA* **96**, 8762–8767.

Hildebrandt P., Griebenow K., Holzwarth A. R. and Schaffner K. (1991), 'Resonance Raman spectroscopic evidence for the identity of the bacteriochlorophyll *c* organization in protein-free and protein-containing chlorosomes from *Chloroflexus aurantiacus*', *Z. Naturforsch.* **46C**, 228–232.

Holzwarth A. R. (1986), 'Fluorescence lifetimes in photosynthetic systems', *Photochem. Photobiol.* **43**, 707–725.

Holzwarth A. R., Griebenow K. and Schaffner K. (1990), 'A photosynthetic antenna system which contains a protein-free chromophore aggregate', *Z. Naturforsch.* **45C**, 203–206.

Holzwarth A. R. (1991), 'Structure-function relationships and energy transfer in phycobiliprotein antennae', *Physiol. Plant.* **83**, 518–528.

Holzwarth A. R., Griebenow K. and Schaffner K. (1992), 'Chlorosomes, photosynthetic antennae with novel self-organized pigment structures', *J. Photochem. Photobiol. A* **65**, 61–71.

Holzwarth A. R. and Roelofs T. A. (1992), 'Recent advances in the understanding of chlorophyll excited state dynamics in thylakoid membranes and isolated reaction centre complexes', *J. Photochem. Photobiol. B* **15**, 45–62.

Holzwarth A. R. and Schaffner K. (1994), 'On the structure of bacteriochlorophyll molecular aggregates in the chlorosomes of green bacteria. A molecular modeling study', *Photosynth. Res.* **41**, 225–233.

Holzwarth A. R., Dorra D., Müller M. G. and Karapetyan N. V. (1998), 'Structure-function relationships and excitation dynamics in Photosystem I', in *Photosynthesis: Mechanism and Effects*, Garab G., ed., Kluwer Academic Publishers, Dordrecht, pp. 497–502.

Hsu C.-P., Walla P. J., Head-Gordon M., and Fleming G. R. (2001), 'The role of the S_1 state of carotenoids in photosynthetic energy transfer: the light-harvesting complex II of purple bacteria', *J. Phys. Chem. B* **105**, 11016–11025.

Hu X., Ritz T., Damjanovic A. and Schulten K. (1997), 'Pigment organization and transfer of electronic excitation in the photosynthetic unit of purple bacteria', *J. Phys. Chem. B* **101**, 3854–3871.

Hu X. and Schulten K. (1998), 'Model for the light-harvesting complex I (B875) of *Rhodobacter sphaeroides*', *Biophys. J.* **75**, 683–694.

Hu X., Damjanovic A., Ritz T. and Schulten K. (1998), 'Architecture and mechanism of the light-harvesting apparatus of purple bacteria', *Proc. Nat. Acad. Sci. USA* **95**, 5935–5941.

Hu X. C., Ritz T., Damjanovic A., Autenrieth F. and Schulten K. (2002), 'Photosynthetic apparatus of purple bacteria', *Quart. Rev. Biophys.* **35**, 1–62.

Jennings R. C., Croce R., Dorra D., Garlaschi F. M., Holzwarth A. R., Rivadossi A. and Zucchelli G. (1998), 'Photosystem I red spectral forms: diffusion-limited energy transfer, optical reorganisation energy and absorption cross section', in *Photosynthesis: Mechanism and Effects*, XI Int. Congr. Photosynthesis Budapest 1998, Garab G., ed., Kluwer Academic Publishers, Dordrecht, pp. 271–276.

Jordan P., Fromme P., Witt H. T., Klukas O., Saenger W. and Krauβ N. (2001), 'Three-dimensional structure of cyanobacterial Photosystem I at 2.5Å resolution', *Nature* **411**, 909–917.

Josue J. S. and Frank H. A. (2002), 'Direct determination of the S_1 excited-state energies of xanthophylls by low-temperature fluorescence spectroscopy', *J. Phys. Chem. A* **106**, 4815–4824.

Kamiya N. and Shen J.-R. (2003), 'Crystal structure of oxygen-evolving Photosystem II from *Thermosynechococcus vulcanus* at 3.7Å resolution', *Proc. Nat. Acad. Sci. USA* **100**, 98–103.

Karapetyan N. V. (1998), 'Organization and role of the long-wave chlorophylls in the Photosystem I of the cyanobacterium *Spirulina*', *Membr. Cell Biol.* **12**, 571–584.

Karapetyan N. V., Holzwarth A. R. and Rögner M. (1999), 'The Photosystem I trimer of cyanobacteria: molecular organisation, excitation dynamics and physiological significance', *FEBS Lett.* **460**, 395–400.

Kargul J., Nield J. and Barber J. (2003), 'Three-dimensional reconstruction of a light-harvesting complex I—Photosystem I (LHCI–PSI) supercomplex from the green alga *Chlamydomonas reinhardtii*—insights into light harvesting for PSI', *J. Biol. Chem.* **278**, 16135–16141.

Karrasch S., Bullough P. A. and Ghosh R. (1995), '8.5Å projection map of the light-harvesting complex I from *Rhodospirillum rubrum* reveals a ring composed of 16 subunits', *EMBO J.* **14**, 631–638.

Katz J. J., Bowman M. K., Michalski T. J. and Worcester D. L. (1991), 'Chlorophyll aggregation: chlorophyll/water micelles as models for *in vivo* long-wavelength chlorophyll', in *Chlorophylls*, Scheer H., ed., CRC Press, Boca Raton, pp. 211–35.

Kennis J. T. M., Gobets B., van Stokkum I. H. M., Dekker J. P., van Grondelle R. and Fleming G. R. (2001), 'Light harvesting by chlorophylls and carotenoids in the Photosystem I core complex of *Synechococcus elongatus:* a fluorescence up-conversion study', *J. Phys. Chem. B* **105**, 4485–4494.

Koepke J., Hu X., Muenke C., Schulten K. and Michel H. (1996), 'The crystal structure of the light-harvesting complex II (B800–850) from *Rhodospirillum molischianum*', *Structure* **4**, 581–597.

Konermann L., Gatzen G. and Holzwarth A. R. (1997), 'Primary processes and structure of the Photosystem II reaction center. V. Modeling of the fluorescence kinetics of the D1–D2–Cyt–b559 complex at 77 K', *J. Phys. Chem. B* **101**, 2933–2944.

Kratky C. and Dunitz J. D. (1977), 'Ordered aggregation states of chlorophyll *a* and some derivatives', *J. Mol. Biol.* **113**, 431–442.

Krueger B. P., Yom J., Walla P. J. and Fleming G. R. (1999), 'Observation of the S_1 state of spheroidene in LH2 by two-photon fluorescence excitation', *Chem. Phys. Lett.* **310**, 57–64.

Kühlbrandt W. (1984), 'Three-dimensional structure of the light-harvesting chlorophyll *a*/*b*-protein complex', *Nature* **307**, 478–480.

Kühlbrandt W. and Wang D. N. (1991), 'Three-dimensional structure of plant light-harvesting complex determined by electron crystallography', *Nature* **350**, 130–134.

Kühlbrandt W. (1994), 'Structure and function of the plant light-harvesting complex, LHCII', *Curr. Opinion Struct. Biol.* **4**, 519–528.

Kühlbrandt W., Wang D. N. and Fujiyoshi Y. (1994), 'Atomic model of plant light-harvesting complex by electron crystallography', *Nature* **367**, 614–621.

Li X.-P., Björkman O., Shih C., Grossman A. R., Rosenquist M., Jansson S. and Niyogi K. K. (2000), 'A pigment-binding protein essential for regulation of photosynthetic light harvesting', *Nature* **403**, 391–395.

Li Y.-F., Zhou W., Blankenship R. E. and Allen J. P. (1997), 'Crystal structure of the bacteriochlorophyll *a* protein from *Chlorobium tepidum*', *J. Mol. Biol.* **271**, 456–471.

Louwe R. J. W., Vrieze J., Aartsma T. J. and Hoff A. J. (1997), 'Toward an integral interpretation of the optical steady-state spectra of the FMO-complex of *Prosthecochloris aestuarii*. 1. An investigation with linear-dichroic absorbance-detected magnetic resonance', *J. Phys. Chem. B* **101**, 11273–11279.

Louwe R. J. W. and Aartsma T. J. (1997), 'On the nature of energy transfer at low temperatures in the BChl *a* pigment–protein complex of green sulfur bacteria', *J. Phys. Chem. B* **101**, 7221–7226.

MacColl R. and Guard-Friar D. (1987), *Phycobiliproteins*, CRC Press, Inc., Boca Raton.

Matthews B. W. and Fenna R. E. (1980), 'Structure of a green bacteriochlorophyll protein', *Acc. Chem. Res.* **13**, 309–317.

McDermott G., Prince S. M., Freer A. A., Papiz M., Hawthornthwaite-Lawless A. M., Cogdell R. J. and Isaacs N. W. (1995), 'Crystal structure of an integral membrane light-harvesting complex from photosynthetic bacteria', *Nature* **374**, 517–521.

Melkozernov A. N., Lin S. and Blankenship R. E. (2000), 'Excitation dynamics and heterogeneity of energy equilibration in the core antenna of Photosystem I from the cyanobacterium *Synechocystis sp.* PCC 6803', *Biochem.* **39**, 1489–1498.

Melkozernov A. N. (2001), 'Excitation energy transfer in Photosystem I from oxygenic organisms', *Photosynth. Res.* **70**, 129–153.

Melkozernov A. N., Bibby T. S., Lin S., Barber J. and Blankenship R. E. (2003), 'Time-resolved absorption and emission show that the CP43 antenna ring of iron-stressed *Synechocystis* sp PCC6803 is efficiently coupled to the Photosystem I reaction centre core', *Biochem.* **42**, 3893–3903.

Miller M., Gillbro T. and Olson J. M. (1993), 'Aqueous aggregates of bacteriochlorophyll *c* as a model for pigment organization in chlorosomes', *Photochem. Photobiol.* **57**, 98–102.

Mizoguchi T., Sakamoto S., Koyama Y., Ogura K. and Inagaki F. (1998), 'The structure of the aggregate form of bacteriochlorophyll *c* showing the μ_y absorption above 740 nm as determined by the ring-current effects on ^{1}H and ^{13}C nuclei and by ^{1}H–^{1}H intermolecular NOE correlations', *Photochem. Photobiol.* **67**, 239–248.

Montano G. A., Wu H.-M., Lin S., Brune D. C. and Blankenship R. E. (2001), 'Isolation and characterization of the B795 baseplate light-harvesting complex from the chlorosomes of *Chloroflexus aurantiacus*', *Biophys. J.* **80**, 126.Möltgen H., Kleinermanns K., Jesorka A., Schaffner K. and Holzwarth A. R. (2002), 'Self-assembly of [Et, Et]-bacteriochlorophyll c_F on highly oriented pyrolytic graphite revealed by scanning tunnelling microscopy', *Photochem. Photobiol.* **75**, 619–626.

Müller M. G., Griebenow K. and Holzwarth A. R. (1993), 'Picosecond energy transfer and trapping kinetics in living cells of the green bacterium *Chloroflexus aurantiacus*', *Biochim. Biophys. Acta* **1144**, 161–169.

Müller M. G., Niklas J., Lubitz W. and Holzwarth A. R. (2003), 'Ultrafast transient absorption studies on Photosystem I reaction centers from *Chlamydomonas reinhardtii*. 1. A new interpretation of the energy trapping and early electron transfer steps in Photosystem I', *Biophys. J.*, to be published Nov. 2003.

Naqvi K. R. (1980), 'The mechanism of singlet-singlet excitation energy transfer from carotenoids to chlorophyll'. *Photochem. Photobiol.* **31**, 523–524.

Nield J., Orlova E., Morris E. P., Gowen B., van Heel M., and Barber J. (2000a), '3D map of plant Photosystem II supercomplex obtained by cryoelectron microscopy and single particle analysis', *Nature Struct. Biol.* 7, 44–47.

Nield J., Kruse O., Ruprecht J., Da Fonseca P., Buchel C., and Barber J. (2000b), '3D structure of *Chlamydomonas reinhardtii* and *Synechococcus elongatus* Photosystem II complexes allows comparison of the OEC organisation', *J. Biol. Chem.* **275**, 27940–27946.

Nield J., Morris E. P., Bibby T. S. and Barber J. (2003), 'Structural analysis of the Photosystem I supercomplex of cyanobacteria induced by iron deficiency', *Biochem.* **42**, 3180–3188.

Novoderezhkin V., Salverda J. M., van Amerongen H. and van Grondelle R. (2003), 'Exciton modeling of energy-transfer dynamics in the LHCII complex of higher plants: a Redfield theory approach', *J. Phys. Chem. B* **107**, 1893–1912.

Nozawa T., Ohtomo M., Suzuki M., Nakagawa H., Shikama Y., Konami H. and Wang Z.-Y. (1994), 'Structures of chlorosomes and aggregated BChl *c* in *Chlorobium tepidum* from solid state high resolution CP/MAS ^{13}C NMR, *Photosynth. Res.* **41**, 211–223.

Olson J. M. (1980), 'Chlorophyll organization in green photosynthetic bacteria', *Biochim. Biophys. Acta* **594**, 33–51.

Olson J. M., Gerola P. D., van Brakel G. H., Meiburg R. F. and Vasmel H. (1985), 'Bacteriochlorophyll *a*- and *c*-protein complexes from chlorosomes of green sulfur bacteria compared with bacteriochlorophyll *c* aggregates in CH_2Cl_2–hexane', in *Antennas and Reaction Centers of Photosynthetic Bacteria*, Springer Ser. Chem. Phys., Vol. 42, Michel-Beyerle M. E., ed., Springer, Berlin, pp. 67–73.

Olson J. M. and Pedersen J. P. (1988), 'Bacteriochlorophyll *c* aggregates in carbon tetrachloride as models for chlorophyll organization in green photosynthetic bacteria', in *Photosynthetic Light-Harvesting Systems*, Scheer H. and Schneider S., eds., de Gruyter, Berlin, pp. 365–73.

Olson J. M., Brune D. C. and Gerola P. D. (1990), 'Organization of chlorophyll and protein in chlorosomes', in *Mol. Biol. of Membrane-Bound Complexes in Phototrophic Bacteria,* Drews G. and Dawes E. A., eds., Plenum Press, New York, pp. 227–34.

Olson J. M. (1998), 'Chlorophyll organization and function in green photosynthetic bacteria', *Photochem. Photobiol.* **67**, 61–75.

Olson J. M. and Pedersen J. P. (1990), 'Bacteriochlorophyll *c* monomers, dimers, and higher aggregates in dichloromethane, chloroform, and carbon tetrachloride', *Photosynth. Res.* **25**, 25–37.

Palsson L.-O., Flemming C., Gobets B., van Grondelle R., Dekker J. P. and Schlodder E. (1998), 'Energy transfer and charge separation in Photosystem I P700 oxidation upon selective excitation of the long-wavelength antenna chlorophylls of *Synechococcus elongatus', Biophys. J.* **74**, 2611–2622.

Paulsen H. and Hobe S. (1992), 'Pigment-binding properties of mutant light-harvesting chlorophyll-*a*/*b*-binding protein', *Eur. J. Biochem.* **205**, 71–76.

Paulsen H., Finkenzeller B. and Kühlein N. (1993), 'Pigments induce folding of light-harvesting chlorophyll *a*/*b*-binding protein', *Eur. J. Biochem.* **215**, 809–816.

Pearlstein R. M. (1982a), 'Chlorophyll singlet excitons', in *Photosynthesis*, Vol. 1, *Energy Conversion by Plants and Bacteria,* Govindjee, ed., Academic Press, New York, pp. 293–331.

Pearlstein R. M. (1982b), 'Exciton migration and trapping in photosynthesis', *Photochem. Photobiol.* **35**, 835–844.

Pearlstein R. M. (1982c), 'Excitons in photosynthetic and other biological systems', in *Excitons*, Rashba E. I. and Sturge M. D., eds., North-Holland Publishing Company, Amsterdam, pp. 735–70.

Pearlstein R. M. (1991), 'Theory of the optical spectra of the bacteriochlorophyll *a* antenna protein trimer from *Prosthecochloris aestuarii*', *Photosynth. Res.* **31**, 213–226.

Pearlstein R. M. and Hemenger R. P. (1978), 'Bacteriochlorophyll electronic transition moment directions in bacteriochlorophyll *a*–protein', *Proc. Nat. Acad. Sci. USA* **75**, 4920–4924.

Peterman E. J. G., Gradinaru C. C., Calkoen F., Borst J. C., van Grondelle R. and van Amerongen H. (1997), 'Xanthophylls in light-harvesting complex II of higher plants: light-harvesting and triplet quenching', *Biochem.* **36**, 12208–12215.

Polivka T., Herek J. L., Zigmantas D., Akerlund H.-E. and Sundström V. (1999), 'Direct observation of the (forbidden) S_1 state in carotenoids', *Proc. Nat. Acad. Sci. USA* **96**, 4914–4917.

Polivka T., Zigmantas D., Herek J. L., He Z., Pascher T., Pullerits T., Cogdell R. J., Frank H. A. and Sundström V. (2002), 'The carotenoid S_1 state in LH2 complexes from purple bacteria *Rhodobacter sphaeroides* and *Rhodopseudomonas acidophila*: S_1 energies, dynamics, and carotenoid radical formation', *J. Phys. Chem. B* **106**, 11016–11025.

Prokhorenko V. I., Steensgaard D. B. and Holzwarth A. R. (2000), 'Exciton dynamics in the chlorosomal antennae of the green bacteria *Chloroflexus aurantiacus* and *Chlorobium tepidum*', *Biophys. J.* **79**, 2105–2120.

Prokhorenko V. I., Holzwarth A. R., Müller M. G., Schaffner K., Miyatake T. and Tamiaki H. (2002a), 'Energy transfer in supramolecular artificial antennae units of synthetic zinc chlorins and co-aggregated energy traps. A time-resolved fluorescence study', *J. Phys. Chem. B* **106**, 5761–5768.Prokhorenko V. I., Holzwarth A. R., Nowak F. R. and Aartsma T. J. (2002b), 'Growing-in optical coherence in the FMO antenna complexes', *J. Phys. Chem. B* **106**, 9923–9933.

Prokhorenko V. I., Steensgaard D. B. and Holzwarth A. R. (2003), 'Exciton theory for supramolecular chlorosomal aggregates. 1. Aggregate size dependence of the linear spectra', *Biophys. J.*, in press for publication in Nov. 2003.Psencik J., Ma Y.-Z., Arellano J. B., Hala J. and Gillbro T. (2003), 'Excitation energy transfer dynamics and excited-state structure in chlorosomes of *Chlorobium phaeobacteroides*', *Biophys. J.* **84**, 1161–1179.

Roelofs T. A., Lee C.-H., and Holzwarth A. R. (1992), 'Global target analysis of picosecond chlorophyll fluorescence kinetics from pea chloroplasts. A new approach to the characterization of the primary processes in Photosystem II α- and β-units', *Biophys. J.* **61**, 1147–1163.

Sakuragi Y., van Walree C. A., Steensgaard D. B., Frigaard N.-U., Matsuura K., Cox R. P., Holzwarth A. R. and Miller M. (1998), 'Alkali treatment of green bacterial chlorosomes, in *Photosynthesis: Mechanism and Effects*, XI Int. Congr. Photosynthesis, Budapest 1998, Garab G., ed., Kluwer Academic Publishers, Dordrecht, pp. 161–4.

Sandona D., Croce R., Pagano A., Crimi M. and Bassi R. (1998), 'Higher plants light-harvesting proteins. Structure and function as revealed by mutation analysis of either protein or chromophore moieties', *Biochim. Biophys. Acta* **1365**, 207–214.

Santini C., Tidu V., Tognon G., Magaldi A. G. and Bassi R. (1994), 'Three-dimensional structure of the higher-plant Photosystem II reaction centre and evidence for its dimeric organization *in vivo*', *Eur. J. Biochem.* **221**, 307–315.

Sauer K. (1979), 'Photosynthesis—the light reactions', *Ann. Rev. Phys. Chem.* **30**, 155–178.

Savikhin S., Buck D. R., Struve W. S., Blankenship R. E., Taisova A. S., Novoderezhkin V. I. and Fetisova Z. G. (1998), 'Excitation delocalization in the bacteriochlorophyll *c* antenna of the green bacterium *Chloroflexus aurantiacus* as revealed by ultrafast pump-probe spectroscopy', *FEBS Lett.* **430**, 323–326.

Savikhin S., van Noort P. I., Blankenship R. E. and Struve W. S. (1995), 'Femtosecond probe of structural analogies between chlorosomes and bacteriochlorophyll *c* aggregates', *Biophys. J.* **69**, 1100–1104.

Savikhin S., Zhu Y. W., Blankenship R. E. and Struve W. S. (1996), 'Ultrafast energy transfer in chlorosomes from the green photosynthetic bacterium *Chloroflexus aurantiacus*', *J. Phys. Chem.* **100**, 3320–3322.

Savikhin S., Xu W., Chitnis P. R. and Struve, W. S. (2000), 'Ultrafast primary processes in PSI from *Synechocystis* sp. PCC 6803: roles of P700 and A_0, *Biophys. J.* **79**, 1573–1586.

Savikhin S., Xu W., Martinsson P., Chitnis P. R., and Struve W. S. (2001), 'Kinetics of charge separation and $A_0 \rightarrow A_1$ electron transfer in Photosystem I reaction centres, *Biochem.* **40**, 9282–9290.

Schaffner K., Braslavsky S. E. and Holzwarth A. R. (1991), 'Protein environment and photophysics and photochemistry of the prosthetic chromophores of biliproteins', in *Frontiers in Supramolecular Organic Chemistry and Photochemistry*, Schneider H.-J. and Durr H., eds., VCH Verlagsgesellschaft, Weinheim, pp. 421–452.

Schatz G. H., Brock H. and Holzwarth A. R. (1987), 'Picosecond kinetics of fluorescence and absorbance changes in Photosystem II particles excited at low photon density', *Proc. Nat. Acad. Sci. USA* **84**, 8414–8418.

Schatz G. H., Brock H. and Holzwarth A. R. (1988), 'A kinetic and energetic model for the primary processes in Photosystem II', *Biophys. J.* **54**, 397–405.

Scheer H. (1981), 'Biliproteine', *Angew. Chem.* **93**, 230–250.

Schmidt K. (1980), 'A comparative study on the composition of chlorosomes (*Chlorobium* vesicles) and cytoplasmic membranes from *Chloroflexus aurantiacus* strain Ok-70-fl and *Chlorobium limicola* f. *thiosulfatophilum* 6230', *Arch. Microbiol.* **124**, 21–31.

Schmidt K., Maarzahl M. and Mayer F. (1980), 'Development and pigmentation of chlorosomes in *Chloroflexus aurantiacus* strain Ok–70–fl.', *Arch. Microbiol.*, **127**, 87–97.

Scholes G. D., Jordanides X. J. and Fleming G. R. (2001), 'Adapting the Förster theory of energy transfer for modeling dynamics in aggregated molecular assemblies', *J. Phys. Chem. B* **105**, 1640–1651.

Sener M. K., Lu D. Y., Ritz T., Park S., Fromme P. and Schulten K. (2002), 'Robustness and optimality of light harvesting in cyanobacterial Photosystem I', *J. Phys. Chem. B* **106**, 7948–7960.

Smith K. M. (1991), in *Chlorophylls*, Scheer H., ed., CRC Press, Boca Raton, pp. 115–43.

Staehelin L. A., Golecki J. R., Fuller R. C. and Drews G. (1978), 'Visualization of supramolecular architecture of chlorosomes (*Chlorobium* type vesicles) in freeze-fractured cells of *Chloroflexus aurantiacus*', *Arch. Mikrobiol.* **119**, 269–277.

Staehelin L. A., Golecki J. R. and Drews G. (1980), 'Supramolecular organization of chlorosomes (*Chlorobium* vesicles) and of their membrane attachment sites in *Chlorobium limicola*', *Biochim. Biophys. Acta* **589**, 30–45.

Stanier R. Y. (1974), 'The origins of photosynthesis in eukaryotes', *Symp. Soc. Gen. Microbiol.* **24**, 219–240.

Steensgaard D. B., van Walree C. A., Baneras L., Borrego C. M., Garcia-Gil J. and Holzwarth A. R. (1999), 'Evidence for spatially separate bacteriochlorophyll *c* and bacteriochlorophyll *d* pools within the chlorosomal aggregate of the green sulfur bacterium *Chlorobium limicola*', *Photosynth. Res.* **59**, 231–241.

Steensgaard D. B., van Walree C. A., Permentier H., Baneras L., Borrego C. M., Garcia-Gil J., Aartsma T. J., Amesz J., and Holzwarth A. R. (2000), 'Fast energy transfer between BChl *d* and BChl *c* in chlorosomes of the green sulfur bacterium *Chlorobium limicola*', *Biochim. Biophys. Acta* **1457**, 71–80.

Strouse C. E. (1974), 'The crystal and molecular structure of ethyl chlorophyllide *a*. 2. H_2O and its relationship to the structure and aggregation of chlorophyll *a*', *Proc. Nat. Acad. Sci. USA* **71**, 325–328.

Uehara K., Ozaki Y., Okada K. and Olson J. M. (1991), 'FT–IR studies on the aggregation of bacteriochlorophyll *c* from *Chlorobium limicola*', *Chem. Lett.* **6**, 909–912. van Amerongen H., Valkunas L. and van Grondelle R. (2000), *Photosynthetic Excitons*, World Scientific Press, Singapore.

van Amerongen H. and van Grondelle R. (2001), 'Understanding the energy transfer function of LHCII, the major light-harvesting complex of green plants', *J. Phys. Chem. B* **105**, 604–617.

van Dorssen R. J., Gerola P. D., Olson J. M. and Amesz J. (1986), 'Optical and structural properties of chlorosomes of the photosynthetic green sulfur bacterium *Chlorobium limicola*', *Biochim. Biophys. Acta* **848**, 77–82.

van Mourik F., Griebenow K., van Haeringen B., Holzwarth A. R. and van Grondelle R. (1990), 'Pigment organization in BChl *a*-free and BChl *a*-containing chlorosomes from *Chloroflexus aurantiacus*, studied by absorption dichroism', in *Current Research in Photosynthesis II*, Baltscheffsky M., ed., Kluwer Academic Publishers, Dordrecht, pp. 141–144.

van Rossum B.-J., van Duyl B. Y., Steensgaard D. B., Balaban T. S., Holzwarth A. R., Schaffner K. and de Groot H. J. M. (1998), 'Evidence from solid-state NMR correlation spectroscopy for two interstack arrangements in the chlorosome antenna system', in *Photosynthesis: Mechanism and Effects*, XI Int. Congr. Photosynthesis Budapest 1998, Garab G., ed., Kluwer Academic Publishers, Dordrecht, pp. 117–20.

van Rossum B.-J., Steensgaard D. B., Mulder F. M., Boender G.-J., Schaffner K., Holzwarth A. R. and de Groot H. J. M. (2001), 'A refined model of chlorosomal antennae of *Chlorobium tepidum* from proton chemical shift constraints obtained with high-field 2-D and 3-D MAS NMR dipolar correlation spectroscopy', *Biochem.* **40**, 1587–1595.

van Rossum B.-J., Schulten E. A. M., Raap J., Oschkinat H. and de Groot H. J. M. (2002), 'A 3-D structural model of solid self-assembled chlorophyll *a*/H_2O from multispin labeling and MAS NMR 2-D dipolar correlation spectroscopy in high magnetic field', *J. Magn. Reson.* **155**, 1–14.

van Walree C. A., Sakuragi Y., Steensgaard D. B., Bösinger C. S., Frigaard N.-U., Cox R. P., Holzwarth A. R. and Miller M. (1999), 'Effect of alkaline treatment on bacteriochlorophyll *a*, quinones, and energy transfer in chlorosomes from *Chlorobium tepidum* and *Chlorobium phaeobacteroides*', *Photochem. Photobiol.* **69**, 322–328.

Vasiliev S., Orth P., Zouni A., Owens T. G. and Bruce D. (2001), 'Excited-state dynamics in Photosystem II: insights from the X-ray crystal structure', *Proc. Nat. Acad. Sci.* **98**, 8602–8607.

Vassiliev S., Lee C.-I., Brudvig G. W. and Bruce D. (2002), 'Structure-based kinetic modeling of excited-state transfer and trapping in histidine-tagged Photosystem II core complexes from *Synechocystis*', *Biochem.* **41**, 12236–12243.

Visser H. M., Kleima F. J., van Stokkum I. H. M., van Grondelle R. and van Amerongen H. (1996), 'Probing the many energy-transfer processes in the photosynthetic light-harvesting complex II at 77 K using energy-selective sub-picosecond transient absorption spectroscopy', *Chem. Phys.* **210**, 297–312.

Vulto S. I. E., Streltsov A. M. and Aartsma T. J. (1997), 'Excited-state energy relaxation in the FMO complexes of the green bacterium *Prosthecochloris aestuarii* at low temperatures', *J. Phys. Chem. B* **101**, 4845–4850.

Vulto S. I. E., Neerken S., Louwe R. J. W., de Baat M. A., Amesz J. and Aartsma T. J. (1998), 'Excited-state structure and dynamics in FMO antenna complexes from photosynthetic green sulfur bacteria', *J. Phys. Chem. B* **102**, 10630–10635.

Vulto S. I. E., de Baat M. A., Neerken S., Nowak F. R., van Amerongen H., Amesz J. and Aartsma T. J. (1999), 'Excited-state dynamics in FMO antenna complexes from photosynthetic green sulfur bacteria: a kinetic model', *J. Phys. Chem. B* **103**, 8153–8161.

Walla P. J., Linden P. A., Hsu C.-P., Scholes G. D. and Fleming G. R. (2000a), 'Femtosecond dynamics of the forbidden carotenoid S_1 state in light-harvesting complexes of purple bacteria observed after two-photon excitation', *Proc. Nat. Acad. Sci. USA* **97**, 10808–10813.

Walla P. J., Yom J., Krueger B. P. and Fleming G. R. (2000b), 'Two-photon excitation spectrum of light-harvesting complex II and fluorescence upconversion after one- and two-photon excitation of the carotenoids', *J. Phys. Chem. B* **104**, 4799–4806.

Walla P. J., Linden P. A., Ohta K. and Fleming G. R. (2002), 'Excited-state kinetics of the carotenoid S_1 state in LHCII and two-photon excitation spectra of lutein and β-carotene in solution: efficient car $S_1 \rightarrow$ Chl electronic energy transfer via hot S_1 states?' *J. Phys. Chem. A* **106**, 1909–1916.

Wendling M., Pullerits T., Przyjalgowski M. A., Vulto S. I. E., Aartsma T. J., van Grondelle R. and van Amerongen H. (2000), 'Electron-vibrational coupling in the Fenna–Matthews–Olson complex of *Prosthecochloris aestuarii* determined by temperature-dependent absorption and fluorescence line-narrowing measurements', *J. Phys. Chem. B* **104**, 5825–5831.

Wendling M., Przyjalgowski M. A., Gullen D., Vulto S. I. E., Aartsma T. J., van Grondelle R. and van Amerongen H. (2002), 'The quantitative relationship between structure and polarized spectroscopy in the FMO complex of *Prosthecochloris aestuarii*: refining experiments and simulations', *Photosynth. Res.* **71**, 99–123.

Wildman R. and Bowen C. C. (1974), 'Phycobilisomes in blue-green algae', *J. Bacteriol.* **117**, 866–881.

Worcester D. L., Michalski T. J. and Katz J. J. (1986), 'Small-angle neutron scattering studies of chlorophyll micelles: models for bacterial antenna chlorophyll', *Proc. Nat. Acad. Sci. USA* **83**, 3791–3795.

Worcester D. L., Michalski T. J., Tyson R. L., Bowman M. K. and Katz J. J. (1989), 'Structure, red-shifted absorption and electron transport properties of specific aggregates of chlorophylls', *Physica B* **156** & **157**, 502–504.

Yang M. and Fleming G. R. (2002), 'Influence of phonons on exciton transfer dynamics: comparison of the Redfield, Förster, and modified Redfield equations', *Chem. Phys.* **275**, 355–372.

Zouni A., Witt H. T., Kern J., Fromme P., Krauβ N., Saenger W. and Orth P. (2001), 'Crystal structure of Photosystem II from *Synechococcus elongatus* at 3.8 Å resolution', *Nature* **409**, 739–743.

Zuber H. (1978), 'Studies on the structure of the light-harvesting pigment–protein complexes from cyanobacteria and red algae', *Ber. Deutsch. Bot. Ges.* **91**, 459–475.

This page intentionally left blank

CHAPTER 3

ELECTRON TRANSFER IN PHOTOSYNTHESIS

WINFRIED LEIBL and PAUL MATHIS
CEA Saclay, Service de Bioénergétique, 91191 Gif-sur-Yvette Cedex, France
Leibl@dsvidf.cea.fr; Mathis@dsvidf.cea.fr

Il faut souffler sur quelques lueurs pour faire de la bonne lumière
René Char, *Les Matinaux*, 1950.

Photosynthesis is the process by which autotrophic biological organisms make use of solar energy for the synthesis of their biomolecules. It has large-scale planetary consequences, such as the storage of fuels and the accumulation of oxygen in the atmosphere. On the molecular scale, all the initial steps of conversion of light energy into chemical energy are carried out in photosynthetic organisms by molecular energy converting proteins called reaction centres (RCs). Several classes of biological organisms perform photosynthesis; some photosynthetic bacteria are prokaryotic (lacking cell nuclei), while algae and higher plants are eukaryotic (possessing cell nuclei). Eukaryotic photosynthesisers fall into two groups, oxygenic (liberating oxygen during photosynthesis) and anoxygenic (liberating no oxygen). Oxygenic organisms (higher plants, algae and cyanobacteria) possess two types of reaction centre called Photosystem I and Photosystem II and often abbreviated as PSI and PSII, respectively. These work in series, allowing oxygenic organisms to use water as a source of electrons to reduce CO_2. Photosynthetic bacteria (purple non-sulphur bacteria, green sulphur bacteria *etc.*) are anoxygenic. These possess only one type of RC, either of the PSI or of the PSII type, and they use sulphur compounds such as H_2S or oxidisable organic molecules as their source of electrons. In other ways, for instance in size and ecological requirements, photosynthetic organisms can be widely different, but their reaction centres all function according to these principles.

To start the process of photosynthesis, pigment molecules must absorb light. These are arranged in some kind of array or antenna system that allows energy transfer, as described by Alfred Holzwarth in the previous chapter. The absorption of a photon by a pigment molecule creates an electronically excited state and excitation energy is transferred from pigment molecule to pigment molecule within the antenna until it reaches the RC, where (in most cases) it is trapped by a specialised pigment

dimer known as the 'special pair' and given the symbol P. Excitation of P initiates the primary photochemistry, which consists of electron transfer from $^1P^*$, the lowest electronically excited singlet state of P, to a primary acceptor, designated A_0. The first electron transfer step creates the initial radical pair ($P^+A_0^-$) which then initiates successive steps of electron transfer: electron transfer from A_0^- to other electron acceptors in the so-called 'acceptor side' of the reaction centre, and further electron transfer to the primary oxidant P^+ on the 'donor side' of the reaction centre.

Reaction centres are located in a lipid bilayer membrane, which makes up closed volumes (vesicles, cells, thylakoids) defining two distinct compartments. The outside is called the periplasmic phase and the inside the cytoplasmic phase (these correspond to the 'stroma' and 'lumen' in the chloroplasts of higher plants). All RCs are oriented transversely in the membrane, as shown in Fig. 3.1, functioning almost like microscopic photocells at a spatial scale of about 50 Å. Further steps in energy conversion involve the formation of more stable reduced species (NADPH or NADH) on the cytoplasmic side. The transfer of electrons from the periplasmic side to the cytoplasmic side, together with the pumping of protons (H^+) in the opposite direction creates a transmembrane electrochemical potential gradient with two components: an

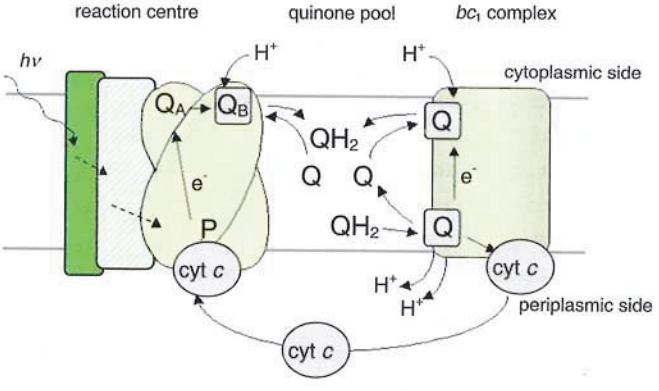


Figure 3.1 Schematic representation of the photosynthetic membrane and electron transfer in purple photosynthetic bacteria, showing the proteins and some of the redox cofactors participating in energy collection, electron transfer and proton transfer. P is the primary electron donor; Q_A, Q_B, Q are quinones and cyt *c* is cytochrome *c*. The antenna proteins absorb light and transfer energy to the RC. The RC ensures electron transfer from a soluble *c*-type cytochrome on the periplasmic side of the membrane, to a quinone acceptor Q_A, which passes its electron to a second quinone, Q_B. In the course of reduction, Q_B picks up two protons on the cytoplasmic side and is then transferred into a pool of many quinone molecules in the membrane. The cycle of electron transfer is completed by the bc_1 complex, where cyt *c* is re-reduced and quinone reoxidised, with concomitant discharge of protons on the perisplasmic side.

electrical part associated with the transmembrane electron transfer and a chemical part arising from the difference in proton concentration on the two sides of the membrane. These two components cooperate in driving the synthesis of ATP from ADP and inorganic phosphate in a membrane protein complex that acts as an ATP-synthase (Mitchell, 1966). Thus the membrane reactions of photosynthesis result in the formation of ATP and NAD(P)H, both of which are required for the incorporation of CO_2 into sugars, the first stable chemical molecules resulting from CO_2 fixation.

The above brief and simplified description of photosynthesis serves to introduce the two themes that underlie this chapter: the complexity of photosynthetic reactions and the central role played by electron transfer, mediated by various redox centres, in the whole sequence of reactions. A wide range of redox entities are involved in mediating electron transfer. These include organic molecules such as quinones, chlorophylls, pheophytins and $NAD(P)^+$ that are more or less tightly bound to a protein, redox-active protein-bound metal ions such as copper ions in plastocyanin and manganese ions in the water-oxidising site of PSII, redox cofactors such as iron–sulphur proteins or *c* cytochromes that are covalently bound to a protein, and even some specific amino acid residues in a protein, such as tyrosines in PSII. Some of these redox centres exchange only electrons while others, especially quinones and $NAD(P)^+$, exchange both electrons and protons, allowing them to couple proton and electron transfer across the membrane.

3.1 Biological electron transfer

Electron-transfer reactions play important roles not only in bioenergetic processes such as photosynthesis and respiration, but also in many other biological processes such as nitrogen metabolism, metabolism of xenobiotics and production of O_2^- in cell defence. At the molecular level, two different situations are encountered:

- electron transfer between different redox centres within a single large protein which includes several such centres. In photosynthesis, this is the case for reaction centres and for cytochrome *bc* complexes. The redox centres have well-defined positions and electrons flow from one to another according to their redox potentials and spatial relationships. A similar situation is encountered in nitrate reductase, hydrogenase, nitrogenase and cytochrome *c* oxidase. Many of these large proteins are membrane-bound.
- electron transfer between a redox centre of a large multi-centre complex and a small partner which diffuses in the medium and transiently docks to the large

complex in order to make electron transfer possible. In this case, electron transfer is determined not only by the properties of the redox-active partners in the transient complex, but also by their concentrations and by properties of the medium such as viscosity and ionic composition. The mechanism of formation of the transient complex and a number of other factors are also important. In photosynthesis, this type of electron transfer occurs between reaction centres or cytochrome *bc* complexes and their various redox-active partners, which may be hydrophilic proteins (cytochrome c_2 or c_6, plastocyanin, ferredoxin, flavodoxin) or hydrophobic quinones. Figure 3.1 illustrates the interplay of multi-centre proteins and diffusible molecules, both inside and outside the membrane, in purple photosynthetic bacteria. Quinones diffuse in the membrane when they are either fully oxidised or fully reduced, carrying electrons from the reaction centre to the *bc* complex and carrying protons from one side of the membrane to the other. Cytochrome *c* diffuses in the solvent of the periplasmic side, bringing back electrons to the RC. Reduction of $NADP^+$ by ferredoxin-$NADP^+$ reductase and oxidation of water by the PSII RC are also cases where one of the redox partners is a small molecule, not a protein.

3.1.1 Energetics and kinetics of electron transfer

Electron flow from one redox centre to another is governed by a molecular thermodynamic parameter known as the *reduction–oxidation potential* or *redox potential.* This term has two meanings. For a medium in thermodynamic equilibrium, the redox potential is a macroscopic electrical property, designated E_h, measured in volts by reference to a reference electrode, normally the standard hydrogen electrode (SHE). The redox potential is also a thermodynamic property of a redox couple (Ox, Red) which measures its tendency to accept or donate n electrons: $Ox + ne^- \leftrightharpoons Red$, according to the Nernst equation:

$$E_h = E_h^\circ + \frac{RT}{nF} \ln \frac{[Ox]}{[Red]} \tag{3.1}$$

where E_h° is the redox potential of the couple measured under standard thermodynamic conditions with respect to the SHE. In practical cases, E_h° is often replaced by the midpoint potential E_m, measured in a non-standard state. E_m is obtained by a redox titration in which E_h is measured as the concentrations of Ox and Red are

varied. When protons are also exchanged in the reaction, for example in the reaction $A + H^+ + e^- \leftrightharpoons AH$, the Nernst equation can be written

$$E_h = E_{m,0} + \frac{RT}{F} \ln \frac{[A][H^+]}{[AH]} \tag{3.2}$$

where $E_{m,0}$ is the midpoint potential at standard conditions. At $T = 302$ K, this equation becomes

$$\begin{aligned} E_h &= E_{m,0} + 0.06 \log \frac{[A]}{[AH]} + 0.060 \log [H^+] \\ &= E_m - 0.06\,\mathrm{pH} \end{aligned} \tag{3.3}$$

Thus increasing the pH lowers the midpoint potential (reached when [A] = [AH]) of a one-electron redox couple by ~60 mV per pH unit.

If electrons are exchanged between the redox couples A/A^- (acceptor) and D/D^+ (donor) under conditions where there is no exchange with the external medium or with other redox couples, the position of equilibrium is given by the equations

$$E^o_A + \frac{RT}{F} \ln \frac{[A]}{[A^-]} = E^o_D + \frac{RT}{F} \ln \frac{[D^+]}{[D]} \tag{3.4}$$

and

$$E^o_A - E^o_D - \frac{RT}{F} \ln \frac{[D][A]}{[D^+][A^-]} = -\frac{RT}{F} \ln K = \frac{\Delta G^\circ}{F} \tag{3.5}$$

where E^o_A and E^o_D are the standard redox potentials of the two couples $A + e^- \leftrightharpoons A^-$ and $D^+ + e^- \leftrightharpoons D$, K is the equilibrium constant of the reaction $D + A \leftrightharpoons D^+ + A^-$, and ΔG° is the standard Gibbs free energy change of this reaction.

These equations show how the free energy change and the equilibrium state of a redox reaction can be calculated on the basis of measured midpoint potentials, but it should be kept in mind that the midpoint potentials depend on the dielectric environment. When the redox potentials of the donor and acceptor are measured *in situ*, they already contain the influence of the surrounding medium, in particular electrostatic interactions with other charged or polarisable groups. However, they may not take the electrostatic interaction between the charged states of the donor and acceptor themselves fully into account. In the above example, coulombic interaction between the opposite charges leads to stabilisation of the product state D^+A^-, to an

extent that depends on the effective dielectric constant and how far apart the redox centres are. Another example is charge-shift reactions of the type $D^-A \rightarrow DA^-$, where the midpoint potential of the D/D^- couple can only be measured when A is reduced. The negative charge on A, however, shifts $E_m(D/D^-)$ to lower values, *i.e.* destabilises the state D^-. These interaction potentials can often be neglected but they may be important for electron transfer between cofactors which possess similar redox potentials and which are relatively close to each other and sited in a low dielectric environment.

Kinetics are another important aspect of electron transfer. As we shall see, photosynthetic reaction centres are constructed to exhibit kinetic properties that are the origin of their efficiency. According to a classical treatment, the rate constant k_{ET} of non-adiabatic electron transfer[1] from D to A is given by

$$k_{ET} = \frac{4\pi^2}{h} V_r^2 FC \tag{3.6}$$

where V_r^2 is the electronic term

$$V_r^2 = V_o^2 \exp(-\beta r) \tag{3.7}$$

and V_o^2 is the electronic coupling between D and A when $r = 0$, r is the edge-to-edge distance between D and A, and β is the coefficient of decay of electronic coupling with distance. FC in eq. 3.6 is the Franck–Condon factor, given by

$$FC = (4\pi\lambda kT)^{-1/2} \exp[-(\Delta G^\circ + \lambda)^2 / 4\lambda kT] \tag{3.8}$$

where λ is the reorganisation energy, and ΔG° is the standard Gibbs free energy change of the electron-transfer reaction from eq. 3.5, neglecting electrostatic interactions between donor and acceptor.

Equations 3.6–3.8 are very helpful in understanding some features of reaction centre structure–function relationships. We shall return to this point in Section 3.4 with a more detailed description of the theory aspects of electron transfer in photosynthesis.

[1] Non-adiabatic electron transfer occurs when D and A are sufficiently far apart to be only very weakly coupled.

3.2 Electron transfer in anoxygenic photosynthesis

The photosynthetic apparatus of anoxygenic organisms is less complex and better characterised than that of oxygenic ones. The discovery of structural themes common to all types of reaction centres and the determination in the mid-1980s of the structure of the reaction centre of purple photosynthetic bacteria to atomic resolution has established this RC as a model for all other types of photosynthetic reaction centres, and in particular as a model for the RC of Photosystem II in oxygenic photosynthetic systems (Nitschke and Rutherford, 1991; Rutherford and Nitschke, 1996).

3.2.1 The electron-transfer chain in anoxygenic photosynthetic systems

The elementary unit of the electron transfer chain of purple photosynthetic bacteria (shown in Fig. 3.1) contains two major membrane-bound enzyme complexes, the reaction centre with its associated antenna proteins (light-harvesting complex) and the bc_1 complex. From an enzymatic point of view, the RC is a light-driven cytochrome oxidase/quinone reductase and the bc_1 complex a quinol oxidase/cytochrome reductase. Lipophilic molecules (quinones) and water-soluble proteins (cytochrome *c*) shuttle as electron carriers between the two large membrane complexes to close the electron transfer cycle. The vectorial orientation of the proteins in the membrane, with the site of quinone reduction close to the cytoplasmic phase and the site of quinol oxidation close to the periplasmic phase, gives rise to a net transfer of protons across the membrane and thereby the reaction cycle builds up the electrochemical potential driving force for ATP synthesis.

3.2.2 The reaction centre of purple photosynthetic bacteria

As outlined above, cyclic electron transfer in purple photosynthetic bacteria requires electrons transfer from cytochrome *c* ($E_m \approx +340$ mV) to ubiquinone ($E_m \approx 0$ mV), as shown in the lefthand diagram of Fig. 3.2. This thermodynamically uphill reaction is driven by the energy of one photon. To accomplish this task, specialised RC complexes of ~100 kDa molecular mass, containing about one thousand amino acids and 8–12 cofactors, have evolved. The isolation and purification procedures for the RC protein complexes of several purple photosynthetic bacterial species have long been established (Reed and Clayton, 1968; Feher 1971; Feher and Okamura, 1978) and this has allowed their structure and function to be studied in detail. Furthermore,

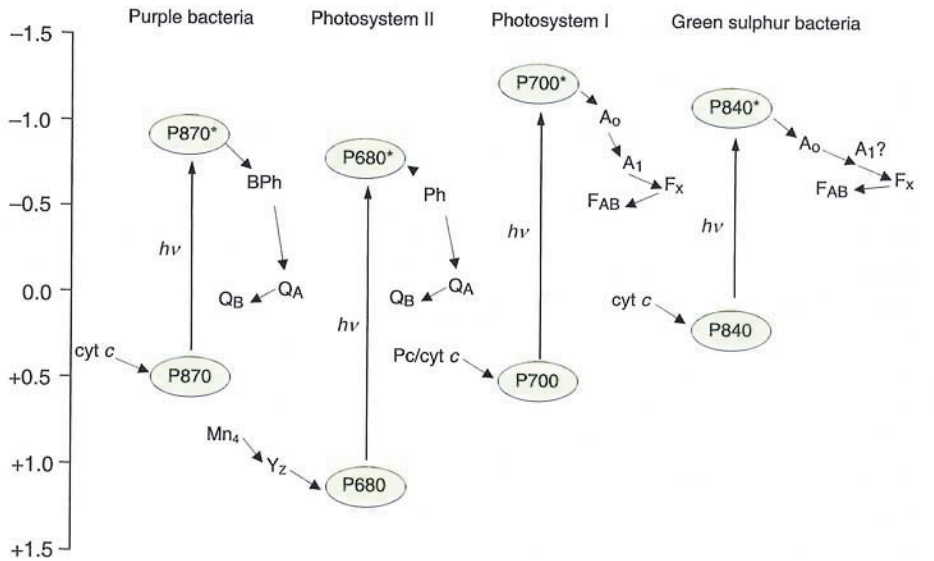


Figure 3.2 Sequence of redox centres participating in electron transfer in the four main classes of photosynthetic reaction centre. The redox centres are placed at a vertical position that corresponds to their mid-point redox potentials. Light ($h\nu$) allows upward endergonic reactions while spontaneous (exergonic) reactions go downwards.

these isolated photosynthetic reaction centres have the considerable advantage that their reaction sequence is naturally triggered by light, allowing kinetic studies down to femtosecond time resolution. As a consequence, a large body of experimental data is available and the function of the RC of purple photosynthetic bacteria is now understood in great detail (Hoff and Deisenhofer, 1997).

The structure of the reaction centre The determination of the three-dimensional structure of the reaction centre of the purple photosynthetic bacteria *Rp. viridis* and *Rb. sphaeroides* provided the first atomic view of a membrane protein (Deisenhofer *et al.*, 1984; Michel *et al.*, 1986; Feher *et al.*, 1989; Ermler *et al.*, 1994). Essentially this confirmed the overall models made on the basis of functional studies, but it also brought a lot of new and astonishing insights. The architecture of the RC, shown in Fig. 3.3 for *Rp. viridis*, is very similar in these two purple photosynthetic bacterial species. Two large protein subunits (L and M) form the core of the RC. Both subunits possess five transmembrane helices and the high degree of homology of their amino acid sequences is reflected in their similar architecture in the membrane. The L and M subunits form a symmetric X-shaped protein heterodimer and are related by a two-

fold symmetry axis perpendicular to the plane of the membrane. A third subunit, H, has only one membrane-spanning α-helical segment, but it nearly completely covers the plasmic side of the core complex.

The central subunits L and M bind non-covalently a number of cofactors, namely four bacteriochlorophylls BChl (P, which is a dimer; B_L, B_M), two bacteriopheophytins BPh (H_L, H_M), two quinones (Q_A, Q_B), one non-haem iron Fe, and one carotenoid (not shown in Fig. 3.3). The approximate two-fold symmetry of the protein subunits extends to these cofactors. From the periplasmic side, where a pair of two BChl molecules form the 'special pair' P, two chains extend to the cytoplasmic side, each composed of one BChl, one BPh and one quinone. The non-haem iron is situated between the two quinones and on the C_2 symmetry axis, which also runs through the special pair. On its periplasmic side, the RC of *Rp. viridis* contains an additional subunit, the tetrahaem cytochrome complex cyt, with four haems organised in a linear arrangement (see Fig. 3.6 for greater detail). This tightly bound cytochrome subunit is missing in *Rb. sphaeroides*, where a soluble *c*-type cytochrome bound electrostatically at a similar position plays the same role.

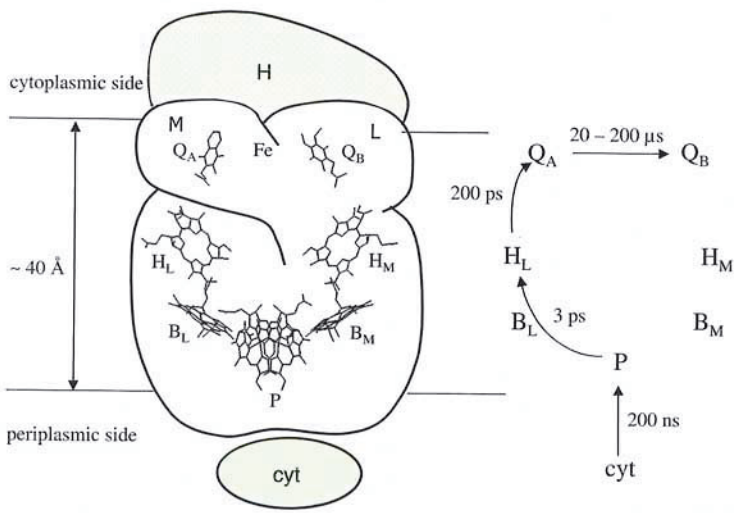


Figure 3.3 Structure of the RC of the purple bacterium *Rp. viridis* (left), showing the rough shape of the three polypeptides (L, M, H), as well as the pigments and redox cofactors, the special pair of BChl (P), two BChl (B_L, B_M), two BPh (H_L, H_M), two quinones (Q_A, Q_B), and the non-haem iron Fe. The cytochrome is shown in a more realistic manner in Fig. 3.6. The scheme on the right shows the primary electron transfer steps and rates at room temperature.

Trapping of excitation energy by primary charge separation The sequence of photochemical reactions starts after an exciton in the antenna has been transferred to the special pair of BChl in the reaction centre, exciting it to the singlet state P^*. The absorption maximum of P is red-shifted compared with monomeric BChl because of the strong interaction of the two pigment molecules in the dimer. This red shift corresponds to a lower excitation energy for P^*, which favours the localisation of the excitation from the antenna onto P. P^* is a reactive, unstable state which can decay by several mechanisms, including back transfer of the excitation to the antenna or wasteful decay to the ground state, with or without fluorescence emission. However, the redox potential of the state P^* is sufficiently low to make it a strong reductant, able to transfer an electron very quickly to a nearby electron acceptor. The primary photochemical event in photosynthesis is therefore a very fast charge separation, the transfer of an electron from the excited primary donor P to one of the BPh molecules (H_L). The 'trapping' of the excitation energy is usually defined by formation of the primary radical pair $P^+H_L^-$. In isolated RCs, this occurs within about 3 ps after excitation (Martin *et al.*, 1986; Schmidt *et al.*, 1993; Holzwarth and Müller, 1996). In intact systems, where the RC is coupled to a light-harvesting complex, equilibration of the excitation in the antenna and transfer to the RC slow down the apparent time constant of trapping to 20–60 ps.

Two aspects of the primary charge-separation step are not fully understood. The first concerns the role of the monomeric BChl molecule B_L located between P and H_L (Fig. 3.3). There is agreement among researchers that this BChl is involved in the electron transfer, but there is disagreement concerning the precise mechanism. This so-called 'accessory' BChl could promote electron transfer from P to H_L by means of the virtual state P^+BChl^- in a 'superexchange' mechanism (Bixon *et al.*, 1991). Alternatively, it was proposed to be the real primary acceptor, the transient concentration of the $BChl^-$ anion being low because its reoxidation (by electron transfer to H_L) is faster than its reduction by P^* (0.9 ps vs. 3.6 ps; Holzapfel *et al.*, 1989; Arlt *et al.*, 1993). Besides these two extreme models, superexchange and sequential transfer, alternative theoretical models have been developed to describe electron transfer mediated by a midway molecule as a unified single process (Sumi, 1997; Sumi and Kakitani, 2001).

The second intriguing aspect of the primary charge-separation step is that the remarkable C_2 symmetry of redox cofactors apparent in the structure is not found in the function: charge separation occurs essentially along only one of the two branches, the one which is held mainly by subunit L. No significant reduction of H_M is observed in native RCs but up to 40% M side electron transfer has been observed in mutants that alter the envirenment or the chemical identity of the cofactors (Heller *et al.*, 1996;

Kirmaier *et al.*, 1999; Katilius *et al.*, 1999). The protein is presumably responsible for this asymmetric function. It is still unclear exactly what the origin of this asymmetry is. It might be small differences in distances between cofactors, different distribution of charged amino acids, asymmetry in the structure of P or a number of other factors (Michel-Beyerle *et al.*, 1988; Parson *et al.*, 1990; Steffen *et al.*, 1994; Ivashin *et al.*, 1998; Hasegawa and Nakatsuji, 1998). However, a possible rationale of the need for unidirectional electron transfer may be the very different function of the two quinones Q_A and Q_B (see below).

Stabilisation of the charge-separated state In the primary radical pair state $P^+H_L^-$, the charges have been separated by 17 Å within 3 ps. Under normal conditions the electron is then transferred from H_L to the quinone Q_A in 200 ps (Kirmaier and Holten, 1987; Williams *et al.*, 1992). Unlike the primary radical pair, the state $P^+Q_A^-$ is stable for milliseconds, whereas the former would decay to the ground state or the triplet state in about 10 ns, as has been shown by kinetic measurements when forward electron transfer is blocked by chemical reduction or removal of Q_A. As electron transfer to Q_A is connected with a slowing down of the back reaction by five orders of magnitude, this reaction is the major charge-stabilisation step. Further stabilisation is achieved by reduction of P^+ by a secondary donor (cytochrome) on a microsecond time scale. As shown in Fig. 3.2 for the example of purple photosynthetic bacteria, each step is accompanied by a decrease of the amount of stored energy. The following scheme summarises the sequence of primary reactions (the time constants are for *Rp. viridis* at room temperature):

$$P\,B_L\,H_L\,Q_A \xrightarrow{h\nu} P^*\,B_L\,H_L\,Q_A \xrightarrow{3.5\ ps} P^+\,B_L^-\,H_L\,Q \xrightarrow{0.65\ ps} P^+\,B_L\,H_L^-\,Q_A \xrightarrow{200\ ps} P^+\,B_L\,H_L\,Q_A^-$$

It is a remarkable feature of the photosynthetic RC that the rate of the primary electron transfer reactions from P to Q_A are essentially temperature-independent and the kinetics of primary charge separation even speed up at very low temperatures. With a quantum yield close to unity, about 30% of the energy of an absorbed red photon is converted within 1 ns into the charge pair $P^+\,Q_A^-$ separated by about 30 Å. The state $P^+\,Q_A^-$ is stable for milliseconds, long enough for efficient coupling to slower, chemical reactions that are often limited by diffusion and binding of reactants. In this context it is worth recalling that even in bright sunlight only a few photons are absorbed per second per RC.

Interesting events take place when electron transfer from H_L to Q_A is made impossible, either because Q_A is missing as a result of an artificial treatment, or because it is reduced by chemical redox poising or as a result of a previous charge

separation. The radical pair $P^+H_L^-$ then lasts for about 10 ns, instead of 200 ps when Q_A is functioning, and it decays by charge recombination. The radical pair, being created from a singlet excited state, initially has a singlet character, *i. e.*, the two unpaired electrons on the radicals H_L^- and P^+ remain coupled with antiparallel spins. The radical pair then oscillates, at a frequency of $\sim 10^8\ s^{-1}$, between the singlet state (with antiparallel spins) and a triplet state (with parallel spins). When the charges recombine, the radical pair returns to a neutral state, either singlet or triplet according to the spin state of the pair (Fig. 3.4). The singlet state is either P* or a vibrationally excited ground state of P. The triplet state is ^3P. This has three nearly degenerate energy levels, T_{+1}, T_0 and T_{-1}, of which practically only T_0 is populated because the singlet and the T_0 states of the radical pair are closest in energy. This situation is very far from Boltzmann equilibrium. In EPR experiments, where a magnetic field of about 3000 Gauss is applied, the energy differences between triplet sublevels are increased and the non-Boltzmann distribution can be maintained for milliseconds at low temperature. This situation is reflected in the EPR spectra as a very intense signal with a specific shape (shown in Fig. 3.13 for a similar state in PSII). This is the signature of a triplet state arising from a radical-pair recombination (Budil and Thurnauer, 1991; Volk *et al.*, 1995).

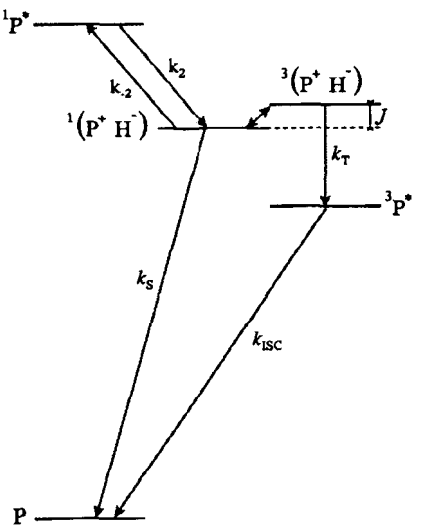


Figure 3.4 Energy levels of the states involved in primary reactions, with corresponding rates in RC where forward electron transfer is blocked. The radical pair P^+H^- can have two states, singlet or triplet, which are separated by the exchange interaction J (the relative size of this is exaggerated by a factor of about 10^6 compared to the energy of other states). k_S and k_T are rate constants for the radical pair to recombine to the ground state or the triplet state of P, respectively.

From Q_A to Q_B : the two-electron gate The function of Q_A in charge stabilisation implies that it is always available when a charge separation occurs, and therefore that it is firmly bound. The function of the second quinone Q_B is very different: it has to accept two electrons successively from Q_A as well as two protons from the cytoplasmic phase before leaving its binding site and diffusing as a neutral quinol in the membrane to the bc_1 complex, where it is reoxidised. The quinone acceptor complex of purple photosynthetic bacteria thus operates as a two-electron gate, requiring electrons to be exported in packets of two. The binding affinities of the RC protein for the different redox states of Q_B are nicely tuned to the functional requirements: the oxidised and doubly reduced states (Q_B and Q_BH_2) are weakly bound and the semiquinone state Q_B^- is strongly bound. But the role of the protein goes even further: it also modifies the effective midpoint potential of the quinones and provides a proton pathway from the RC surface to the Q_B site. The first function is most clearly seen in *Rb. sphaeroides*, where quinones Q_A and Q_B are the same chemical species, ubiquinone (Q_A is a menaquinone in *Rp. viridis*), but with E_m of –20 mV and +40 mV respectively for reduction to the semiquinone form. The midpoint potential of the ubiquinone pool is about +90 mV. To permit efficient double reduction of Q_B by electron flow from Q_A^-, the midpoint potential of the first *and* second reduction steps of Q_B must be higher than that of Q_A/Q_A^-, which indeed is the case. It is not completely established what the main factors controlling the redox potentials of the quinones are. Certainly the accessibility of Q_A and Q_B to protons plays an important role. It is also significant that the protein environment of Q_A is more aprotic than that of Q_B. Other possible factors include a difference in the strengths of the hydrogen bonds to the carbonyl oxygens of the quinones, conformational constraints, especially on the methoxy groups of the quinones, and electrostatic interactions with charged amino acids near the binding site or with the non-haem Fe^{2+} ion located between Q_A and Q_B. The unusually small difference between the midpoint potentials for the first and second electron reduction of Q_B contrasts strongly with the behaviour of quinones both in aqueous solution and in aprotic solvents, and highlights the role of the protein as an optimised solvent (see Trumpower, 1982 for a review of quinone chemistry *in vitro* and *in vivo*).

The sequence of electron and proton transfers to Q_B leading to the formation of the fully reduced quinol Q_BH_2 is not completely established but a rather detailed model has emerged (Okamura and Feher, 1995; Okamura *et al.*, 2000). The first electron transfer to Q_B ($Q_A^-Q_B \rightarrow Q_AQ_B^-$) is virtually independent of the driving force of the reaction, as assayed by replacing the native ubiquinone in the Q_A site by quinones having different redox potentials (Graige *et al.*, 1998). However, this reaction is not activationless and it is therefore thought that the electron transfer is rate-limited by a

conformational gating step. Stowell *et al.* (1997) proposed that this gating step involves movement of Q_B from a distal to a proximal binding site, only the latter being competent in electron transfer. Another possibility is that stabilisation of the semiquinone state of Q_B requires proton rearrangement near the Q_B binding site. It has been shown that the reaction centre takes up protons from the medium when Q_A^- and Q_B^- are formed. However, their absorption spectra indicate that Q_A^- and Q_B^- are not protonated in this state. It therefore seems that protons are taken up by the *protein*, rather than the quinones themselves, in response to the formation of Q_A^- or Q_B^-. This uptake is attributed to pK shifts of protonatable amino acid residues induced by coulombic interaction with the negative charge on the semiquinones (see Fig. 3.3b for an example). This interpretation explains the observed behaviour of the proton uptake: it is not stoichiometric (only $0.4H^+$ is taken up at pH 7, for both $Q_A \rightarrow Q_A^-$ and $Q_B \rightarrow Q_B^-$) and it is pH-dependent. Such 'electrostatic' protons contrast with 'chemical' protons, the uptake or release of which is the result of a chemical reaction involving protons in stoichiometric amounts. For Q_B then to be doubly protonated after transfer of the second electron, two protons must be supplied. However, the protein might already have taken up a certain fraction on the first reduction step, so that only the complementary fraction appears as proton uptake from the medium accompanying the second electron transfer. Different protonation states of the protein as a function of pH also affect the equilibrium constant for the reaction $Q_A^-Q_B \rightarrow Q_AQ_B^-$ which is shifted to the left at high pH.

The second electron transfer to Q_B ($Q_A^-Q_B^- \rightarrow Q_AQ_B^{2-}$) displays a strong pH-dependence, indicating that it is intimately related to proton uptake (Kleinfeld *et al.*, 1985; Leibl and Breton, 1991). This means that stabilisation of a second electron on Q_B is not possible without concomitant protonation and that the transfers of the second electron and the first proton to Q_B are strongly coupled. Two mechanisms are considered possible. Either electron transfer precedes protonation (intermediate Q_B^{2-}) or protonation precedes electron transfer (intermediate $Q_BH^{\bullet}$). In both cases the intermediate state is unstable. Presently the second of the described possibilities is favoured (Graige *et al.*, 1996). The high pK of the doubly reduced, singly protonated state of the quinone Q_BH^- provides the driving force for the second protonation of Q_B to form Q_BH_2.

With the structure of the purple photosynthetic bacterial RC resolved, this protein has become an important target for modification by site-directed mutagenesis. This technique, which is very useful in studying the relationships between protein structure and function, involves well-defined modifications of a protein resulting from specific manipulations of the gene coding for that protein. A large number of mutations in the Q_B binding site have been constructed and the functional characterisation of these

mutants has contributed significantly to the current understanding of the role of the protein environment, especially with respect to proton transfer to Q_B. Unlike electrons, protons cannot be transferred over distances larger than a few Ångstroms in a vacuum in the microsecond time domain. For protons to travel from the surface to a site buried in the interior of a protein, a proton-conductive pathway must exist, presumably a sequence of protonatable amino acids, hydrogen bonds or internal water molecules. From the reaction centre structure it is evident that the protein around Q_B is significantly more polar and contains more protonatable groups than that around Q_A. Site-directed mutagenesis has identified several residues, shown in Fig. 3.5a, which are essential for proton delivery to Q_B, although it is not always clear whether these residues are directly involved in proton transfer. The essential residues include Ser L223 and Glu L212, which are conserved in all purple photosynthetic bacteria. The replacement of these residues by the non-protonatable residues Ala or Gln lowers the rates of first and second proton transfers by two and three orders of magnitude, respectively. Besides these residues, which are in close contact with Q_B, many other mutations affect proton transfer, and the importance of clusters of interacting charged amino acid side chains, such as Asp L210, Asp L213 and Glu L212 in Fig. 3.5a, is acknowledged. These clusters of ionised residues show complicated pH titration behaviour but evidently provide an electrostatic potential favouring proton transfer. Finally, Lancaster and Michel (1997) have shown that internal water (located inside the protein) is likely also to play a role in proton transfer to Q_B.

The weak binding of the quinone at the Q_B site is the basis for the action of a group of inhibitors (herbicides) containing small molecules of the triazine or urea class. These have a higher affinity for the Q_B site than ubiquinone and can competitively replace the latter, thereby inhibiting photosynthetic electron transfer. Due to the strong overlap of the binding site for Q_B and these inhibitors, it is not surprising that spontaneously occurring mutations, which lead to herbicide resistance, also affect the binding of quinones or the rate of proton transfer.

Re-reduction of P^+ by cytochrome In order to complete the cycle of electron flow, P^+ has to be re-reduced by electrons coming from the bc_1 complex, on the periplasmic side of the RC. In purple photosynthetic bacteria, this is undertaken by a *c*-type cytochrome, but two different situations are found, as shown in Fig. 3.6 (Tiede and Dutton, 1993; Meyer and Donohue, 1995; Nitschke and Dracheva, 1995): a) P^+ is reduced directly by a monohaem, soluble cytochrome (cytochrome c_2), which docks transiently to the bc_1 complex, is reduced, diffuses to the RC where it docks, transfers an electron to P^+, unbinds, *etc.* This situation is found in a minority of species, such as *Rb. sphaeroides*; b) P^+ is reduced by a tetrahaem cytochrome which is permanently

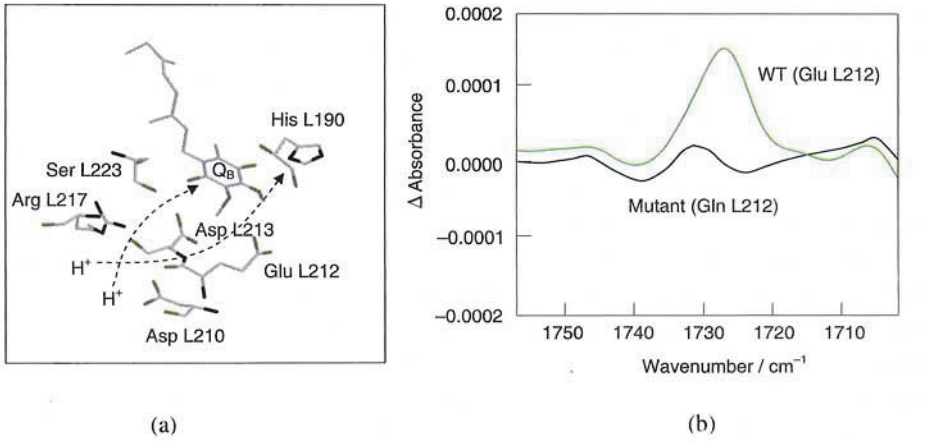


Figure 3.5 (a) Part of the Q_B binding pocket of *Rb. sphaeroides* as determined by X-ray crystallography (Yeates *et al.*, 1987) showing several protonatable amino acid residues that have been identified to be involved in proton transfer to Q_B. Arrows indicate proposed proton transfer pathways from a surface exposed region to the two carbonyl groups of the quinone. A considerable number of water molecules have been found near Q_B in recent X-ray structures (not shown here; see Lancaster and Michel, 1997); (b) Light-induced FTIR difference spectrum due to the transition $Q_B \rightarrow Q_B^-$ in the region of absorption of protonated carboxylic groups (Nabedryk *et al.*, 1995). An absorption band appearing on Q_B^- formation in WT is absent in a mutant where Glu L212 was replaced by a non-protonatable residue (Gln). This band is attributed to the protonated carboxylic side chain of Glu L212. The most direct interpretation is a (partial) protonation of Glu L212 due to a pK shift induced by the charge on Q_B^-.

bound to the RC. This situation occurs in many species, the best known of which is *Rp. viridis*. Another protein is required for shuttling electrons from the bc_1 complex to the tetrahaem cytochrome. This function can be fulfilled by *c*-type cytochromes (*e.g.* cytochrome c_2) or iron–sulphur proteins (HiPIP, high-potential iron–sulphur proteins).

Reduction of P^+ by cytochrome c_2 has been studied *in vitro* in isolated RC by flash absorption spectroscopy (Tiede *et al.*, 1993; Venturoli *et al.*, 1993; Tetreault *et al.*, 2001). Cytochrome c_2 is a small monohaem cytochrome with a MW of 9.0 kDa and a midpoint potential of around +340 mV. It is very soluble in water, but it also forms complexes with RC. Electron transfer to P^+ in the cytochrome c_2-RC complex has a half-time of about 1 μs. Association of these partners is strongly disfavoured by addition of monovalent ions such as Na^+, showing that it arises mainly from electrostatic interactions. In *Rb. sphaeroides*, the periplasmic face of the RC in the neighbourhood of P includes several carboxylic amino acids residues (aspartic and glutamic acids) which are negatively charged and are thought to interact in the complex through coulombic coupling with lysine residues located at the periphery of cyt c_2, close to the haem group. Several docking models have been proposed, which

take into consideration more or less localised electric charge interactions. The cytochrome c_2-RC complex has also been crystallised, and recently an improved structural model has been proposed on the basis of X-ray diffraction data with a resolution of 2.4 Å (Adir *et al.*, 1996; Axelrod *et al.*, 2002).

For the reduction of P^+ by a tetrahaemic cytochrome *c*, *Rp. viridis* offers an excellent model, for both structural and functional aspects. The cytochrome is an integral subunit of the RC, which cannot be dissociated from it. It has a MW of 40 kDa, and its N-terminal amino acid, a cysteine, is linked by a thioether bond to a diglyceride which ensures its anchoring in the RC. The arrangement of the haems is roughly linear (Deisenhofer and Michel, 1989), as shown in Fig. 3.6. There is a pair of high-potential haems (*c-559*, E_m = +380 mV; *c-556*, E_m = +310 mV) and a pair of low-potential ones (*c-552*, E_m = +10 mV; *c-554*, E_m = –60 mV), which are located as shown in Fig. 3.6, in such a way that their redox potentials alternate high – low – high – low. The reason for this alternation is still not understood. It is assumed that cyt c_2 docks close to the tetrahaem cytochrome *c-556*, to which it donates an electron. The physiological electron transfer path is therefore: P^+ is reduced by haem *c-559* in about 200 ns, and *c-559* is re-reduced by *c-556* in about 2 μs via *c-552*. The uphill electron transfer step from *c-556* to *c-552* has been proposed to be the rate-limiting step in this reaction (Chen *et al.*, 2000). The subsequent step is the reduction of oxidised *c-556* by cytochrome c_2 in about 100 μs. Within our present knowledge, no function has been attributed to the low-potential haems. It seems, however, that a large majority of purple photosynthetic bacteria have a bound tetrahaem cytochrome, and that the four

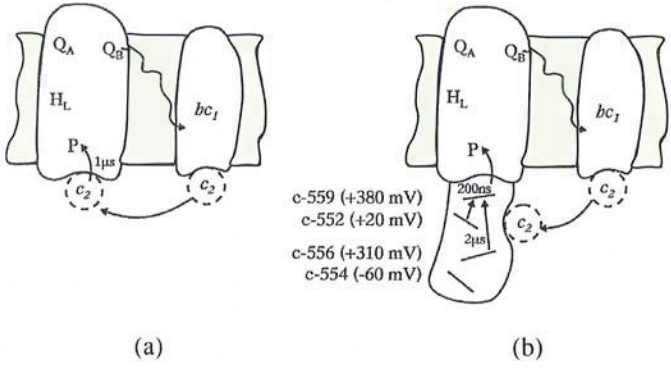


Figure 3.6 Cyclic electron transfer path in two species of purple photosynthetic bacteria: (a) *Rb. sphaeroides*, where the soluble cytochrome c_2 docks to the RC and delivers an electron directly to P^+; (b) *Rp. viridis*, where cytochrome c_2 docks to the tetrahaem cytochrome which feeds the electron to P^+. Also shown are the half-times of a few electron transfer steps, and the midpoint potentials of the four haems (each haem is designated by the wavelength of its alpha absorption band).

haems are always grouped in two high-potential and two low-potential ones. This general property should have a biological reason. From a theoretical point of view it has been shown that electron tunnelling through a high-energy intermediate state like *c-552* is possible due to the proximity of the redox centers (Page *et al.*, 1999).

The donor–acceptor couple cytochrome *c* (monohaem or tetrahaem)/P^+ is an interesting system for an in-depth study of electron transfer in proteins. Two parameters, temperature and driving force $\Delta G°$, have been studied in detail. The effect of temperature on the rate of electron transfer to P^+ from the proximal haem of tetrahaem cytochromes and from docked cytochrome c_2 has been studied thoroughly by flash absorption spectroscopy (Ortega and Mathis, 1993; Ortega *et al.*, 1998). When temperature is decreased, the rate varies very little, by at most a factor of ten. Electron transfer, however, becomes blocked rather suddenly at temperatures between 240 and 100 K, according to the nature or the redox state of the complex. The blockage occurs for unknown reasons. It cannot be attributed to a change in the respective positions of P^+ and of the haem, which would be induced by the cooling. The effect of changing the free energy $\Delta G°$ is much better understood in terms of electron-transfer theory. This has been studied for electron transfer from cytochrome c_2 to P^+ in the RC of *Rb. sphaeroides* in which the redox potential of the P/P^+ couple was varied by site-directed mutagenesis (Lin *et al.*, 1994; Venturoli *et al.*, 1998). The redox potential of P/P^+ is strongly influenced by the number of hydrogen bonds between the bacteriochlorophylls of P and histidine amino acids residues in its surrounding. The number of histidines can be varied from one to four, and E_m varies accordingly from +430 to +735 mV. With $\Delta G° = E_m$ (cyt c_2^+/cyt c_2) – E_m (P^+/P) (see eq. 3.5; the factor F is not necessary if $\Delta G°$ and E_m are expressed in mV), all variations in the P^+/P potential are translated into variations of $\Delta G°$. The electron-transfer rate varies by a factor of ~100, nicely following the predictions from the Marcus theory, with a reorganisation energy of 600 meV.

3.2.3 The bc_1 complex

Under normal conditions ($E_h \approx 100$ mV), the pool of quinines in the photosynthetic membrane is partly reduced. The quinones, of which there are about 20 per reaction centre, are therefore present in the oxidised (Q) and reduced form (QH_2) in comparable amounts. As we described above, the photocycle in the RC feeds QH_2 into the pool and oxidises cyt c_2 in the periplasmic phase. These two electron carriers act as energetically 'charged' substrates for another multi-protein complex, the bc_1 complex, where QH_2 is reoxidised and Q is rereduced. These redox reactions drive a

reaction cycle that results in additional pumping of protons from the cytoplasmic to the periplasmic phase and contributes to the build-up of the electrochemical potential gradient used for ATP synthesis. Similar complexes of cytochromes *b* and *c* are found in oxygenic photosynthetic organisms (cyt b_6f, see Fig. 3.9), and also in the electron transfer chains of mitochondria of non-photosynthetic bacteria.

The structure of a bc_1 complex from purple photosynthetic bacteria has not yet been determined. However, the structures of mitochondrial bc_1 complexes from vertebrates and yeast have been determined to atomic resolution (Xia *et al.*, 1997; Zhang *et al.*, 1998; Iwata *et al.*, 1998; Hunte *et al.*, 2000). This has provided a strong structural basis to earlier tentative models derived from functional and biochemical studies, which identified the subunits and prosthetic groups and gave rise to the so-called Q-cycle description of the reaction sequence (for reviews, see Gennis *et al.*, 1993 or visit the website of A. R. Croft's lab at *www.life.uiuc.edu/crofts/bc-complex_site*). The bc_1 complex in purple photosynthetic bacteria consists of four protein subunits, as shown in Fig. 3.7: a cyt *b* subunit binding two haems, b_H and b_L (where H and L refer to high and low redox potential), a cyt c_1 subunit binding one *c*-type haem, a Rieske iron–sulphur protein binding a 2Fe–2S centre, and a fourth subunit without a prosthetic group (not shown in Fig. 3.7). The largest subunit, cyt *b*, extends across the membrane, whereas the cyt c_1 and the Rieske protein are on the periplasmic side of the membrane. Two quinone-binding sites exist, the Q_o- and Q_i-sites, both sited on the cyt *b* subunit, with the Q_i-site closer to the cytoplasmic surface and the Q_o-site closer to the periplasmic surface. The two cyt *b* haems are similarly arranged, with b_H close to the Q_i-site and b_L close to the Q_o-site.

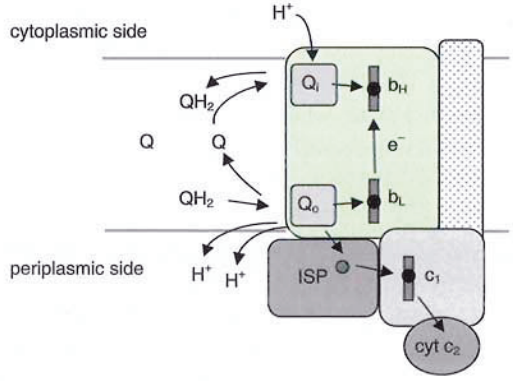


Figure 3.7 Schematic structure of the bc_1 complex with the cofactors involved in electron and proton transfer via the 'Q-cycle' mechanism. Green, cyt *b* subunit; ISP: iron–sulphur protein; c_1: cyt c_1 subunit.

The redox centres in the bc_1 complex can be grouped into two categories. The haem c_1 and the iron–sulphur centre have relatively high midpoint potentials (high-potential chain; E_m = +270 and +290 mV, respectively), and therefore they are reduced under normal conditions, whereas haems b_H and b_L (low-potential chain; E_m = +50 and –90 mV) are oxidised. Cytochrome c_2, after its oxidation by the primary donor in the RC, binds on the periplasmic side of the bc_1 complex and oxidises the high potential chain. A quinol, reduced as Q_B in the RC, binds to the Q_o-site and feeds one electron into the high-potential chain and one into the low-potential chain, releasing two protons into the periplasmic phase. Two different locations for the extrinsic head of the Rieske iron–sulphur protein have been identified in X-ray crystal structures indicating that domain movement might be important for this bifurcated electron transfer reaction (Zhang *et al.*, 1998; Crofts and Berry, 1998; Brugna *et al.*, 2000). An oxidised quinone bound to the Q_i-site can be reduced to the semiquinone form via haem b_H. After oxidation of another quinol in the Q_o-site, a second electron arrives at b_H, which then reduces the semiquinone to the quinol, with uptake of two protons from the cytoplasmic phase. This complex sequence of events is called the 'Q-cycle'. The stoichiometry of additional proton pumping by the Q-cycle mechanism in the bc_1 complex depends on the redox state of the quinone pool.

As in reaction centres, long-range electron transfer between cofactors plays an important role in the reaction mechanism of the bc_1 complex. However, the optimisation of the rate is much more crucial for the primary reactions in the RC, where excited states are involved. The direct determination of the rates of electron-transfer reactions in the bc_1 complex is considerably more difficult than for the RC, as the former reactions can only be indirectly triggered by light. With a time constant of about 1 ms, the oxidation of QH_2 at the Q_o-site of the bc_1 complex is thought to be the rate-limiting step of photosynthetic membrane processes under high light conditions. Under normal light conditions this reaction is fast enough to prevent accumulation of the quinone pool in the reduced state, which would lead to a blocking of the primary reaction in the RC and result in reduced efficiency and perhaps in photodamage.

3.2.4 The reaction centre of green sulphur bacteria and Heliobacteria

A second class of anoxygenic photosynthetic organisms has reaction centres that are distinctively different from the purple photosynthetic bacterial RC. To this class belong green sulphur bacteria (*Chlorobiaceae*) and *Heliobacteria*, families of species that all live in reducing conditions. Whereas purple photosynthetic bacterial RC catalyse the photoreduction of ubiquinone as terminal acceptors ($E_m \approx 0$ mV), the RC

of green sulphur bacteria and of *Heliobacteria* possess very low-potential iron–sulphur centres ($E_m \approx -600$ mV) as terminal electron acceptors and they are hence called Fe–S-type reaction centres. Although the midpoint potential of the primary donor P is also lower than in purple photosynthetic bacteria ($E_m \approx +225$ mV instead of +500 mV), the free energy available for export from the RC is significantly higher than in purple photosynthetic bacteria (as shown in Fig. 3.2). However, as no direct reduction of a quinone pool is involved, the mechanism for the build-up of a transmembrane proton gradient is more complicated than in the 'quinone-type' RC, implying additional electron transfer steps with inherent energy losses.

The basic structure of Fe-S type reaction centres The atomic structure of the Fe–S-type RC of anoxygenic bacteria is unknown, although an X-ray structure of the closely related PSI RC of oxygenic photosynthesis is available (and will be discussed in Section 3.3.3). Also biochemical and biophysical characterisation of Fe–S-type RC is less advanced than that of purple photosynthetic bacterial RC and molecular genetics on 'green' anoxygenic systems is only now starting. Nevertheless, a rough picture of the structure and function of Fe–S-type RC has emerged, often somewhat biased by the assumption of analogy with PSI.

As in all RC known so far, two large protein subunits make up the core of the RC of green sulphur bacteria and *Heliobacteria*. However, unlike the other systems, the two large subunits seem to be identical, so that the RC core is a true protein homodimer (Liebl *et al.*, 1993; for reviews see Amesz, 1995*a*, *b*; Feiler and Hauska, 1995; Neerken and Amesz, 2001). As in the RC of purple photosynthetic bacteria, the core proteins bind the cofactors involved in primary charge separation, but in addition they bind a relatively large number of antenna pigments (at least 30 molecules). This seems to be a characteristic feature of Fe–S-type RC and sequence comparisons suggest that these large RC evolved by fusion of an ancient, purple photosynthetic bacterial-like RC and an antenna protein, forming an antenna–RC complex. A subunit corresponding to the subunit H in purple photosynthetic bacteria is missing, but a small, ferredoxin-like protein is bound to the core complex on the cytoplasmic side (see Fig. 3.8). There is evidence that cytochromes also act as secondary electron donors in these bacterial systems, but the nature of the immediate electron donor to P^+ (a mono, di- or tetrahaeme cytochrome, c_{553}, presumably bound to the RC) is still a matter of debate (Okkels *et al.*, 1992; Oh-oka *et al.*, 1993; Nitschke *et al.*, 1995; Kusumoto *et al.*, 1999). The main difference between green sulphur bacteria and *Heliobacteria* reaction centres lies in the chemical nature of the pigments and the antenna composition. The main chlorophyll species in *Heliobacteria* is BChl *g* and all pigments seem to be bound to the RC. No additional antenna protein is found. In

contrast, green sulphur bacteria essentially have BChl *a* bound to the RC and possess in addition special, huge antenna complexes called chlorosomes. Each of these contains thousands of pigment molecules (BChl *c*, *d*, *e*) and is attached on the cytoplasmic side of the membrane. Excitation energy absorbed in the chlorosomes is transferred to the RC via two BChl *a*-proteins (the FMO-proteins; for details of the antenna see the previous chapter by Holzwarth).

Cofactors and electron transfer reactions The primary photochemistry in Fe–S type reaction centres does not seem to be essentially different from the purple photosynthetic bacterial systems. The primary donor P is a special pair of BChl with a midpoint potential of about +225 mV. P is composed of two molecules of BChl *g* in *Heliobacteria*, and two molecules of BChl *a* in *Chlorobiaceae*. These are known as P798 and P840 respectively, by reference to their absorption bleaching maxima when photooxidised. Comparing these two RC, no other significant difference apart from the chemical nature of the pigments constituting the primary donor is apparent.

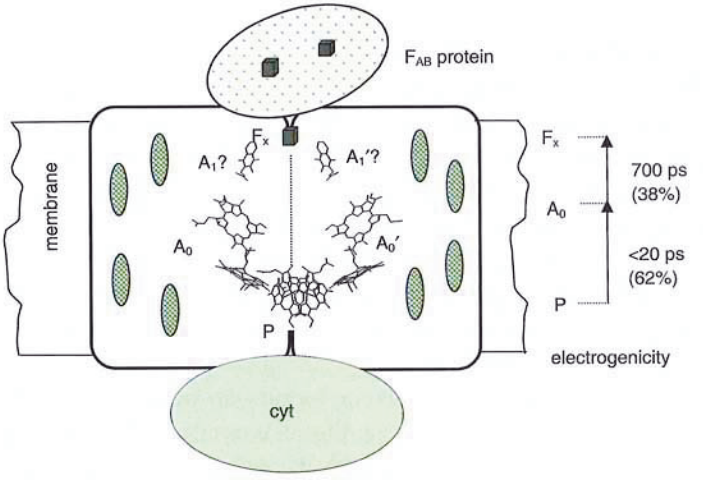


Figure 3.8 Schematic structure of the RC of green sulphur bacteria and *Heliobacteria*. This contains four essential polypeptides (the two large core polypeptides, the cytochrome and the F_{AB} iron–sulphur protein). Ellipsoids represent chlorin pigments with an antenna function (there are about 30 in total); the cubes are 4Fe–4S clusters, two of which are in the F_{AB} protein, and the third one, F_X, is held at the interface between the two core polypeptides. The cofactors of the RC core (special pair P; four chlorin molecules, including the primary acceptor A_0; two quinones, including A_1) are placed in a symmetric manner. At the right are shown the primary electron transfer steps as observed by photovoltage measurements (Brettel *et al.*, 1998). This technique detects kinetically distinct phases of flash-induced rise in membrane potential and thus gives information on relative transmembrane distances between cofactors.

Primary charge separation occurs in a few picoseconds by electron transfer from P^* to a primary acceptor A_0, which is a Chl *a* molecule absorbing at 670 and 663 nm in *Heliobacteria* and *Chlorobiaceae*, respectively. This acceptor plays a similar role to that of BPh H_L in the RC of purple photosynthetic bacteria, and back reaction similarly occurs from A_0^- to P^+ in ~20 ns if forward electron transfer is blocked. There is no indication of any involvement of an 'accessory' intermediate acceptor analogous to the monomeric BChl B_L in purple photosynthetic bacteria. Under normal conditions, A_0^- is reoxidised in ~700 ps by forward electron transfer to a secondary acceptor. The chemical nature of this acceptor is controversial. By analogy with PSI, where the secondary acceptor A_1 is vitamin K_1 (see Section 3.3.3), a menaquinone was proposed to function as secondary acceptor in *Heliobacteria* and *Chlorobium*. Although the photosynthetic membrane of these bacteria and isolated antenna-RC complexes does contain menaquinone, and although reduced quinones can be photoaccumulated under strongly reducing conditions (Muhiuddin *et al.*, 1995), the RC photochemistry is not affected when all quinones seem to be extracted (Kleinherenbrink *et al.*, 1993; Frankenberg *et al.*, 1996). Therefore it is uncertain whether a menaquinone functions in normal electron transfer in a manner analogous to A_1 in PSI, and time-resolved absorption and photovoltage measurements indicate that electron transfer from A_0^- probably occurs directly to F_X within 700 ps (Lin *et al.*, 1995; Brettel *et al.*, 1998; Kusumoto *et al.*, 1999). F_X is a 4Fe–4S iron–sulphur centre that is closely bound to the cytoplasmic surface by both large RC subunits. A motif of cysteine residues forming the binding site of F_X is conserved in all Fe–S-type reaction centres. The position of F_X resembles that of the non-haem iron between the two quinone acceptors in the purple photosynthetic bacterial RC. By bridging the two parts of the RC protein dimer, F_X might, like the non-haem iron, play a structural role, but in contrast to the latter it is redox-active with a midpoint potential of less than –600 mV. However, F_X is not the terminal acceptor. The small ferredoxin-like subunit on the cytoplasmic side of the RC complex contains two other 4Fe–4S clusters, centres F_A and F_B, together constituting the F_{AB} protein. Indeed, photoreduction of three different Fe–S centres at room temperature has been reported in RCs from green sulphur bacteria (Sétif *et al.*, 2001). In the X-ray structure obtained for PSI, the three Fe–S centres are clearly visible and it is likely that these centres occupy similar positions in all Fe–S-type reaction centres. Besides the definition of the three iron–sulphur centres by their characteristic low temperature EPR spectra (*e.g.* Hager-Braun *et al.*, 1997), not much more information about the assignment of the EPR signature to individual iron–sulphur centres or the pathway of electron transfer between them is available for these anoxygenic systems (but more is known about the PSI reaction centre, as described in Section 3.3.3).

The low potential of the two 4Fe–4S clusters carried by the F_{AB} protein, the terminal acceptors of Fe–S-type reaction centres, allows the reduction of soluble ferredoxin, and subsequently reduction of $NAD(P)^+$ via a flavoprotein (FNR, ferredoxin-$NADP^+$-reductase). As in purple photosynthetic bacteria, a *bc* complex has been identified in green sulphur bacteria and *Heliobacteria* (Nitschke and Liebl, 1992; Schütz *et al.*, 1994). This complex, which probably receives electrons from a menaquinone pool, contributes to the formation of a transmembrane proton gradient, again as in purple photosynthetic bacteria. The mechanism of light-induced reduction of the quinone pool is not completely clear. It probably involves another membrane-bound enzyme, the NADH-dehydrogenase complex, which transfers reducing equivalents from NADH to the quinone pool and contributes to transmembrane proton transfer (Kolpasky *et al.*, 1995). On the donor side, a soluble cytochrome (cyt *c555*) transfers electrons from the *bc* complex to the RC, probably via RC-bound cytochromes (cyt *c551*/*c553*). It appears that the redox potentials of the cytochromes and the cofactors in the *bc* complex are adjusted to the lower midpoint potential of the primary donor in the RC. They are therefore lower than the potentials of their purple photosynthetic bacterial counterparts (Kramer *et al.*, 1997).

Homodimeric versus heterodimeric reaction centres When the two families of RC of anoxygenic photosystems are compared, similarities and differences can be observed. First, all RC are protein dimers, but these are heterodimers in the case of the quinone-type RC and homodimers in the case of Fe–S-type reaction centres. A dimeric protein might be necessary to form the special pair of strongly coupled pigments which act as the primary electron donor, the dimeric structure of which is one of the most conserved features in all RC (in the special case of PSII, the primary donor is probably a weakly coupled multimer; see Section 3.3.2). Differences between the two families of RC exist essentially on the acceptor side. It was argued above that in purple photosynthetic bacteria the very different properties of the two quinone acceptors Q_A and Q_B bound to the L and M protein subunits might demand two different protein subunits, that is a heterodimeric RC. This constraint does not exist in the Fe–S-type RC, where no two-electron gate is necessary since all acceptors transfer electrons one by one. They are firmly bound and their function does not involve molecular diffusion.

It is generally accepted that the RC of green sulphur bacteria and *Heliobacteria* are homodimeric. Evidence comes mainly from the fact that only a single gene for a large subunit was found in these two systems and that partial polypeptide sequencing did not reveal any disagreement with this (unique) gene sequence (Büttner *et al.*, 1992; Liebl *et al.*, 1993). This raises interesting questions about the symmetry of

these RC: are all cofactors present in two copies and do they form two symmetric branches for electron transfer from P to F_X? There is little doubt that P and F_X are bound by both subunits on the putative symmetry axis of the RC. By contrast, the cofactors involved in electron transfer between them are likely to be buried in one or other of the two large subunits. In view of the apparent high degree of structural symmetry, it is difficult to explain the inactivity of one branch in the heterodimeric purple photosynthetic bacterial RC. A symmetric function of two identical electron transfer pathways in homodimeric RC has to be considered as a serious possibility, although there is no experimental evidence for this. It may be, however, that the symmetry is broken by the asymmetric binding of other protein subunits, for instance cytochromes on the donor side or the F_{AB} protein or other small subunits on the acceptor side.

3.3 Electron transfer in oxygenic photosynthesis

3.3.1 Overall electron transfer: the Z-scheme

Oxygenic photosynthesis occurs in plants, algae and cyanobacteria under natural conditions. Its main characteristic is its overall electron-transfer process, which abstracts electrons from water to reduce $NADP^+$, forming two products: oxygen, which is released to the atmosphere, and NADPH, which serves to reduce CO_2. Thus electrons flow from the redox reaction $2H_2O \rightarrow O_2 + 4\ H^+ + 4e^-$, ($E_m$ = +0.82 V at pH7) to the redox couple $NADP^+ + 2e^- + H^+ \rightarrow$ NADPH (E_m = –0.32 V at pH7). This reaction is endergonic by 1.14 V and the Gibbs free energy increase comes from absorbed light.

In the development of the understanding of photosynthesis, there has been a serious debate about the number of photons needed to evolve one oxygen molecule or to reduce one CO_2 molecule: is it four or eight? There is now a clear consensus on eight as a minimum number under optimum conditions. This number, which is based on difficult quantum yield measurements, is in good agreement with our present understanding of the thermodynamics of photosynthesis and with the biochemical data which are incorporated in the so-called 'Z-scheme' shown in Fig. 3.9 (and in less detail in Fig. 3.2). This scheme, formulated by Hill and Bendall (1960), proposes that electrons flow from water to $NADP^+$ through two reaction centres (PSI and PSII) and a series of electron carriers, described below. Formation of one O_2 molecule requires the removal of four electrons from water, and the scheme requires two photons to transfer one electron from water to $NADP^+$; it follows that eight photons are needed to

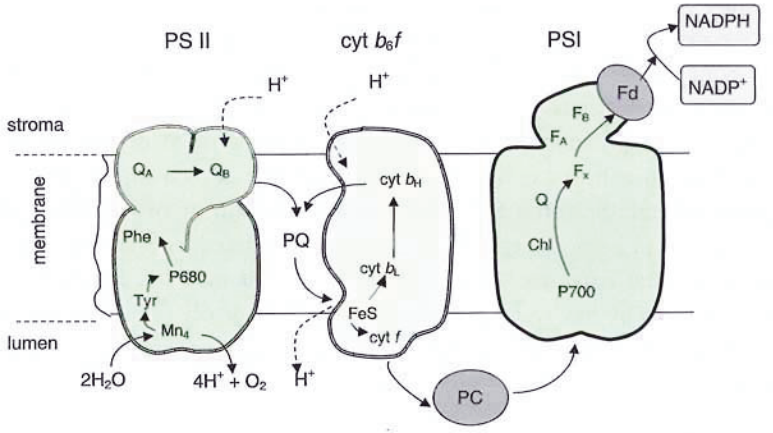


Figure 3.9 The scheme of electron flow from H_2O to $NADP^+$ in the Z-scheme of oxygenic photosynthesis, showing the three membrane complexes (the PSII RC, the cyt b_6f complex and the PSI RC), with their redox centres in simplified form. Links are ensured by plastoquinone (PQ) in the membrane, by plastocyanin (PC) in the lumenal volume and by ferredoxin (Fd) in the stromal side.

produce one O_2 molecule (and to reduce one CO_2, since there is a 1:1 stoichiometry between the two processes in the steady state). This electron flow is coupled to transmembrane proton transfer from the outside of the thylakoid (stroma) to its inner surface (lumen), creating a transmembrane electrochemical potential gradient of protons, which is used for the conversion of ADP to ATP. The total Gibbs free energy stored when one electron flows from water to $NADP^+$ is the sum of the redox energy (1.14 eV) and of the contribution to building up the electrochemical potential gradient $\Delta\mu(H^+)$ across the thylakoid membrane. This potential is the sum of the transmembrane electrical potential, which can be neglected in chloroplasts under steady-state conditions, and the difference in chemical potential of protons on the two sides of the membrane (ΔpH), given by

$$\Delta\mu(H^+) = 2.3\,RT\,\Delta\text{pH} \tag{3.9}$$

At 298 K, $\Delta\mu(H^+)$ (in eV) = 0.059 ΔpH. In order to calculate the Gibbs free energy stored per electron transferred, the operating value of ΔpH must be known, as well as the number n of protons transferred across the thylakoid membrane per electron transferred. ΔpH is around 3.5, and n is between 2 and 3. Taking n = 2.5, it follows that the energy stored as a proton chemical potential difference is 0.059 x 2.5 x 3.5 = 0.52 eV per electron transferred. The total Gibbs free energy stored in electron transfer and coupled H^+ transfer is thus approximately 1.14 + 0.52 = 1.66 eV per electron transferred, *i.e.* per two photons absorbed, one by PSI and one by PSII.

Active photons have a minimum energy of 1.8 eV and the energy conversion yield of the Z–scheme is thus 46%.

We start the discussion of electron transfer in oxygenic photosynthesis with the motors that drive the system, the two reaction centres PSII and PSI. For both, a strong homogeneity with only very limited differences is found among all organisms from cyanobacteria to higher plants, indicating an evolutionary relationship.

3.3.2 Photosystem II reaction centre

By contrast with purple photosynthetic bacteria and PSI, the structural description of the PSII RC (shown in Fig. 3.9 and in more detail in Fig. 3.10) could for a long time not rely on a detailed structure determined by X-ray crystallography. The difficulty in obtaining 3D crystals of PSII RC of a quality allowing high resolution X-ray crystallography has often been attributed to a structural heterogeneity, possibly arising from natural instability of the reaction centre. PSII RCs are usually surrounded by light-harvesting complexes. These have a dimeric organisation that was first studied by freeze-etched, freeze-fractured and single particle images by electron microscopy (Hankamer *et al.*, 1997; Nield *et al.*, 2000). The functional significance of this dimeric structure is still unknown. Further advances have been made in the preparation and analysis by electron crystallography of 2D crystals of PSII from which part of the antenna complexes have been removed (Rhee *et al.*, 1997; Morris *et al.*, 1997). The analysis of these crystals has already provided a very useful image of the PSII, its overall shape and the way several proteins are organised. A little later, Rhee *et al.* (1998) obtained a 3D structure at 8 Å resolution that shows the helices of the sub-units and the position of many pigment molecules. Recently, crystals of PSII that are fully active in water oxidation have been obtained from the thermophilic cyanobacterium *Synechococcus elongatus* and the structure resolved to 3.8 Å resolution (Zouni *et al.*, 2001). These data have confirmed many aspects of previous 3D models, including a model based on homology with the RC of purple photosynthetic bacteria (Svensson *et al.*, 1996), but they have also provided new structural details, especially on the position of the manganese cluster that catalyses water oxidation. In fact, despite the many structural similarities between PSII and the RC of purple photosynthetic bacteria (described in Section 3.2.2), it should always be remembered that PSII is unique insofar as the primary reaction generates a very strong oxidant, able to oxidise water to molecular oxygen. As we shall see, this functional difference has important structural consequences, especially on the oxidising (donor) side of the RC.

Redox centres: electron transfer The primary donor of PSII has been identified by spectroscopic techniques to be a chlorophyll *a* species, long known as P680 from the 680 nm wavelength of its absorption maximum. This is excited by energy transfer from neighbouring pigments, thus becoming able to transfer an electron through a series of electron acceptors that are very similar to those in purple photosynthetic bacteria. Pheophytin *a* (Ph in Fig. 3.10) is the primary acceptor, followed by two plastoquinones acting in series: the first, Q_A, is a one-electron carrier, while the second, Q_B, must be fully reduced to the hydroquinone state by the addition of two electrons and two protons before it leaves the RC to reduce the cytochrome b_6f complex. A second pheophytin *a* molecule is in a position analogous to H_M in purple photosynthetic bacteria suggesting an 'inactive branch' is also present in PSII, as shown in Fig. 3.10. The redox potentials of Ph, Q_A and Q_B are quite similar to those of the corresponding species in purple photosynthetic bacteria (shown in Fig. 3.2). However, spectroscopic data suggested that the coupling of the chlorophyll pigments making up the primary donor is significantly weaker in PSII compared with all other RCs. This difference has often been related to the much higher midpoint potential of P680, and it led Durrant *et al.* (1995) to propose a multimer model for P680. Our

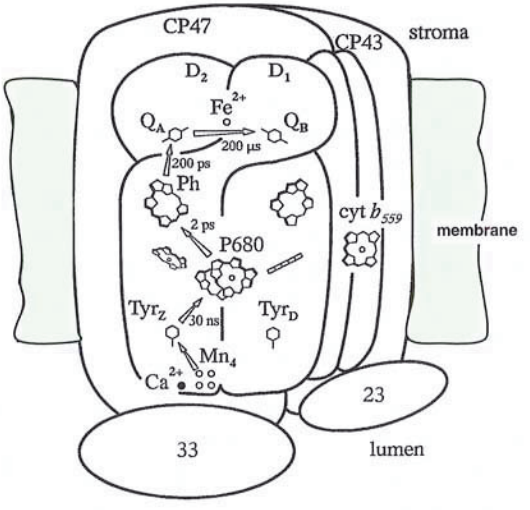


Figure 3.10 Structural model of the PSII RC, showing the core polypeptides D_1 and D_2, the core antenna complexes CP47 and CP43, the two subunits of cytochrome b_{559} and the two extrinsic lumenal polypeptides of 33 and 23 kDa. D_1 and D_2 hold the redox cofactors involved in electron transfer from water to plastoquinone. The overall structure of D_1, D_2 and CP47, as well as the detailed positions of the cofactors can be found in Rhee *et al.* (1998) and Zouni *et al.* (2001). See also Hankamer *et al.* (2001) and Rutherford and Faller (2001) for reviews.

present knowledge of the structure of P680 and the details of primary charge separation are discussed below.

Once it has been oxidised by light, P680 is re-reduced by a tyrosine residue, Tyr_Z, belonging to one of the polypeptides, and the oxidised Tyr_Z then oxidises the cluster of four manganese ions (Mn_4 in Fig. 3.10). This cluster is the catalytic site for water oxidation. It is located about 15 Å off the pseudo-symmetry axis of the RC, close to Tyr_Z on the D_1 subunit. The properties of P680, Tyr_Z and the Mn_4 cluster are still largely unknown and are the object of active research and debate (Diner and Babcock, 1996; BBA, 2001). Tyr_Z is probably the best-known species in this so-called 'donor side'. This residue is located at position 161 in the D_1 polypeptide. Following its oxidation, it transfers a proton to a neighbouring amino acid, yielding the neutral radical $Tyr_Z^{\bullet}$, which then oxidises the manganese cluster. A second tyrosine radical, known as $Tyr_D^{\bullet}$, has been known for a long time from its EPR spectrum, denoted Signal II. $Tyr_D^{\bullet}$ is very stable, sometimes existing for hours, whereas $Tyr_Z^{\bullet}$ is normally short-lived. The function of Tyr_D is still unknown, although it is possible that it participates in the redox reactions accompanying the biosynthesis of the manganese cluster. Direct participation of amino acid residues in electron transfer is quite rare in proteins. In that respect Tyr_Z and Tyr_D seem to be unique in photosynthetic electron transfer.

Figure 3.10 also shows the halftimes of the most important electron transfer reactions in PSII; some of these vary with the redox states of Q_B and the Mn cluster. The redox potentials of all the species on the PSII donor side have not been measured directly, but clearly they must be high enough to permit the irreversible oxidation of water.

PSII proteins The redox centres in PSII are held in a well-defined conformation by the protein, which also holds the redox-active tyrosines Tyr_Z and Tyr_D. In its simplest form, the PSII RC probably also includes two polypeptides D_1 and D_2 (Fig. 3.10), which carry P680, Ph, Q_A and Q_B, Tyr_Z and Tyr_D. D_1 and D_2 also hold another pheophytin *a* and 2–4 additional chlorophyll *a* molecules. This hypothetical reaction centre has never been isolated: the simplest effectively isolated PSII complex, D_1–D_2–cyt b_{559}, does not retain Q_A and Q_B although their native sites are provided by D_2 and D_1, respectively (Satoh, 1996). The complex also includes a small polypeptide, called polypeptide I, of molecular weight 4.2 kDa (the function of this polypeptide is not known and it is not shown in Fig. 3.10) and the two subunits (MW 9.3 and 4.5 kDa) of cytochrome b_{559}. This cytochrome, which apparently does not participate in electron transfer from water to Q_B, is always present in PSII, intimately belonging to the RC core; it is thought to have an essential, although still unknown, function.

In the thylakoid membrane, the natural photosynthetic membrane, the PSII RC has a much more complex accompaniment of polypeptides (Barber *et al.*, 1997; Hankamer *et al.*, 2001). In addition to the five polypeptides of the D_1–D_2–cyt b_{559} complexes, a further twelve or so polypeptides contribute to the native structure. Several of these carry pigment molecules (chlorophyll *a*, carotenoids): these include CP47 and CP43, which belong to the PSII core, and several others (CP29, CP26, CP24) that are more remote and ensure contact with the LHCII antenna complexes. The main function of these pigment–protein complexes seems to be light absorption and energy transfer, but they may also contribute to the structural stability of the edifice, to the binding of plastoquinones at the Q_A and Q_B sites and to the binding of manganese cluster. A few small polypeptides, which are largely hydrophobic, have no known function. In higher plants, several subunits (D_1, D_2, H, CP29, CP43) display a reversible phosphorylation that may have a regulatory function. Three polypeptides, of MW 16, 23, and 33 kDa, are extrinsic to the membrane, whereas all the others are intrinsic. These are located in the thylakoid lumen (two of these, with MW 23 and 33 kDa, are shown in Fig. 3.10). These polypeptides seem to participate in stabilising the manganese cluster, by screening the water oxidation site from redox-active molecules and providing it with optimal levels of Ca^{2+} and Cl^- (Seidler, 1996). However, the 16 and 23 kDa polypeptides are missing in cyanobacteria, in which they are perhaps replaced by cytochrome c-550 and a 12 kDa polypeptide.

In summary, it appears that all redox centres, including the four manganese ions, are held by the D_1 and D_2 proteins, which have a structure and function homologous to the L, M pair of polypeptides in purple photosynthetic bacteria.

Quinone acceptors, Fe^{2+}, herbicides We shall examine electron transfer in PSII in some detail. This RC must produce both stable and mobile reduced species in order to deliver electrons to the cytochrome b_6f complex. This is the function of plastoquinones, as it is of ubiquinones in purple photosynthetic bacteria and other quinones in respiration. The quinones of PSII are organised in three subsets, exactly as they are in purple photosynthetic bacteria: Q_A, Q_B and a pool of PQ (Mathis and Rutherford, 1987). Q_A is bound to the RC and normally functions as a one-electron carrier. It accepts an electron from the pheophytin Ph in about 200 ps, short enough to avoid the wasteful and perhaps damaging back-reaction between Ph^- and $P680^+$, which has a half-time of a few nanoseconds. Q_B can accept two electrons, one by one, from Q_A. It serves as the required interface between the primary photochemical events, which deliver electrons one by one, and hydroquinone formation, which requires two electrons and two protons. Q_B is the two-electron gate of PSII. After its full reduction and protonation, the plastoquinone Q_B loses its affinity for the protein; leaves it and

becomes part of the plastoquinone pool in the thylakoid membrane, where it diffuses more or less freely to reach a site on the cytochrome b_6f complex. Here it binds and delivers its two electrons one by one, together with the two protons, which are released in the thylakoid lumen. Another oxidised plastoquinone binds to the Q_B site in the meanwhile. Diffusion of plastoquinone within the membrane is probably severly hindered by the high concentration of protein complexes and restricted to small domains (Joliot and Joliot, 1992; Kirchhoff *et al.*, 2000).

The redox potential of the Q_A/Q_A^- couple has been measured by various methods (fluorescence, EPR, flash absorption spectroscopy), which unfortunately give highly scattered values. Krieger *et al.* (1995) showed that the state of the redox machinery involved in the re-reduction of $P680^+$ influences the Q_A redox potential, providing a possible explanation for the disagreement. It seems that, under physiological conditions, E_m is –80 mV for the Q_A/Q_A^- redox couple and this is independent of pH. For the Q_B/Q_B^- couple, E_m is thought to lie between 0 and +50 mV.

The function of Q_B raises the interesting question of the coupling between electron and proton transfer, which can be studied in much more detail in purple photosynthetic bacteria. Its site can also be occupied by competitive inhibitors (such as DCMU or triazines) that are of considerable importance in agriculture since they are widely used as herbicides. At submicromolar concentrations, these inhibitors have an affinity for the Q_B site higher than that of oxidised plastoquinone. In their presence, electron transfer therefore stops at Q_A^-. These inhibitors are hence useful tools in the study of primary PSII reactions, for mapping electron transfer that goes no further than Q_A. These inhibitors, as well as mutants that are insensitive to their action, have been extensively used in structure–activity studies of the Q_B site.

Like the RC of purple photosynthetic bacteria, PSII also possesses a non-haem Fe^{2+} ion. This perturbs the EPR spectra of Q_A^- and Q_B^- similarly and so it has tentatively been assigned a position mid-way between the two quinones, as shown in Fig. 3.10. This position, at the interface of subunits D_1 and D_2, was confirmed by X-ray crystallography by Zouni *et al.* (2001). The present structure does not resolve the amino acid side chains, but according to the present models each subunit has two histidine residues, which provide four of the ligands for Fe^{2+}. In purple photosynthetic bacteria, the two side-chain oxygens of a glutamic residue form the fifth and sixth ligands of Fe^{2+}. An equivalent residue is absent in D_1 and D_2, and it is proposed that a bicarbonate ion is bound as a bidentate ligand to complete the Fe^{2+} coordination. This may indirectly be the reason for the well-known need for bicarbonate in electron transfer from Q_A to the plastoquinone pool. It should be added that the exact role of the Fe^{2+} in PSII is not known. At variance with what happens in purple photosynthetic bacteria, this Fe^{2+} can experience changes in its redox state: provided it is first

oxidised, it can very quickly accept an electron from Q_A^-. The midpoint potential of the Fe^{2+}/Fe^{3+} couple is, however, fairly high (about +400 mV at pH 7), so that Fe^{2+} cannot reduce Q_B and cannot be involved directly in electron transfer from Q_A^- to Q_B. This ion may have a structural role in the stability of the D_1, D_2 complex.

P680, the primary electron donor As long ago as the 1960s, the flash absorption studies of Witt and co-workers (see Witt, 1991) revealed a bleaching of chlorophyll in PSII, centred around 680 nm. This was attributed to the photooxidation of the primary electron donor, a chlorophyll *a* species named P680 by analogy with the corresponding species (P870, P700) in other reaction centres, also named according to the wavelengths of their maximum bleaching. Flash kinetic studies revealed that $P680^+$ is always very short lived: from 50 to 200 ns under physiological conditions to ~2 ms at low temperature (Mathis and Rutherford, 1987). This instability is in agreement with the estimated (but never measured) midpoint potential of the $P680^+/P680$ redox couple of about +1.1 V, permitting irreversible oxidation of water. After its formation by the primary photochemical event, $P680^+$ can decay by the same routes as in other reaction centres, by secondary electron transfer from Tyr_Z or charge recombination with Ph^- or Q_A^- when forward electron transfer is inhibited. But being very oxidising, $P680^+$ can also be reduced in pathological reactions by any neighbouring molecule within a distance of 15 Å or so: candidates include β-carotene and accessory or antenna Chl *a* molecules, as well as amino acid residues. All these possible reactions contribute to the instability of $P680^+$, a property which in turn renders a detailed study of P680 very difficult: differential absorption spectra are ambiguous, as are all the spectroscopic data (CD, EPR, FTIR *etc.*) about the $P680^+/P680$ couple.

The triplet state of P680 ($^3P680$) can be populated by the radical-pair mechanism when forward electron transfer is blocked; a similar event occurs in other types of reaction centres, as described in Section 3.2.2. The lifetime of $^3P680$ varies from about 10 μs at room temperature to about 2 ms at low temperature. More precise values cannot be given, because the lifetime depends on oxygen concentration in the medium (it is a widespread property of triplet states that they are deactivated by energy transfer to oxygen), on temperature, and also on the state of the acceptor side (presence of singly or doubly reduced Q_A, or absence of Q_A; presence or absence of Fe^{2+}). $^3P680$ has been detected by EPR at low temperature, by time-resolved flash absorption, by ADMR, and more recently by FTIR.

The detailed molecular structure of P680 has been a matter of long debate (Durrant *et al.*, 1995; Hillmann *et al.*, 1995; Diner and Babcock, 1996). Folding models of the D_1 and D_2 polypeptides have pinpointed conserved histidine residues that are close to the lumenal side and possible ligands for P680. Based on the known

dimeric structure of the primary donor in purple photosynthetic bacteria, P680 has also been assumed to have a special pair structure, with nearly parallel tetrapyrrolic planes perpendicular to the membrane plane. However, this model has been challenged by several experimental observations. First, compared with the primary donors in other RC, P680 must have a specific structure that endows it with a very high redox potential (at least 600 mV more oxidising than P700, which is also a Chl *a* species and the structure of which is rather similar to P in purple photosynthetic bacteria). Moreover, $P680^+$ accepts an electron from Tyr_Z, which is located in the membrane-spanning region of the RC, whereas other primary donors are re-reduced by extrinsic metalloproteins, the redox centres of which are about 7 Å from the lumenal membrane surface.

Other structural models for P680 have been proposed to account for properties such as the optical and infrared absorption spectra of P680 and the EPR spectra of $P680^+$ and $^3P680$. These differ in the orientation of Chl *a* molecules with respect to the membrane, in the distance between their tetrapyrrolic planes and even in the number of Chl *a* molecules that make up P680. The recent 3D structure (Zouni *et al.*, 2001) shows that the cofactors in the D_1/D_2 complex have positions analogous to those in the purple bacterial RC, except that the distance between the chlorophylls corresponding to the putative 'special pair' is larger. The four Chl *a* molecules that make up P680 are nearly equidistant and the interaction between them is certainly much weaker than in other reaction centres. This suggests that all four have a high redox potential. It is also quite possible that the three observable entities (P680*, $P680^+$ and $^3P680$) correspond to different subsets of Chl *a* molecules in the reaction centre. To summarise, there is now agreement that PS II is unique insofar as P680 is not a 'special pair' as in all other known RCs, but is instead probably composed of a cluster of four weakly coupled monomeric chlorophylls, sometimes called a 'quasi-multidimer' or tetramer (Prokhorenko and Holzwarth, 2000; Barber, 2002). The uniquely high redox potential of $P680^+$ may be a consequence of its more monomeric structure (Barber and Archer, 2001).

Water oxidation Water oxidation is a terminal electron-transfer reaction in oxygenic photosynthesis. It is absolutely unique to that process, although the inverse reaction is performed in respiration:

Photosynthesis	$2H_2O \rightarrow O_2 + 4H^+ + 4e^-$	(catalysed by the water-oxidising site of PSII)
Respiration	$O_2 + 4e^- + 4H^+ \rightarrow 2H_2O$	(catalysed by cytochrome oxidase)

In the case of cytochrome oxidase, electrons are provided by cytochrome *c*. In spite of the formal similarity of these reactions, the enzymes that catalyse them are fairly different in terms of redox centres: Mn_4 and Tyr_Z in PSII, copper and haems in cytochrome oxidase. But they show the common property of coupling a one-electron step to a four-electron reaction, and therefore they can both be considered as devices for the storage of redox equivalents, with five redox states in a cycle, the last one leading to formation of the product. It is not an exaggeration to say that oxygen production is a key process on Earth. We do not know exactly when this process first evolved (Blankenship and Hartman, 1998), but there is no doubt that photosynthesis is the source of atmospheric oxygen and that an oxygen-rich atmosphere was necessary for the advent for the majority of present forms of life.

The thermodynamics of water oxidation shows that the concerted four-electron mechanism is the only one that does not include any step requiring a very oxidising species. It is therefore the only one that is compatible with the known redox properties of PSII redox centres: with an E_m of –0.6 V for the acceptor Ph, and a photon input energy of 1.8 eV, it follows that the maximum possible E_m of the $P680^+/P680$ redox couple is +1.2 V, supposing that all the photon energy is converted into redox energy. No single step of the water oxidation mechanism can therefore have a redox potential higher than +1.2 V. Our present knowledge of the site of water oxidation in PSII and of the catalytic mechanism is based on many experimental results. We shall mention a few of the most important ones (Debus, 1992; Yachandra *et al.*, 1996; BBA, 2001).

S states Excitation of PSII in photosynthetic membranes by a train of short, intense flashes, which photoinduce one-electron transfer reactions in all RC, leads to a periodic pattern of O_2 evolution as a function of flash number (Joliot *et al.*, 1969). The periodicity is four, and the maximum emission is at the third, seventh, *etc.* flash. This fundamental result is interpreted by a model in which four oxidising equivalents need to be stored, one by one, before two molecules of water are oxidised and one O_2 is produced. Successive states of the enzymes are named S states. There are five of them, as shown in Fig. 3.11. The S_1 state is the most stable and it is therefore the most abundant when PSII has been kept in darkness. One of the objectives of research on PSII has been to describe the S states in molecular terms, and more precisely in terms of the oxidation state and the structure of the manganese cluster. As discussed below, various tools have been used in that respect.

EPR Electron Paramagnetic Resonance (EPR) spectroscopy is a very valuable technique for the study of metalloenzymes. Dismukes and Siderer (1981) made the important discovery that PSII membranes, excited by one flash and thus put into the

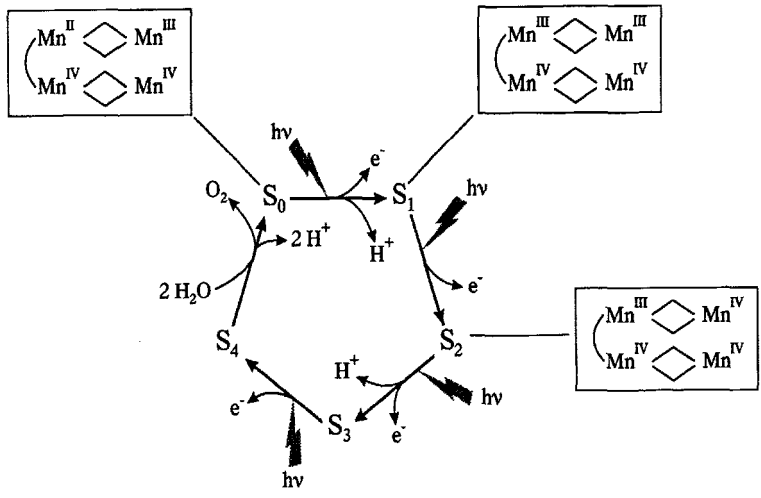


Figure 3.11 The cycle of S states of the water-oxidising catalytic site in PSII RC ('Kok–Joliot model'). The reactions giving rise to the release of a proton are not yet confirmed and nor are the valencies of manganese atoms in states S_0, S_1 and S_2, here drawn on the basis of two manganese dimers. One of the dimers is thought to remain in the state (IV, IV), while the other one becomes progressively more oxidised during the cycle. In states S_3 and S_4, it is controversial whether the manganese cluster becomes more oxidised or other redox centres are oxidised (Yachandra *et al.*, 1996; Hoganson and Babcock, 1997; Messinger *et al.*, 2001; Dau *et al.*, 2001; Nugent *et al.*, 2001; Renger, 2001; Robblee *et al.*, 2001; Vrettos *et al.*, 2001; BBA, 2001).

S_2 state, which was then trapped at low temperature, display a complex EPR spectrum with about nineteen lines centred at $g = 2$, called the 'S_2 multiline signal'. The best interpretation of this signal is that it originates from a cluster of at least two Mn ions in the mixed valence state Mn^{III}–Mn^{IV}. Further work by EPR showed that the S_2 state can also display a single broad line at $g \approx 4$. Remarkably, the multiline signal can be converted to the broad-line state at $g \approx 4$ by illumination with near-infrared light, which does not induce photosynthetic electron transfer (Boussac *et al.*, 1996). A complete analysis of the shapes and relaxation behaviour of EPR spectra leads to the conclusion that at least three, and more probably four, Mn ions are involved, and that the cluster can have several slightly different structures.

Besides the S_2-state multiline signal, EPR spectra from the states S_0, S_1 and S_3 have been reported. The S_1 signal, with an effective g value of 12 and at least 18 hyperfine lines, is only observed with parallel mode detection, indicating that this state has integral spin (Campbell *et al.*, 1998). Recently EPR signals arising from the

S_3-state have been observed both with perpendicular and parallel mode detection (Matsukawa *et al.*, 1999; Ioannidis and Petrouleas, 2000). A signal of the S_3 state can also be induced *in vitro* by removal of the chloride or calcium ions that are normally bound to the RC. Both ions are necessary for oxygen evolution: their depletion blocks the S states cycle at the state S_3. This abnormal S_3 state displays an EPR spectrum that is attributed to the manganese cluster in interaction with an oxidised organic radical (Boussac *et al.*, 1990), probably $Tyr_Z^{\bullet}$ (Tang *et al.*, 1996). An EPR spectrum of the S_0

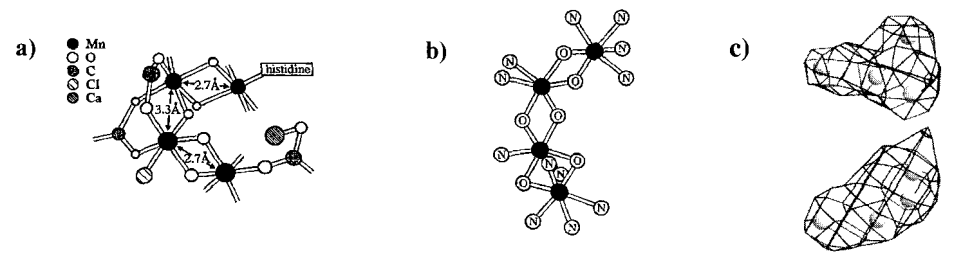


Figure 3.12 (a) Tentative structure of the manganese cluster in the PSII RC (adapted from Yachandra *et al.*, 1996); (b) structure of a synthetic tetranuclear manganese cluster modelling the manganese cluster of PSII (adapted from Philouze *et al.*, 1994); (c) structure of PSII at 3.8 Å resolution, showing the manganese cluster as an electron density in the form of a 'Y' (Zouni *et al.*, 2001). The precise geometry of the manganese cluster and the location of the Ca^{2+} ion(s) remain unclear.

state was first observed after addition of methanol to PSII membranes (Messinger *et al.*, 1997) and more recently without methanol addition in the PSII from cyanobacteria (Boussac *et al.*, 1999). EPR spectroscopy (high-field EPR, electron spin echo, ENDOR) is in progress and it seems probable that this will give more and more detailed structural information about the catalytic site of water oxidation.

EXAFS X-ray absorption spectroscopy of the Mn ions is extremely useful for probing the position of close neighbour atoms (Yachandra *et al.*, 1996). Measurements on several S states indicate distances of 2.7 Å and 3.3 Å between manganese ions, leading to the model shown in Fig. 3.12a. Lighter atoms (oxygen and nitrogen) are located at 1.9–2.0 Å from the manganese centres, in agreement with the structure of di-μ-oxo dimers[2] shown in Fig. 3.12b, which is the basic model for the structural interpretation of spectroscopic data. X-ray absorption edge measurements have also been used to establish the redox changes of manganese ions shown in Fig. 3.11, and

[2] A μ-oxo bridge consists of one oxygen, formally in the state O^{2-}, sited between two metal ions. Figure 3.12 shows two oxygen ions between each Mn.

in particular to establish that the transition from state S_1 to S_2 effectively corresponds to the oxidation of a Mn ion from Mn^{III} to Mn^{IV}.

Proton release Several models have been put forward for the chemical mechanism of water oxidation. In an extreme case where water molecules are involved only at the S_4 state, it could be envisioned that four H^+ are released only after full oxidation of the charge-accumulating device. However, proton release can be measured on flash excitation of PSII, and the results indicate much more complex behaviour (Haumann and Junge, 1996). It appears that oxidation of manganese ions and Tyr_Z induces an electrostatic shift in the pK_a of nearby amino acids (including Tyr_Z), mostly in the upward (weaker acid) direction, leading to a non-integral and flash-dependent number of protons being released during a sequence of flashes. In the whole sequence, it is not known precisely which protons come from water oxidation, which would yield four protons released per cycle, and which come from electrostatic influences, the balance of which per cycle would be neutral.

Synthetic models. The multiline EPR spectrum of state S_2 is highly reminiscent of the spectrum of a synthetic manganese complex in which two ions in oxidation states Mn^{III} and Mn^{IV} are bound by two μ-oxo bridges. This observation was the start of a fruitful feedback between the study of PSII and the synthesis of multinuclear manganese complexes that seek to mimic the properties of the enzyme as an aid to understanding its structure (Armstrong, 1992; Frapart *et al.*, 1996). The binuclear mixed-valence Mn complex has an EPR spectrum with 16 lines, whereas the multiline signal has at least 19 lines. It is known moreover that four Mn ions are present in PSII. So, in order to be more realistic, the present synthetic work deals mainly with tetranuclear complexes. Several structures have been proposed, but none of them behaves exactly as in PSII itself. Figure 3.12 shows a typical model, with a tetranuclear complex consisting of two dimers. Each dimer has a di-μ-oxo structure, and they are bridged by ligands that ensure a Mn–Mn distance of about 3.0 Å, as found by EXAFS in PSII. A few years ago, a binuclear manganese complex that can produce molecular oxygen from hypochlorite has been synthesised (Limburg *et al.*, 1999). This molecule may help understanding the mechanism of formation of the O–O bond, a critical step in photosynthetic water oxidation.

In spite of all the experimental information mentioned above, and much more that we have not cited, the molecular structure of the catalytic site for water oxidation is not established, and its mechanism remains one of the major unknowns in photosynthesis. All proposed models, including the one shown in Fig. 3.12, will have to be examined

for consistency with the shape of the electron density revealed in the crystal structure (Zouni *et al.*, 2001; Robblee *et al.*, 2002). Hoganson and Babcock (1997) have advanced a more complete structural and functional model with the main feature that hydrogen atoms are abstracted from water by the Tyr_Z radical. A major experimental difficulty is that the S_4 state is not stable, with the consequence that we have no clue as to its properties: once it is formed, it exists for just a few milliseconds during the final step of water oxidation. Let us mention a few questions that need to be addressed:

- how are the manganese ions held by the polypeptides of the RC?
- what are the roles of the Ca^{2+} and Cl^- ions that are necessary for completing the catalytic cycle?
- what are the roles of the extrinsic 33, 23, and 16 kDa polypeptides in higher plants and green algae?
- what exactly are the oxidation states of the four Mn ions in the various S states? Does an amino acid (Tyr_Z or histidine) participate directly in the storage of oxidising equivalents in some of the S states?
- when and how is the 0–0 bond of dioxygen formed, and how are the two substrate water molecules involved?

Primary PSII photochemistry, chlorophyll fluorescence, triplet state The primary processes in PSII resemble those in other types of RC. Specific aspects, however, arise from the nearly isoenergetic levels of the PSII pigments, including P680 and Ph, and the antenna chlorophyll *a* molecules (Holzwarth, 1991). These all absorb light around 678–680 nm, so there is no low-lying singlet excited state of the PSII pigments that could be considered as a trap for excitation energy. There are indications that a Chl *a* monomer, analogous to the 'accessory' BChl B_L of purple photosynthetic bacteria, functions as the primary electron donor in PSII, at least at low temperatures (Prokhorenko and Holzwarth, 2000). Initial charge separation between this pigment and the neighbouring pheophytin is probably followed by rapid migration of the positive charge to another of the four weakly coupled chlorophylls. With the assumption that Ph is the primary electron acceptor and ignoring the transfer step of the positive charge within P680, the primary reactions are as shown below:

$$\text{(Antenna)}^* \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \text{P680}^* \text{ Ph } Q_A \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} \text{P680}^+ \text{Ph}^- Q_A \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} \text{P680}^+ \text{Ph } Q_A^-$$

The rates of the various reactions of this scheme have been measured by flash absorption, fluorescence decay and the generation of photovoltage. There is clear consensus about the stabilisation step, the electron transfer from Ph^- to Q_A with $k_3 = (250\ ps)^{-1}$ and $k_{-3} \approx (150\ \mu s)^{-1}$, the latter decreasing to about $(2\ ms)^{-1}$ at low temperatures. The primary electron transfer step has a rate constant $k_2 \approx (3\ ps)^{-1}$, but there is no consensus about the relative rates of energy and electron transfer (Greenfield and Wasielewski, 1996). Some data indicate that k_2 is much larger than the other rates, leading to a 'diffusion-limited' model in which energy arrives relatively slowly at the primary reactants P680 and Ph, where electron transfer takes place quickly and nearly irreversibly. Most data, however, favour the view that k_2 is not much larger than other rates, leading to the 'trap-limited' or 'reversible radical pair' model, in which formation of the primary radical pair ($P680^+$ Ph^-) has a high probability of being followed by reformation of an excited state which is returned to the antenna. In agreement with this model is the finding that the size of the PSII antenna strongly influences the kinetic behaviour.

It is well known that the fluorescence yield of Chl *a* in thylakoids is higher when Q_A is reduced than when it is oxidised. The time course of this variation in fluorescence yield is termed fluorescence induction. Its study is an important experimental tool for assessing the properties of Q_A and electron transfer to Q_B. When fluorescence induction was discovered in the 1950s, it was thought quite natural that the Chl *a* excited state should decay more by fluorescence when photochemistry is blocked by Q_A reduction. Things became more complex when it was found that Ph precedes Q_A as an electron acceptor, and that primary photochemistry should still take place even when Q_A is reduced. Fluorescence induction can be explained by two mechanisms: 1) According to the scheme above, strong decrease of k_3 (blocking of electron transfer from Ph to Q_A) should lead to some return of excitation to the antenna, followed by fluorescence; 2) Prereduction of Q_A to semiquinone may increase the energy level of ($P680^+$ Ph^-) and decrease k_2 and/or increase k_{-2}, resulting in increased concentration of excitation in the antenna and a higher fluorescence yield. Both mechanisms probably contribute to the fluorescence induction. However, it is important to keep in mind that the effective free energy of the equilibrated excited state is strongly temperature-dependent (van Mieghem *et al.*, 1995).

Electron transfer from Ph^- to Q_A is in fact blocked not only when Q_A is singly reduced as Q_A^- (a situation which can occur naturally under sunlight), but also when Q_A is absent (as in isolated RC) or when Q_A is doubly reduced to the quinol form Q_AH_2. The latter is a pathological case that can be created *in vitro*, but may also occur *in vivo* under strong light. In this condition, the radical pair ($P680^+$ Ph^-) has a lifetime of about 10 ns, instead of only 250 ps when Q_A is oxidised, and it may recombine to

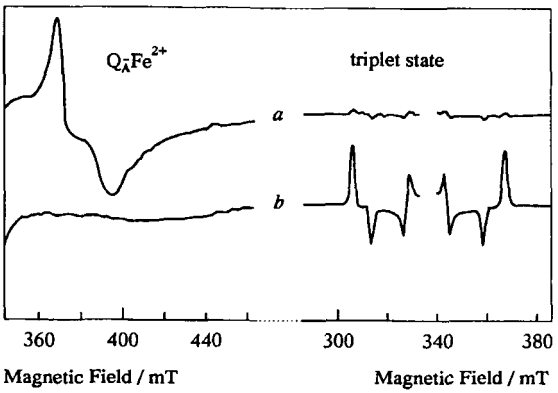


Figure 3.13 EPR spectra of PSII membranes with reduced Q_A. The left-hand spectra are recorded under conditions allowing observation of the $Q_A^-Fe^{2+}$ state (recorded in the dark), whereas the right-hand spectra display the triplet state (recorded under illumination). (a) singly reduced Q_A; (b) doubly reduced Q_A. Reproduced from van Mieghem (1993).

form a triplet state, as already discussed for purple photosynthetic bacteria. Striking behaviour has been observed for the triplet state in PSII: its EPR spectrum is very intense when Q_A is either absent or doubly reduced, but quite small when Q_A is in the semi-reduced form Q_A^- (Fig. 3.13) (van Mieghem *et al.*, 1989). It is now known that the difference originates partly from a lower yield of triplet formation, but mostly from a difference in triplet lifetime: about 2 ms against 10 μs, at 20 K, resulting in a very different steady-state population. The reason for this kinetic difference is not yet understood but it might be part of a protection mechanism.

Photoactivation and photodamage In addition to promoting electron transfer from water to plastoquinone, light has two other remarkable effects on PSII, photoactivation and photodamage, both of which are related to electron transfer.

Photoactivation Light-induced PSII electron transfer is required for the assembly of the manganese cluster *in vivo*. This requirement is clearly observed in algae that can synthesise chlorophyll in the dark: a PSII RC is assembled, but the manganese cluster is not. The inactive thylakoids, however, do contain Mn^{II}, which needs to bind to the RC and to be oxidised to Mn^{III} by Tyr_Z (or Tyr_D). Two successive photoreactions are needed, presumably to form a binuclear ($Mn^{III}Mn^{III}$) complex (Ananyev and Dismukes, 1997). A similar photoactivation can be observed when reconstitution experiments are conducted with isolated PSII preparations previously treated to

remove the manganese cluster. Associated with the photoactivation of the formation of the Mn cluster and O_2 evolution are other phenomena (conversion of cytochrome b_{559} from a low-potential to a high-potential form, binding of Ca^{2+} and of Cl^-, and a shift of the E_m of Q_A to a low value) that could be part of an ensemble of protective mechanisms.

Photodamage Photodamage of PSII is an important and highly complex phenomenon that has been studied in great detail. It will be described only briefly in this chapter; Baker and Bowyer (1994) give a comprehensive review. Photodamage seems to occur all the time, even under physiological conditions, albeit with very low quantum yield, and some pathological conditions increase the yield strongly. Following the initial light-induced event, a limited cleavage of the amino acid backbone of the D_1 polypeptide occurs. This leads to the disassembly of the PSII RC, which is then reassembled as new D_1 protein is synthesised. *In vivo*, the process is visualised as a light-dependent turnover of the D_1 polypeptide. *In vitro*, however, the process is blocked at an early stage that depends on experimental conditions. The initial reactions leading to PSII photodamage fall in two classes, acceptor-side or donor-side, according to the nature of the initial events (Andersson and Barber, 1996; Melis, 1999).

Acceptor-side reactions lead to damage at the stromal side of the D_1 protein and require aerobic conditions. They result from a block in electron transfer away from Q_A or Q_B, so that light-induced charge separation is followed by charge recombination. It is assumed that a triplet state ^{3}P680 is formed, by the mechanism discussed above, and that this reacts with oxygen to form singlet oxygen by the reaction

$$^3\text{P680} + {}^3\text{O}_2 \rightarrow \text{P680} + {}^1\text{O}_2 \qquad (3.10)$$

Singlet oxygen is a very reactive species and is believed directly or indirectly to cause damage to the D_1 protein. Results from several laboratories indicate that charge recombination takes place under two widely different situations: i) high light intensity, which could result in an overreduction of Q_A to the quinol state (similar events can occur *in vitro* when Q_A is lost); the ($P680^+$ Ph^-) radical pair then has a high probability of decaying directly to the triplet state ^{3}P680; ii) very low light intensity, which results in a high yield of damage (Keren *et al.*, 1997), presumably via a back reaction between Q_B^- and the S_2 or S_3 state, ultimately leading to the same process as in i). Some herbicides act at that level, through a change in the redox potential of Q_A (Krieger-Liszkay and Rutherford, 1998).

Turning to donor-side reactions, these have been well identified *in vitro*, when the manganese cluster is destroyed. They occur under anaerobic as well as aerobic conditions, and the damage is localised at the lumenal side of the D_1 protein. These reactions are attributed to highly oxidising species such as $P680^+$, which are normally short-lived but may accumulate when they cannot be re-reduced by electrons coming from water. There are two circumstances in which this type of damage can occur *in vivo*: i) when the PSII RC is not fully built; the Mn cluster is the last cofactor to be inserted and its absence may result in photodamage; ii) when thylakoids function correctly, a large ΔpH can be generated across the membrane, with a low pH (about 4.0) inside, which may induce a loss of bound Ca^{2+} and destabilise the Mn cluster.

It remains to be fully understood why PSII is more sensitive to photodamage than other parts of the photosynthetic apparatus. The donor-side damage obviously originates in the high oxidising power of $P680^+$, since the redox potential needed to oxidise water renders $P680^+$ also able to oxidise amino acid residues, forming radicals that could be the sites for polypeptide cleavage. The manganese cluster is certainly one of the least stable redox centres in the electron-transfer machinery and, as shown above, its disruption is the most probable origin of donor-side photodamage. The occurrence of acceptor-side damage may also have its origin in the oxidising power of $P680^+$. Indeed it is probably the consequence of triplet state formation in the recombination of the primary radical pair. Triplet states can normally be deactivated by energy transfer to a neighbouring carotenoid molecule. This process

$$^3(B)Chl + Car \rightarrow (B)Chl + {}^3Car \qquad (3.11)$$

(where (B)Chl denotes the primary donor or any chlorophyll-type pigment) has been well established in both purple photosynthetic bacteria reaction centres and antenna complexes. ^3Car decays harmlessly to the ground state. Triplet-triplet energy transfer, however, requires a close proximity of the partners, and the occurrence of such a mechanism would require that a carotenoid molecule be localised very close to P680. Experiments with isolated PSII RC show that this kind of energy transfer does not take place, although these particles do contain carotenoids (Takahashi *et al.*, 1987). One may speculate that the carotenoids are not close to P680 because they would not only quench the triplet state, but also donate an electron to $P680^+$, a reaction that should be avoided in order to maintain a high yield of electron transfer from the water-oxidising site.

These hypotheses leave open the question of the roles of carotenoids in PSII reaction centres. One obvious possible role is the deactivation of singlet oxygen, which could be formed by reaction of ground-state oxygen with triplet chlorophyll.

Other experiments indicate that, together with cyt b_{559} (Stewart and Brudvig, 1998) and the Chl a_Z molecule of the RC (de Paula *et al.*, 1985), a β-carotene may be part of a cyclic electron-transfer pathway that allows the re-reduction of $P680^+$ when other electron donors are not functional (Hanley *et al.*, 1999).

3.3.3 Photosystem I

Photosystem I, the other photosystem involved in oxygenic photosynthesis is an Fe–S type reaction centre. It is better characterised than its relatives in anoxygenic systems (such *Heliobacteria* and *Chlorobiaceae*, discussed in Section 3.2.4) but it performs essentially the same function: the light-driven transfer of electrons from reduced plastocyanin or cytochrome *c* in the lumen to ferredoxin in the stroma. The redox span between the external donors ($E_m \approx +360$ mV) and ferredoxin ($E_m \approx -420$ mV) is about 780 mV (see Fig. 3.2) making the maximum energy conversion yield of PSI about 43%. The low redox potential of reduced ferredoxin allows the reduction of $NADP^+$ to NADPH ($E_m \approx -350$ mV) via another enzyme, flavoprotein ferredoxin–NADP reductase. Under certain conditions, PSI can also perform cyclic electron transfer by feeding electrons via ferredoxin into the cyt b_6f complex, thereby contributing to the build-up of a transmembrane proton gradient. The photooxidised donors, plastocyanin or cytochrome, are re-reduced by the cyt b_6f complex. As in other Fe–S type reaction centres, PSI also binds a large number of chlorophylls that serve as undissociable antenna. In higher plants, additional light-harvesting complexes (LHC1) are associated with PSI, whereas in cyanobacteria large external pigment complexes, the phycobilisomes, serve to increase the absorption cross-section of PSI (and of PSII). Holzwarth discussed these in Chapter 2.

Structure of the PSI reaction centre The PSI reaction centre is a multiprotein complex that seems to exist as a trimer in the native membrane of cyanobacteria. The first structural models of PSI were obtained from the cyanobacterium *Synechococcus* by X-ray crystallography (Krauß *et al.*, 1996; Schubert *et al.*, 1997; Klukas *et al.*, 1999). Recently the resolution has been improved to 2.5 Å (Jordan *et al.*, 2001; Fromme *et al.*, 2001), providing an atomically detailed picture of 12 protein subunits and 127 cofactors. The latter comprise 96 chlorophylls, 2 phylloquinones, 3 Fe_4S_4 clusters and 22 carotenoids. In addition, 4 lipids, a putative Ca^{2+} ion and 201 water molecules have been identified. This structural information provides a solid basis for understanding the function of this enzyme, in particular electron transfer in the PSI RC (Golbeck and Bryant, 1991; Brettel, 1997; Brettel and Leibl, 2001).

The core of the RC, shown in Fig. 3.14, consists of two large membrane-integral protein subunits of 80 kDa each, forming a heterodimer. As in all other RC, these two subunits bind the primary donor. In PSI, this is a special pair of Chl a molecules absorbing at 700 nm and hence called P700. However, as judged from the larger distance between the two chlorophylls, this special pair is less strongly coupled than its counterpart in purple photosynthetic bacteria. From P700, two roughly symmetrical potential electron-transfer chains, each containing two chlorophyll molecules, extend across the membrane. On the stromal side, the 4Fe–4S cluster F_X is bound to two cysteine residues on each subunit, bridging the two large subunits. Two additional 4Fe–4S centres, known as F_A and F_B, are clearly resolved in the X-ray structure. These are bound to a small, ferredoxin-like protein subunit known as the F_{AB} protein, which is relatively firmly attached to the RC core on its stromal surface.

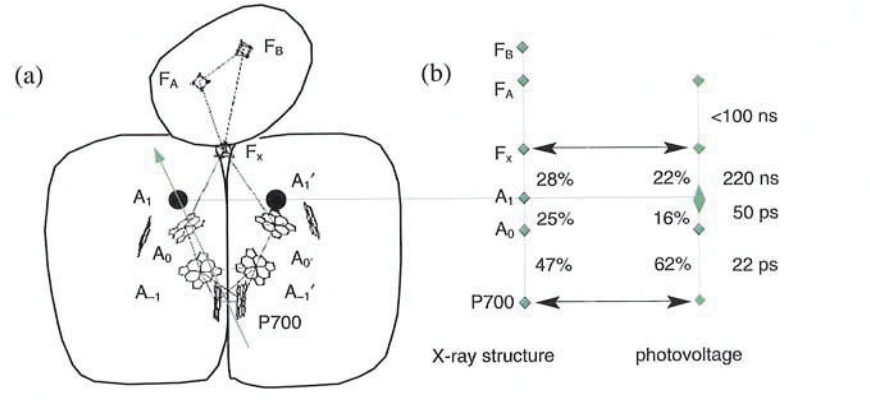


Figure 3.14 (a) Model of the PSI RC (only one RC of the trimer is shown). The position of chlorophyll cofactors is taken from X-ray diffraction data of PSI trimers from *Synechococcus* (Krauss *et al.*, 1996) and the position of the two phylloquinones A_1 and A_1' is indicated according to Klukas *et al.* (1999). On the left and right of the cofactor chains are shown two symmetrically positioned chlorophylls, which are thought to function in the transfer of excitation energy from the RC-bound antenna chlorophylls (not shown) to P700; (b) Fractional transmembrane distances between cofactors (relative to the transmembrane distance P700–F_X) as deduced from the X-ray structure (lefthand column of figures) and fractional electrogenicity of electron-transfer steps as determined by time-resolved photovoltage measurements (middle column of figures) with time constant of electron transfer (righthand column of figures). Assuming a dielectrically homogeneous RC, the electrogenicity of an electron-transfer step is proportional to the transmembrane distance between the cofactors involved. This allows determination of a plane in the membrane (shown as a horizontal dotted line) where A_1 must be located. Further structural information came from EPR measurements, *e.g.* the angle of the vector P700–A_1 with the membrane normal (27°, shown as a green arrow) as well as values for the P700–A_1 and A_1–F_X distances (see text). For a recent review of the structure of PSI with details of the cofactor–protein interactions, see Fromme *et al.* (2001).

Two quinone molecules, at least one of which is involved in electron transfer to F_X, are positioned between the chlorophylls in the middle of the membrane and F_X. As is typical for Fe–S-type reaction centres, the core proteins also bind a large number of pigments (90 chlorophylls and 22 carotenoids have been resolved in the structure of *Synechococcus*) as an intrinsic antenna. The two core protein subunits each contain 11 transmembrane α-helices. Five of these are proposed to be homologous to the five transmembrane helices of the L and M subunit of the purple bacterial RC, whereas the remaining six show homology with the antenna proteins CP43 and CP47 of PSII (Rhee *et al.*, 1998). This indicates an evolutionary relationship between the different types of RC and antenna proteins (Fromme *et al.*, 1996; Barber and Kühlbrandt, 1999; Baymann *et al.*, 2001). Besides the two large core proteins and the F_{AB} protein, about 10 subunits with relatively low molecular mass have also been identified in PSI. Some of these seem to be involved in the binding of external electron carriers, such as ferredoxin and plastocyanin, some are probably involved in the formation of PSI trimers in the membrane, and others have no known function.

Redox centres and electron transfer rates The primary photochemical events in PSI follow the universal scheme valid for all types of RCs. Transfer of excitation energy from the antenna results in electronic excitation of P700, followed by primary charge separation by electron transfer from the primary donor, here P700, to the primary acceptor. In PSI, the primary acceptor is a monomeric Chl *a* molecule known as A_0. The intrinsic time constant of this reaction cannot be measured directly since an intact PSI RC core devoid of antenna pigments is difficult to isolate. However, it seems that primary charge separation occurs at least as fast as in RC from purple photosynthetic bacteria, that is in <3 ps. The acceptor chlorophyll A_0 thus plays a similar role to the bacteriopheophytin H_L in purple photosynthetic bacteria. It would be reasonable to assign A_0 to one of the Chl *a* molecules close to the middle of the membrane (labelled A_0 and A_0' in Fig. 3.14), which are about 16 Å from P. However, this distance is too long to be compatible with picosecond electron transfer. It can thus be hypothesised that the interpositioned Chl *a* (A_{-1} and A_{-1}' in Fig. 3.14) probably mediates electron transfer as does the 'accessory' BChl B_L in the purple bacterial RC. When forward electron transfer is blocked, the primary radical pair ($P700^+ A_0^-$) decays in 20–30 ns by charge recombination, leading to a high yield of triplet state formation.

Under normal conditions, the primary charge separation in PSI is stabilised by electron transfer from A_0 to A_1 and then to F_X. A_1 has been identified as one of the two phylloquinone (vitamin K_1) molecules present in PSI. Since one of the phylloquinone molecules can be removed without impairing electron transfer, it has been thought that the PSI RC has one active and one inactive branch, like the RC of purple

photosynthetic bacteria. However, this model has been questioned and the possibility of two active branches is presently a matter of discussion (see below). With a halftime of about 30 ps, reduction of A_1 is nearly an order of magnitude faster than charge stabilisation on Q_A in quinone-type RCs and twenty times faster than reoxidation of A_0^- in other Fe–S-type RCs. The quinone A_1 has a much lower midpoint potential (about –800 mV) than the quinone acceptors Q_A in quinone-type RCs, which implies a special protein environment. A large body of experimental data demonstrates that A_1 is an intermediate acceptor in electron transfer to F_X. Therefore it was proposed that A_1 is located between A_0 and F_X long before the phylloquinones were identified in the X-ray structure. Consideration of the angle between the membrane normal, the vector P700 → A_1 determined by transient EPR (MacMillan *et al.*, 1997; van der Est *et al.*, 1997; Bittl *et al.*, 1997) and the relative transmembrane distances between cofactors determined by picosecond photovoltage measurements (Leibl *et al.*, 1995) predicts a position for A_1 close to that determined from the X-ray structure. This is also consistent with distance information obtained from pulsed EPR measurements, which indicate distances of 25.4 Å between $P700^+$ and A_1^-, and about 15 Å between A_1^- and F_X (Bittl and Zech, 1997; Deligiannakis *et al.*, 1998). The phylloquinone A_1 is firmly bound to the RC but can be removed by a hexane/ methanol treatment. Like Q_A in quinone-type RC, A_1 normally accepts only one electron but it can be doubly reduced under artificial, highly reducing conditions.

The Fe–S centres as terminal electron acceptors The three 4Fe–4S centres F_X, F_A and F_B that function as terminal RC-bound electron acceptors in PSI are mainly characterised by their different low-temperature EPR spectra. Of the three, F_X has the lowest E_m (of about –750 mV) and cannot be chemically reduced, although F_X^- can be photoaccumulated under reducing conditions. The midpoint potentials of F_A and F_B are around –550 mV, with some variation between different species. The study of electron transfer between the Fe–S centres is difficult because their absorption difference spectra are so similar. However, establishment of biochemical procedures to remove or more or less selectively destroy certain clusters, and then reconstitute them, has significantly facilitated functional characterisation of the complex under physiological conditions at room temperature. For example, the F_{AB} protein is readily removed by treatment with chaotropic agents that disorganise the water structure, F_X can be oxidatively denatured after removal of the F_{AB} protein and F_B is most susceptible to destruction by Hg.

In the absence of external donors and acceptors, flash-induced charge separation is followed by charge recombination, which can easily be followed by kinetic absorption spectroscopy. The kinetics of the recombination depend on the nature of

the electron acceptor from P700, and its half-time varies from about 30 ms in the intact system to 10 μs in the absence of all Fe–S centres. Forward electron transfer from A_1^- has been studied by several techniques, including transient EPR, absorption spectroscopy and photovoltage measurements. Reoxidation of A_1^- has a half-time of about 200 ns[3], independent of the presence of the F_{AB} protein, establishing F_X as an intermediate electron acceptor which is reduced in 200 ns. As regards the pathway of electron flow towards and between the two Fe–S centres F_A and F_B, there are now good arguments for electron flow along the sequence $F_X \rightarrow F_1 \rightarrow F_2 \rightarrow$ Fd, where F_1 is either F_A or F_B and F_2 is either F_B or F_A. To summarise these arguments, first, Fd binds to the F_{AB} protein with its 2Fe–2S centre close to F_2 (Lelong *et al.*, 1996); second, electron transfer from F_X to the next acceptor is very fast (<100 ns; Leibl *et al.*, 1995); third, reduction of Fd is very rapid, with a fastest phase of <1 μs (Sétif and Bottin, 1994). Moreover, in view of the distances between the three Fe–S centres, F_1 has to be involved in electron transfer to F_2. Recent studies on PSI complexes with destroyed F_B suggest that F_A reduction is not affected whereas Fd reduction is (Diaz-Quintana *et al.*, 1998). These observations strongly favour the assignments $F_A = F_1$ and $F_B = F_2$. This assignment is in agreement with the conclusions derived from X-ray crystallography (Fromme *et al.*, 2001 and references therein).

3.4 Photosynthetic electron transfer: importance of kinetics

The description of the primary processes of photosynthetic energy conversion presented above shows that essentially the same strategy is realised in all systems, which we can summarise as follows: large protein complexes hold a certain number of pigments and redox-active cofactors in well-defined positions. Absorption of a photon leads to an excited state that is rapidly and efficiently converted to a charge-separated state. The separated charges are stabilised by several successive electron-transfer reactions that further increase the distance between them. The overall quantum yield of these processes is very nearly unity and, although the energetic yield

[3] The finding of that the reoxidation rate of A_1^- is biphasic, not only in preparations of spinach but also in whole cells of the algae *Chlorella*, has led to two interesting hypotheses. The biphasicity can be explained either by the establishment of an equilibrium between the states $A_1^-F_X$ and $A_1F_X^-$ which is close to one (Sétif and Brettel, 1993) followed by electron transfer to F_A and F_B, or by two classes of phylloquinones, which differ in their reoxidation rates (Joliot and Joliot, 1999). The latter possibility could indicate that in PSI both branches of cofactors are active in electron transfer from P700 to F_X. Support for the model of two active branches comes from the effect on the kinetics of A_1^- reoxidation induced by mutations of residues near the two quinones (Guergova-Kuras *et al.*, 2001).

necessarily decreases with every reaction step, energy conversion yields for a photosynthetic reaction centre, measured as the difference in redox potential of the primary donor and the terminal acceptor, typically reach 40%. These high yields demonstrate ingenious optimisation during evolution. In the following we will discuss some basic features of photosynthetic electron transfer and especially of the primary photochemical reactions taking place before chemistry takes over.

The free energy change of an electron-transfer reaction determines its driving force and also the energy loss in the multistep electron transfers of photosynthetic systems. However, thermodynamics does not take kinetic constraints into account, albeit they are of primordial importance in the primary reactions of photosynthesis, controlling reaction rates over a huge time range from femtoseconds to seconds. For example, from a simple energetic point of view, direct electron transfer from the excited primary donor to the first quinone acceptor is highly favourable, but the distance between them is so great that this reaction would be much too slow to compete with wasteful deexcitation; an intermediate electron acceptor is needed to prevent this occurring. To understand the necessary complexity of electron transfer in photosynthetic energy conversion, we shall now consider some basic principles of electron-transfer theory.

3.4.1 Electron transfer theory: factors governing kinetics

Each electron transfer implies some change in chemical structure. Besides the actual transfer of the electron from a donor D to an acceptor A, and the changes in geometry of D and A that this may entail, nuclear motions within and around the molecules themselves have to be considered. Even when the surrounding environment is treated as a homogeneous dielectric, calculation of structural changes can be fairly complicated. However, in most cases electron transfer can be understood by modelling the reactant (D A) and product (D^+A^-) states as two intersecting parabolas describing the free energy of each as a function of a (multidimensional) reaction coordinate, as shown in Fig. 3.15. In this diagram, the free energy change of reaction is the vertical displacement of the minima of the parabolas. Electron transfer occurs at the intersection point of the two parabolas, where the reactant and the product states have the same energy. The activation energy of the reaction is the energy difference between this intersection point and the energy minimum of the reactant parabola. The reorganisation energy λ is the energy required to move the system from the reactant equilibrium coordinates to the product equilibrium coordinates without actually transferring the electron, that is, while staying on the reactant potential energy curve.

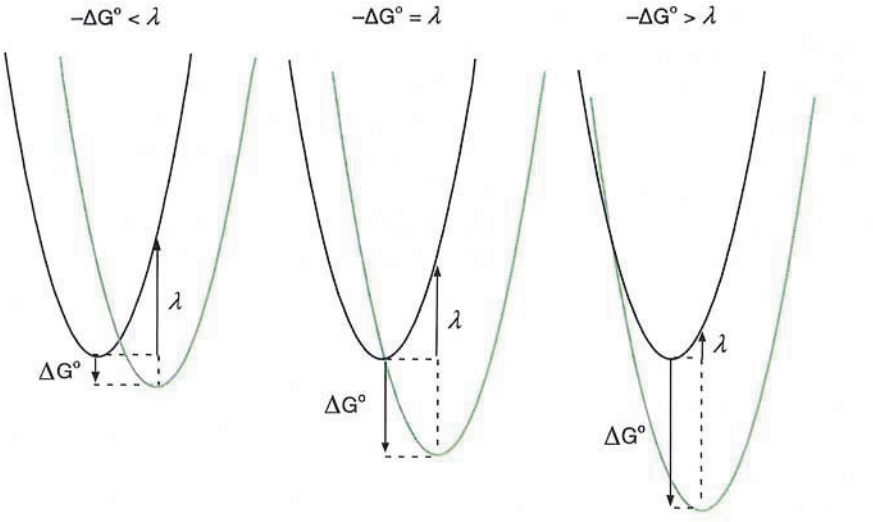


Figure 3.15 Schematic representation on a one-dimensional reaction coordinate of the parabolic free energy surfaces of the reactant (left parabola) and product (right parabola) electronic states of an electron-transfer reaction. $-\Delta G^\circ < \lambda$: normal region; $-\Delta G^\circ = \lambda$: special case of activationless reaction; $-\Delta G^\circ > \lambda$: inverted region (see text).

Long-range electron transfer between weakly coupled partners is well described by Marcus theory (Marcus, 1956; Marcus and Sutin, 1985). In the classical approximation, the activation energy U_a is given by

$$U_a = \frac{(\Delta G^\circ + \lambda)^2}{4\lambda} \tag{3.12}$$

and the rate constant for electron transfer k_{ET} by

$$k_{ET} = \frac{2\pi}{\hbar} V_r^2 \frac{1}{\sqrt{4\pi\lambda kT}} \exp\left(-\frac{U_a}{kT}\right) \tag{3.13}$$

where V_r is the electronic coupling term (tunnelling matrix element) (compare eqs. 3.6–3.8). V_r depends on the overlap between the molecular orbitals of the electron donor and acceptor. This decreases exponentially with increasing donor–acceptor distance r (eq. 3.7), so k_{ET} falls off very strongly with increasing distance. The Franck–Condon factor describes a weighted density of states and contains the dependence of the rates on the free energy of electron transfer (ΔG°), temperature (T) and reorganisation energy (λ). At low temperature, a quantum mechanical treatment of one or several vibrational modes coupled to electron transfer has to be applied in

the expression for the Franck–Condon factor (Bixon and Jortner, 1986; Weintraub and Bixon, 1994). This gives the rates a weaker temperature dependence so long as the characteristic frequencies of the vibrational modes are much larger than kT. We will restrict our discussion to physiological temperatures for which a classical treatment is sufficient (but see for instance Barbara *et al.*, 1996 for quantum-mechanical treatments).

Moser and Dutton (1992) have fitted the rates of many electron-transfer reactions in photosynthetic reaction centres at room temperature to the following parameterised form of the Marcus equation:

$$\log k_{ET} = 15 - 0.6r - 3.1\frac{(\Delta G^{\circ} + \lambda)^2}{\lambda} \tag{3.14}$$

This formula gives the rate constant of electron transfer k_{ET} (s^{-1}) as a function of the edge-to-edge distance r (Å) between the cofactors, the free energy change ΔG° and the reorganisation energy λ (both in eV). The rate of intraprotein long-range electron transfer is essentially determined by these three parameters, ΔG°, λ and r. We shall look at each of these in turn.

3.4.2 *The role of the driving force ΔG°*

The Marcus equation predicts that a plot of ln k_{ET} *vs.* the driving force $-\Delta G^{\circ}$ will be a parabola, the curvature depending on the reorganisation energy, as shown in Fig. 3.16. The rate constant increases with increasing driving force until it reaches a maximum, and decreases when $-\Delta G^{\circ}$ increases further. From eq. 3.13 or 3.14 it can be seen that the maximum rate is observed when $-\Delta G^{\circ} = \lambda$. This condition is fulfilled when the parabolas for the reactant and the product states intersect at the minimum of the reactant curve (as in the middle diagram of Fig. 3.15). At this point the reaction is activationless (eq. 3.12) and the rate depends essentially only on the distance. Reactions with $-\Delta G^{\circ} > \lambda$ are again activated and this is called the 'inverted region'.

As outlined above (eq. 3.5), ΔG° is determined by the midpoint potentials of the donor and the acceptor, which can themselves be controlled by electrostatic or hydrogen-bonding interactions with the protein. For electron-transfer reactions in the normal region ($-\Delta G^{\circ} < \lambda$), the variation of the rate with ΔG° is relatively weak: typically the change in the rate of an activated reaction is around a factor of ten for a change in ΔG° of –200 mV. Near-activationless reactions are particularly insensitive to small changes in ΔG°, and this is true of most electron-transfer reactions in photosynthesis. An exception is the charge-stabilisation reaction on Q_A in PSII and

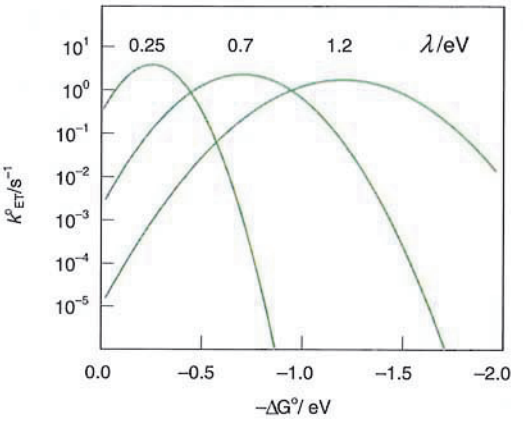


Figure 3.16 Effect of the driving force $-\Delta G^{\circ}$ on the 'standard' electron-transfer rate k^{0}_{ET} for three different values of the reorganisation energy λ. The rate constant k^{0}_{ET} corresponds only to the FC factor, *i.e.* it is assumed that $2\pi/\hbar V_{fi}^{2} = 1$ in eq. 3.13.

purple photosynthetic bacteria (see Fig. 3.2). This can be rationalised by considering the function of the Q_A/Q_B acceptor couple: interacting with Q_B, a soluble and weakly bound redox partner, it cannot be expected that Q_A^- can always transfer its electron rapidly. Therefore the lifetime of Q_A^- has to be relatively long (much longer than the lifetime of A_1 in PSI, where the next electron acceptor is firmly bound). Under such conditions, back transfer to (B)Ph and subsequently to P^+ has to be avoided and this is best achieved by maximising the rate of forward reaction (which is activationless) and giving the reaction a large driving force. The free energy difference ΔG° determines the equilibrium constant K ($= \exp[-\Delta G^{\circ}/kT]$) of the reaction. A large driving force therefore results in an equilibrium that is very much in favour of the state Q_A^- and a rate of back-electron transfer many orders of magnitude smaller than the rate of forward transfer. For example, the driving force of ~650 mV for electron transfer from BPh to Q_A in purple photosynthetic bacteria (Fig. 3.2) implies a 'forward' equilibrium constant of $K = 2 \times 10^{11}$ and a rate constant of $(40\ s)^{-1}$ for endergonic back transfer compared with $(200\ ps)^{-1}$ for the exergonic forward reaction. In summary, a large driving force makes forward electron transfer strongly irreversible.

It is interesting to note that ΔG° may not be a well-fixed quantity for a given reaction but might depend on the history of the system. For example, a significant increase in ΔG° from picoseconds to nanoseconds between the exited singlet state and the charge-separated state has been reported (Peloquin *et al.*, 1994; Holzwarth and Müller, 1996), attributed to relaxations of the surrounding protein that follow an electron-transfer reaction and stabilise the product state. Besides such a passive role, a

possible active role of the intervening protein has been proposed giving rise to self-organised electron transfer (Pohlmann and Tributsch, 1996; Tributsch and Pohlmann, 1997). This model is based on the idea that in systems operating far from thermodynamic equilibrium part of the input energy can be used as negative friction energy to facilitate the electron-transfer process by preparing a favourable activation barrier through a well-adjusted transient change of the intervening protein structure.

3.4.3 The role of the reorganisation energy λ

According to the pioneering work of Marcus, the reorganisation energy λ, which reflects the importance of the conformational changes that accompany the reaction, is the sum of two contributions, the outer (solvent or medium) reorganisation energy λ_{out} and the internal (or vibrational) reorganisation energy λ_{in} (Marcus, 1956; Marcus and Sutin, 1985). The solvent reorganisation energy depends on the size of the donor and acceptor, the distance between them and the polarity of the intervening medium. When the donor and acceptor molecules are approximated by spheres of effective radii r_D and r_A, the solvent reorganisation energy can be expressed as

$$\lambda_{out} = q^2 (1/2r_D + 1/2r_A - 1/r_{DA}) (1/\epsilon_{op} - 1/\epsilon_s) \qquad (3.15)$$

where r_{DA} is the centre-to-centre distance between donor and acceptor, and ϵ_{op}, ϵ_s are the optical (electronic polarisation) and static dielectric constants of the medium. Effective radii of cofactors of RC have been determined, for instance: (B)Chl and (B)Ph, 5.56 Å; $(BChl)_2$, 6.89 Å; MQ, 2.84 Å; UQ, 2.66 Å. The quantity $(1/\epsilon_{op} - 1/\epsilon_s)$ describes the influence of the solvent and is sometimes called the coupling constant. The low (static) dielectric constant of proteins leads to a low value of the factor $(1/\epsilon_{op} - 1/\epsilon_s)$, typically 0.15–0.20, less than half the value for water (Krishtalik, 1989; Krishtalik and Cramer, 1996). Equation 3.15 indicates that long-range electron transfer between large cofactors in a low-dielectric protein environment will be characterised by small solvent reorganisation energies. This has been experimentally confirmed, at least as far as the (fast) primary reactions of photosynthesis are concerned.

The internal part λ_{in} of the reorganisation energy describes the conformational changes that occur at the redox centres themselves. This reflects contributions from the change of all (or the most important) internal vibrational modes accompanying electron transfer and is more difficult to estimate than λ_{out}. The changes in the normal modes can be expected to be weak for large molecules with an extended conjugated

electronic structure, like chlorophylls or pheophytins, leading to a relatively small contribution of λ_{in}, of the order of 100–300 meV, to λ. In contrast, λ_{in} may be much larger in iron–sulphur centres or other redox-active metal ions.

The variation of k_{ET} with λ is relatively small, typically around a factor of 10 for a 200 mV change in λ. The reorganisation energy contributes significantly to the activation energy ($U_a = \lambda/4$ for an isoenergetic reaction) and determines the driving force ($-\Delta G^\circ = \lambda$) for activationless transfer. Hence, engineering a suitable value of λ is important for optimising functionality, especially as the possibility of modifying ΔG° is usually limited for energetic reasons. However, there are constraints. Specific functions, such as intra-protein proton transfer, require a polar environment, necessarily leading to larger values of the reorganisation energy of electron-transfer reactions. Moreover, some regions of the RC are more exposed to the aqueous phase than others which are deeply buried within the membrane. As a consequence, the reorganisation energy varies widely for different electron-transfer reactions in photosynthesis, with values ranging from ~0.2 eV for primary reactions to more than 1 eV, but typical values are around 700 mV. It is also possible that slower (protein) conformational changes that occur on the time scale of electron transfer have some influence on apparent values for the reorganisation energy (see, *e.g.*, Cherepanov *et al.*, 2001).

3.4.4 The role of the distance r

We have seen that the nuclear terms in the Franck–Condon factor generally have a rather modest influence on the electron-transfer rate. By contrast, the electronic coupling, which depends on the distance between donor and acceptor, clearly dominates the rate and determines its strong distance dependence (although λ_{out} may also contribute some distance dependence, as explained in the previous section). The exponential decrease with distance of the rate of electron transfer has its origin in the exponential radial decay of electronic wavefunctions and is expected to be sensitive to the material in the region between donors and acceptors. The distance dependence of k_{ET} can be described by

$$k_{ET} = k_0 \exp\left[-\beta\left(r - r_0\right)\right] \tag{3.16}$$

where r_0 is the van der Waals distance, r is the edge-to-edge distance between donor and acceptor (nearest atoms) and k_0 is the electron-transfer rate at the contact distance $r = r_0$.

The distance dependence of long-range intra-protein electron transfer has been the subject of intense investigation. In the simplest description, the parameter β describes the average effect of the intervening medium. In this model, a vacuum is characterised by $\beta = 2.8$ Å^{-1}, covalent bonds by $\beta = 0.7$ Å^{-1}, and an extended conjugated π-bond system by an infinite value of β. By investigating the dependence of the optimal rate, which depends only on the electronic coupling, on the edge-to-edge distance between cofactors for a series of electron transfer reactions in the bacterial RC, Moser *et al.* (1992) derived the value $\beta = 1.4$ Å^{-1} and a rate of $k_0 = 10^{13}$ s^{-1} at van der Waals distance. Thus the β value for the RC protein appears to be intermediate between those for covalently linked systems and a vacuum. It implies that the rate changes by a factor of 10 for a 1.7 Å change in donor–acceptor distance. To give some numbers, an optimal rate of (1 ns)$^{-1}$ is compatible with an edge-to-edge distance of 10 Å, one of (1 μs)$^{-1}$ with 15 Å, and one of (1 ms)$^{-1}$ with 20 Å.

Simple approaches such as the one described above treat the intervening medium as an average over through-space and through-bond electron-transfer steps, or consider the packing density of protein atoms in the volume between redox cofactors (Page *et al.*, 1999). But more sophisticated calculations of through-bond transfer pathways have also been developed and applied to model systems with known structure (Beratan *et al.*, 1991; Curry *et al.* 1995; Gray and Winkler, 1997; Newton, 1997; Ungar *et al.*, 1999; Sisido *et al.*, 2001). In most cases, these predict exponential distance dependence for long-range electron transfer, suggesting that in proteins there are generally many equivalent pathways for electron transfer.

Figure 3.17 demonstrates the dominant influence of the distance on the electron transfer rates in the RC. It shows the predicted $\beta = 1.4$ Å^{-1} dependence of ln k_{ET} on distance r as a straight line, together with some points for actual rates of exergonic electron-transfer reactions in *Rp. viridis*. The relatively small deviations of the measured rates from the predicted ones indicate that nuclear factors play only a minor role in determining the rates of these reactions.

It is worth noting that distances between cofactors seem to be well conserved in RCs from different organisms belonging to the same family (PSI- or PSII-type). In terms of direction of electron transfer, the distance between two cofactors is a neutral factor. But the linear (transmembrane) arrangement of a co-factor chain, leading to a successive increase in distance between the positive and the negative charges, is an efficient way to favour forward transfer against charge recombination. This is apparent from the data points in Fig. 3.17, which show that increasing the distance from 10 to 23 Å decreases the rate by seven orders of magnitude. In this way, electron transfer to the nearest neighbour acceptor is overwhelmingly favoured despite the presence of eight or more cofactors in the RC protein.

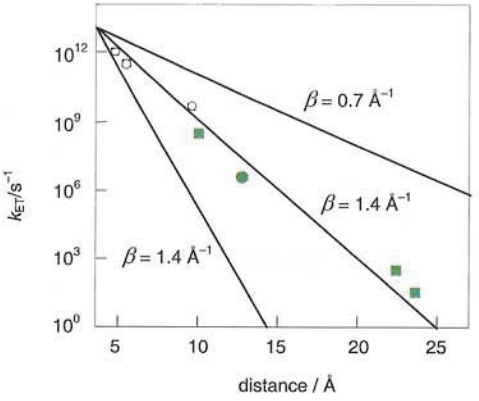
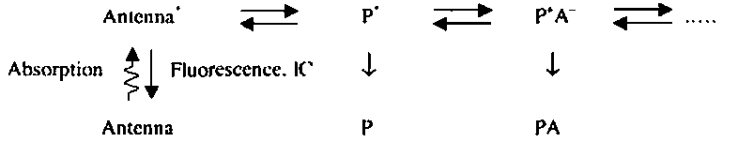


Figure 3.17 Effect of the edge-to-edge distance between redox centres on the electron-transfer rate constant k_{ET}. The squares show experimentally determined rate constants for exergonic electron transfer reactions in the reaction centre of *Rp. viridis*. Open symbols denote forward reactions and closed symbols back reactions. The straight lines show the exponential decay of the rate with distance according to eq. 3.15 with a rate at van der Waals contact of $k_{vw} = 10^{13}$ s^{-1} and $\beta = 0.7$ Å^{-1}, 1.4 Å^{-1} and 2.8 Å^{-1}.

3.4.5 *Primary charge separation*

In native photosynthetic systems, light is absorbed in the antenna complexes and transferred as a singlet excited state to the primary donor P, where it induces primary charge separation. In general, these reaction steps are reversible and can be expressed by the following reaction scheme



The existence of inherent channels for energy loss, such as fluorescence and radiationless deactivation, demand that the effective electron-transfer rate from P* to A be significantly faster than 10^9 s^{-1}. This can only be achieved when the distance between P and the primary acceptor A is small. In the reaction centre of purple bacteria, the edge-to-edge distance between the special pair and BPh is 10.1 Å, which would allow an electron-transfer time constant of ~1 ns under optimum conditions. This is much longer than the measured time of 3–4 ps for reduction of BPh, which shows that the accessory BChl must be involved as an intermediate acceptor.

Fast charge separation is necessary but not sufficient for a high quantum yield. In general there is equilibrium between the excited state and the charge-separated state, and the entropic contribution of a large antenna system could shift this equilibrium towards the excited state. The energetic difference between the excited and the charge-separated states cannot be too great or too much energy will be lost. However, in many cases there seems to be a certain degree of kinetic decoupling between the antenna and the reaction centre. Although energy transfer within the antenna in general is very fast, transfer between the antenna and P might be slower than primary charge separation, making the trapping process 'diffusion-limited' rather than 'trap-limited'. In this way, back transfer of excitation energy to the antenna might be limited. An exception seems to be PSII, probably the 'slowest' reaction centre in terms of energy transfer in the antenna, and also showing the highest fluorescence yield. When this RC is coupled to its relatively large antenna it is a 'shallow trap' and primary charge separation is freely reversible. This might be related to the fact that PSII is the first RC in a linear electron-transfer chain, implying some need for regulation of its activity as part of a protection mechanism against over-excitation that would lead to photodamage.

In fact, reformation of an excited state P^* from the radical pair state P^+A^- is not the main problem in maintaining high yield of charge separation, because the energy is not lost in the excited state and might be used again to induce charge separation. The main challenge is to avoid the energetically very favourable charge recombination to the ground state (with or without triplet formation) and concomitant loss of photon energy. The key to energy conversion in photosynthesis can be found in the characteristics of this process of primary charge separation: it is activationless and has very low reorganisation energy ($\lambda = -\Delta G^\circ \approx 0.1$–$0.2$ eV). The first fact makes the forward rate optimal, and the second puts the back reaction to the ground state with its enormous driving force (~1.3 eV) far in the inverted region ($-\Delta G^\circ \gg \lambda$), giving this reaction a much slower rate. This allows enough time for charge stabilisation by electron transfer to another, more distant acceptor. As pointed out earlier, energy is dissipated in secondary charge-stabilisation reactions to prevent thermally activated decay channels.

It is not completely clear how such a low reorganisation energy for the primary charge separation is achieved, but several factors can be tentatively identified. These include the large size of the donor (a 'special pair' dimer of chlorophylls in most cases) as well as of the acceptor molecules involved in primary charge separation, and the low dielectric constant of the protein around the special pair. The orientation of co-factors might also be important. The symmetry of the electronic wavefunctions of the ground and excited donor states may be different, so that the optimal orientation

for electron transfer from the excited state to an acceptor might be different from the optimal orientation for wasteful back reactions (to the ground state), further favouring formation of the charge-separated state (Hasegawa and Nakatsuji, 1998).

In summary, reaction centres, with their array of redox co-factors arranged in a protein matrix of tuned polarity so as strongly to favour forward electron transfer and disfavour wasteful back reactions, is the engine that permits photosynthesis to harness solar energy.

Editors' note added in proof

During the final stages of preparing this book, Ferreira *et al.* (2004) reported a highly refined X-ray structure of PSII isolated from *Thermosynechococcus elongatus.* Their new structural model is a considerable improvement on those reported by Zouni *et al.* (2001) and Kamiya and Shen (2003). The structure of Ferreira *et al.* (2004) contains most of the amino acid residues of the PSII complex and shows details of the protein environments of the various cofactors, providing answers to many of the questions raised in this chapter. For example, the role of bicarbonate as a ligand for the non-haem iron is clearly seen in the new structure. However, the most exciting new information is that the catalytic site responsible for water oxidation consists of 4 Mn ions and one Ca^{2+}. The metals are arranged as a cubane-like $Mn_3Ca^{2+}O_4$ cluster bridged to the fourth Mn by a mono-μ-oxo bond. The metal-to-metal distances within the cluster are 2.7 Å Mn–Mn and 3.4 Å for Mn–Ca^{2+}, which are typical for the oxo bridges proposed and consistent with distances derived from the EXAFS studies mentioned in Section 3.3.2. The distances between the Mn ions in the cubane and the fourth Mn is ~3.3 Å, typical of a mono-μ-oxo bridge. Six amino acids ligate the Mn ions, while the Ca^{2+} does not directly interact with protein. Five of the amino acid ligands belong to the D1 protein (Asp170, Glu189, His332, Glu333, Asp342) while the sixth, surprisingly, is a residue of the chlorophyll-binding protein CP43 (Glu354). A number of other amino acids lie very close to the metal cluster and probably play an important part in stabilising intermediates of the water oxidation process and providing a channel for protons and water molecules between the catalytic centre and the external lumenal phase. This X-ray structure gives only a snapshot of the catalytic centre, probably in its S_1 (dark) state. Interestingly, a non-protein species seems to form ligands with Ca^{2+} and the adjacent fourth Mn, and this density has been tentatively assigned to bicarbonate, an anion which is known to be important as a cofactor for the water oxidation process. Overall the structure of the metal cluster suggests that only one Mn is directly involved in the chemistry of water oxidation and

that the Ca^{2+} provides a binding site for the second water substrate molecule. It is possible that prior to dioxygen formation (S_4 state), a highly reactive electrophilic Mn(V) oxo or an Mn(IV) oxyl radical is formed and undergoes a nucleophilic attack from the second substrate molecule ligated to Ca^{2+}. Although further information will be required, this recent X-ray structure provides a major step forward in revealing the precise molecular details of the water oxidation process.

References

Adir N., Axelrod H. L., Beroza P., Isaacson R. A., Rongey S. H., Okamura M. Y. and Feher G. (1996), 'Co-crystallization and characterization of the photosynthetic reaction center–cytochrome c_2 complex from *Rhodobacter sphaeroides*', *Biochem.* **35**, 2535–2547.

Amesz J. (1995a), 'The antenna–reaction center complex of *Heliobacteria*', in Blankenship *et al.* (1995, eds.), pp. 687–697.

Amesz J. (1995b), 'The heliobacteria, a new group of photosynthetic bacteria', *J. Photochem. Photobiol. B* **30**, 89–96.

Ananyev G. M. and Dismukes G. C. (1997), 'Calcium induces binding and formation of a spin-coupled dimanganese (II, II) center in the apo-water oxidation complex of Photosystem II as precursor to the functional tetra-Mn/Ca cluster', *Biochem.* **36**, 11342–11350.

Andersson B. and Barber J. (1996), 'Mechanisms of photodamage and protein degradation during photoinhibition of PSII', *Adv. Photosynth.* **5**, 101–121.

Arlt T., Schmidt S., Kaiser W., Lauterwasser C., Meyer M., Scheer H. and Zinth W. (1993), 'The accessory bacteriochlorophyll: a real electron carrier in primary photosynthesis', *Proc. Nat. Acad. Sci. USA* **90**, 11757–11761.

Armstrong W. H. (1992), 'Polynuclear manganese complexes as models for the Photosystem II water oxidation catalyst', in Pecoraro V. L., ed., *Manganese Redox Enzymes*, VCH Publishers, New York, pp. 261–286.

Axelrod H. L., Abresch E. C., Okamura M. Y., Yeh A. P., Rees D. C. and Feher G. (2002), 'X-ray structure determination of the cytochrome c_2: reaction center electron transfer complex from *Rhodobacter sphaeroides*', *J. Mol. Biol.* **319**, 501–515.

Baker N. R. and Bowyer J. R. (1994), *Photoinhibition of Photosynthesis. From Molecular Mechanisms to the Field*, Bios Scientific Publishers, Oxford, UK.

Barber J., Nield J., Morris E. P., Zheleva D. and Hankamer B. (1997), 'The structure, function and dynamics of Photosystem II', *Physiol. Plant.* **100**, 817–827.

Barber J. and Kühlbrandt W. (1999), 'Photosystem II', *Curr. Opin. Struct. Biol.* **9**, 469–475.

Barber J. and Archer M. D. (2001), 'P680, the primary electron donor of Photosystem II', *J. Photochem. Photobiol. A* **142**, 97–106.

Barber J. (2002), 'P680: what is it and where is it?', *Bioelectrochem.* **55**, 135–138.

Barbara P. F., Meyer T. J. and Ratner M. A. (1996), 'Contemporary issues in electron transfer research', *J. Phys. Chem.* **100**, 13148–13168.

Baymann F., Brugna M., Mühlenhoff U. and Nitschke W. (2001), 'Daddy, where did (PS)I come from?', *Biochim. Biophys. Acta* **1507**, 291–310.

BBA (2001), *Biochim. Biophys. Acta* **1503**, 1–259, special issue on photosynthetic water oxidation.

Beratan D. N., Betts J. N. and Onuchic J. N. (1991), 'Protein electron transfer rates set by the bridging secondary and tertiary structure', *Science* **252**, 1285–1287.

Bittl R. and Zech S. G. (1997), 'Pulsed EPR study of spin-coupled radical pairs in photosynthetic reaction centers: measurement of the distance between P_{700}^+ and A_1^- in Photosystem I and between P_{865}^+ and Q_A^- in bacterial reaction centers', *J. Phys. Chem. B* **101**, 1429–1436.

Bittl R., Zech S. G., Fromme P., Witt H. T. and Lubitz W. (1997), 'Pulsed EPR structure analysis of Photosystem I single crystals: localization of the phylloquinone acceptor', *Biochem.* **36**, 12001–12004.

Bixon M. and Jortner J. (1986), 'Coupling of protein modes to electron transfer in bacterial photosynthesis', *J. Phys. Chem.* **90**, 3795–3800.

Bixon M., Jortner J. and Michel-Beyerle M. E. (1991), 'On the mechanism of the primary charge separation in bacterial photosynthesis', *Biochim. Biophys. Acta* **1056**, 301–315.

Blankenship R. E., Madigan M. T. and Bauer C. E. (1995, eds.), *Anoxygenic Photosynthetic Bacteria*, Kluwer Academic Publishers, Dordrecht,

Blankenship R. E. and Hartman H. (1998), 'The origin and evolution of oxygenic photosynthesis', *Trends Biochem. Sci.* **23**, 94–97.

Boussac A., Zimmermann J.-L., Rutherford A. W. and Lavergne J. (1990), 'Histidine oxidation in the oxygen-evolving Photosystem II enzyme', *Nature* **347**, 303–306.

Boussac A., Girerd J.-J. and Rutherford A. W. (1996), 'Conversion of the spin state of the manganese complex in Photosystem II induced by near-infrared light', *Biochem.* **35**, 6984–6989.

Boussac A., Kuhl H., Ghibaudi E., Rögner M. and Rutherford A. W. (1999), 'Detection of an electron paramagnetic resonance signal in the S_0 state of the manganese complex of Photosystem II from *Synechococcus elongatus*', *Biochem.* **38**, 11942–11948.

Brettel K. (1997), 'Electron transfer and arrangement of the redox cofactors in Photosystem I', *Biochim. Biophys. Acta* **1318**, 322–373.

Brettel K., Leibl W. and Liebl U. (1998), 'Electron transfer in the heliobacterial reaction center: evidence against a quinone-type electron acceptor functioning analogous to A_1 in Photosystem I', *Biochim. Biophys. Acta* **1363**, 175–181.

Brettel K. and Leibl W. (2001), 'Electron transfer in Photosystem I', *Biochim. Biophys. Acta* **1507**, 100–114.

Brugna M., Rodgers S., Schricker A., Montoya G., Kazmeier M., Nitschke W. and Sinning I. (2000), 'A spectroscopic method for observing the domain movement of the Rieske iron–sulfur protein', *Proc. Nat. Acad. Sci. USA* **97**, 2069–2074.

Budil D. E. and Thurnauer M. C. (1991), 'The chlorophyll triplet state as a probe of structure and function in photosynthesis', *Biochim. Biophys. Acta* **1057**, 1–41.

Büttner M., Xie D.-L., Nelson H., Pinther W., Hauska G. and Nelson N. (1992), 'The Photosystem I-like reaction center of green S-bacteria is a homodimer', *Biochim. Biophys. Acta* **1101**, 154–156.

Campbell K. A., Gregor W., Pham D. P., Peloquin J. M., Debus R. J. and Britt R. D. (1998), 'The 23 and 17 kDa extrinsic proteins of Photosystem II modulate the magnetic properties of the S_1-state manganese cluster', *Biochem.* **37**, 5039–5045.

Chen I.-P., Mathis P., Koepke J. and Michel H. (2000), 'Uphill electron transfer in the tetraheme cytochrome subunit of the *R. viridis* photosynthetic reaction center: evidence from site-directed mutagenesis', *Biochem.* **39**, 3592–3602.

Cherepanov D. A., Krishtalik L. I. and Mulkidjanian A. Y. (2001), 'Photosynthetic electron transfer controlled by protein relaxation: analysis by Langevin stochastic approach', *Biophys. J.* **80**, 1033–1049.

Crofts A. R. and Berry E. A. (1998) 'Structure and function of the cytochrome bc_1 complex of mitochondria and photosynthetic bacteria', *Curr. Opin. Struct. Biol.* **8**, 501–509.

Curry W. B., Grabe M. D., Kurnikov I. V., Skourtis S. S., Beratan D. N., Regan J. J., Aquino A. J. A., Beroza P. and Onuchic N. (1995) 'Pathways, pathway tubes, pathway docking, and propagators in electron transfer proteins', *J. Bioen. Biomembr.* **27**, 285–293.

Dau H., Iuzzolino L. and Dittmer J. (2001), 'The tetra-manganese complex of Photosystem II during its redox cycle—X-ray absorption results and mechanistic implications', *Biochim. Biophys. Acta* **1503**, 24–39.

Debus R. J. (1992), 'The manganese and calcium ions of photosynthetic oxygen evolution', *Biochim. Biophys. Acta* **1102**, 269–352.

Deisenhofer J., Epp O., Miki K., Huber R. and Michel H. (1984), 'X-ray structure analysis of a membrane protein complex. Electron density map at 3 Å resolution

and a model of the chromophores of the photosynthetic reaction center from *Rhodopseudomonas viridis*', *J. Mol. Biol.* **180**, 385–398.

Deisenhofer J. and Michel H. (1989), 'The photosynthetic reaction centre from the purple bacterium *Rhodopseudomonas viridis*', *EMBO J.* **8**, 2149–2170.

Deligiannakis Y., Hanley J. and Rutherford A. W. (1998), 'Spin-lattice relaxation of the phyllosemiquinone radical of Photosystem I', *Biochem.* **37**, 3329–3336.

de Paula J. C., Innes J. B. and Brudvig G. W. (1985) 'Electron transfer in Photosystem II at cryogenic temperatures', *Biochem.* **24**, 8114–8120.

Diaz-Quintana A., Leibl W., Bottin H. and SÛtif P. (1998), 'Electron transfer in Photosystem I reaction centers follows a linear pathway in which iron–sulfur cluster F_B is the immediate electron donor to soluble ferredoxin', *Biochem.* **37**, 3429–3439.

Diner B. A. and Babcock G. T. (1996), 'Structure, dynamics, and energy conversion efficiency in Photosystem II', in Ort *et al.* (1996, eds.), pp. 213–247.

Dismukes G. C. and Siderer Y. (1981), 'Intermediates of a polynuclear manganese center involved in photosynthetic oxidation of water', *Proc. Nat. Acad. Sci. USA* **78**, 274–278.

Durrant J. R., Klug D. R., Kwa S. L. S., van Grondelle R., Porter G. and Dekker J. P. (1995), 'A multimer model for P680, the primary electron donor of Photosystem II', *Proc. Nat. Acad. Sci. USA* **92**, 4798–4802.

Ermler U., Fritsch G., Buchanan S. K. and Michel H. (1994), 'Structure of the photosynthetic reaction centre from *Rhodobacter sphaeroides* at 2.65 Å resolution: cofactors and protein-cofactor interactions', *Structure* **2**, 925–936.

Feiler U. and Hauska G. (1995), 'The reaction center from green sulfur bacteria', in Blankenship *et al.* (1995, eds.), pp. 665–685.

Feher G. (1971), 'Some chemical and physical properties of a bacterial reaction center particle and its primary photochemical reactants', *Photochem. Photobiol.* **14**, 373–387.

Feher G. and Okamura M. Y. (1978), 'Chemical composition and properties of reaction centers', in Clayton R. K. and Sistrom W. R., eds., *The Photosynthetic Bacteria*, Plenum Press, New York, pp. 349–386.

Feher G., Allen J. P., Okamura M. Y. and Rees D. C. (1989), 'Structure and function of bacterial photosynthetic reaction centres', *Nature* **339**, 111–116.

Ferreira K. N., Iverson T., Maghlaoui K., Barber J. and Iwata S. (2004), 'Architecture of the photosynthetic oxygen-evolving center', *Sciencexpress*, 5 February 2004/ *Science* 1093087.

Frankenberg N., Hager-Braun C., Feiler U., Fuhrmann M., Rogl H., Schneebauer N., Nelson N. and Hauska G. (1996), 'P840 reaction centers from *Chlorobium*

tepidum—quinone analysis and functional reconstitution into lipid vesicles', *Photochem. Photobiol.* **64**, 14–19.

Frapart Y.-M., Boussac A., Albach R., Anxolabéhère-Mallart, Delroisse M., Verlach J.-B., Blondin G., Girerd J.-J., Guilhem J., Césario M., Rutherford A. W. and Lexa D. (1996), 'Chemical modeling of the oxygen-evolving center in plants. Synthesis, structure, and electronic and redox properties of a new mixed-valence Mn–Oxo cluster: $[Mn_2^{III,IV}O_2(bisimMe_2en)_2]^{3+}$(bisimMe$_2$en=N,N'-dimethyl-N,N'-bis (imidazol-4-ylmethyl) ethane-1,2-diamine). EPR detection of an imidazol radical induced by UV irradiation at low temperature', *J. Am. Chem. Soc.* **118**, 2669–2678.

Fromme P., Witt H. T., Schubert W.-D., Klukas O., Saenger W. and Krauß N. (1996), 'Structure of Photosystem I at 4.5 Å resolution: a short review including evolutionary aspects', *Biochim. Biophys. Acta* **1275**, 76–83.

Fromme P., Jordan P. and Krauß N. (2001), 'Structure of Photosystem I', *Biochim. Biophys. Acta* **1507**, 5–31.

Gennis R. B., Barquera B., Hacker B., van Doren S. R., Arnaud S., Crofts A. R., Davidson E., Gray K. A. and Daldal F. (1993), 'The bc_1 complexes of *Rhodobacter sphaeroides* and *Rhodobacter capsulatus*', *J. Bioenerg. Biomembr.* **25**, 195–209.

Golbeck J. H. and Bryant D. A. (1991), 'Photosystem I', in *Current Topics in Bioenergetics* **16**, 83–177.

Graige M. S., Paddock M. L., Bruce J. M., Feher G. and Okamura M. Y. (1996), 'Mechanism of proton-coupled electron transfer for quinone (Q_B) reduction in reaction centers of *Rb. sphaeroides*', *J. Am. Chem. Soc.* **118**, 9005–9016.

Graige M. S., Feher G. and Okamura M. Y. (1998), 'Conformational gating of the electron transfer reaction $Q_A^- Q_B \rightarrow Q_A Q_B^-$ in bacterial reaction centers of *Rhodobacter sphaeroides* determined by a driving force assay', *Proc. Nat. Acad. Sci. USA* **95**, 11679–11684.

Gray H. B. and Winkler J. R. (1997), 'Electron tunnelling in structurally engineered proteins', *J. Electroanal. Chem.* **438**, 43–47.

Greenfield S. R. and Wasielewski M. R. (1996), 'Excitation energy transfer and charge separation in the isolated Photosystem II reaction center', *Photosynth. Res.* **48**, 83–97.

Guergova-Kuras M., Boudreaux B., Joliot A., Joliot P. and Redding K. (2001), 'Evidence for two active branches for electron transfer in Photosystem I', *Proc. Nat. Acad. Sci. USA* **98**, 4437–4442.

Hager-Braun C., Jarosch U., Hauska G., Nitschke W. and Riedel A. (1997), 'EPR studies of the terminal electron acceptors of the green sulfur bacterial reaction

centre revisited', *Photosynth. Res.* **51**, 127–136.

Hankamer B., Barber J. and Boekema E. J. (1997), 'Structure and membrane organization of Photosystem II in green plants', *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **48**, 641–671.

Hankamer B., Morris E., Nield J., Carne A. and Barber J. (2001), 'Subunit positioning and transmembrane helix organisation in the core dimer of Photosystem II', *FEBS Lett.* **504**, 142–151.

Hanley J., Deligiannakis Y., Pascal A., Faller P. and Rutherford A. W. (1999), 'Carotenoid oxidation in Photosystem II', *Biochem.* **38**, 8189–8195.

Hasegawa J. and Nakatsuji H. (1998), 'Mechanism and unidirectionality of the electron transfer in the photosynthetic reaction center of *Rhodopseudomonas viridis*: SAC–CI theoretical study', *J. Phys. Chem. B* **102**, 10420–10430.

Haumann M. and Junge W. (1996), 'Protons and charge indicators in oxygen evolution', in Ort *et al.* (1996, eds.), pp. 165–192.

Heller B. A., Holten D. and Kirmaier C. (1996), 'Effects of Asp residues near the L-side pigments in bacterial reaction centers', *Biochem.* **35**, 15418–15427.

Hill R. and Bendall F. (1960), 'Function of two cytochrome components in chloroplasts: a working hypothesis', *Nature* **186**, 136–137.

Hillmann B., Brettel K., van Mieghem F., Kamlowski A., Rutherford A. W. and Schlodder E. (1995), 'Charge recombination reactions in Photosystem II. 2. Transient absorbance difference spectra and their temperature dependence', *Biochem.* **34**, 4814–4827.

Hoff A. J. and Deisenhofer J. (1997), 'Photophysics of photosynthesis', *Physics Reports* **287**, 1–247.

Hoganson C. W. and Babcock G. T. (1997), 'A metalloradical mechanism for the generation of oxygen from water in photosynthesis', *Science* **277**, 1953–1956.

Holzapfel W., Finkele U., Kaiser W., Oesterhelt D., Scheer H., Stilz H. U. and Zinth W. (1989), 'Observation of a bacteriochlorophyll anion radical during the primary charge separation in a reaction center', *Chem. Phys. Lett.* **160**, 1–7.

Holzwarth A. R. (1991), 'Excited-state kinetics in chlorophyll systems and its relationship to the functional organization of the Photosystems', in Scheer H., ed., *Chlorophylls*, CRC Press, Boca Raton, pp. 1125–1151.

Holzwarth A. R. and Müller M. G. (1996), 'Energetics and kinetics of radical pairs in reaction centers from *Rhodobacter sphaeroides*. A femtosecond transient absorption study', *Biochem.* **35**, 11820–11831.

Hunte C., Koepke J., Lange C., Rossmanith T. and Michel H. (2000), 'Structure at 2.3 Å resolution of the cytochrome bc_1 complex from the yeast *Saccharomyces cerevisiae* co-crystallized with an antibody Fv fragment', *Structure* **8**, 669–684.

Ioannidis N. and Petrouleas V. (2000), 'Electron paramagnetic resonance signals from the S_3 state of the oxygen-evolving complex. A broadened radical signal induced by low-temperature near-infrared light illumination', *Biochem.* **39**, 5246–5254.

Ivashin N., Källebring B., Larsson S. and Hansson Ö. (1998), 'Charge separation in photosynthetic reaction centers', *J. Phys. Chem. B* **102**, 5017–5022.

Iwata S., Lee J. W., Okada K., Lee J. K., Iwata M., Rasmussen B., Link T. A., Ramaswamy S. and Jap B. K. (1998), 'Complete structure of the 11-subunit bovine mitochondrial cytochrome bc_1 complex', *Science* **281**, 64–71.

Joliot P., Barbieri G. and Chabaud R. (1969), 'Un nouveau modèle des centres photochimique du système II', *Photochem. Photobiol.* **10**, 309–329.

Joliot P. and Joliot A. (1992), 'Electron transfer between Photosystem II and the cytochrome *b/f* complex: mechanistic and structural implications', *Biochim. Biophys. Acta* **1102**, 53–61.

Joliot P. and Joliot A. (1999) '*In vivo* analysis of the electron transfer within Photosystem I: are the two phylloquinones involved?', *Biochem.* **38**, 11130–11136.

Jordan P., Fromme P., Witt H. T., Klukas O., Saenger W. and Krauß N. (2001), 'Three-dimensional structure of cyanobacterial Photosystem I at 2.5 Å resolution', *Nature* **411**, 909–917.

Kamiya N. and Shen J. R. (2003), 'Crystal structure of oxygen-evolving photosystem II from *Thermosynechococcus vulcanus* at 3.7 Å resolution', *Proc. Nat. Acad. Sci. USA* **100**, 989–102.

Katilius E., Turanchik T., Lin S., Taguchi A. K. W. and Woodbury N. W. (1999), 'B-side electron transfer in a *Rhodobacter sphaeroides* reaction center mutant in which the B-side monomer bacteriochlorophyll is replaced with bacteriopheophytin', *J. Phys. Chem. B* **103**, 7386–7389.

Keren N., Berg A., van Kan P. J. M., Levanon H. and Ohad I. (1997), 'Mechanism of Photosystem II photoinactivation and D1 protein degradation at low light: the role of back electron flow', *Proc. Nat. Acad. Sci. USA* **94**, 1579–1584.

Kirchhoff H., Horstmann S. and Weis E. (2000), 'Control of the photosynthetic electron transport by PQ diffusion microdomains in thylakoids of higher plants', *Biochim. Biophys. Acta* **1459**, 148–168.

Kirmaier C. and Holten D. (1987), 'Primary photochemistry of reaction centers from the photosynthetic purple bacteria', *Photosynth. Res.* **13**, 225–260.

Kirmaier C., Weems D. and Holten D. (1999), 'M-side electron transfer in reaction center mutants with a lysine near the nonphotoactive bacteriochlorophyll', *Biochem.* **38**, 11516–11530.

Kleinfeld D., Okamura M. Y. and Feher G. (1985), 'Electron transfer in reaction

centers of *Rhodobacter sphaeroides*. II. Free energy and kinetic relations between the acceptor states $Q_A^-Q_B^-$ and $Q_AQ_B^{2-}$', *Biochim. Biophys. Acta* **809**, 291–310.

Kleinherenbrink F. A. M., Ikegami I., Hiraishi A., Otte S. C. M. and Amesz J. (1993), 'Electron transfer in menaquinone-depleted membranes of *Heliobacterium chlorum*', *Biochim. Biophys. Acta* **1142**, 69–73.

Klukas O., Schubert W.-D., Jordan P., Krauß N., Fromme P., Witt H. T. and Saenger W. (1999), 'Localization of two phylloquinones, QK and QK', in an improved electron density map of Photosystem I at 4 Å resolution', *J. Biol. Chem.* **274**, 7361–7367.

Kolpasky A., Mühlenhoff U., Atteia A. and Nitschke W. (1995), 'Purification and characterisation of an NADH-dehydrogenase (NDH–1) from *Heliobacillus mobilis*', in Mathis P., ed., *Photosynthesis: from Light to Biosphere*, Kluwer Academic Publishers, Vol. II, pp. 951–954.

Kramer D. M., Schoepp B., Liebl U. and Nitschke W. (1997), 'Cyclic electron transfer in *Heliobacillus mobilis* involving a menaquinol-oxidizing cytochrome *bc* complex and an RCI-type reaction center', *Biochem.* **36**, 4203–4211.

Krauβ N., Schubert W.-D., Klukas O., Fromme P., Witt H. T. and Saenger W. (1996), 'Photosystem I at 4 Å resolution represents the first structural model of a joint photosynthetic reaction centre and core antenna system', *Nature Struct. Biol.* **3**, 965–973.

Krieger A., Rutherford A. W. and Johnson G. N. (1995), 'On the determination of redox midpoint potential of the primary quinone electron acceptor, Q_A, in Photosystem II', *Biochim. Biophys. Acta* **1229**, 193–201.

Krieger-Liszkay A. and Rutherford A. W. (1998), 'Influence of herbicide binding on the redox potential of the quinone acceptor in Photosystem II: relevance to photodamage and phytotoxicity', *Biochem.* **37**, 17339–17344.

Krishtalik L. I. (1989), 'Activationless electron transfer in the reaction centre of photosynthesis', *Biochim. Biophys. Acta* **977**, 200–206.

Krishtalik L. I. and Cramer W. A. (1996), 'Basic aspects of electron and proton transfer reactions with applications to photosynthesis', in Ort *et al.* (1996, eds.), pp. 399–411.

Kusumoto N., Sétif P., Brettel K., Seo D. and Sakurai H. (1999), 'Electron transfer kinetics in purified reaction centers from the green sulfur bacterium *Chlorobium tepidum* studied by multiple-flash excitation', *Biochem.* **38**, 12124–12137.

Lancaster C. R. D. and Michel H. (1997), 'The coupling of light-induced electron transfer and proton uptake as derived from crystal structures of reaction centres from *Rhodopseudomonas viridis* modified at the binding site of the secondary quinone, Q_B', *Structure* **5**, 1339–1359.

Leibl W. and Breton J. (1991), 'Kinetic properties of the acceptor quinone complex in *Rhodopseudomonas viridis*', *Biochem.* **30**, 9634–9642.

Leibl W., Toupance B. and Breton J. (1995), 'Photoelectric characterization of forward electron transfer to iron–sulfur centers in Photosystem I', *Biochem.* **34**, 10237–10244.

Lelong C., Boekema E. J., Kruip J., Bottin H., Rögner M. and Sétif P. (1996), 'Characterization of a redox active cross-linked complex between cyanobacterial Photosystem I and soluble ferredoxin', *EMBO J.* **15**, 2160–2168.

Liebl U., Mockensturm-Wilson M., Trost J. T., Brune D. C., Blankenship R. E. and Vermaas W. (1993), 'Single core polypeptide in the reaction center of the photosynthetic bacterium *Heliobacillus mobilis*: structural implications and relations to other Photosystems', *Proc. Nat. Acad. Sci. USA* **90**, 7124–7128.

Limburg J., Vrettos J. S., Liable-Sands L. M., Rheingold A. L., Crabtree R. H. and Brudvig G. W. (1999), 'A functional model for O–O bond formation by the O_2-evolving complex in Photosystem II', *Science* **283**, 1524–1527.

Lin S., Chiou H.-C. and Blankenship R. E. (1995), 'Secondary electron transfer processes in membranes of *Heliobacillus mobilis*', *Biochem.* **34**, 12761–12767.

Lin X., Williams J. C., Allen J. P. and Mathis P. (1994), 'Relationship between rate and free energy difference for electron transfer from cytochrome c_2 to the reaction center in *Rhodobacter sphaeroides*', *Biochem.* **33**, 13517–13523.

MacMillan F., Hanley J., van der Weerd L., Knüpling M., Un S. and Rutherford A. W. (1997), 'Orientation of the phylloquinone electron acceptor anion radical in Photosystem I', *Biochem.* **36**, 9297–9303.

Marcus R. A. (1956), 'On the theory of oxidation–reduction reactions involving electron transfer', *J. Chem. Phys.* **24**, 966–978.

Marcus R. A. and Sutin N. (1985), 'Electron transfer in chemistry and biology', *Biochim. Biophys. Acta* **811**, 265–322.

Martin J.-L., Breton J., Hoff A. J., Migus A. and Antonetti A. (1986), 'Femtosecond spectroscopy of electron transfer in the reaction center of the photosynthetic bacterium *Rhodopseudomonas sphaeroides* R-26: direct electron transfer from the dimeric bacteriochlorophyll primary donor to the bacteriopheophytin acceptor with a time constant of 2.8 ± 0.2 psec', *Proc. Nat. Acad. Sci. USA* **83**, 957–961.

Mathis P. and Rutherford A. W. (1987), 'The primary reactions of Photosystems I and II of algae and higher plants', in Amesz J. (1987, ed.), *Photosynthesis*, Elsevier Science Publishers, pp. 63–96.

Matsukawa T., Mino H., Yoneda D. and Kawamori A. (1999), 'Dual-mode EPR study of new signals from the S_3-state of oxygen-evolving complex in Photosystem II', *Biochem.* **38**, 4072–4077.

Melis A. (1999), 'Photosystem II damage and repair cycle in chloroplasts: what modulates the rate of photodamage *in vivo*?', *Trends Plant Sci.* **4**, 130–135.

Messinger J., Nugent J. H. A. and Evans M. C. W. (1997), 'Detection of an EPR multiline signal for the S_0^* state in Photosystem II', *Biochem.* **36**, 11055–11060.

Messinger J., Robblee J. H., Bergmann U., Fernandez C., Glatzel P., Visser H., Cinco R. M., McGarlane K. L., Bellacchio E., Pizarro S. A., Cramer S. P., Sauer K., Klein M. P. and Yachandra V. K. (2001), 'Absence of Mn-centered oxidation in the $S_2 \rightarrow S_3$ transition: implications for the mechanism of photosynthetic water oxidation', *J. Am. Chem. Soc.* **123**, 7804–7820.

Meyer T. E. and Donohue T. J. (1995), 'Cytochromes, iron–sulfur, and copper proteins mediating electron transfer from cyt bc_1 complex to photosynthetic reaction center complexes', in Blankenship *et al.* (1995, eds.), pp. 725–745.

Michel H., Epp O. and Deisenhofer J. (1986), 'Pigment–protein interactions in the photosynthetic reaction centre from *Rps. viridis*', *EMBO J.* **5**, 2445–2451.

Michel-Beyerle M. E., Plato M., Deisenhofer J., Michel H., Bixon M. and Jortner J. (1988), 'Unidirectionality of charge separation in reaction centers of photosynthetic bacteria', *Biochim. Biophys. Acta* **932**, 52–70.

Mitchell P. (1966), 'Chemiosmotic coupling in oxidative and photosynthetic phosphorylation', *Biol. Rev.* **41**, 445–502.

Morris E. P., Hankamer B., Zheleva D., Friso G. and Barber J. (1997), 'The three-dimensional structure of a Photosystem II core complex determined by electron crystallography', *Structure* **5**, 837–849.

Moser C. C. and Dutton P. L. (1992), 'Engineering protein structure for electron transfer function in photosynthetic reaction centers', *Biochim. Biophys. Acta* **1101**, 171–176.

Muhiuddin I. P., Rigby S. E. J., Evans M. C. W. and Heathcote P. (1995), 'Electron-nuclear-nuclear special triple resonance studies of a quinone electron acceptor in the photosynthetic reaction centres of green sulphur photosynthetic bacteria', in Mathis P., ed., *Photosynthesis: from Light to Biosphere,* Kluwer Academic Publishers, Vol. II, pp. 159–162.

Nabedryk E., Breton J., Hienerwadel R., Fogel C., Mäntele W., Paddock M. L. and Okamura M. Y. (1995), 'Fourier transform infrared difference spectroscopy of secondary quinone acceptor photoreduction in proton transfer mutants of *Rhodobacter sphaeroides*', *Biochem.* **34**, 14722–14732.

Neerken S. and Amesz J. (2001), 'The antenna reaction center complex of heliobacteria: composition, energy conversion and electron transfer', *Biochim. Biophys. Acta* **1507**, 278–290.

Newton M. D. (1997), 'Medium reorganization and electronic coupling in long-range

electron transfer', *J. Electroanal. Chem.* **438**, 3–10.

Nield J., Orlova E. V., Morris E. P., Gowen B., Van Heel M. and Barber J. (2000), '3D map of the plant Photosystem II supercomplex obtained by cryoelectron microscopy and single particle analysis'; *Nature Struct. Biol.* **7**, 44–47.

Nitschke W. and Rutherford A. W. (1991), 'Photosynthetic reaction centres: variations on a common structural theme?', *Trends Biochem. Sci.* **16**, 241–245.

Nitschke W. and Liebl U. (1992), 'The cytochromes in *Heliobacillus mobilis*', in Murata N., ed., *Research in Photosynthesis*, Vol. III, Kluwer Academic Publishers, pp. 507–510.

Nitschke W. and Dracheva S. M. (1995), 'Reaction center associated cytochromes', in Blankenship *et al.* (1995, eds.), pp. 775–805.

Nitschke W., Liebl U., Matsuura K. and Kramer D. M. (1995), 'Membrane-bound *c*-type cytochromes in *Heliobacillus mobilis*. *In vivo* study of the hemes involved in electron donation to the photosynthetic reaction center', *Biochem.* **34**, 11831–11839.

Nugent J. H. A., Rich A. M. and Evans M. C. W. (2001), 'Photosynthetic water oxidation: towards a mechanism', *Biochim. Biophys. Acta* **1503**, 138–146.

Oh-oka H., Kakutani S., Matsubara H., Malkin R. and Itoh S. (1993), 'Isolation of the photoactive reaction center complex that contains three types of Fe–S centers and a cytochrome *c* subunit from the green sulfur bacterium *Chlorobium limicola* f *thiosulfatophilum*, strain Larsen', *Plant Cell Physiol.* **34**, 93–101.

Okamura M. Y. and Feher G. (1995), 'Proton-coupled electron transfer reactions of Q_B in reaction centers from photosynthetic bacteria', in Blankenship *et al.* (1995, eds.), pp. 577–594.

Okamura M. Y., Paddock M. L., Graige M. S. and Feher G. (2000), 'Proton and electron transfer in bacterial reaction centers', *Biochim. Biophys. Acta* **1458**, 148–163.

Okkels J. S., Kjær B., Hansson Ö., Svendsen I., Lindberg-Møller B. and Scheller H. V. (1992), 'A membrane-bound monoheme cytochrome c_{551} of a novel type is the immediate electron donor to P840 of the *Chlorobium vibrioforme* photosynthetic reaction center complexes', *J. Biol. Chem.* **267**, 21139–21145.

Ort D. R. and Yocum C. F (1996, eds.), *Oxygenic Photosynthesis: The Light Reactions*, Kluwer Academic Publishers, Dordrecht.

Ortega J. M. and Mathis P. (1993), 'Electron transfer from the tetraheme cytochrome to the special pair in isolated reaction centers of *Rhodopseudomonas viridis*', *Biochem.* **32**, 1141–1151.

Ortega J. M., Dohse B., Oesterhelt D. and Mathis P. (1998), 'Low-temperature electron transfer from cytochrome to the special pair in *Rhodopseudomonas*

viridis: role of the L–162 residue', *Biophys. J.* **74**, 1135–1148.

Page C. C., Moser C. C., Chen X. and Dutton P. L. (1999), 'Natural engineering principles of electron tunnelling in biological oxidation–reduction', *Nature* **402**, 47–52.

Parson W. W., Chu Z.-T. and Warshel A. (1990), 'Electrostatic control of charge separation in bacterial photosynthesis', *Biochim. Biophys. Acta* **1017**, 251–272.

Peloquin J. M., Williams J. C., Lin X., Alden R. G., Taguchi A. K. W., Allen J. P. and Woodbury N. (1994), 'Time-dependent thermodynamics during early electron transfer in reaction centers from *Rb. sphaeroides*', *Biochem.* **33**, 8089–8100.

Philouze C., Blondin G., Girerd J.-J., Guilhem J., Pascard C. and Lexa D. (1994), 'Aqueous chemistry of high-valent manganese. Structure, magnetic, and redox properties of a new type of Mn-oxo cluster, $[Mn^{IV}_4O_6(bpy)_6]^{4+}$: relevance to the oxygen-evolving center in plants', *J. Am. Chem. Soc.* **116**, 8557–8565.

Pohlmann L. and Tributsch H. (1996), 'Self-organized electron transfer as a possible element in the photosynthetic process', *Ber. Bunsenges. Phys. Chem.* **100**, 2097–2102.

Prokhorenko V. I. and Holzwarth A. R. (2000), 'Primary processes and structure of the Photosystem II reaction center: a photon echo study', *J. Phys. Chem. B* **104**, 11563–11578.

Reed W. and Clayton R. K. (1968), 'Isolation of a reaction center fraction from *Rhodopseudomonas sphaeroides*', *Biochem. Biophys. Res. Commun.* **30**, 471–475.

Renger G. (2001), 'Photosynthetic water oxidation to molecular oxygen: apparatus and mechanism', *Biochim. Biophys. Acta* **1503**, 210–228.

Rhee K.-H., Morris E. P., Zheleva D., Hankamer B., Kühlbrandt W. and Barber J. (1997), 'Two-dimensional structure of plant Photosystem II at 8 Å resolution', *Nature* **389**, 522–526.

Rhee K.-H., Morris E. P., Barber J. and Kühlbrandt W. (1998), 'Three-dimensional structure of the PSII reaction centre at 8 Å resolution', *Nature* **396**, 283–286.

Robblee J. H., Cinco R. M. and Yachandra V. K. (2001), 'X-ray spectroscopy-based structure of the Mn cluster and mechanism of photosynthetic oxygen evolution', *Biochim. Biophys. Acta* **1503**, 7–23.

Robblee J. H., Messinger J., Cinco R. M., McFarlane K. L., Fernandez C., Pizarro S. A., Sauer K. and Yachandra V. K. (2002), 'The Mn cluster in the S_0 state of the oxygen-evolving complex of Photosystem II studied by EXAFS spectroscopy: are there three di-μ-oxo-bridged Mn_2 moieties in the tetranuclear Mn complex?', *J. Am. Chem. Soc.* **124**, 7459–7459.

Rutherford A. W. and Nitschke W. (1996), 'Photosystem II and the quinone-iron-containing reaction centers: comparisons and evolutionary perspectives', in

Baltscheffsky H. (1996, ed.), *Origin and Evolution of Biological Energy Conservation*, VCH Publishers, pp. 177–203.

Rutherford A. W. and Faller P. (2001), 'The heart of photosynthesis in glorious 3D', *Trends Biochem. Sci.* **26**, 341–344.

Satoh K. (1996), 'Introduction to the PSII reaction center—isolation and biochemical and biophysical characterization', in Ort *et al.* (1996, eds.), pp. 193–211.

Schubert W.-D., Klukas O., Krauß N., Saenger W., Fromme P. and Witt H. T. (1997), 'Photosystem I of *Synechococcus elongatus* at 4 Å resolution: comprehensive structure analysis', *J. Mol. Biol.* **272**, 741–769.

Schmidt S., Arlt T., Hamm P., Lauterwasser C., Finkele U., Drews G. and Zinth W. (1993), 'Time-resolved spectroscopy of the primary photosynthetic processes of membrane-bound reaction centers from an antenna-deficient mutant of *Rhodobacter capsulatus*', *Biochim. Biophys. Acta* **1144**, 385–390.

Schütz M., Zirngibl S., le Coutre J., Büttner M., Xie D.-L., Nelson N., Deutzmann R. and Hauska G. (1994), 'A transcription unit for the Rieske FeS protein and cytochrome *b* in *Chlorobium limicola*', *Photosynth. Res.* **39**, 163–174.

Seidler A. (1996), 'The extrinsic polypeptides of Photosystem II', *Biochim. Biophys. Acta* **1277**, 35–60.

Sétif P. and Brettel K. (1993) 'Forward electron transfer from phylloquinone A1 to iron–sulfur centers in spinach Photosystem I', *Biochem.* **32**, 7846–7854.

Sétif P. Q. Y. and Bottin H. (1994), 'Laser flash absorption spectroscopy study of ferredoxin reduction by Photosystem I in *Synechocystis* sp. PCC 6803: evidence for submicrosecond and microsecond kinetics', *Biochem.* **33**, 8495–8504.

Sétif P., Seo D. and Sakurai H. (2001), 'Photoreduction and reoxidation of the three iron–sulfur clusters of reaction centers of green sulfur bacteria', *Biophys. J.* **81**, 1208–1219.

Sisido M., Hoshino S., Kusano H., Kuragaki M., Makino M., Sasaki H., Smith T. A. and Ghiggino K. P. (2001), 'Distance dependence of photoinduced electron transfer along α-helical polypeptides', *J. Phys. Chem. B* **105**, 10407–10415.

Steffen M. A., Lao K. Q. and Boxer S. G. (1994), 'Dielectric asymmetry in the photosynthetic reaction center', *Science* **264**, 810–816.

Steward D. H. and Brudvig G. W. (1998), 'Cytochrome b_{559} of Photosystem II', *Biochim. Biophys. Acta* **1367**, 63–87.

Stowell M. H. B., McPhillips T. M., Rees D. C., Soltis S., Abresch E. and Feher G. (1997), 'Light-induced structural changes in photosynthetic reaction center: implications for mechanism of electron-proton transfer', *Science* **276**, 812–816.

Sumi H. (1997), 'Electron transfer via a midway molecule as seen in primary processes in photosynthesis: superexchange or sequential, or unified?',

J. Electroanal. Chem. **438**, 11–20.

Sumi H. and Kakitani T. (2001), 'Unified theory on rates for electron transfer mediated by a midway molecule, bridging between superexchange and sequential processes', *J. Phys. Chem. B.* **105**, 9603–9622.

Svensson B., Etchebest C., Tuffery P., van Kan P., Smith J. and Styring S. (1996), 'A model for the Photosystem II reaction center core including the structure of the primary donor P_{680}', *Biochem.* **35**, 14486–14502.

Takahashi Y., Hansson Ö., Mathis P. and Satoh K. (1987), 'Primary radical pair in the Photosystem II reaction centre', *Biochim. Biophys. Acta* **893**, 49–59.

Tang X.-S., Randall D. W., Force D. A., Diner B. A. and Britt R. D. (1996), 'Manganese–tyrosine interaction in the Photosystem II oxygen-evolving complex', *J. Am. Chem. Soc.* **118**, 7638–7639.

Tetreault M., Rongey S. H., Feher G. and Okamura M. Y. (2001), 'Interaction between cytochrome c_2 and the photosynthetic reaction center from *Rhodobacter sphaeroides*: effects of charge-modifying mutations on the binding and electron-transfer', *Biochem.* **40**, 8452–8462.

Tiede D. M. and Dutton P. L. (1993), 'Electron transfer between bacterial reaction centers and mobile *c*-type cytochromes', in Deisenhofer J. and Norris J. R., eds., *The Photosynthetic Reaction Center*, Academic Press, New York, pp. 257–288.

Tiede D. M., Vashishta A.-C. and Gunner M. R. (1993), 'Electron-transfer kinetics and electrostatic properties of the *Rhodobacter sphaeroides* reaction center and soluble *c*-cytochromes', *Biochem.* **32**, 4515–4531.

Tributsch H. and Pohlmann L. (1997), 'Synergetic electron transfer in molecular electronic and photosynthetic mechanisms', *J. Electroanal. Chem.* **438**, 37–41.

Trumpower B. L. (1982, ed.), *Function of Quinones in Energy-Conserving Systems*, Academic Press, New York.

Ungar L. W., Newton M. D. and Voth G. A. (1999), 'Classical and quantum simulation of electron transfer through a polypeptide', *J. Phys. Chem. B* **103**, 7367–7382.

van der Est A., Prisner T., Bittl R., Fromme P., Lubitz W., Möbius K. and Stehlik D. (1997), 'Time-resolved X-, K-, and W-band EPR of the radical pair state $P_{700}^{+}A_1^{-}$ of Photosystem I in comparison with $P865^{+}Q_A^{-}$ in bacterial reaction centers', *J. Phys. Chem. B* **101**, 1437–1443.

van Mieghem F. J. E. (1993), 'Photochemistry and structural aspects of the Photosystem II reaction centre', Thesis, University of Wageningen.

van Mieghem F. J. E., Nitschke W., Mathis P. and Rutherford A. W. (1989), 'The influence of the quinone–iron electron acceptor complex on the reaction centre photochemistry of Photosystem II', *Biochim. Biophys. Acta* **977**, 207–214.

van Mieghem F. J. E., Brettel K., Hillmann B., Kamlowski A., Rutherford A. W. and Schlodder E. (1995), 'Charge recombination reactions in Photosystem II. 1. Yields, recombination pathways, and kinetics of the primary pair', *Biochem.* **34**, 4798–4813.

Venturoli G., Mallardi A. and Mathis P. (1993), 'Electron transfer from cytochrome c_2 to the primary donor of *Rhodobacter sphaeroides* reaction centers. A temperature dependence study', *Biochem.* **32**, 13245–13253.

Venturoli G., Drepper F., Williams J. C., Allen J. P., Lin X. and Mathis P. (1998), 'Effects of temperature and ΔG° on electron transfer from cytochrome c_2 to the photosynthetic reaction center of the purple bacterium *Rhodobacter sphaeroides*', *Biophys. J.* **74**, 3226–3240.

Volk M., Ogrodnik A. and Michel-Beyerle M.-E. (1995), 'The recombination dynamics of the radical pair $P^{+}H^{-}$ in external magnetic and electric fields', in Blankenship *et al.* (1995, eds.), pp. 595–626.

Vrettos J. S., Limburg J. and Brudwig G. W. (2001), 'Mechanism of photosynthetic water oxidation: combining biophysical studies of Photosystem II with inorganic model chemistry', *Biochim. Biophys. Acta* **1503**, 229–245.

Weintraub O. and Bixon M. (1994), 'Entropy of reaction and number of active modes in the theory of electon-transfer reactions', *J. Phys. Chem.* **98**, 3407–3411.

Williams J. C., Alden R. G., Murchison H. A., Peloquin J. M., Woodbury N. W. and Allen J. P. (1992), 'Effects of mutations near the bacteriochlorophylls in reaction centers from *Rhodobacter sphaeroides*', *Biochem.* **31**, 11029–11037.

Witt H. T. (1991), 'Functional mechanism of water-splitting photosynthesis', *Photosynth. Res.* **29**, 55–77.

Yachandra V. K., Sauer K. and Klein M. P. (1996), 'Manganese cluster in photosynthesis: where plants oxidize water to dioxygen', *Chem. Rev.* **96**, 2927–2950.

Yeates T. O., Komiya H., Rees D. C., Allen J. P. and Feher G. (1987), 'Structure of the reaction center from *Rhodobacter sphaeroides* R-26: membrane–protein interactions', *Proc. Nat. Acad. Sci. USA* **84**, 6438–6442.

Xia D., Yu C.-A., Kim H., Xia J.-Z., Kachurin A. M., Zhang L., Yu L. and Deisenhofer J. (1997), 'Crystal structure of the cytochrome bc_1 complex from bovine heart mitochondria', *Science* **277**, 60–66.

Zhang Z., Huang L., Shulmeister V. M., Chi Y.-I., Kim K. K., Hung L.-W., Crofts A. R., Berry E. A. and Kim S.-H. (1998), 'Electron transfer by domain movement in cytochrome bc_1', *Nature* **392**, 677–684.

Zouni A., Witt H.-T., Kern J., Fromme P., Krauß N., Saenger W. and Orth P. (2001), 'Crystal structure of Photosystem II from *Synechococcus elongatus* at 3.8 Å resolution', *Nature* **409**, 739–743.

CHAPTER 4

PHOTOSYNTHETIC CARBON ASSIMILATION

GERALD E. EDWARDS
School of Biological Sciences, Washington State University, Pullman, WA 99164–4236, USA
edwardsg@wsu.edu
and
DAVID A. WALKER
Robert Hill Institute, Animal and Plant Sciences, University of Sheffield S10 2TN, UK
http://www.shef.ac.uk/uni/academic/A-C/aps/
david@alegba.demon.co.uk

I have discovered what I have long been in quest of, viz, the process in nature by which air rendered noxious by breathing, is restored to its former salubrious condition.
Joseph Priestley, letter to Philus Lindsey, August 1771.

Green plants, and therefore ultimately all other higher forms of life, depend on organic carbon derived from carbon dioxide in processes driven by light energy. Central to every aspect of such photosynthetic carbon assimilation by leaves lies the Calvin cycle. In this cyclic sequence of reactions, carbon dioxide is added to an acceptor molecule. The newly formed addition compound then undergoes a series of changes, including reduction, so that a stable product is formed and set aside. At the same time, the CO_2-acceptor is regenerated and new molecules of acceptor formed so that the process may continue and the green plant may grow.

4.1 Environmental and metabolic role

Remarkably, there is a still a popular belief, reinforced by gardeners who habitually talk about 'feeding' their plants, that green plants obtain most of their nutriment from the soil. In fact, the fallacy of this supposition was demonstrated as long ago as the seventeenth century by van Helmont, who grew a willow in a tub of weighed soil. After watering for five years, the willow weighed 164 lbs. more and the soil only

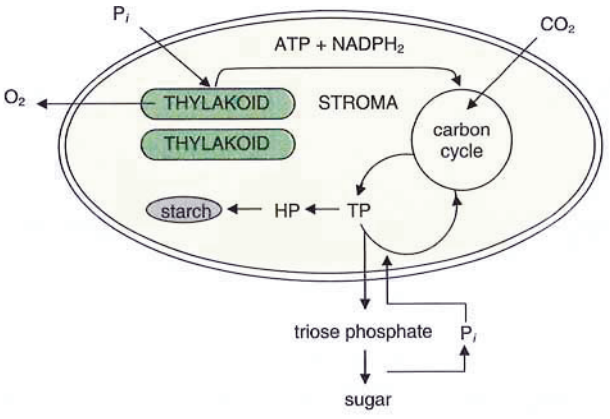


Figure 4.1 A stylised chloroplast bounded by an inner and outer envelope of protein/polar lipid membranes. Because these are devoid of chlorophyll but retain a carotenoid composition similar to that of the thylakoids, they appear yellow when isolated. The inner envelope houses the phosphate translocator that controls the movement of metabolites into and out of the chloroplast. The thylakoid membranes, in which the chlorophylls and other components of the photosynthetic electron transport system are located, are embedded in the stroma, which also contains the soluble enzymes of the Calvin cycle (Section 4.3). The Calvin cycle utilises ATP and $NADPH_2$, produced by photosynthetic electron transport, to form 3-carbon sugar phosphates (triose phosphates) from CO_2. Such triose phosphates (TP) are either exported through the chloroplast envelopes, via the phosphate translocator, in exchange for external inorganic phosphate (P_i), or utilised in the Calvin cycle or in starch synthesis. Exported triose phosphates are mostly consumed in sucrose synthesis in the cytosol (the aqueous gel, containing many more soluble enzymes, in which the chloroplasts, mitochondria and other cellular organelles are suspended). Sucrose synthesis, like starch synthesis and some of the reactions of the Calvin cycle, liberates P_i needed for the continuation of ATP formation by photochemistry in the thylakoids.

2 ounces less. This is in accord with contemporary measurements, which show that about 90% of the dry weight of green plants is comprised of carbon and oxygen derived from the air by photosynthesis and usually less than 5% (nitrogen and minerals) from the soil.

Photosynthesis, more properly called 'oxygenic photosynthesis' to distinguish it from forms of bacterial photosynthesis that do not liberate oxygen, is not only by far the major source of organic carbon in the biosphere but also the principal mechanism of biological energy transduction. It is the means by which light energy is converted into electrical energy and then into chemical energy. In essence this is achieved by breaking H–O bonds in water (H_2O), liberating the oxygen (O_2) and passing the hydrogen (H_2) through a series of intermediates to carbon dioxide (CO_2), in the process forming carbohydrates such as starch and sucrose, as shown in Fig. 4.1. In plant and animal respiration, this process is largely reversed: oxygen is taken up,

carbohydrates are consumed, carbon dioxide is liberated, H–O bonds are re-established and energy is made available for other metabolic processes. It was oxygenic photosynthesis that created our present atmosphere and continues to maintain it. Oxygenic photosynthesis was also the source of our fossil fuels (Edwards and Walker, 1983; Walker, 1993).

4.2 Chloroplast and cell

In the final stages of photosynthesis, atmospheric carbon dioxide is assimilated and carbohydrates are formed (for details on mechanisms of acquiring and assimilating carbon dioxide, see Edwards and Walker, 1983; Winter and Smith, 1996; Sage and Monson, 1999 and Leegood *et al.*, 2000). As shown in Fig. 4.1, these events are separated in time and location from the photochemical events that occur in the thylakoid membranes of the chloroplast. Carbon dioxide fixation is sometime called the 'dark biochemistry' of photosynthesis, as shown in Fig. 4.2, in order to distinguish it from the 'photochemistry', or light reactions but, while it is true that many of the component reactions of CO_2 assimilation can be made to proceed in the dark, they are

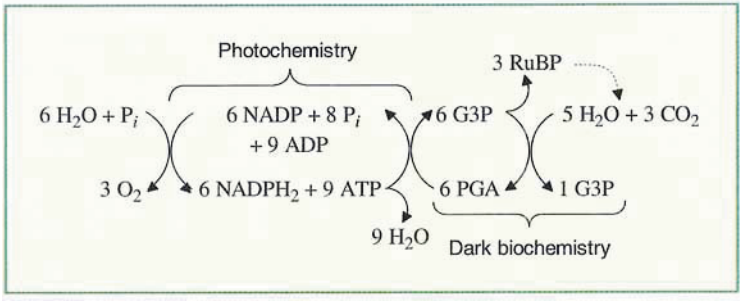


Figure 4.2 Light and dark events in photosynthesis. The first product of photosynthetic carbon assimilation (CO_2 fixation) in the Calvin cycle is 3-phosphoglycerate (PGA), a three-carbon acid. This is converted to a corresponding three-carbon sugar phosphate called glyceraldehyde 3-phosphate (G3P) in a reductive process that consumes two-thirds of the ATP and all of the $NADPH_2$ generated by the photochemistry. The rest of the ATP is utilised in the regeneration of the CO_2-acceptor, ribulose-1,5-bisphosphate (RuBP) in a process by which five 3-C compounds (triose phosphates) are rearranged to give three 5-C carbon compounds (this is discussed in more detail in Section 4.4.4). The ATP and $NADPH_2$ generated by photochemical events in the light are consumed in the 'dark biochemistry'—so called because none of the biochemical reactions involved have an absolute requirement for light even though, in a photosynthetic context, they normally occur only in the light and are often made more active in the light by direct or indirect means (Woodrow and Berry, 1988). Triose phosphates not used in regenerating the CO_2-acceptor undergo subsequent transformations to starch, sucrose *etc*.

all an integral part of the photosynthetic apparatus within the living organism. This chapter describes the manner in which carbon dioxide assimilation is linked to the photochemistry on which it depends and which, in turn, it influences.

Many of the component reactions of CO_2 assimilation and their associated enzymes are physically located in the stroma in which the thylakoid membranes are embedded (Fig. 4.1). All are separated from the surrounding cytosol by the chloroplast envelopes. Nevertheless, events such as sucrose synthesis that occur in the cytosol beyond the chloroplast may be properly regarded as aspects of photosynthesis. Similarly, all aspects of photosynthetic carbon assimilation and much of the photochemistry depend on, or are influenced to a greater or smaller extent by, events that occur outside the chloroplasts.

4.3 C_3 photosynthesis in its relation to the photochemistry

In 'C_3 photosynthesis', the first product of carbon assimilation contains 3 atoms of carbon, as illustrated in Fig. 4.2. In 'C_4 photosynthesis' (C_4 and CAM plants, discussed in Sections 4.7.1 and 4.7.2), we shall see that the first product of CO_2 fixation contains 4 atoms of carbon. Central to both C_3 and C_4 photosynthesis lies the Calvin or Benson–Calvin cycle, the complex metabolic pathway by which plants incorporate CO_2 as carbohydrates.[1] Why, we might ask, did evolution favour the complexity of the Calvin cycle when paper chemistry offers simpler alternatives such as the direct formation of formaldehyde (HCHO), as first proposed by Baeyer in 1870 (eq. 4.1; see Rabinowitch, 1945 for a fuller discussion).

$$\begin{aligned} 2CO_2 &\rightarrow 2CO + O_2 \\ 2CO + 4H &\rightarrow 2HCHO \\ 6HCHO &\rightarrow C_6H_{12}O_6 \end{aligned} \qquad (4.1)$$

It is tempting to suppose that the more complex solution was favoured because the energy required for carbon assimilation could be provided by ATP, already utilised by anaerobic bacteria as a means of energy conservation at the time that these cyanobacteria, destined to be the precursors of modern chloroplasts, evolved. (For discussion of the unique position of ATP in intermediary metabolism, see Banks and Vernon, 1970.)

---

[1] The late Melvin Calvin received the Nobel Prize for his work on the elucidation of this metabolic sequence; Andy Benson's name is added by those who rightly wish to acknowledge the contribution of this, his most senior, colleague.

Photosynthetic carbon assimilation (PCA) has often been summarised by the equation

$$6CO_2 + 6H_2O \rightarrow C_6H_{12}O_6 + 6O_2 \qquad (4.2)$$

This portrays a reaction sequence, driven by light energy, in which carbon dioxide (CO_2) and water (H_2O) are consumed, oxygen (O_2) is liberated and glucose ($C_6H_{12}O_6$) is the end product. However, although sugars such as glucose and fructose (both of which have the same empirical formula) may eventually appear in plant leaves as a result of photosynthesis, they are not formed directly from carbon dioxide and are not even particularly important compounds in leaf metabolism. A more realistic equation, in which $\{CH_2O\}$ represents a nominal carbohydrate, and molecules of water are included on both sides of the equation, takes account of the fact that the oxygen that is released is derived, not from CO_2, but from water.

$$CO_2 + 2H_2O \rightarrow \{CH_2O\} + O_2 + H_2O \qquad (4.3)$$

Indeed, with one minor addition, such an equation adequately summarises the combined reactions of the Calvin cycle (Edwards and Walker, 1983; Walker, 1992a) but any such simplification inevitably masks important features, not least the question of precisely where in a leaf photosynthesis occurs and how it is driven and controlled. Here we shall take what we regard as the conventional view: that photosynthetic electron transport is driven by light, that it leads to the formation of ATP and $NADPH_2$, and that these are then consumed in the reduction of CO_2 to triose phosphate within the chloroplast.

In terms of energy inputs, we can ascribe a value ($\Delta G'$) of about 29 kJ (7 kcal) to ATP formation (*cf.* Banks and Vernon, 1970) and 218 kJ (52 kcal) to $NADPH_2$ formation. As represented below, the Calvin cycle consumes 9 molecules of ATP and 6 molecules of $NADPH_2$ in order to produce one molecule of triose phosphate product (designated G3P in Fig. 4.2) In terms of ATP and $NADPH_2$, this would be equivalent to an energy consumption of 1569 kJ (375 kcal). Burning glucose ($C_6H_{12}O_6$) in a calorimeter yields 2822 kJ (672 kcal). This would allow us to put a value of 2822/6 = 470 kJ (112 kcal) on $[CH_2O]$ in eq. 4.3 and a corresponding value of 1569/3 = 523 kJ (125 kcal) of energy consumed for each molecule of CO_2 incorporated into product in Fig. 4.2. This is consistent with what is known about partial reactions in the entire sequence and implies a high degree of efficiency. However, calculating free energy changes *in vivo* is inevitably something of an approximation depending, as it does, on factors such as the prevailing Mg^{2+} concentration that can rarely be determined with certainty.

4.4 The Calvin cycle

The experiments in which Calvin and his colleagues fed radioactive carbon dioxide to *Chlorella*[2] implicated the participation of a number of sugar phosphates and related acids in photosynthetic carbon assimilation. Several schemes were considered before everything finally fell into place and the now well-established features of the Calvin cycle became clear. These features are the carboxylation of a 5-carbon sugar phosphate (the CO_2-acceptor), the reduction of the immediate product to yield a 3-carbon sugar phosphate, and the regeneration of the acceptor, as shown in Fig. 4.3.

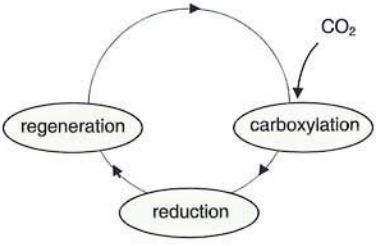


Figure 4.3 Principal features of the Calvin cycle

4.4.1 Carboxylation

A 'carboxylation' is the addition of CO_2 to an acceptor molecule in such a way that a new carboxyl (–COOH) group is formed. In the Calvin cycle, the CO_2-acceptor is a 5-carbon sugar phosphate called ribulose-1,5-bisphosphate (RuBP). The name 'ribulose' indicates that it is a ketose sugar containing a C=O group rather than the aldehyde (CHO) group that characterises the corresponding aldose sugar, ribose. RuBP has two phosphate groups, one at either end (hence '*bis*phosphate'). As shown in Fig. 4.4, its carboxylation involves the incorporation of a molecule of water and separation of the addition product into two molecules of 3-phosphoglycerate (PGA). This reaction, in common with others, involves breaking and re-forming bonds. Energy is always used when chemical bonds are broken and released when they are

[2] Like Warburg, Emerson, and many others before him, Calvin chose to work with *Chlorella*, a unicellular green alga of great experimental repute. Its advantage was that highly reproducible and uniform cultures could be grown with great ease. Such an algal suspension is, to all effects and purposes, like a green liquid that can be evenly illuminated in a glass 'lollipop' and then run into a killing medium such as boiling ethanol. However, it should be noted that, although the pathway of photosynthesis is the same in algae and higher plants, its exact mode of operation and regulation may not be the same in the two tissues.

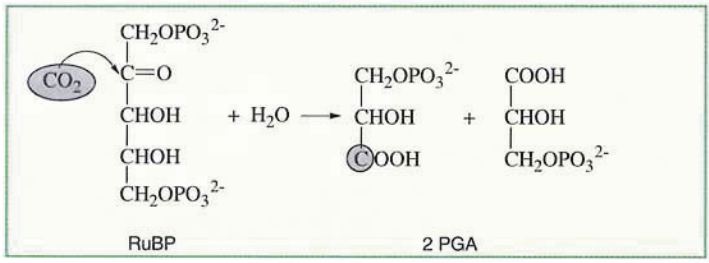


Figure 4.4 The carboxylation of ribulose-1,5-bisphosphate (RuBP). The addition of one molecule of water yields two molecules of 3-phosphoglycerate (PGA).

formed. In this instance the energy released exceeds the energy used by about 30 kJ. This large decrease in 'free' energy ensures (Robinson and Walker, 1981) that the reaction goes largely to completion—that it is not freely reversible. The carbon dioxide molecule has mobile π-electrons not required for keeping the atoms together and their existence confers stability on the molecule (for a classic discussion of CO_2 stability, see Pauling, 1960). The bond dissociation energy required to break open CO_2 in such a way that it becomes possible to form a new carboxyl (–COOH) group is about 30 kJ, equivalent to the free energy ($\Delta G'$) of hydrolysis of ATP. Energy is therefore inevitably expended in metabolic carboxylation reactions and this energy bill has to be met (Edwards and Walker, 1983; Walker *et al.*, 1986). In this instance, the energy required at the moment of carboxylation derives from the fact that RuBP is not a 'comfortable' molecule, 'at ease with itself' like carbon dioxide. On the contrary, the molecular structure of RuBP is such that a great deal of internal stress is present. This is relieved by the carboxylation and associated addition of water. As we shall see in Section 4.4.4, this 'uncomfortable' twisting within RuBP, which enables the carboxylation reaction to proceed so effectively in the biological context, is largely imposed by the consumption of ATP in the final stages of regeneration.

Given that the CO_2 concentration in the atmosphere is low (around 0.035%), in addition to having a favourable equilibrium position a biological carboxylation needs to be catalysed by an enzyme with a high affinity for CO_2 (but see sections on C_4 and CAM). When fully activated, the ubiquitous enzyme ribulose-1,5-bisphosphate carboxylase (rubisco) meets that requirement, but it is also subject to competitive inhibition by O_2. The reaction that follows when O_2 wins this competition yields one molecule of PGA and one of 2-phosphoglycolate (Keys, 1986). This oxygenation is the biochemical initiator of a form of photorespiration (Section 4.6) and it is for this reason that rubisco is more properly referred to as ribulose-1,5-bisphosphate carboxylase-*oxygenase*. Rubisco occurs in large amounts in leaves and is believed to be the most abundant protein on earth (Ellis, 1979; Ellis and Gray, 1986).

4.4.2 Mechanism

During catalysis by rubisco, the ketose form of RuBP binds to the enzyme and is converted to the enol form (see Fig. 4.5). CO_2 and O_2 then compete to react with the enzyme-bound enol form of RuBP. Carboxylation of enol-RuBP generates 3-keto-carboxyarbinitol bisphosphate, which is hydrated, and then two molecules of PGA are formed by C–C cleavage between C-2 and C-3. Oxygenation at the C-2 position of RuBP produces a hydroperoxide, which is then converted to P-glycolate and 3-PGA by C–C cleavage between C-2 and C-3, as shown in Fig. 4.6 (for details of other proposed intermediates, see Taylor and Andersson, 1997).

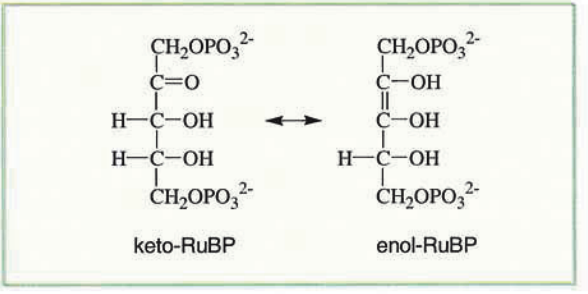


Figure 4.5 Conversion of keto-RuBP to enol-RuBP, the first step in rubisco catalysis.

The capacity of rubisco to function as a catalyst is controlled through a unique mechanism of interconversion of the enzyme between active and inactive forms. The enzyme is converted to an inactive form in the dark and an active form in the light. This, along with control of other enzymatic steps, assures that the substrate-consuming reactions of photosynthesis are shut down at night. Control of the degree of activation during the day can prevent the RuBP pool from being depleted in the face of changing environmental conditions. For example, as the light intensity decreases and the energy-dependent process of regeneration of RuBP slows, decreased activation of rubisco can maintain the RuBP pool. The catalytically active form of rubisco is carbamylated by a molecule of CO_2 at a lysine residue in the active site, and that CO_2 is different from the one used as a substrate in carboxylation. This allows binding of Mg^{2+} ion, which is essential for catalysis and completes the activation process, creating the so-called ECM (enzyme-CO_2-Mg) complex.

The catalytically active ECM complex uses RuBP and CO_2/O_2 as substrates in the light. In addition to its role in metal binding, the carbamate is thought to catalyse the conversion of keto-RuBP to enol-RuBP, the first step in the catalytic process. X-ray

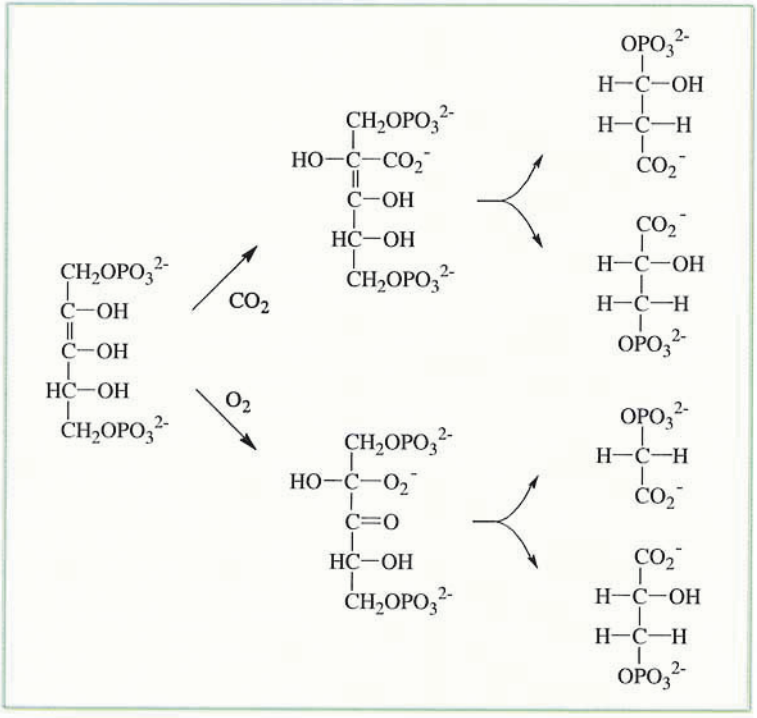


Figure 4.6 Reaction of CO_2 or O_2 with enol-RuBP.

crystallographic analysis of rubisco indicates flexible loop regions close around the active site, sequestering sugar phosphates from the solvent and protecting reaction intermediates; as reaction products are formed the active site reopens. Decarbamylation, and inactivation of the enzyme at night is favoured by the lower Mg^{2+} concentration and pH in the chloroplast stroma. Binding of RuBP to the decarbamylated form of the enzyme in the light has a role in controlling the level of inactive enzyme. RuBP binds to this inactive form of the enzyme, resulting in closure of the active site and formation of a dead-end complex that traps RuBP and prevents carbamylation of the enzyme. ATP and a protein called activase are required to open the traps and dissociate RuBP from this complex. ATP may convert activase to an active conformation so that it can open the closed catalytic site, allow RuBP to dissociate and spontaneous carbamylation to proceed. This indicates that ATP, which is formed in the chloroplast in the light, probably plays an important role in controlling the state of activation of rubisco (Andrews, 1996; Taylor and Andersson, 1997).

4.4.3 Reduction

Once formed by carboxylation, PGA is rapidly reduced to triose phosphate in the two-stage reaction shown in Fig. 4.7. The first stage consumes all of the $NADPH_2$ and two-thirds of the ATP used in the cycle. Nevertheless it is energetically unfavourable and gives rise to no significant quantities of free glycerate 1,3-bisphosphate (GBP); the overall sequence is made energetically favourable by the subsequent hydrolysis of this bisphosphate. The resulting triose phosphate, glyceraldehyde 3-phosphate (G3P), can be regarded as the immediate end product of the Calvin cycle. Some of it may be stored within the chloroplast, as starch (thereby releasing P_i *in situ*), though not all plant species make leaf starch. Some triose phosphate is exported from the chloroplast (via the 'phosphate translocator') as G3P or dihydroxyacetonephosphate (DHAP) in exchange for inorganic phosphate (P_i) from the cytosol and, once in this compartment, it is commonly consumed producing sucrose which is transported to other parts of the plant. *Sucrose synthesis*, from triose phosphate, releases P_i, which re-enters the chloroplast, enabling photosynthetic phosphorylation and the continuation of the Calvin cycle. Isolated chloroplasts are not able to synthesize sucrose because they lack the necessary enzymes and would, if not supplied with the P_i that is made available by sucrose synthesis in the living cell, soon cease to evolve O_2 at normal rates (Edwards and Walker, 1983; Walker, 1992a).

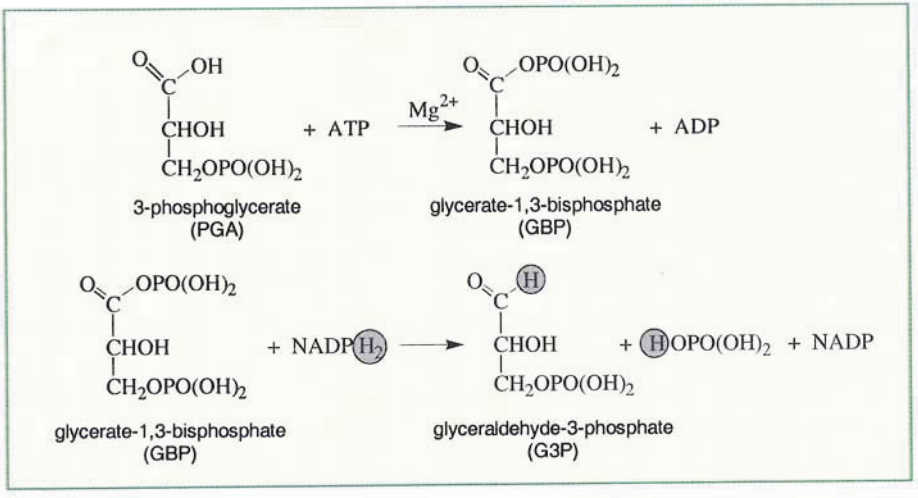


Figure 4.7 The reduction of PGA to triose phosphate (G3P) at the expense of ATP and $NADPH_2$ produced by photosynthetic electron transport.

4.4.4 Regeneration

Each time CO_2 is added to RuBP, a molecule of that compound is consumed. In order that this process can continue it is necessary to regenerate new RuBP from the newly formed product. Arithmetic demands that, in order to make a 5-carbon CO_2 acceptor from 3-carbon products without loss, it is necessary to convert five threes into three fives, as shown in Fig. 4.8. Accordingly, a large fraction of the triose phosphate formed by reduction of the PGA formed in the initial carboxylation is retained within the chloroplast for this purpose. There follows a sequence of reactions that may at first see very complex. On closer examination a repeated pattern emerges.

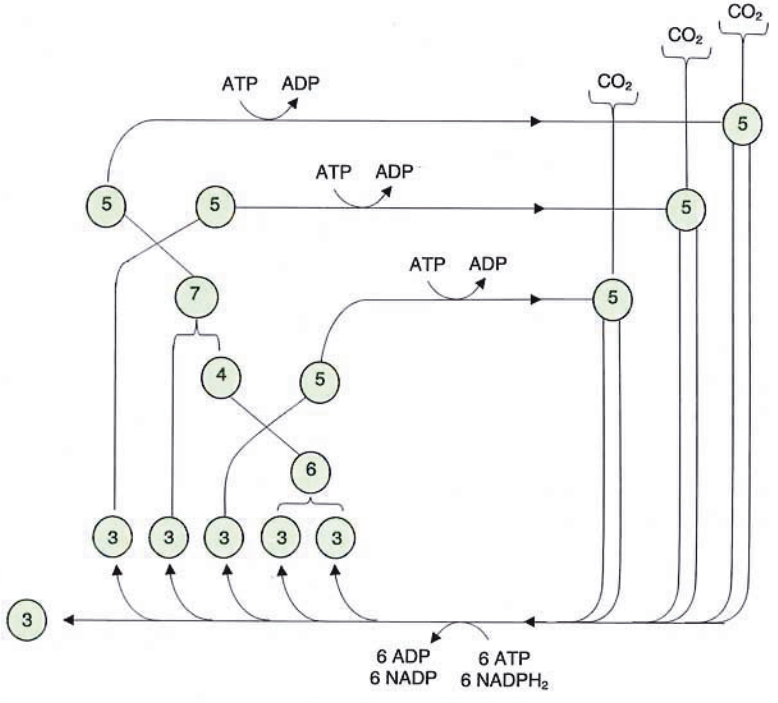


Figure 4.8 The Calvin cycle in outline, showing how three molecules of CO_2 give rise to one molecule of 3-carbon product and how five 3-C molecules are rearranged to give three 5-C acceptors. The 2-C unit transferred (e.g. from a 7-C molecule to a 3-C to give two 5-C molecules) is the ketose glycoaldehyde (CH_2OHCHO), and the enzyme that catalyses its transfer is called transketolase.

Figure 4.9 shows the metabolites involved in these molecular rearrangements. Two types of reaction are involved. The first (eq. 4.4) joins (or condenses) two triose (3-C) phosphates to give one hexose (6-C) bisphosphate

$$3\text{-C} + 3\text{-C} \rightarrow 6\text{-C} \tag{4.4}$$

The triose phosphates that are condensed at this stage are G3P and its isomer dihydroxyacetonephosphate (DHAP).

$$\text{G3P} + \text{DHAP} \rightarrow \text{FBP} \tag{4.5}$$

The condensation product is fructose-1,6-bisphosphate (FBP). Reaction 4.5 is classified as an aldol condensation because it involves an aldehyde and an alcohol group. Accordingly the enzyme which catalyses it is called an aldolase. Once formed, FBP is hydrolysed by a specific phosphatase.

$$\text{FBP} + \text{H}_2\text{O} \rightarrow \text{F6P} + \text{P}_i \tag{4.6}$$

A 2-C (ketose) unit is then transferred (as shown in Figs. 4.8 and 4.9) from the top of F6P to G3P, creating new pentose (5-C) and tetrose (4-C) phosphates called xylulose-5-phosphate (Xu5P) and erythrose-4-phosphate (E4P) respectively.

$$\text{F6P} + \text{GBP} \rightarrow \text{Xu5P} + \text{E4P} \tag{4.7}$$

This sequence (eqs. 4.5–4.7) of three reactions (aldol condensation, hydrolysis and 2-C transfer), which is portrayed in the centre of Fig. 4.9, is now repeated, at least in principle. This time, although the condensation involves G3P as before, the 4-C molecule (E4P) also participates (eq. 4.8) so that the product is the 7-C compound sedoheptulose-1,7-bisphosphate (SBP).

$$\text{E4P} + \text{G3P} \rightarrow \text{SBP} \tag{4.8}$$

This is then hydrolysed by a specific phosphatase to give sedoheptulose-7-phosphate (S7P) and P_i

$$\text{SBP} + \text{H}_2\text{O} \rightarrow \text{S7P} + \text{P}_i \tag{4.9}$$

Finally, a 2-C unit (carbons 1 and 2) is transferred (Fig. 4.9, top left) from the 'top' of S7P to the last (G3P) of the five molecules of triose phosphate that participate in this process in which five 3-C sugar phosphates are rearranged to give three 5-C sugar phosphates.

$$\text{S7P} + \text{G3P} \rightarrow \text{Xu5P} + \text{R5P} \tag{4.10}$$

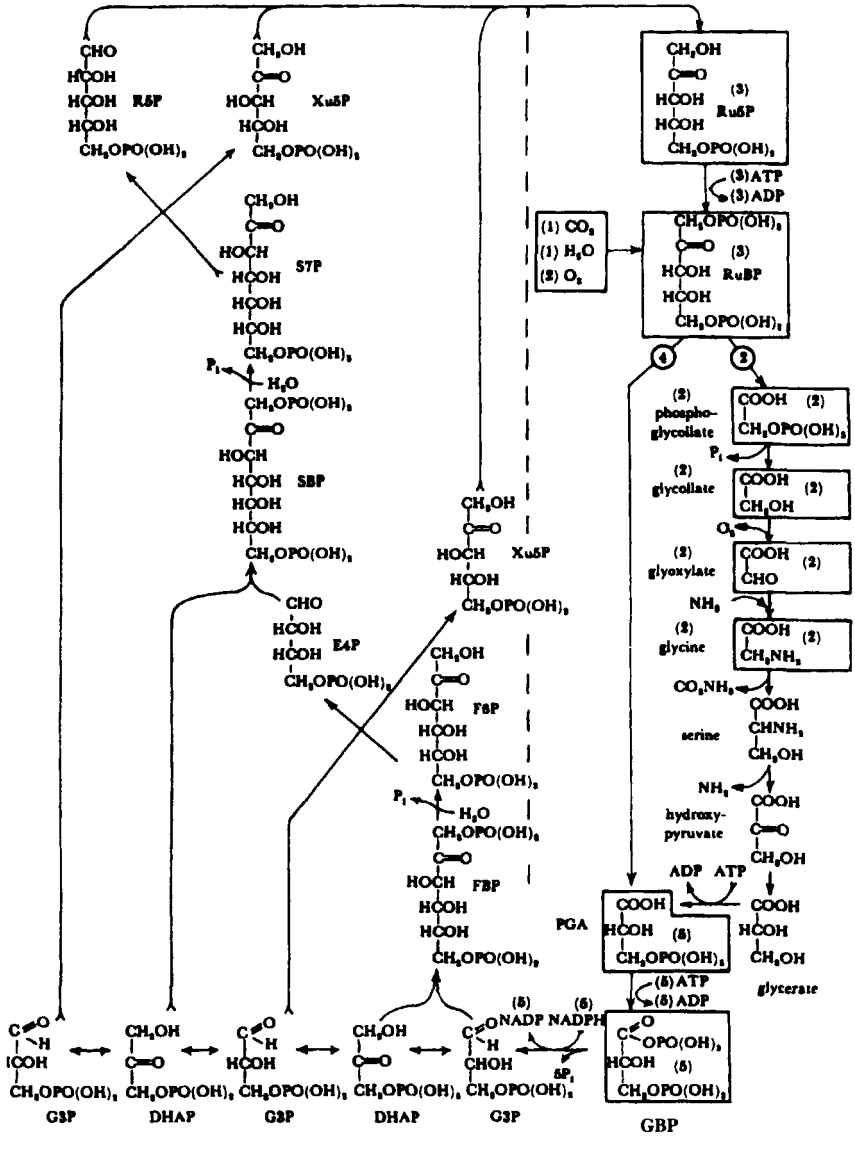


Figure 4.9 Calvin Cycle in more detail (*cf.* Fig. 4.8), with the intermediates identified. All of the left-hand side of the sequence is concerned with the regeneration of the CO_2 acceptor as five 3-C molecules are converted to three 5-C molecules. The reduction of the CO_2 fixation product (PGA), shown in more detail in Fig. 4.7, is at the bottom right.

Since G3P is again the recipient of the 2-C ketose unit, one of the products is Xu5P as before (in eq. 4.7), but what is left of a 7-C sugar that loses two of its carbons must be a 5-C compound and, indeed, the second product is the pentose sugar ribose-5-phosphate (R5P).

All three pentose phosphates (*i.e.* two molecules of Xu5P and one of R5P) are now converted to ribulose-5-phosphate (Ru5P) in one of two reactions. One of these converts Xu5P to its epimer, and the enzyme concerned is hence called an epimerase.

$$2\text{Xu5P} \rightarrow 2\text{Ru5P} \tag{4.11}$$

The other converts R5P to its isomer (eq. 4.12) and the catalyst is called an isomerase

$$\text{R5P} \rightarrow \text{Ru5P} \tag{4.12}$$

As a consequence of reactions 4.11 and 4.12, three molecules of Ru5P have been created, largely by rearranging five molecules of triose phosphate. All that remains is the final phosphorylation, brought about by ATP, which re-creates the CO_2 acceptor, RuBP.

$$3\text{Ru5P} + 3\text{ATP} \rightarrow 3\text{ADP} + 3\text{RuBP} \tag{4.13}$$

As we have seen, RuBP is an intrinsically unstable molecule. In effect, the energy required to drive the carboxylation in an energetically favourable reaction (*i.e.* one with an equilibrium position which overwhelmingly favours carboxylation, despite the very low concentrations of CO_2 in the atmosphere) is provided by ATP at the time of phosphorylation of Ru5P (eq. 4.13). The remaining one-third of the ATP consumed in the Calvin cycle is used at this point.

4.4.4 The phosphate translocator

It is important not to lose sight of the role played by the phosphatases in regeneration of RuBP (eqs. 4.6 and 4.9) because, in addition to their direct role in this rearrangement, they also contribute to another key aspect of photosynthetic carbon assimilation, *i.e.* the maintenance of P_i availability for ATP formation. The Calvin cycle does not itself produce free sugars. Starch is a sugar polymer and, to the extent that it is formed within the chloroplast stroma in many species, it constitutes a means of releasing P_i for further photophosphorylation of ADP to ATP. Nevertheless, such starch formation is largely a mechanism by which a plant maintains a supply of

metabolites in darkness and in any other circumstances in which the rate of photosynthesis is insufficient to meet consumption of photosynthetic products in other cellular processes. That part of the newly created triose phosphate pool that is not consumed in re-creating or increasing the amount of the CO_2 acceptor (RuBP) is directly exported to the cytosol through the P_i translocator located in the innermost chloroplast envelope. Such export occurs by exchange and this is the means by which P_i released from triose phosphate in cytosolic sucrose synthesis re-enters the chloroplast. Photosynthesis, depending as it does on P_i for ATP synthesis, can only meet this requirement by internal release of P_i (*e.g.* in starch synthesis and in the phosphatase-catalysed reactions) and by importing P_i in exchange for triose phosphate. The Calvin cycle, as portrayed in Fig. 4.9, requires nine molecules of ATP and therefore nine molecules of P_i must be made available if it is to continue. Two are released by the hydrolysis of FBP and SBP (via eqs. 4.6 and 4.9) and a further six in the reduction of GBP (as shown in Figs. 4.8 and 4.9). Only one is made re-available by import of P_i from the cytosol (in exchange for every molecule of triose phosphate exported for sucrose synthesis *etc.*) or for triose phosphate consumed in internal starch synthesis. Even so, it is these molecules of P_i that play a pivotal role in regulation (Woodrow and Berry, 1988) and poising of the entire system. There is no doubt that, in some circumstances, experimental manipulation of cytosolic P_i availability can have profound effects on photosynthesis, which reach right back to the early events of the photochemistry (Walker and Sivak, 1985).

4.5 Autocatalysis: adding to the triose phosphate pool

Had carbon assimilation evolved in such a way that it involved direct reduction of CO_2 to formaldehyde, there would have been no need to regenerate a CO_2 acceptor. Since in fact it involves addition of CO_2 to a pre-existing acceptor (RuBP), there is not only a requirement for regeneration of this acceptor but a need to create more. The Calvin cycle is a mechanism by which one molecule of triose phosphate product is produced from three molecules of CO_2. The original amount of acceptor is then regenerated as five molecules of triose phosphate (G3P) are rearranged and transformed to give three molecules of pentose bisphosphate (RuBP). However, it will be immediately apparent from Fig. 4.9 that, if more than one out of every six molecules of triose phosphate were withdrawn as product, the entire sequence would rapidly spiral to a halt. Conversely, if a proportion of the potential triose phosphate product is retained within the cycle, the amount of acceptor formed will automatically increase. It is this 'autocatalytic' increase that allows both for growth and the rapid

changes in RuBP quantities that are necessary if the plant is to optimise CO_2 assimilation in response to changes in light, temperature, availability of water *etc.* When leaves are abruptly illuminated after a period of darkness, PCA does not start immediately. Instead there is a period of induction, a lag before the full rate of assimilation is achieved. Induction lags are longer at lower than at high temperatures (Edwards and Walker, 1983). They are due in part to delays imposed while light activation of enzymes and the building up of Calvin cycle intermediates occurs. In all of this there is a need to regulate the operation of the cycle in such a way that an appropriate balance between export of triose phosphate from the cycle and regeneration and utilisation of RuBP within the cycle is maintained. In certain circumstances, abrupt illumination initiates pronounced (but dampening) oscillations in the rate of carbon assimilation, as regulatory responses appear to struggle to regain control. These are particularly marked when concentrations of P_i in the cytosol are limiting, implying that the ability of electron transport to supply ATP at optimal rates may be constrained in these circumstances (Walker, 1992b).

The autocatalytic nature of the Calvin cycle is also apparent when maximum rates of CO_2 fixation by isolated chloroplasts are measured at different temperatures. The rates of other metabolic processes, such as respiration, double for every 10 degrees rise in temperature between about 1 C and 30 C, whereas increasing temperatures does not increase the rates of purely photochemical events rates. Photosynthetic carbon assimilation falls into neither category. At temperatures close to 0 C, its rate may increase eight- or ten-fold. This has been attributed to the fact that, whereas individual reactions within the Calvin cycle may double for every 10 C increase in temperature, the amount of substrate made available for fixation by autocatalysis will also increase so that the effects will be multiplicative (Baldry *et al.*, 1966).

4.6 Photorespiration

In order for plants to exist as autotrophic organisms,[3] assimilation of inorganic matter including C, N, S, P is required by anabolic processes (in which biomolecules are synthesized from simpler components). However, plants also have metabolic processes that produce inorganic matter in catabolic (degradative) processes. For example, in non-photosynthetic tissue (*e.g.* roots and seeds), respiration and some loss of CO_2 occurs out of the necessity for converting soluble carbohydrates and amino acids into other forms of organic matter, including starch, protein and lipids. In

[3] Autotrophic organisms are those able to synthesize organic materials from inorganic sources.

general terms, the net rate of gain of organic matter equals the rate of assimilation of inorganic matter minus the rate of production of inorganic matter. Respiration, a catabolic process, is known to occur in the light in photosynthetic tissue. Unlike dark mitochondrial-type respiration, additional types of respiration occur in photosynthetic tissue in the light. Besides considering the extent of occurrence of photorespiration, there are questions about the purpose of different types of photorespiration.

4.6.1 Photorespiration via the Mehler-peroxidase reaction

One type of photorespiration occurs when O_2 acts as an alternative to NADP by accepting electrons from Photosystem I (PSI) in the so-called Mehler-peroxidase reaction. This results in the production of the superoxide radical O_2^-, which is subsequently reduced to water. The reductant is ascorbate, and the overall process is called the Mehler-peroxidase reaction (MPR). This reaction occurs through the following combined steps: (1) the transfer of electrons from splitting of water at Photosystem II (PSII) through the photosynthetic electron transport chain to oxygen to form superoxide at PSI; (2) the dismutation of the superoxide radical by superoxide dismutase (SOD) to form H_2O_2; (3) the reduction of H_2O_2 to water by ascorbate via the enzyme ascorbate peroxidase (APO), resulting in the formation of monodehydroascorbate (MDHA); and (4) the regeneration of ascorbate. The regeneration of ascorbate from MDHA occurs by donation of electrons from the electron transport chain via ferredoxin, by an NAD(P)-dependent MDHA reductase. Alternatively, if the disproportionation reaction 2 MDHA $\rightarrow$ ascorbate + DHA generates dehydroascorbate (DHA), then ascorbate can be regenerated through the combined actions of DHA reductase and glutathione reductase (Miyake and Asada, 1994). In the process, O_2 is evolved at PSII and consumed at PSI, resulting in no net change in O_2 levels. Obviously, the net rate of O_2 evolution from plants will be lower than the true rate of O_2 evolution from PSII when the MPR is functional. In this form of photorespiration, there is no release of CO_2 in the process. There are two potential benefits of the MPR. One is to provide additional ATP (via the proton-motive force generated from the associated electron flow) when there is insufficient ATP generated from linear electron flow associated with NADP reduction (Ivanov and Edwards, 1997). The other is to provide a means of safely dissipating energy under high irradiance, when the energy absorbed is in excess of what can be used for carbon assimilation (Osmond and Grace, 1995). The magnitude of the MPR reaction in plants continues to be investigated; during C_3 photosynthesis under non-stressful conditions, its contribution to photorespiration is considered to be very low (Badger *et al.*, 2000).

4.6.2 *Photorespiration via RuBP oxygenase*

Another unique form of respiration in the light occurs as a consequence of the reaction of RuBP with O_2. This leads to formation of glycolate and metabolism in the PCO (Photosynthetic Carbon Oxidation) cycle (Fig. 4.9). Metabolism of products of this form of photorespiration results in release of CO_2 and ammonia. The relation between the carboxylase and oxygenase activity of rubisco is given by (Jordan and Ogren, 1984)

$$v_c/v_o = S_{rel}\,[CO_2]/[O_2] \tag{4.14}$$

where v_c is the velocity of reaction with carboxylase, v_o the velocity of reaction with oxygenase, and S_{rel} the specificity factor, which indicates the specificity of the enzyme for reaction with CO_2 rather than O_2. A specificity factor of 100 (a typical value for rubisco from a terrestrial C_3 plant) indicates that, at equal concentrations of CO_2 and O_2 around rubisco, the rate of carboxylation (v_c) would be 100 times faster than the rate of oxidation (v_o). Obviously the enzyme is reasonably effective as a carboxylase at equivalent gas concentrations. However, under current atmospheric conditions, the v_c/v_o ratio is only about 2.5 in C_3 plants because the concentration of O_2 is so much higher than CO_2; the exact value is affected by leaf diffusive resistance to CO_2 and temperature. The greater the stomatal limitation to photosynthesis (*e.g.* as may occur under water stress), the lower the supply of CO_2 to rubisco. This decreases the v_c/v_o ratio and increases partitioning of RuBP into the PCO cycle. The v_c/v_o ratio decreases with increasing temperature, partly because S_{rel} decreases as the kinetic properties of rubisco change in response to a large decrease in affinity for CO_2, and partly because the solubility of CO_2 decreases more than that of O_2 (Jordan and Ogren, 1984; Leegood and Edwards, 1996). The 2,3-enediol intermediate has a higher free energy of activation for reaction with O_2 than with CO_2, making oxygenase activity increase more with temperature than carboxylation activity, and causing S_{rel} to decrease (Chen and Spreitzer, 1992). Under atmospheric CO_2 concentrations, C_3 photosynthesis has a relatively flat response to temperature, in part because the rate of photorespiration increases with increasing temperature. Also with increasing temperature, k_c (the Michaelis constant for CO_2 as substrate) increases, and this limits carboxylation under atmospheric levels of CO_2 (Farquhar and von Caemmerer, 1982; Jordan and Ogren, 1984). Thus, in C_3 plants, CO_2-saturated photosynthesis has a steep response to temperature because RuBP oxygenase activity is low and high CO_2 levels overcome the limitation of decreasing affinity for CO_2.

When RuBP reacts with O_2, the products are P-glycolate and 3-PGA, as shown in Fig. 4.9. 3-PGA is converted to triose-P through the reductive phase, the same as when 3-PGA is produced as a result of carboxylation. The P-glycolate is metabolised through the glycolate pathway, resulting in the synthesis of PGA. Thus RuBP carboxylase and RuBP oxygenase have in common the synthesis of PGA. The difference is in the number of PGA molecules synthesized. When 2 CO_2 react with RuBP in the carboxylase reaction, 4 PGA are synthesized. When 2 O_2 react with 2 RuBP, 3 PGA molecules are synthesized, as shown in Fig. 4.10. Carboxylation is an assimilatory pathway, while oxygenation is a respiratory pathway. Through carboxylation, CO_2 and P_i are assimilated into organic-P. Through the oxygenase reaction, inorganic matter—CO_2, P_i and NH_3—is produced. Since CO_2 and O_2 compete for reaction with RuBP, the relative rates of these two processes depends on the relative concentrations of CO_2 and O_2 at the catalytic site. Since for each 2 O_2 reacting with 2 RuBP, one CO_2 is produced in the glycolate pathway, the net rate of CO_2 assimilation is $(v_c - 0.5v_o)$, in the absence of dark-type mitochondrial respiration in the light.

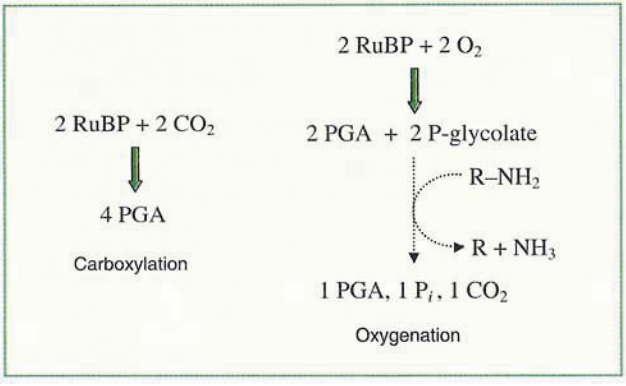


Figure 4.10 Fixation of CO_2 via RuBP carboxylase and generation of inorganic matter via respiration using RuBP as substrate. R–NH_2 is an amino acid and R is a keto-acid.

The metabolism of P-glycolate to PGA has its origin and termination in the chloroplast, but the pathway is very circuitous and involves metabolism in other organelles, namely peroxisomes and mitochondria. The sequence of reactions whereby two molecules of P-glycolate are metabolised in the glycolate pathway is as follows: in the first step phosphate is removed from phosphoglycolate (reaction catalysed by a phosphatase located in the chloroplast) and the product glycolate is exported from the chloroplast and imported by the peroxisomes.

$$2\ \text{P-glycolate} \rightarrow 2\ \text{glycolate} + 2\ P_i \quad (4.15)$$

In the peroxisomes the glyoxylate is aminated to form glycine. First, glycolate is oxidised to glyoxylate by glycolate oxidase and the hydrogen peroxide formed is converted to water and O_2 by catalase.

$$2\ \text{glycolate} + 2O_2 \rightarrow 2\ \text{glyoxylate} + 2H_2O_2 \quad (4.16)$$

$$2H_2O_2 \rightarrow 2H_2O + O_2 \quad (4.17)$$

Then, by two transaminase reactions glyoxylate is aminated to form glycine which is exported from the peroxisomes and imported by the mitochondria.

$$\text{glyoxylate} + \text{glutamate} \rightarrow \text{glycine} + \text{2-oxoglutarate} \quad (4.18)$$

$$\text{glyoxylate} + \text{serine} \rightarrow \text{glycine} + \text{hydroxypyruvate} \quad (4.19)$$

In the mitochondria, two molecules of glycine are converted to serine, CO_2 and NH_3 by two reactions which are catalysed by glycine synthase and serine hydroxymethyltransferase respectively.

$$\text{glycine} + \text{THF} + NAD^+ \rightarrow \text{NADH} + H^+ + \text{methylene-THF} + CO_2 + NH_3 \quad (4.20)$$

$$\text{methylene-THF} + \text{glycine} + H_2O \rightarrow \text{serine} + \text{THF} \quad (4.21)$$

The serine is imported to the peroxisomes, where it is converted to hydroxypyruvate (eq. 4.19), and then the hydroxypyruvate reduced to glycerate (4.22). The glycerate is imported to the chloroplast and phosphorylated to form PGA.

$$\text{hydroxypyruvate} + \text{NADH} + H^+ \rightarrow NAD^+ + \text{glycerate} \quad (4.22)$$

$$\text{glycerate} + \text{ATP} \rightarrow \text{PGA} + \text{ADP} \quad (4.23)$$

$$\text{ADP} + P_i \rightarrow \text{ATP} \quad (4.24)$$

The sum of reactions 4.15–4.24 is

$$2\ \text{P-glycolate} + O_2 + \text{glutamate} \rightarrow \text{PGA} + CO_2 + NH_3 + P_i + \text{2-oxoglutarate} + H_2O$$

What is clear is that the relative partitioning between CO_2 fixation and O_2 reaction with RuBP is dependent on the relative concentration of the two gases around rubisco. It is also evident that the rate of CO_2 assimilation can be limited under current atmospheric levels of CO_2 because of competition by O_2 and photorespiration. During PCO cycle activity CO_2 is produced and energy is required to reduce the PGA and to assimilate the NH_3 and P_i that are formed. However, it is also evident that decreasing the level of PCO cycle activity, *e.g.* by CO_2 enrichment, does not guarantee that there

will be an increase in photosynthesis and growth. Obviously plants cannot assimilate triose-P at a rate above which it can be utilised. Thus, in situations where the rate of photosynthesis is limited by the rate of triose-P utilisation, for example the rate it can be used for synthesis of starch and sucrose, then the rate of photosynthesis will not increase by eliminating photorespiration (*e.g.* by increasing CO_2 level or lowering O_2). Cases in which transient increases in growth of C_3 plants under elevated levels of CO_2 have been observed may reflect lack of ability for sustained use of the additional photosynthate. PCO cycle activity is least likely to limit plant growth under conditions where the rate of production of photosynthate (triose-P) nearly matches or exceeds the capacity to utilise it. This condition is favoured under conditions where stomatal conductance and access to atmospheric CO_2 is high. It is favoured by low temperatures which increases the solubility of CO_2, increases S_{rel}, and decreases the K_c for CO_2. As plants face periods of stress which limits the supply of CO_2 to the chloroplast, increased PCO cycle activity may be beneficial in providing a mechanism to safely dissipate excess energy (Osmond and Grace, 1995).

4.7 CO_2-concentrating mechanisms

It is clear that CO_2 is limiting for photosynthesis in certain environments since some species have evolved mechanisms for active accumulation of CO_2. CO_2-concentrating mechanisms have been discovered in a range of organisms, from microalgae to higher plants. Although there are differences in the mechanisms, there are certain common features, most notably the ATP-dependent accumulation of CO_2 at the site of rubisco (Fig. 4.11). In microalgae, including green algae and cyanobacteria, a CO_2-concentrating mechanism is induced when growth occurs under low CO_2. Growth of microalgae under low CO_2 induces synthesis of the enzyme carbonic anhydrase (which catalyses the interconversion of CO_2 and bicarbonate) and the appearance of new proteins in the plasmamembrane and chloroplast envelope, some of which are believed to be essential components of the CO_2 uptake mechanism.

In higher plants, there are two types of CO_2-concentrating cycles, namely the CAM and C_4 cycles. Although these two photosynthetic cycles differ in detailed mechanism, they have in common the C_4 pathway of photosynthesis, involving enzymes that are orders of magnitude more active at capturing and concentrating CO_2 than are those in the leaves of C_3 plants.

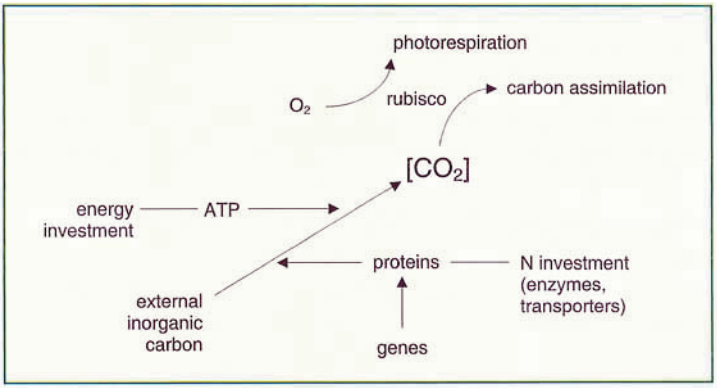


Figure 4.11 Illustrations of common features of CO_2-concentrating mechanisms in cyanobacteria, microalgae and higher plants. External inorganic carbon refers to a limiting concentration available to higher plants in the atmosphere and to photosynthetic microorganisms in aquatic environments.

4.7.1 CAM plants

CAM, an abbreviation for Crassulacean acid metabolism, is named after the family Crassulaceae since the mechanism was first identified in this family. Terrestrial CAM plants have been found within 33 plant families (Smith and Winter, 1996). In CAM plants, the CO_2-concentrating mechanism functions by fixing atmospheric CO_2 in the dark into organic acids (primarily malate) which are stored in the vacuole. This is a type of C_4 photosynthesis in which the primary initial product of fixation of atmospheric CO_2 is malate, a C_4 acid. On the following day, malate is exported from the vacuole to the cytoplasm, where it is enzymatically decarboxylated, resulting in the production of CO_2. The production of CO_2 in the leaf causes the stomata to close, so that the leaf becomes relatively gas-tight. As a result there is a rise in CO_2 in the leaf, which allows for efficient fixation by RuBP carboxylase with minimal photorespiration. Thus, there is temporal separation of fixation of atmospheric CO_2 into C_4 acids and their utilisation to provide a high level of CO_2 to rubisco. This mechanism in land plants is thought to be more important for its conservation of water than for its reduction in photorespiration since it allows stomata to be closed during the day when the potential for water loss is highest. However, in aquatic CAM plants (*e.g. Isoetes howellii*), no one can argue that water is limiting; rather the CAM mechanism allows the plant to assimilate CO_2 in the dark, when the supply of CO_2 is higher than in the day, and thus avoid day-time competition with other photosynthetic organisms for CO_2 (see Keeley's review in Winter and Smith, 1996).

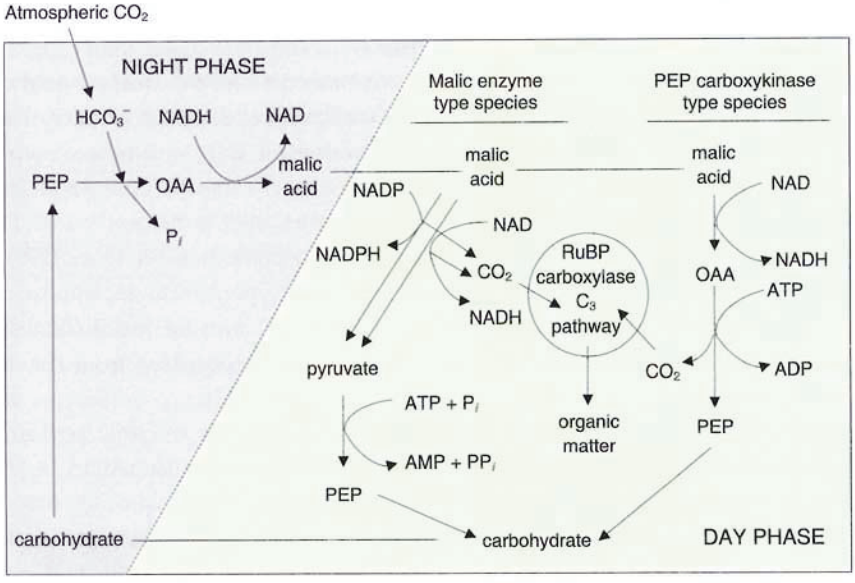
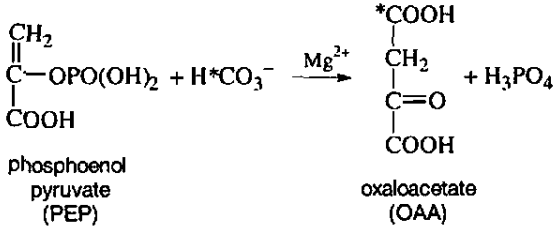


Figure 4.12 Key metabolic steps in Crassulacean acid metabolism.

Figure 4.12 shows the key metabolic steps in CAM plants. During the night, carbohydrate reserves (*e.g.* starch and sucrose) are metabolised via glycolysis to produce phosphoenolpyruvate (PEP). Atmospheric CO_2 entering the leaf is converted to bicarbonate through carbonic anhydrase.

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^- \qquad (4.25)$$



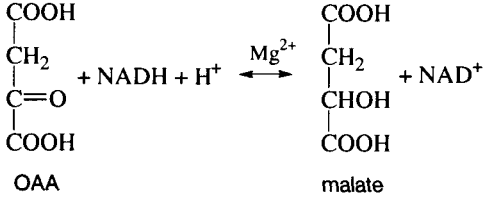
(4.26)

Phosphoenolpyruvate, like RuBP, is a high-energy substrate in the sense that the addition to it of CO_2, which gives rise to the formation of oxaloacetate and P_i, leads to a large decrease in free energy. About 25 kJ is needed to drive the carboxylation but the decrease in free energy associated with the overall reaction is sufficiently large to give a net change of about –29 kJ. Accordingly the reaction is, like the action of

rubisco, virtually irreversible. Once fixed, the CO_2 stays fixed (Walker, 2000). Unlike RuBP carboxylase, PEP carboxylase utilises HCO_3^- as substrate rather than CO_2, and O_2 does not affect catalysis. The reaction that combines HCO_3^- and PEP to yield the C_4 acid oxaloacetate (OAA) is catalysed by PEP carboxylase. The high affinity of the enzyme for bicarbonate results in an apparent k_c value for CO_2 well below current atmospheric levels (He and Edwards, 1996). The catalytic mechanism starts with binding of metal^{2+} (Mg^{2+} or Mn^{2+}), PEP and HCO_3^- in this order to the active site. The chemical steps may be summarised as follows: (1) phosphate transfer from PEP to form carboxyphosphate and enolate of pyruvate; (2) carboxyphosphate decomposes to form enzyme-bound CO_2 and phosphate; (3) CO_2 combines with the metal-stabilised enolate; and (4) the products oxaloacetate and phosphate are released from enzyme (for details, see Chollet *et al.*, 1996).

Diurnal changes in activity of PEP carboxylase in CAM plants and light activation in C_4 plants and associated changes in kinetic properties were shown to be via phosphorylation/dephosphorylation at a Ser residue near the C terminal of the protein by a specific protein kinase/phosphatase system. The phosphorylated enzyme is the active form occurring in the dark in CAM plants and in the light in C_4 plants (Carter *et al.*, 1996; Chollet *et al.*, 1996). Unlike rubisco, where O_2 competes with CO_2 for reaction with the substrate RuBP, O_2 does not compete with bicarbonate for reaction with PEP in the reaction catalysed by PEP carboxylase. Thus, atmospheric levels of O_2 do not inhibit PEP carboxylase activity.

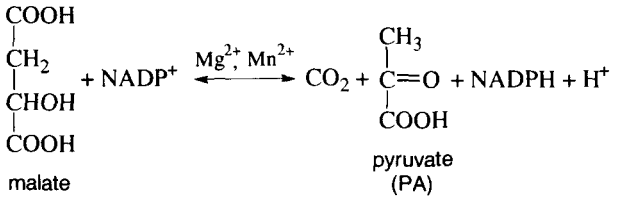
OAA, which is formed via PEP carboxylase, is reduced to malate via NAD-malate dehydrogenase using NADH generated in glycolysis and the malate is stored in the vacuole.



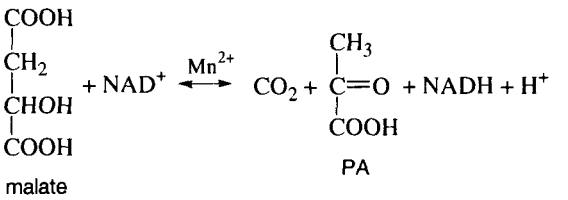
(4.27)

During the day the malate is exported from the vacuole and decarboxylated by C_4 acid decarboxylases. Two subgroups of CAM species exist, those which primarily use malic enzymes and those which primarily use PEP carboxykinase as the decarboxylation mechanism.

Malic enzyme type CAM species have high activities of malic enzymes; malate is decarboxylated by NADP-malic enzyme located in the cytoplasm and by NAD-malic enzyme located in the mitochondria.

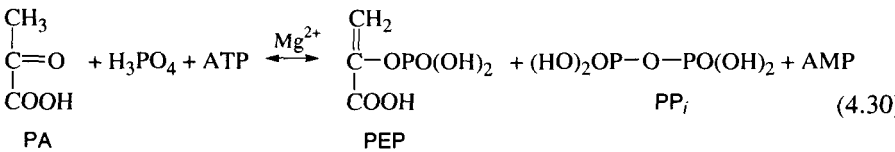


(4.28)



(4.29)

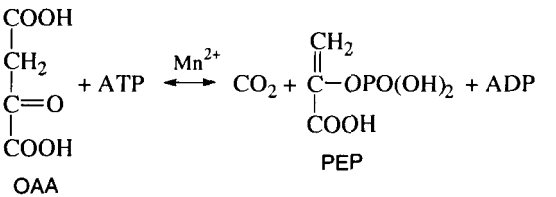
CO_2 generated from decarboxylation of malate enters the chloroplast, where it is assimilated into organic matter by the C_3 pathway and the pyruvate formed is converted to carbohydrate by gluconeogenesis. A key enzyme in this process is pyruvate-phosphate dikinase, which converts pyruvate to PEP.



(4.30)

This enzyme, which was first reported as 'PEP synthetase' by Hatch and Slack in C_4 plants in 1967, was identified as pyruvate, orthophosphate dikinase, a new key enzyme in CAM and C_4 plants which is activated by illumination (See Edwards *et al.*, 1985). Although the reaction itself is reversible, it proceeds to form PEP *in vivo* because the active pyrophosphatase (catalysing the reaction $PP_i \rightarrow 2P_i$) and adenylate kinase (catalysing the reaction AMP + ATP $\rightarrow$ 2ADP) in the chloroplasts consume the products AMP and PP_i. The reaction mechanism includes phosphorylation of P_i to form pyrophosphate, and histidine residues at the active site of the enzyme with γ- and β-P of ATP, respectively, and then pyruvate reacts with the His-P residue to form PEP. As for light/dark regulation, the active enzyme having His-P is inactivated by phosphorylation of a specific threonine residue with β-P of ADP, and it is reactivated by phosphorolysis of Thr-P to form pyrophosphate. Interestingly, a single regulatory protein mediates both phosphorylation and dephosphorylation of the Thr residue (Edwards *et al.*, 1985).

In PEP-CK type CAM species, the malate is converted to OAA by NAD-malate dehydrogenase during the day (eq. 4.27). The oxaloacetate is then decarboxylated by PEP carboxykinase.



(4.31)

PEP carboxykinase is located in the cytoplasm of CAM and C_4 plants and phosphorylation/dephosphorylation of the enzyme may play a role in its regulation (Walker and Leegood, 1996).

4.7.2 C_4 plants

C_4 plants have been found among at least 18 families of higher plants (Edwards and Walker, 1983; Sage and Monson, 1999). Photosynthesis in C_4 plants consists of the coordinated function of two cell types in the leaves, usually designated mesophyll cells (MC) and bundle sheath cells (BSC). In C_4 plants, atmospheric CO_2 enters leaves through stomata and is first accessible to MC, where it is fixed by phosphoenolpyruvate (PEP) carboxylase to form oxaloacetate, and then malate and aspartate. These C_4 dicarboxylic acids are transported to BSC where they are decarboxylated, the released CO_2 concentrated, and refixed by RuBP carboxylase and assimilated through the Calvin cycle to form sucrose and starch. Thus, in C_4 plants there is a spatial separation of CO_2 fixation into malate (occurring in MC) and utilisation of C_4 acids as donors of CO_2 to the C_3 pathway (occurring in BSC), whereas in CAM, there is a temporal separation of CO_2 fixation into malate (occurring at night) and utilisation of C_4 acids as donors of CO_2 to the C_3 pathway (occurring during the day). In C_4 plants, although anatomical differentiation is apparent in BSC, they are functionally similar to C_3 MC in carbon assimilation except for the presence of enzymes concerned with decarboxylation of C_4 acids. For reviews of the biochemistry of C_4 photosynthesis, see Edwards and Walker (1983) and Hatch (1987).

The physiological significance of separate, but coordinate, function of the two cell types in C_4 plant photosynthesis is the specialisation of MC towards generation of a high concentration of CO_2 in BSC in order to reduce the oxygenase activity of rubisco, and consequential reduction of photorespiration. Photosynthesis in C_4 plants

can be visualised as a mechanism to provide rubisco with near saturating CO_2 when C_4 plants can afford a high stomatal conductance, or to provide sufficient CO_2 for survival and growth when stomatal conductance is low.

C_4 photosynthesis functions in both C_4 and CAM plants to capture atmospheric CO_2, and to supply it to rubisco. Even though the mechanism in CAM and C_4 plants is very different, as noted earlier some of the key enzymes are the same. Three subgroups have been found in C_4 plants, distinguished by differences in the decarboxylation step in BSC. These are the NADP-malic enzyme (NADP-ME), NAD-malic enzyme (NAD-ME) and PEP carboxykinase (PEP-CK) types. As noted earlier, the same decarboxylases function in CAM in two types: malic enzyme-type species and PEP-CK-type species. In Fig. 4.13, the key metabolic steps during C_4 photosynthesis are illustrated in one of the C_4 subtypes, a NADP-ME species.

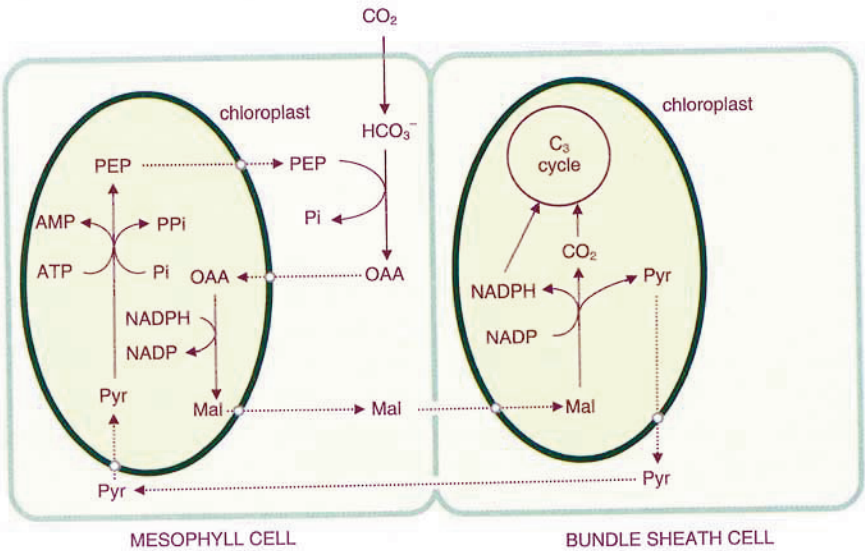
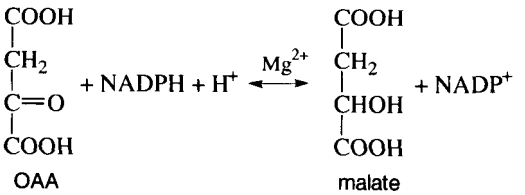


Figure 4.13 Key metabolic steps during C_4 photosynthesis in NADP-malic enzyme-type C_4 species. Members of this subgroup include maize, sugarcane and sorghum. Illustration provided by Dr. R. Kanai.

C_4 plants are considered to have evolved from C_3 plants. Common to all C_4 plants is the initial fixation of HCO_3^- by PEP carboxylase (eq. 4.26) to form oxaloacetate in the MC cytoplasm. Carbonic anhydrase (eq. 4.25) in the same compartment helps to equilibrate atmospheric CO_2, entering MC via stomata, to HCO_3^-. Malate is formed from oxaloacetate in MC by the enzyme NADP-malate dehydrogenase (eq. 4.32).



(4.32)

In the course of evolution of C_4 plants from C_3 plants, the MC developed a high level of carbonic anhydrase and PEP carboxylase in the cytosol for initial CO_2 fixation in the cytoplasm, and pyruvate, orthophosphate (P_i) dikinase (eq. 4.30) in the chloroplasts for provision of PEP, the HCO_3^- acceptor. It is equally important that the synthesis of some key photosynthetic enzymes in carbon metabolism of C_3 photosynthesis is repressed in MC of C_4 plants. This includes rubisco and phosphoribulokinase of the Calvin cycle in MC chloroplasts, and enzymes of glycine decarboxylation in the PCO cycle in MC mitochondria.

4.8 Survival and efficiencies of photosynthesis

The obvious advantage for C_4 photosynthesis is to supply CO_2 to rubisco under any condition where it would be most limiting for photosynthesis. The solubility of CO_2 in photosynthetic tissue decreases with increasing temperature, and the carboxylase to oxygenase specificity of rubisco decreases with increasing temperature, all of which increases the relative RuBP oxygenase/carboxylase activity and photorespiration via the PCO cycle in C_3 plants. Thus, C_4 plants have an advantage at higher temperatures and they have a much higher temperature optimum for photosynthesis than C_3 plants.

Under water stress caused by drought or high salinity, low stomatal conductance provides resistance to CO_2 uptake, which favours PCO cycle activity. CAM and C_4 plants have adaptations in photosynthesis, which along with modifications in life forms (*e.g.* leaf forms, leaf succulence, rooting systems) enable certain species to survive in very harsh environments where C_3 plants are not found. CAM plants (*e.g.* cacti) use water efficiently, and can survive in arid conditions. CAM appears most advantageous when the differential between day and night temperature is high since the potential for water loss at a given water vapour concentration in the air increases with increasing temperature of photosynthetic tissue. Low stomatal conductance of CAM plants helps conserves water in arid environments, but is unfavourable for the direct fixation of atmospheric CO_2 by C_3 photosynthesis.

C_3 plants and C_4 plants can survive and coexist in moderate environments, although C_4 photosynthesis may be advantageous under warm temperatures and high-light conditions. In cool climates, where the potential for photorespiration is minimal, it is more effective for the plant to invest in the components for C_3 photosynthesis. There are clear trade-offs: plants that invest in CAM or C_4 have lower levels of components of the C_3 pathway, *e.g.* rubisco protein, and these trade-offs can result in a similar capacity for photosynthesis under moderate environmental conditions.

The efficiency of use of solar energy for CO_2 assimilation (quantum yield, mole CO_2 fixed or O_2 evolved per mole quanta absorbed by the leaf) depends on light intensity, other environmental factors and the photosynthetic mechanism of carbon assimilation. Under high light, where the capacity to produce assimilatory power is high (ATP and NADPH), it is more likely that photosynthesis will be limited in C_3 than in C_4 plants due to the limiting supply of CO_2. Under limiting light, where the rate of photosynthesis is limited by the production of assimilatory power, the rate of CO_2 assimilation will depend on the efficiency of use of ATP and NADPH. This efficiency under limiting light can be calculated as the maximum quantum yield. In C_3 plants, the maximum quantum yield is limited due to photorespiration; and for that reason the quantum yield decreases with increasing temperature, with decreasing CO_2, or with increasing O_2, all of which causes increased PCO cycle activity. In C_4 plants, the rate of photorespiration is low, but there is an additional investment in supply of energy (ATP) to accumulate CO_2. Under moderate temperatures, and adequate water, the quantum yield is similar in C_3 and C_4 plants, but under CO_2 limitation (higher temperature and decreased stomatal conductance), C_3 plants have a lower quantum yield. Whether considering ability to survive, maximum capacity for photosynthesis under higher light, or maximum quantum yield under limiting light in C_3 versus C_4 photosynthesis, environmental conditions must be carefully considered. Since plants are prone to CO_2 limitation in hot, dry, and/or saline habitats, C_4 plants are expected to have an advantage over C_3 plants for conversion of solar energy into biomass in such conditions. CAM plants can utilise solar energy and grow under extremes of high temperature and drought.

References

Andrews T. J. (1996), 'The bait in the rubisco mousetrap', *Nature Struct. Biol.* **3**, 3–7.

Badger M. R., von Caemmerer S., Ruuska S. and Nakano H. (2000), 'Electron flow to oxygen in higher plants and algae: rates and control of direct photoreduction (Mehler reaction) and rubisco oxygenase', *Phil. Trans. Roy. Soc.* **355**, 1433–1446.

Baldry C. W., Bucke C. and Walker D. A. (1966), 'Temperature and photosynthesis. Some effects of temperature on carbon dioxide fixation by isolated chloroplasts', *Biochim. Biophys. Acta* **126**, 207–213.

Banks E. C. and Vernon C. A. (1970), 'Reassessment of the role of ATP in *vivo*', *J. Theor. Biol.* **29**, 301–326.

Carter P. J., Fewson C. A., Nimmo G. A., Nimmo H. G. and Wilkins M. B. (1996), 'Roles of circadian rhythms, light and temperature in regulation of phosphoenolpyruvate carboxylase in CAM', in *Crassulacean Acid Metabolism*, Winter K. and Smith J. A. C., eds., Springer-Verlag, Berlin, pp. 46–52.

Chen Z. and Spreitzer R. (1992), 'How various factors influence the CO_2/O_2 specificity of ribulose-1,5-bisphosphate carboxylase/oxygenase', *Photosyn. Res.* **31**, 157–164.

Chollet R., Vidal J. and O'Leary M. H. (1996), 'Phosphoenolpyruvate carboxylase: a ubiquitous, highly regulated enzyme in plants', *Ann. Rev. Plant Physiol. Mol. Biol.* **47**, 273–298.

Edwards G. E. and Walker D. A. (1983), *C_3, C_4, Mechanisms, and Cellular and Environmental Regulation of Photosynthesis*, Blackwell Scientific Publications Ltd., Oxford.

Edwards G. E., Nakamoto H., Burnell J. N. and Hatch M. D. (1985), 'Pyruvate, P_i dikinase and NADP-malate dehydrogenase in C_4 photosynthesis: properties and mechanism of light/dark regulation', *Ann. Rev. Plant Physiol.* **36**, 255–286.

Ellis R. J. (1979), 'The most abundant protein in the world', TIBS **4**, 241–244.

Ellis R. J. and Gray J. C. (1986), *Ribulose Bisphosphate Carboxylase–Oxygenase.* Royal Society, London.

Farquhar G. E. and von Caemmerer S. (1982), 'Modeling of photosynthetic response to environmental conditions', pp. 550–587 in Lange O. L., Nobel P. S., Osmond C. B. and Ziegler H. (1982), eds., *Physiological Plant Ecology II. Water Relations and Carbon Assimilation* **12B**, Springer-Verlag, Berlin.

Hatch M. D. (1987), 'C_4 photosynthesis: a unique blend of modified biochemistry, anatomy and ultrastructure', *Biochim. Biophys. Acta* **895**, 81–106.

He D. and Edwards G. E. (1996), 'Estimation of diffusive resistance of bundle sheath cells to CO_2 from modeling of C_4 photosynthesis', *Photosynthesis Res.* **49**, 195–208.

Ivanov B. and Edwards G. E. (1997), 'Electron flow accompanying the ascorbate peroxidase cycle in maize mesophyll chloroplasts and its cooperation with linear electron flow to $NADP^+$ and cyclic electron flow in thylakoid membrane energization', *Photosynthesis Res.* **52**, 187–198.

Jordan D. B. and Ogren W. L. (1984), 'The CO_2/O_2 specificity of ribulose 1,5-bisphosphate carboxylase/oxygenase. Dependence on ribulose bisphosphate concentration, pH and temperature', *Planta* **161**, 308–313.

Keys A. J. (1986), 'Rubisco—its role in photorespiration', *Phil. Trans. Roy. Soc. B* **313**, 325–336.

Leegood R. C. and Edwards G. E. (1996), Carbon metabolism and photorespiration: Temperature dependence in relation to other environmental factors. In: *Photosynthesis and the Environment*, Baker N. R., ed., *Advances in Photosynthesis Series*, Kluwer Academic Publishers, Dordrecht, pp. 191–221.

Leegood R. C., Sharkey T. D. and von Caemmerer S. (2000), *Photosynthesis: Physiology and Metabolism*, Kluwer Academic Publishers, Dordrecht, pp. 1–624.

Miyake C. and Asada K. (1994), 'Ferredoxin-dependent photoreduction of the monodehydroascorbate radical in spinach thylakoids', *Plant Cell Physiol.* **35**, 539–549.

Osmond C. B. and Grace C. E. (1995) Perspectives on photoinhibition and photorespiration in the field: quintessential inefficiencies of the light and dark reaction of photosynthesis?', *J. Exp. Botany* **46**, 1351–1362.

Pauling L. (1960), *The Nature of the Chemical Bond*, Cornell University Press, Ithaca, New York, 3rd. ed., pp. 1–644.

Rabinowitch E. I. (1945), *Photosynthesis and Related Processes*, Vols. I and II, Wiley Interscience, New York.

Robinson S. P. and Walker D. A. (1981), 'Photosynthetic carbon reduction cycle', in Hatch M. D. and Boardman N. K. *The Biochemistry of Plants: a Comprehensive Treatise*, Vol. 8, Academic Press, London & New York, pp. 193–236.

Sage R. F. and Monson R. K. (1999), *C_4 Plant Biology*, Academic Press, New York, pp. 1–596.

Taylor T. C. and Andersson I. (1997), 'The structure of the complex between rubisco and its natural substrate ribulose-1,5-bisphosphate', *J. Mol. Biol.* **265**, 432–444.

Walker D. A., Leegood R. C. and Sivak M. N. (1986), 'Ribulose bisphosphate carboxylase-oxygenase: its role in photosynthesis', *Phil. Trans. Roy. Soc. B* **313**, 305–324.

Walker D. A. (2nd. ed., 1992a), *Energy, Plants and Man*, Oxygraphics, Brighton.

Walker D. A. (1992b), 'Concerning oscillations', *Photosynthesis Res.* **34**, 387–395.

Walker R. P. and Leegood R. C. (1996), 'Phosphorylation of phosphoenolpyruvate carboxykinase in plants. Studies in plants with C_4 photosynthesis and Crassulacean acid metabolism and in germinating seeds', *Biochem. J.* **317**, 653–658.

Walker D. A. (2000), *Like Clockwork*, Oxygraphics, Sheffield, pp. 1–29.

Walker D.A. and Sivak M. (1986), 'Can phosphate limit photosynthetic carbon assimilation *in vivo*?', *Physiol. Veg.* **23**, 829–841.

Winter K. and Smith J. A. C. (1996), *Crassulacean Acid Metabolism, Biochemistry, Ecophysiology and Evolution,* Ecological Studies, Vol. 114, Springer-Verlag Berlin.

Woodrow I. E. and Berry J. A. (1988), 'Enzymatic regulation of photosynthetic CO_2 fixation in C_3 plants', *Ann. Rev. Plant. Physiol. Plant Mol. Biol.* **39,** 533–594.

CHAPTER 5

REGULATION OF PHOTOSYNTHESIS IN HIGHER PLANTS

DORIS GODDE
Merveldstr.472
45665 Recklinghausen, Germany
doris.godde@t-online.de
and
JANET F. BORNMAN
Department of Plant Biology, Research Centre Flakkebjerg
Danish Institute of Agricultural Sciences (DIAS)
DK-4200 Slagelse, Denmark
Janet.Bornman@agrsci.dk

Laudato sie, mi' Signore, cum tucte le Tue creature,
spetialmente messor lo frate Sole,
lo qual è iorno, et allumini noi per lui.
Et ellu è bellu e radiante cum grande splendore:
de Te, Altissimo, porta significatione.

(Praised be Thou, my Lord, through all that Thou hast made,
And first my lord Brother Sun,
Who brings the day, and light Thou givest us through him.
How beautiful is he, how radiant in all his splendour:
Of Thou, Most High, he bears witness.)
St. Francis of Assisi, *Canticle of the Sun*, 1225.

Higher plants are well adapted to life on earth. The development of seeds responsible for the distribution of succeeding generations was an invaluable evolutionary advantage, since it made the process of fertilisation independent of access to atmospheric water. The 240,000 known species of higher plants that have developed during the last 500 million years dominate every biotype from the tropical rainforests to the very hostile dry or cold deserts (Strassburger, 1991).

Plants surpass all animals and microorganisms with respect to mass production, storing about 99% of all biomass on Earth. Plant biomass consists mainly of polysaccharides, and its protein and lipid contents are relatively low. Biomass production is determined by the difference between the assimilation of CO_2 and its loss by dark

respiration and photorespiration. Biomass production and growth are therefore largely determined by the length of the growth period. When this is sufficiently long, considerable increases in biomass are possible even when photosynthetic yields are moderate. The length of the vegetation period is largely determined by the climate. In arctic and temperate regions, low temperatures limit the vegetation period, whereas in the subtropics the supply of water is the limiting factor for growth (Larcher, 1994).

Additional factors affecting plant growth are the quality of the soil and its inorganic nutrient supply. Often the availability of nitrogen limits plant growth. However, owing to the increasing use of fertilisers and the production of NO_x by traffic, cationic nutrients may become rate-limiting for plant growth in many European ecosystems. This effect is enhanced by the substantial and long-lasting acid deposition that has substantially decreased the content of cations in the soils of these regions (Schulze, 1989; Ulrich, 1995). In large areas of the world, particularly in subtropical regions, the build-up of salt from non-optimal irrigation reduces soil quality and restricts plant growth. Other anthropogenic factors with increasing impact on plant growth are the substantial loads of air pollutants such as ozone. Furthermore, the effects of rising atmospheric CO_2 concentrations and increased levels of ultraviolet–B (UV–B, 280–320 nm) radiation on plant growth are not yet well understood, especially when they are accompanied by other stressors such as high loads of air pollutants and mineral deficiencies.

To understand the influence of such stress factors on plant growth, it seems logical to investigate their effects on photosynthesis, which is the central anabolic pathway responsible for the production of the energy-rich compounds necessary for growth. Numerous studies have now provided evidence that adverse conditions seldom directly interfere with photosynthesis. Instead, they seem to act indirectly on the photosynthetic apparatus by disturbing the balance between production and consumption of assimilates. This chapter attempts to give an overview of some of the central processes involved in the acclimation and regulation of photosynthesis, especially photosynthetic electron transport, and how this determines the demand for assimilates such as carbohydrates, and thus ultimately the physiological needs of the whole plant.

5.1 Anatomy, morphology and genetic basis of photosynthesis in higher plants

5.1.1 Genetic basis

Photosynthesis in higher plants, as in other eukaryotes, is restricted to special organelles, the chloroplasts. The chloroplast is surrounded by an outer double

membrane, the envelope, which encloses an inner membrane system, the thylakoids. The protein complexes of the photosynthetic electron transport chain, such as PSII, PSI, the cytochrome b_6/f complex and the CF_1/CF_o ATP synthase are localised in the thylakoid membrane. During photosynthetic electron transport, light energy is used to drive the reduction of NADP to NADPH and the phosphorylation of ADP to ATP. These energy-rich substrates are needed by the Calvin cycle enzymes in the stroma for the fixation and reduction of CO_2 to carbohydrates, which are then transported through the outer envelope by the triose phosphate translocator into the cytoplasm (Flügge and Heldt, 1981).

As long ago as 1909, Bauer found that all plastids, including chloroplasts, have their own genetic information. Later, it was established that chloroplasts contain multiple DNA molecules, which are concentrated near the thylakoid membrane or at the inner membrane of the envelope (see Westhoff, 1996). The plastid DNA contains genetic information for proteins involved in CO_2 fixation, such as the *rbcL* gene that encodes for the large subunit of the ribulose–1,5–bis-phosphate carboxylase (rubisco), and also for several proteins involved in photosynthetic electron transport, such as the *psaA*, *psaB* and *psaC* genes, which encode for PSI subunits, and *psbA* and *psbD*, which encode for the D1 and D2 protein of PSII and several others. In PSII, all the polypeptides of the reaction centre and the inner antennae are chloroplast-encoded, whereas the information for the polypeptides of the water-splitting system and the light-harvesting polypeptides is nuclear-encoded. Furthermore, several genes encoding for the cytochrome b_6/f complex, ATP synthase and a thylakoid-bound NADH dehydrogenase have been identified in the chloroplast genome. The genetic information of a clip-protease has also been found to be chloroplast-encoded. However, although chloroplasts possess a variety of photosynthetic genes, they are not capable of expressing them autonomously. For their expression, several nuclear genes which are mainly involved in post-transcriptional processes are necessary (Herrmann *et al.*, 1992; Mayfield *et al.*, 1995; Mullet, 1993).

Further information on the expression and regulation of the chloroplast genome of higher plants will become more easily available with the sequencing of the nuclear genome of *Arabidopsis thaliana* and that of maize and rice in the near future (The Arabidopsis Genome Initiative, 2000).

5.1.2 Anatomical and morphological leaf features

In higher plants, chloroplasts are mainly found in the cells of the leaf palisade and spongy mesophyll. Uptake of CO_2 and evaporation of water are controlled by the

stomata, highly specialised epidermal cells. Stomata opening is regulated by the interaction of a variety of ion channels and is mainly controlled by plant hormones, CO_2 and light. This ensures that the aperture of the stomatal apparatus rapidly acclimates to external environment and physiological requirements (Assmann, 1993).

Leaf anatomy and photosynthetic characteristics vary with species and growth irradiance. Even the same plant may experience steep gradients of light. Shade leaves, for instance, located inside the crown of a tree, may receive 10% of the irradiance that sun leaves are exposed to. Under low light conditions, the low rate of production of assimilates limits plant growth. Thus plants or leaves under shady conditions have to ensure efficient use of the incoming light. Typical 'shade' leaves have fewer layers of palisade parenchyma and less chlorophyll (Chl) per unit area compared with leaves grown in full sunlight. However, a clear distinction between sun and shade leaves is not always found.

Plant species adapted to high irradiances often develop certain morphological properties to protect the leaf and the photosynthetic apparatus by reducing the light penetrating to the inner leaf tissue. Normally 5–10% of the incident radiation is reflected from the leaf surface. In some desert species, the proportion of reflected light is increased up to 20–25% by epicuticular waxes, trichomes (hairs) or salt bladders (Ehleringer *et al.*, 1981; Ehleringer and Björkman, 1978; Mooney *et al.*, 1977). A few plant species also selectively reflect certain wavelengths, *e.g.* in the UV and blue regions, owing to an extensive layer of epicuticular wax (Clark and Lister, 1975; see also Vogelmann, 1993) and or trichomes. Removal of trichomes from olive leaves results in increased inactivation of PSII by UV-B (Grammatikopoulos *et al.*, 1994). Such structures may serve to protect plants from extensive photoinhibition, especially in young, developing leaves, in high-irradiance regions (Karabourniotis and Bornman, 1999).

Steep light gradients exist not only at the plant level, but also at the level of the leaf, where most of the light may be attenuated in the upper 20% of the leaf (Vogelmann *et al.*, 1989). The epidermal cells play an important role in modifying light gradients within leaves. The shape of these cells can vary from a fairly uniform flat surface to a continuum of convexity. The convex-shaped epidermal cells tend to focus light at a point just below the epidermal layer (Bone *et al.*, 1985; Martin *et al.*, 1989, 1991; Poulson and Vogelmann, 1990). The ability of epidermal cells to focus light was previously thought to be confined to understorey forest plants (Bone *et al.*, 1985) as a means of increasing light capture. A survey of 47 species, however, indicates that some degree of focussing occurs in most plants (Vogelmann *et al.*, 1996). The internal light distribution is also determined by the arrangement of the photosynthetic palisade and spongy mesophyll tissue (Bornman and Vogelmann, 1988; Terashima and Saeki, 1983; Knapp *et al.*, 1988; Cui *et*

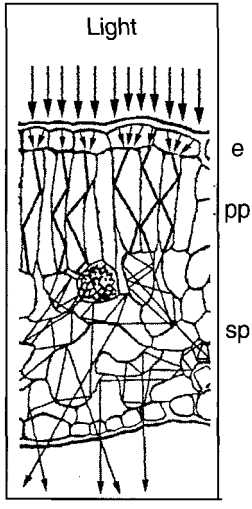


Figure 5.1 Path of radiation through a bifacial leaf. The epidermal cells (e) may focus the incoming light and the long palisade cells (pp) may partially conduct the light towards the spongy mesophyll (sp), where it is scattered and partly reflected back to the palisade cells.

al., 1991). The long, parallel-arranged palisade cells tend to conduct the light towards the spongy mesophyll, whereas these cells, because of their irregular arrangement and high incidence of intercellular spaces, tend to scatter the light-reflecting part of it back to the palisade cells, as shown in Fig. 5.1.

The spectral quality of the incoming light changes on penetration inside the leaf as a consequence of absorption, scattering and internal reflection. In alfalfa leaves, blue and red light can be reduced by 90% by the first 50 μm of tissue, leaving an internal environment enriched in green and far-red light (Vogelmann *et al.*, 1989). Thus it is not unexpected to find that the photosynthetic activities of the chloroplasts inside an individual leaf may vary greatly. Nishio *et al.* (1993) found the highest rates of CO_2 fixation in the mid-region of leaves, where the cells had the highest content of Chl and of rubisco. Radio-labelling showed that 60% of the carbon was fixed by the palisade mesophyll, and the remaining 40% in the spongy mesophyll. Chl fluorescence is most intense in the boundary region between the palisade and spongy mesophyll, towards the middle of the leaf (Bornman *et al.*, 1991). This region of high photosynthetic activity was also detected when a chlorophyll fluorimeter was coupled to a fibre optic microprobe (Schreiber *et al.*, 1996).

5.1.3 *Chloroplast ultrastructure and composition of the photosynthetic apparatus*

In addition to changes in leaf anatomy and morphology, the ultrastructure of the chloroplast and the composition of the photosynthetic apparatus also change in response to external conditions in order to optimise assimilate production. To ensure optimal capturing of photons at low irradiances, there is a tendency in 'shade' leaves to increase the ratio of light-harvesting complex (LHCII) to PSII reaction centres. This is structurally reflected by an increased ratio of stacked grana thylakoids versus unstacked stroma membranes. Furthermore, the ratio of PSII reaction centres to PSI is increased, favouring linear electron transport (Anderson, 1986). An increased PSII to PSI ratio also occurs in chloroplasts in intact leaves if changing light quality favours the activation of PSI. To achieve a balanced activation of the two photosystems, light capturing by PSII is enhanced by an increased antenna size. With increasing irradiance, however, a high degree of light capturing is not necessary, and in many species the ratio between LHCII and PSII reaction centres is reduced. Moreover, energy transfer from LHCII to PSII is decreased during state transitions, a process, in which, owing to redox-regulated phosphorylation, LHCII moves from PSII, and probably docks at PSI (Allen, 1992, 1995). Coupled to this are structural changes in the thylakoid membrane under high irradiances, which often result in an increased amount of non-appressed thylakoid membranes relative to appressed ones. Partial control of photosystem stoichiometry by blue-light receptors and by photosynthetic metabolism has been indicated (Walters and Horton, 1995). At the molecular level, the rate of gene transcription for the reaction centre of PSI (psaAB) and for PSII (psbA) is different, since it is influenced by the light quality and thus is linked to the redox state of the plastoquinone pool (Pfannschmidt *et al.*, 1999).

Acclimatory mechanisms seem to work to optimise photosynthetic efficiency under a given irradiance, but are also necessary to down-regulate certain processes in order to prevent, or at least to minimise, damage to cells and chloroplasts.

5.2 Adaptation of photosynthetic electron transport to excess irradiance

Although low irradiances are often limiting to photosynthesis, plants also have to cope with irradiances exceeding the light-saturation point of photosynthesis. Under high irradiances, enzymatic processes in the Calvin cycle become rate-limiting and photosynthetic electron transport, which can potentially lead to the formation of active oxygen species, has to be down-regulated. Numerous studies have provided evidence that linear photosynthetic electron transport is regulated by the activity of

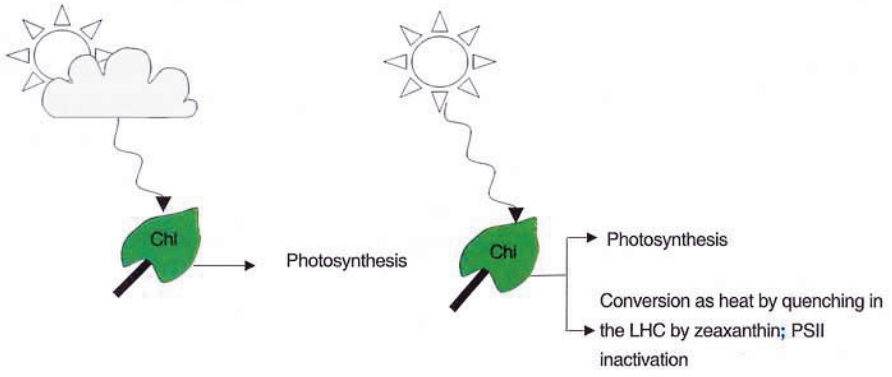


Figure 5.2 Under shady conditions, all or most of the incident sunlight is used for photosynthesis. Under high light conditions, excess solar energy is dissipated reversibly, either directly in the LHC or via zeaxanthin (see Section 5.2.1) or irreversibly by inactivated PSII centres (see Section 5.2.2).

PSII, which can regulate its ability to initiate photosynthetic electron transport (Powles, 1984; Osmond, 1994). So far, two different mechanisms for the inactivation of PSII are known, one reversible and one irreversible. Both rely on a conversion of excess light-energy into heat to reduce the quantum efficiency of PSII in the process known as *photoinhibition* (see Fig. 5.2).

5.2.1 *Reversible down-regulation of Photosystem II by non-radiative quenching of excitation energy*

Although PSII can use even low photon flux densities optimally, it is the only photosystem whose quantum efficiency can be reversibly down-regulated at high irradiances (Horton *et al.*, 1996). This is primarily enabled by its physical properties. The energy difference between the excited Chls in the antennae and the reaction centre Chl is small, resulting in a 'shallow' trap. Moreover, the charge separation is itself a reversible process (Schatz *et al.*, 1988), so excitation energy can be transferred back to the antenna.

In PSII antennae, optical excitation energy can be non-radiatively transformed into heat (Horton *et al.*, 1996; Demmig-Adams and Adams, 1992). Two processes, which both depend on the acidification of the thylakoid lumen, seem to be responsible for this quenching of excitation energy. In the first mechanism, a decrease in lumenal pH is thought to protonate acidic residues of the LHCII polypeptides, resulting in a destabilisation of the excited chlorophylls (Horton *et al.*, 1996, Wentworth *et al.*,

2000). This mechanism is enhanced by a quenching process involving carotenoids of the xanthophyll cycle (Demmig-Adams and Adams, 1992; Pfündel and Bilger, 1994). Based on its chemical structure, zeaxanthin is energetically favoured to accept energy from excited singlet Chl and to dissipate it as heat (Owens, 1994). However, it is not yet certain that the differences in energy levels between zeaxanthin and violxanthin are sufficient to account for the overall quenching effect (Frank *et al.*, 2000). Zeaxanthin is synthesised during the xanthophyll cycle in the light by the de-epoxidation of violaxanthin via antheraxanthin (Demmig-Adams and Adams, 1992; Pfündel and Bilger, 1994). The de-epoxidase is largely pH-dependent and an acidification of the thylakoid lumen favours the formation of zeaxanthin. By this mechanism, the energy reaching PSII reaction centres is decreased and PSII quantum efficiency is lowered in the light. Down-regulation of PSII activity by zeaxanthin is assumed to be a reversible process. At low irradiances or in the dark, when the pH gradient is dissipated, zeaxanthin back-reacts to violaxanthin and PSII quantum efficiency is restored.

There is evidence that the PsbS antenna protein, a minor part of the antenna complex of PSII, may be the site of most of the non-photochemical quenching processes (Li *et al.*, 2000). However, the location of zeaxanthin in the thylakoid membrane is still an open question. There are numerous reports that zeaxanthin is bound to the polypeptides of the light-harvesting complex, so it is assumed that quenching of excitation energy by zeaxanthin takes place in the outer antennae of PSII. However, zeaxanthin is also found in plants grown in intermittent light that are devoid of most of the light-harvesting polypeptides (Jahns and Krause, 1994) and it seems as if a considerable amount of zeaxanthin is bound to the reaction centre core of PSII. The accumulation of zeaxanthin in this region is accompanied by a loss of PSII activity which can probably only be restored by D1-protein turnover (Jahns and Miehe, 1996). It also seems that the xanthophyll, lutein, may play a role in non-photochemical quenching (Niyogi *et al.*, 2001).

5.2.2 *Irreversible inactivation of PSII*

Apart from the reversible down-regulation of PSII activity in the light, limitation of photosynthetic electron transport by CO_2 fixation can also result in irreversible inactivation of PSII. *In-vitro* studies with isolated thylakoids and PSII reaction centres have revealed two possible mechanisms for PSII inactivation (Telfer and Barber, 1994; Styring and Jegerschöld, 1994; Ohad *et al.*, 1994). In the so-called 'acceptor side' mechanism, excess light leads to complete reduction of the plastoquinone pool. As a consequence, the Q_B-binding site remains unoccupied and electron flow between

Q_A and Q_B is inhibited. On further excitation, Q_A becomes doubly reduced and protonated, and leaves its binding site. Such inactivated PSII centres without Q_A are still capable of charge separation, but since the separated charge cannot be stabilised, this favours an increased back reaction between the $P680^+$ and $Pheo^-$, the primary electron acceptor of PSII. In this way, P680 triplets that convert the excitation energy to heat are formed. However, P680 triplets also react with oxygen to form highly reactive singlet oxygen. The formation of singlet oxygen has been demonstrated in isolated reaction centres, where it induces photooxidative damage to pigments and proteins (Telfer and Barber, 1996). The first direct experimental evidence *in vivo*, using the double sensor, DanePy, showed that photoinhibition is accompanied by singlet oxygen production, and that this singlet oxygen was proportional to the amount of inactive PSII centres, indicative of acceptor side-induced photoinhibition (Hideg *et al.*, 1998), seen previously in *in vitro* studies.

PSII can also be irreversibly damaged when water splitting is impaired during the so-called 'donor-side' mechanism. The inhibition of water splitting prolongs the lifetime of the highly reactive $P680^+$, which can easily damage proteins and pigments in isolated reaction centres. Furthermore, back-reaction of $P680^+$ with Q_A is favoured, destabilising charge separation. Studies of Krieger *et al.* (1993) have revealed that acidification of the thylakoid lumen, observed under strong illumination, leads to inhibition of the oxygen-evolving complex owing to a specific release of Ca^{2+} that might favour donor-side inhibition *in vivo*. Although this seems to be a very slow reaction *in vivo*, van Wijk and van Hasselt, (1993) concluded that reversible down-regulation of PSII by increased pH would finally lead to irreversibly inactivated PSII centres. Chl fluorescence studies indicate that Q_A in a leaf seldom becomes fully reduced even under excess light (Godde and Dannehl, 1994; Russell *et al.*, 1995; Park *et al.*, 1996). Thus, electron transfer from Q_A to Q_B always remains possible. Moreover, considerable electron transport from water to oxygen is still possible either during photorespiration or via the Mehler reaction (discussed in Section 5.5.1) even when the stomata are closed (Heber *et al.*, 1996). Based on experiments with transgenic plants with a reduced level of cytochrome b_6/f complex, Hurry *et al.* (1996) have proposed that both a high pH gradient over the thylakoid membrane and a more highly reduced Q_A state are needed to establish photoinhibition.

Although the mechanisms of photoinhibition have been intensively studied during the past decade, its significance for plant growth has not been unequivocally clarified. According to Ögren (1994), photoinhibition as it occurs in non-stressed plants on clear days with high irradiance may reduce photosynthetic efficiency by about 10%. However, under stress conditions such as low temperatures, an accumulation of photoinactivated PSII centres has no effect on plant growth (Hurry *et al.*, 1992). This

has led to the assumption that photoinhibition itself should not be regarded as a damage, but as a long-term down-regulation of excess PSII activity (Hurry *et al.*, 1992; Öquist *et al.*, 1992), necessary for maintaining the balance between the formation of reducing equivalents during linear electron transport and their utilisation during CO_2 fixation.

5.2.3 *Inactivation of the PSI reaction centre*

PSI can also be irreversibly inhibited under special conditions. For example, PSI is inhibited in leaves of *Cucumis* at low temperatures by weak illumination, conditions under which there is almost no damage to PSII (Havaux and Davaux, 1994; Terashima *et al.*, 1994). The inactivation of PSI was reported to have been caused by destruction of its terminal iron–sulphur centres (Sonoike *et al.*, 1995), the possible sites of active oxygen formation, which are bound to the *psaC*-encoded subunit on top of the two reaction centre proteins on the stromal face of the thylakoid membrane. Inactivation is followed by degradation of the reaction centre subunits and the destruction of the PSI special pair P700. Even though PSI is not very sensitive to stress conditions in most plants, several earlier studies using isolated thylakoid membranes have shown susceptibility of PSI to photoinhibition (Satoh 1970, Inoue *et al.*, 1986; Sonoike, 1995). Some loss of PSI activity was also reported in intact spinach plants under high light stress (Godde *et al.*, 1992; Schmid *et al.*, 1995) and under mineral deficiencies (Godde and Dannehl, 1994). Further studies have shown that PSI inhibition occurs not only in certain chilling-sensitive species, such as cucumber, but also in less chilling-sensitive species (potato; Havaux and Davaud, 1994) as well as in some chilling-tolerant plants (barley; Tjus *et al.*, 1999; Teicher *et al.*, 2000). In barley, results indicated subsequent regulation within the cyclic electron transport around PSI (Teicher *et al.*, 2000). Several lines of evidence point to the involvment of active oxygen in the inactivation of both photosystems. While damage to PSII usually occurs through endogenous formation of singlet oxygen (Vass *et al.*, 1992; Hideg *et al.*, 1998) or through long-lived $P680^+/Tyr_Z^+$ at the donor side (Jägerschöld *et al.*, 1990), an interesting aspect is that superoxide and hydrogen peroxide produced at the reducing side of PSI may also damage PSII (Tjus *et al.*, 2001).

In contrast to PSII, which is repaired within a few hours, repair of inactivated PSI reaction centres is extremely slow, and recovery from PSI photoinhibition may take several days (Sonoike, 1996).

5.2.4 Repair of inactivated PSII centres by D1 protein turnover

Unlike PSI, inactivated PSII centres can be easily repaired. Their repair mechanism involves the degradation and replacement of one of the PSII reaction centre polypeptides, the D1 protein (Aro *et al.*, 1993; van Wijk *et al.*, 1994). The rapid turnover of the D1 protein, one of the characteristic features of PSII (Mattoo *et al.*, 1984), is closely related to the process of photoinhibition (Ohad *et al.*, 1994). Telfer and Barber (1994) proposed that D1 protein degradation is induced during the process of photoinactivation by oxidative damage of the protein matrix, probably by singlet oxygen. Damage and the initial breakdown of the D1 protein take place in the grana lamellae of the thylakoids (Barbato *et al.*, 1992). Synthesis and replacement of D1 occurs on the stromal membranes and the newly assembled PSII centres have to migrate back to the grana (Mattoo and Edelman, 1987; van Wijk *et al.*, 1996). *In vivo* PSII activity is determined by the rate of PSII inactivation as well as by the rate of its repair by D1 protein turnover. Thus, it is possible that photoinhibition of PSII is prevented by an increased turnover of D1 (Godde and Dannehl, 1994). Accumulation of inactivated PSII centres is only observed when the rate of PSII inactivation is faster than the rate of D1 degradation (Aro *et al.*, 1994, Kim *et al.*, 1993). Recovery of PSII activity is therefore linked to a large increase in D1 turnover (van Wijk *et al.*, 1994; Dannehl *et al.*, 1996).

Chl molecules are probably set free as a consequence of the disintegration of PSII during the rapid turnover of the D1 protein. In the light, these can easily react with molecular oxygen, forming extremely toxic singlet oxygen (Asada, 1994). It was suggested that Early Light Induced Proteins (ELIPs), the rapidly induced nuclear-encoded thylakoid membrane proteins (related to the light-harvesting chlorophyll a/b-binding gene family), bind these Chl together with lutein (Adamska, 1997), which can rapidly quench excited Chl. The Chl bound to the ELIPs might either be transferred to chlorophyllases to be degraded, or alternatively, it might be rescued and integrated with newly synthesised polypeptides. ELIPs were first identified during pea greening (Meyer and Kloppstech, 1984). The strong induction of ELIPs by light stress suggests that they function in the protection of the reaction centres from excess light, rather than fulfilling a light-harvesting role. This appears to occur through the dissipation of the absorbed light energy in the form of heat or fluorescence (Montané and Kloppstech, 2000). However, different stress events may activate transcription of the ELIP gene through different pathways, as has been reported for heat shock and light (Harari-Steinberg *et al.*, 2001).

Regulation of D1 protein degradation Regulation of D1 protein turnover is only partly understood. It seems that degradation of the D1 protein requires a certain conformation of the protein moiety. It does not take place when electron flow from PSII is inhibited and the plastoquinone pool is mostly reduced (Gong and Ohad, 1991; Hollinderbäumer *et al.*, 1997). Under these conditions, the Q_B site is unoccupied and does not allow any proteolytic attack of the D1 protein. In higher plants, but not in mosses, ferns or algae, D1 protein degradation is also controlled by thylakoid protein phosphorylation (Aro *et al.*, 1992; Elich *et al.*, 1992). The phosphorylation of the PSII reaction centre polypeptides seems to be under the redox control of the plastoquinone pool (Allen, 1992; Ebbert and Godde, 1994), whereas their dephosphorylation is catalysed by a PSII-specific phosphatase (Carlberg and Andersson, 1996; Ebbert and Godde, 1994; Rintamäki *et al.*, 1995), which is inhibited at high ATP levels (Rintamäki *et al.*, 1996). Under such conditions, phosphorylation becomes irreversible and creates a stable pool of PSII centres not involved in the turnover of the D1 protein (Ebbert and Godde, 1994, 1996). These centres are located in the grana membranes. Once these phosphorylated centres are inactivated, they cannot be repaired and instead accumulate. Thus it is very likely that they are the PSII centres responsible for the loss of PSII activity. However, the quantity of phosphorylated centres does not control the D1 protein turnover of non-phosphorylated ones (Ebbert and Godde, 1996; Hollinderbäumer *et al.*, 1997). The PSII centres involved in D1 protein turnover are located in the stroma membranes (Ebbert and Godde, 1996), where D1 protein is synthesised on ribosomes bound to the thylakoids.

The degradation, subsequent resynthesis and assembly of new polypeptides and their cofactors is a continuous process in response to changing environmental stimuli. This process of maintenance includes proteolysis, protein targeting and molecular chaperones (Bailey *et al.*, 2001). Among the proteases known to be associated with the thylakoids and involved in D1 protein degradation is the FtsH protease, an ATP-dependent zinc metalloprotease (Lindahl *et al.*, 2000). There is also evidence that the FtsH protease acts as a chaperone. It was previously proposed that the primary cleavage step of the D1 protein is a GTP-dependent process, leading to accumulation of a 23-kDa N-terminal fragment, and that the chloroplast FtsH protease is likely to be involved in the secondary degradation steps (Spetea *et al.*, 1999). These authors also noted that the breakdown pattern was indicative of the primary cleavage occurring in the stromal loop between the transmembrane D and E helices exposed at the outer thylakoid surface.

Regulation of D1 protein synthesis Degradation and synthesis of the D1 protein are strongly coupled (Komenda and Barber, 1995). Only under conditions inducing

chlorosis is the balance between degradation and synthesis of the D1 protein disturbed and the protein degraded faster than it is synthesised (Godde and Dannehl, 1994). This led to the assumption that D1 protein synthesis controls its own degradation (Komenda and Barber, 1995) or *vice versa* (Adir *et al.*, 1990; Tyystjärvi *et al.*, 1996). It is accepted that light-dependent expression of the plastidal *psbA* gene encoding for the D1 protein is controlled on the translational level (Fromm *et al.*, 1985; Mayfield *et al.*, 1995). Changes at the transcript level are observed during senescence (Humbeck *et al.*, 1996), under high light (Constant *et al.*, 1997; Shapira *et al.*, 1997) and under other stress conditions (Dietz and Harries, 1997; see also Fig. 5.6). It seems very likely that phosphatases and kinases are involved in the regulation of *psbA* transcription (Christopher *et al.*, 1997). However, the amount of transcript has only a small influence on protein synthesis itself (Shapira *et al.*, 1997). The translation of the *psbA* mRNA is initiated by the binding of nuclear-encoded binding factors to the 5' end of the untranslated region (Mayfield *et al.*, 1995). There is evidence that the binding of the nuclear-encoded factors is under the redox control of the thioredoxin system and controlled ADP-dependent phosphorylation (Danon and Mayfield, 1994a and 1994b). However, the further progress of translation also seems to be a light-dependent process (van Wijk and Eichacker, 1996), since the assembly of newly synthesised D1 protein to PSII complexes requires the presence and synthesis of Chl (Eichacker *et al.*, 1990; Feierabend and Dehne, 1996) and carotene (Trebst and Depka, 1997). Recent evidence has shown that apart from translation initiation, the translation elongation of D1 is also regulated by redox status (Zhang *et al.*, 2000).

In vivo, D1 protein turnover seems to be additionally under the control of the carbohydrate status of the cell (Kilb *et al.*, 1996). When supplied via the petiole, soluble carbohydrates inhibit the synthesis of the D1 protein. This inhibition takes place even at moderate irradiances before any change in photosynthetic activities can be observed. It is responsible for the subsequent accumulation of inactivated PSII centres and the chlorosis typical of plants accumulating carbohydrates in their leaves (see Section 5.2.4). Thus inhibition of D1 protein synthesis is part of the feedback control mechanism of photosynthesis. A similar anabolic inhibition is also known from the expression of the nuclear *rbcS* and *cab* genes (Sheen, 1990, 1994; Krapp *et al.*, 1993). Initial experiments have shown that glucose feeding does not affect the amount of *psbA* mRNA.

Wounding also affects D1 protein synthesis (Kilb *et al.*, 1996; also Fig. 5.3). In young source leaves of pea, wounding results in a rapid stimulation of D1 protein synthesis within two hours, whereas in old leaves D1 protein synthesis becomes inhibited. The soluble sugar content is not changed. It is assumed that the changes in *psbA* gene expression are caused by jasmonates, which are synthesised on wounding

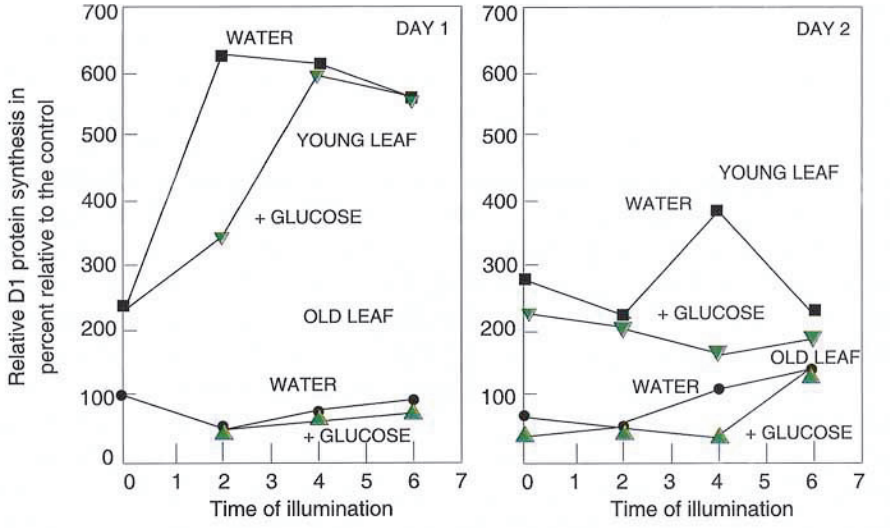


Figure 5.3 D1 protein turnover is influenced by leaf age, wounding and carbohydrates. Pea plants were cut below the third leaf pair and were transferred into water with or without 50 mM glucose. On the first day, D1 protein synthesis, measured as light-dependent incorporation of ^{14}C leucine, was stimulated in the young leaves, independently of whether the medium contained glucose or not. On the second day of the experiment, glucose resulted in a repression of D1 protein synthesis. In old leaves, cutting resulted in an inhibition of D1 protein synthesis, which was released on the second day of the experiment.

(Creelman and Mullet, 1995). This is confirmed by the fact that treatment of leaf disks with methyl jasmonate results in stimulation of D1 protein turnover (Reinbothe *et al.*, 1993). Under these conditions, degradation and synthesis of the D1 protein are no longer balanced, leading to a decreased content of this protein in the thylakoid membrane. Figure 5.4 summarises the main mechanisms and factors influencing D1 protein turnover. It is not yet clear how and under what conditions these work together. However, the great flexibility found in the regulation of D1 protein turnover may be necessary to adapt photosynthetic activity and size of the photosynthetic apparatus to the physiological needs of the plant.

Recovery from photoinhibition Recovery of PSII activity after photoinactivation relies on D1 protein turnover and thus requires certain prerequisites. First, the D1 protein of the inactivated PSII centres has be in a conformation that can be degraded. Therefore the plastoquinone pool has to be largely oxidised and the Q_B site occupied by oxidised plastoquinone. This is only possible when photosynthetic electron transport is possible. Furthermore, the D1 protein should be dephosphorylated. This

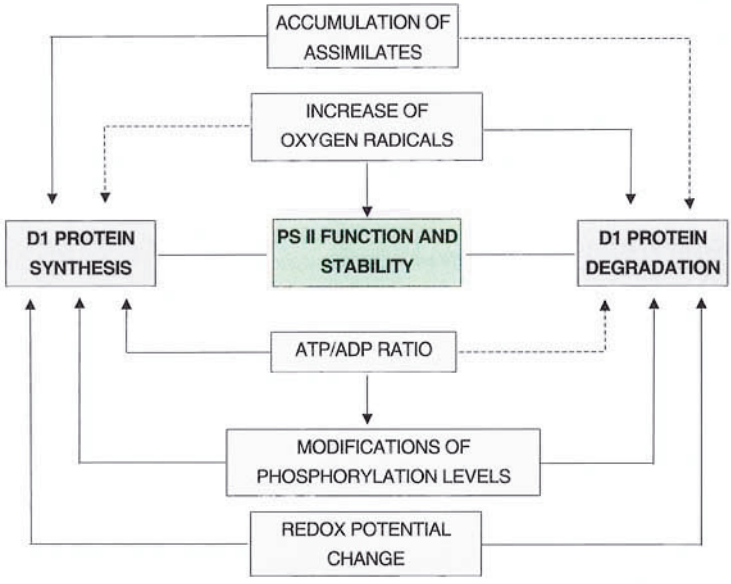


Figure 5.4 Schematic representation of possible mechanisms regulating D1 protein turnover. Solid and dotted lines represent primary and secondary effects, respectively.

requires an active PSII-specific phosphatase and low ATP levels in the chloroplast. Additionally, thioredoxin should be reduced to allow the initiation of translation. All these prerequisites are fulfilled in weak light when reduction equivalents and ATP are optimally consumed during CO_2 fixation and when optimal recovery from photoinhibition is observed (Aro *et al.*, 1994).

Molecular mechanism of stress-induced chlorosis Apart from reversible down-regulation and irreversible photoinactivation, another means by which photosynthetic electron transport can adapt to reduced demand for assimilates is by reducing the photosynthetic apparatus. The degradation of the photosynthetic apparatus, visible as chlorosis, is observed under chronic high irradiances or under stress conditions. Previously, chlorosis was interpreted simply as the photooxidative breakdown of both pigments and proteins of the photosynthetic membrane following photoinhibition (Elstner and Osswald, 1994; Demmig-Adams and Adams, 1992). However, the effects of stress on the photosynthetic apparatus show that degradation of the photosynthetic apparatus precedes any functional disturbances of the photosynthetic electron transport chain (Godde and Dannehl, 1994; Konopka *et al.*, 1996; Ottander *et al.*, 1995). Stress-induced chlorosis therefore has to be regarded as a strongly

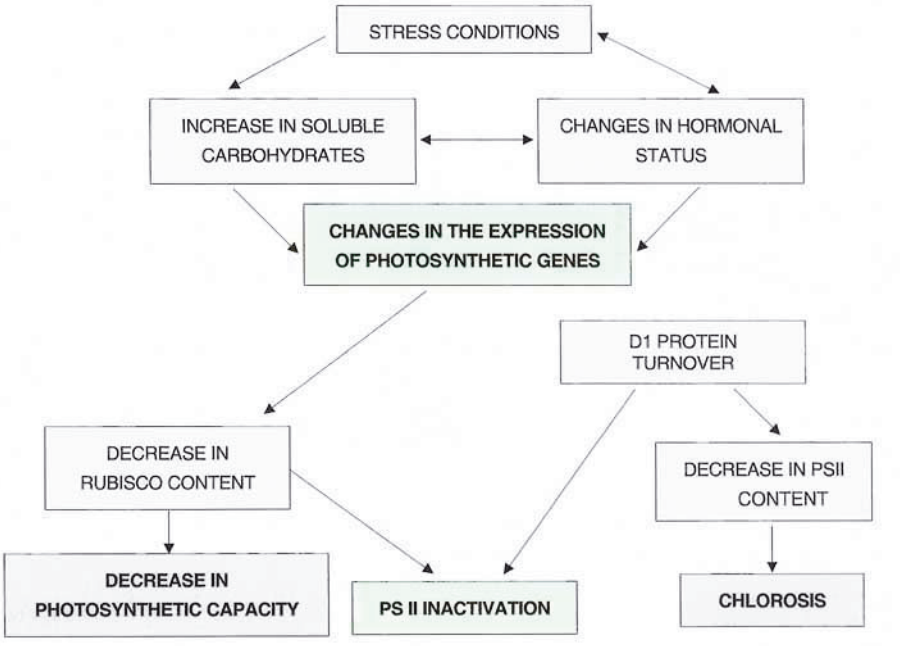


Figure 5.5 Schematic representation of a possible mechanism of stress-induced chlorosis induced by imbalances in D1 protein turnover.

regulated process during which only the size of the photosynthetic apparatus is reduced while the function of its remaining components is retained (Dannehl *et al.*, 1995; see also Fig. 5.5).

The character of stress-induced chlorosis as a regulated process is substantiated by the finding that different photosynthetic polypeptides are not degraded at the same rate or to the same extent. Immunological determinations of polypeptide content during stress-induced chlorosis have revealed that PSII, together with the polypeptides of its inner antennae and the proteins stabilising water splitting, are preferentially degraded (Godde and Hefer, 1994; Godde and Dannehl, 1994; Ottander *et al.*, 1995; Nie *et al.*, 1995c; Giardi *et al.*, 1996). Their loss is as high as 60–70%, when related to Chl, and up to 90% when based on leaf fresh weight. Also, PSII is the first component of the photosynthetic machinery to become degraded during senescence-related chlorosis (Humbeck *et al.*, 1996). PSI is degraded to a slightly greater extent than Chl, but its loss is only about 10–20% when based on equal amounts of Chl (Godde and Dannehl, 1994). The polypeptides of the LHCII, which bind about 40–50% of the total Chl, are lost together with the bulk Chl (Godde and

Dannehl, 1994). The specific degradation of PSII in comparison to LHCII is reflected by the pigment composition. In contrast to fully green plants, yellowing plants show an increased ratio of lutein to Chl, whereas the ratio of Chl to β-carotene remains constant (Kandler and Miller, 1990; Zimmer-Rinderle and Lichtenthaler, 1995). This suggests that stress-induced degradation of the photosynthetic apparatus differs mechanistically from photooxidative processes, where β-carotene is specifically lost (Kandler and Miller, 1990; Sironvall and Kandler, 1958). Even the rubisco seems to be preferentially degraded, with its loss resulting in the often-observed decrease of photosynthetic capacity (Section 5.5).

Since many stress conditions result in accumulation of assimilates (as we shall see in Section 5.5), the high specific degradation of PSII can be regarded as an adaptation of photosynthetic electron transport to a reduced requirement for assimilates. However, the hormonal status of a plant is also affected by stress conditions, and stress hormones like abscisic acid or jasmonates may be involved as signals in the degradation of the photosynthetic apparatus. Since photosynthesis itself remains undisturbed at least during the initial phase of chlorosis, it has to be assumed that these factors act directly on the expression of photosynthetic genes and protein turnover. By diminishing the amount of PSII, not only is the production of reducing equivalents necessary for CO_2 fixation reduced, but also the production of active oxygen species. Thus, the chlorotic process protects the still-existing cell components and allows a rapid regeneration of the photosynthetic machinery when environmental conditions improve (Dannehl *et al.*, 1996). Excess excitation energy is dissipated non-radiatively by the xanthophyll in still-existing light-harvesting complexes, which are degraded to a much lesser extent than PSII itself. The relatively high amounts of PSI are necessary to build up the pH during the cyclic electron flow needed for non-radiative energy quenching in the antennae.

The increased degradation of PSII can be ascribed to imbalances in the turnover of the D1 protein from the reaction centre of PSII (Godde and Dannehl, 1994; Konopka *et al.*, 1996). Under stress conditions, the rate of D1 protein synthesis at first increases (Godde and Dannehl, 1994) to keep up with the increased degradation. In this way, the D1 protein content stays constant and degradation of the photosynthetic apparatus is prevented. However, if the stress continues, the degradation of D1 protein becomes even more stimulated and outbalances its synthesis (Dannehl *et al.* 1996). The loss of PSII seems to initiate the degradation of LHCII. It appears that the degradation of LHCII is an enzymatic process catalysed by an ATP-dependent protease localised in the soluble stroma of the chloroplasts (Andersson and Aro, 1997; Lindahl *et al.*, 1995). It may involve destabilisation of the trimeric LHCII into monomeric forms (Takeuchi and Thornber, 1994; Nie *et al.*, 1995c), which is probably regulated by

phosphorylation (Nilsson *et al.*, 1997). Additionally, expression of *cab* genes and LHCII synthesis is restricted under high light conditions (Maxwell *et al.*, 1995; Escoubas *et al.*, 1995). It is assumed that the repression of the nuclear *cab* genes is under redox poise by the intersystem electron transport chain.

5.3 Regulation of photosynthetic electron transport by CO_2 and oxygen

When rubisco, the first enzyme of the Calvin cycle, accepts O_2 instead of CO_2, a cycle of reactions known as photorespiration is initiated. This results in a loss of carbon as CO_2 and of ATP and NADPH used to regenerate ribulose–1,5–bisphosphate, suggesting that photorespiration is a wasteful process. However, several studies point to the contrary, as we discuss briefly below. The oxygenase activity of rubisco increases with temperature and decreasing CO_2 concentrations inside the leaves. To reduce carbon losses by photorespiration, many plants growing under tropical or subtropical climate separate carbon fixation from CO_2 reduction, either spatially in the C_4 pathway or in time by the crassulacean acid metabolism (CAM). In these pathways, CO_2 is fixed by PEP-carboxylase (PEPC) and reduced to malate.

In view of these observations, it is not surprising that since the discovery of photorespiration by Björkman (1966) and the elucidation of its pathway by Tolbert and Zelitch (Husic *et al.*, 1987) many attempts have been made to eliminate the oxygenation function of rubisco in C_3 plants. Some studies have demonstrated increased plant performance after modifying the capacity for photorespiration. For instance, the intact gene of maize phospho*enol*pyruvate carboxylase was introduced into a C_3 rice plant, resulting in an improved activity of PEPC in the leaves of some of the transgenic plants (Ku *et al.*, 1999).

Osmond and Björkman (1972) suggested that the oxygenase function of rubisco might be essential for C_3 plants in protecting PSII from photoinactivation, especially when CO_2 uptake is inhibited by stomatal closure. Heber and co-workers (1996) found evidence that a substantial electron flow from water to oxygen takes place in leaves with closed stomata. On the basis of inhibitor studies these authors concluded that this electron flow is due to photorespiration. Inhibition of photorespiration immediately caused an inhibition of electron flow and an increase in the reduction state of Q_A, which is known to cause inactivation of PSII. These observations are in contrast to the results of Brestic *et al.* (1995), who did not find a protective effect of photorespiration on PSII activity under drought conditions. These authors assume that non-radiative energy dissipation is sufficient to protect PSII even in the absence of photorespiration. However, transgenic plants with low levels of glutamine synthetase,

the key enzyme of photorespiration, suffer severe photoinhibition and photooxidation under high irradiances or low CO_2 concentrations (Kozaki and Takeba, 1996). Plants with an increased level of glutamine synthetase and enhanced photorespiration instead exhibited greater tolerance to high irradiances than control tobacco plants. Furthermore, the reduction of glutamine decarboxylase by reverse genetics, although not altering photosynthesis under ambient conditions, favours photoinhibition at low CO_2 concentration (Wingler *et al.*, 1997). These observations tend to confirm the protective role of photorespiration in driving photosynthetic electron transport under unfavourable conditions, thus preventing the formation of highly reactive oxygen species. Using barley mutants with a low capacity for photorespiration, it was shown that the metabolic process plays a significant role in preventing over-reduction in the photosynthetic cells through transfer of redox equivalents from chloroplasts (Igamberdiev *et al.*, 2001). However, the possibility of high amounts of photorespiratory H_2O_2 in the peroxisomes and subsequent potential ROS should not be overlooked (Noctor *et al.*, 2002).

Thus several interesting aspects emerge, one of which is that the apparent inefficiency of photorespiration is in itself useful under stress conditions, and therefore elimination or strong reduction of photorespiration by engineering rubisco may not necessarily lead to better plant performance (Wingler *et al.*, 2000).

5.4 Feedback regulation of photosynthesis

As a consequence of plant architecture and its division into leaf, root and shoot, assimilate production is separated from assimilate consumption. Thus, the assimilates fixed in the chloroplasts of autotrophic parenchymatous cells need to be transported away to heterotrophic cells and organs not capable of photosynthesis. Depending on plant development, such organs may be young developing leaves, flowers, fruits or roots. These organs, which depend on the supply of assimilates, are called sink organs, whereas the photosynthetically active leaves are regarded as source organs for assimilates (Turgeon, 1989). The transport of assimilates, water and nutrients takes place in the vascular bundles in phloem and xylem tissues. In the phloem, the sieve cells and their transfer or companion cells are responsible for the long-distance transport of assimilates. Depending on the morphology of the transfer or companion cells, phloem loading is either a symplastic process, where the assimilates remain in the symplastic continuum, or an apoplastic process, where the assimilates are first transported out of the parenchymatous cells before they are taken up by the companion cells (van Bel, 1993). Evidence is accumulating that phloem loading is a

biochemical pathway with a large impact on plant metabolism and photosynthesis. To balance production and consumption of assimilates, photosynthesis has to be regulated by succeeding metabolic pathways such as the export of triose phosphates out of the chloroplast, sucrose synthesis and phloem loading and unloading by feedback mechanisms. Such feedback mechanisms often result not only in a reduction of photosynthetic capacity, but also in a decrease in PSII quantum efficiency and accumulation of inactivated PSII centres. Under certain circumstances, chlorosis may even be initiated.

Several hypotheses have been postulated to explain feedback control of photosynthesis (see Paul and Pellny, 2003). The coupling of elevated sucrose contents with the induction of changes in gene expression has been shown in several studies, especially during the induction of fructan accumulation in leaves of C_3 grasses and cereals (Farrar *et al.*, 2000). Thus sucrose appears to have an important regulatory function, whereby excess sucrose can "feed forward" to stimulate sink processes and 'feed back' to down-regulate photosynthesis (Neales and Incoll, 1968; Farrar *et al.*, 2000).

5.4.1 Regulation of chloroplast metabolism by phosphate availability

According to Heldt and co-workers (Heldt *et al.*, 1977; Stitt, 1986) feedback inhibition of photosynthesis is caused by a local phosphate deficiency inside the chloroplast. This hypothesis is based on the fact that the transport of assimilates out of the chloroplast, catalysed by the triose phosphate translocator, depends on exchange with inorganic phosphate (Flügge and Heldt, 1981). In the cytosol, triose phosphates are utilised to build up sucrose, the most common transport form of sugars. When sucrose accumulates in the leaf, sucrose-phosphate synthetase is deactivated by protein phosphorylation (Huber and Huber, 1992) and the cytosolic fructose–1,6–bisphosphatase is inhibited by the rising level of fructose–2,6–bisphosphate. As a result, sucrose synthesis decreases and phosphorylated metabolites increase in the cytoplasm while the phosphate concentration inside the chloroplast decreases. Export of triose phosphate is reduced by phosphate limitation and the assimilates are then converted into transitory starch. As a consequence, the ATP/ADP ratio decreases, glycerate-3–phosphate accumulates and ribulose–1,5–bisphosphate-carboxylase becomes deactivated. Finally, photosynthetic electron transport is slowed down. Down-regulation of photosynthesis as induced by phosphate deficiency seems to play an important role under conditions of cold stress (Savitch *et al.*, 1997; see also Section 5.5.1).

Studies with transgenic plants have shown that the rate of photosynthesis is not affected when the amount of triose phosphate translocator is reduced (Riesmeier *et al.*, 1993; Heineke *et al.*, 1994). Although the assimilates are mainly stored as transitory starch during the day, this is degraded into glucose moieties during the night, which are then transported out of the chloroplast by the glucose transporter. This might explain why 'anti-sense' potato plants with strong repression of the chloroplast triose phosphate translocator did not show any significant growth inhibition.

5.4.2 Interaction between photosynthesis and assimilate transport

Impairment of either phloem loading or sugar storage in the vacuole has substantial effects on photosynthesis and leaf Chl content (von Schaewen *et al.*, 1990; Lucas *et al.*, 1993; Frommer and Sonnewald, 1995). Impairment in phloem loading is known to lead to an accumulation of soluble sugars in source leaves, where they are stored mainly in the vacuole. Such an accumulation of carbohydrates has substantial effects on the expression of photosynthetic genes. Numerous studies have shown that an increase in the content of soluble carbohydrates results in a repression of the nuclear *rbcS* and *cab* genes encoding for the small subunit of rubisco and the polypeptides of LHCII (Sheen, 1990; Krapp *et al.*, 1993). The repression of *rbcS* genes is accompanied by a decrease in the content of rubisco, which in transgenic plants has been shown to be the major determinant of photosynthetic flux under high irradiances and atmospheric CO_2 levels (Quick *et al.*, 1991; Furbank *et al.*, 1996). A drop in rubisco levels may therefore explain the reduced photosynthetic capacity in plants with impaired phloem loading. The limitations in CO_2 fixation in turn give rise to the observed increased ATP/ADP ratio and the increased thylakoid membrane energisation. Under these conditions, thermal energy dissipation increases in both the antennae and the reaction centre, favouring the accumulation of inactivated PSII centres (see Section 5.2.2). Another characteristic symptom of feedback inhibition of photosynthesis is the development of chlorosis. Chlorosis, induced by an accumulation of carbohydrates in the apoplast, is based on an inhibition of D1 protein synthesis (see Section 5.2.4) in combination with an increase in its degradation rate (Kilb *et al.*, 1996). The loss of Chl might be accelerated by the repression of the *cab* genes.

Certain plant hexokinases are involved in sugar sensing and signaling, functioning both as a regulator and catalyst (Jang and Sheen, 1994; Pego *et al.*, 1999). The hexokinase initiates a kinase cascade that in turn serves as a regulator of transcription

(Koch 1997). Thus the amount of flux through the hexokinase would determine the extent of up-regulation of genes responding via this cascade (Jang and Sheen, 1994). Recent work suggests that there are three distinct glucose signal transduction pathways in plants and that these pathways are closely linked to developmental stage and environmental conditions (Xiao *et al.*, 2000).

The studies of Sheen and co-workers (Jang and Sheen, 1994; Jang *et al.*, 1997) involving the use of protoplasts and transgenic plants provided evidence that the transport of glucose across the plasma membrane and its subsequent phosphorylation by the hexokinase triggers the anabolic repression of photosynthetic genes. However, the work of Herbers *et al.* (1996) with transgenic tobacco plants expressing increased activities of either cytoplasmic or vacuolar invertase led to the conclusion that the accumulation of soluble sugars in the cytoplasm is not sufficient to induce these changes, but that the transport of sugars either into the phloem or into the vacuole has to be disturbed. These studies are in contrast to the results of Büssis *et al.* (1997) with transgenic potato plants. They observed substantial chlorosis when the amount of cytoplasmic invertase was increased.

5.5 Factors limiting plant growth

We have already noted that numerous natural and anthropogenic factors that restrict plant growth have a large impact on both the function and the size of the photosynthetic apparatus. It seems that these factors seldom interfere directly with components of the photosynthetic machinery. Instead, many of the stress conditions lead to secondary effects. An example is the accumulation of soluble carbohydrates inside the leaf as a result of a change in the carbohydrate metabolism itself or in assimilate allocation. Other examples include the modification of the hormonal status of the plants leading to an increase in stress hormones such as abscisic acid, ethylene or jasmonates. As a consequence, the expression of photosynthetic genes is altered, and this may lead to a decrease in the size of the photosynthetic apparatus, an increase in thermal energy dissipation and an accumulation of inactivated PSII centres.

5.5.1 Low temperatures

Plant performance at different temperature extremes is often strongly dependent on the type of photosynthesis mechanism—C_3, C_4 or CAM. In addition, temperature effects are markedly influenced by CO_2 and light levels. Low-temperature stress

frequently has a negative effect on the photosynthetic apparatus, especially when accompanied by exposure to high irradiance (Öquist *et al.*, 1987), which produces chlorosis and increases the inactivation of PSII. Low temperature inhibits sucrose synthesis, and this in turn leads to a restriction of phosphate recycling and photophosphorylation (Sage and Sharkey, 1987; Labate and Leegood, 1988). The ADP/ATP ratio is then lowered, causing the reduction of photosynthetic electron transport (Savitch *et al.*, 1997; see Section 5.4.1) and thereby increasing the probability of photoinhibition. However, acclimation and adaptation mechanisms alleviate some of the stress. These processes include an array of modifications such as post-translational activation and increased expression of enzymes of the sucrose synthesis pathway and enzymes of the Calvin cycle, as well as changes in the leaf protein content (Gray *et al.*, 1996; Hurry *et al.*, 1994; Stitt and Hurry, 2002). Thus sugar metabolism plays a pivotal role during cold acclimation. Low temperatures also result in an accumulation of sugars due to reduced transport, which may be responsible for cryoprotection.

Species originating from tropical or subtropical regions, like maize, are generally of the C_4 type (Ortiz-Lopez *et al.*, 1990). These species are usually more susceptible to cold-stress-induced photoinhibition than those from temperate or cold climates, which mainly belong to the C_3 type (Foyer *et al.*, 2002). In the latter regions, most species have the ability to acclimate photosynthesis to low temperatures, including an increased tolerance to photoinhibition. Studies of such cold-hardened plants, especially different types of rye and wheat, have shown that carbon metabolism and the capacity for non-radiative energy dissipation play an important role in susceptibility to cold-stress-induced photoinhibition. Cold-hardened leaves have a higher photosynthetic capacity than non-hardened leaves at the same temperature (Öquist and Huner, 1993). The increase in the activity and activation state of photosynthetic carbon reduction cycle enzymes such as rubisco, stromal fructose–1,6–bisphosphatase and the enzymes responsible for sucrose synthesis ensures photosynthetic electron transport and keeps the reduction level of Q_A low, thus preventing inactivation of PSII (Gray *et al.*, 1996; Öquist and Huner, 1993). However, even when photoinactivated PSII centres accumulate, growth is not affected (Hurry *et al.*, 1992). In addition to the changes in carbohydrate metabolism, cold-hardened plants are characterised by an increase in protective mechanisms including non-radiative energy dissipation by the xanthophyll cycle (Fryer *et al.*, 1995; Koroleva *et al.*, 1994; Schöner and Krause, 1990). In evergreen conifers, the zeaxanthin content is stable and does not show any light dependency (Adams *et al.*, 1994). In this way, a steady, high non-radiative conversion of excitation energy is achieved.

It has been proposed that the increased photoinhibition at low temperatures is caused by an inhibition of the PSII repair cycle, *i.e.* degradation and *de novo* synthesis of the reaction centre D1 protein (Aro *et al.*, 1990; Gong and Nilsen, 1989; Ottander *et al.*, 1993) since D1 protein turnover is strongly temperature dependent (Bredenkamp and Baker, 1994). Apart from protein synthesis itself, the rates of migration, protein assembly and ligation of cofactors are slowed down at low temperature and might restrict the repair of inactivated PSII centres. This is substantiated by the fact that mutants of *Synechocystis* with increased amounts of unsaturated fatty acids show less sensitivity towards photoinhibition and faster recovery, which is thought to be due to an increased D1 protein turnover (Gombos *et al.*, 1994; Nishida and Murata, 1996). A similar effect has been observed in cyanobacterial mutants transformed to synthesise glycine betaine, which shifts the lipid phase transition to lower temperatures (Nishida and Murata, 1996).

Conifers, winter cereals and maize reduce their levels of photosynthetic apparatus (Ottander *et al.*, 1995; Nie and Baker, 1991) during cold periods. As under various other stress conditions (see Section 5.3.4), cold-induced chlorosis precedes the accumulation of inactive PSII centres. The amount of PSII is specifically reduced (Ottander *et al.*, 1995; Nie and Baker, 1991; Robertson *et al.*, 1993), much more so than that of PSI or LHCII. In this way, the capacity for linear electron flow is reduced and production of active oxygen species can be largely prevented. The incoming excitation energy can be dissipated non-radiatively by zeaxanthin in the remaining light-harvesting complexes. Recovery of both the activity and amount of PSII centres in evergreen conifers is rather rapid (Ottander *et al.*, 1995), but in subtropical plants like maize such a complete recovery does not seem to be possible (Nie *et al.*, 1995c).

5.5.2 *High temperatures*

Plant species from tropical latitudes typically show a temperature optimum greater than 30 C, whereas that for temperate species usually lies between 20 C and 30 C. While the kinetics of certain rate-limiting enzymes such as rubisco largely restrict photosynthesis at low temperatures, the higher temperature tolerance of respiration and the sensitivity of PSII electron transport are key factors in plant response to high temperature (Berry and Björkman, 1980). As in several studies of stress effects, PSII is more sensitive than PSI (Berry and Björkman, 1980; Havaux *et al.*, 1991), with deactivation of PSII by heat involving impairment of the oxygen-evolving complex (Enami *et al.*, 1994) and separation of the peripheral LHCII pigments from the PSII complexes (Schreiber and Berry, 1977; Sundby *et al.*, 1986). These changes are

probably associated with membrane integrity, increased lipid fluidity at high temperatures resulting in a loss of structural stability of key protein complexes within the thylakoid membrane.

Increased rates of protein degradation occur at high temperatures (35–45 C) as well as decreases in protein synthesis, with the exception of the heat-shock proteins (HSP). Certain of these HSPs may protect protein complexes of the electron transport chain, as suggested by Coleman and McConnaughay (1994), since low molecular weight HSPs, notably HSP21 and HSP24, are transported to the chloroplast upon a rise in temperature (Osteryoung and Vierling, 1994). Thermotolerance may be afforded through binding of the low molecular HSP to PSII (Kloppstech *et al.*, 1985), although this has been challenged by reports showing that HSP21 is not associated with the thylakoid membranes, but rather is found in the stroma (Chen *et al.*, 1990). Several HSPs function as molecular chaperones (Hartl *et al.*, 1992, Waters *et al.*, 1996), refolding previously denatured proteins, while others have a proteolytic role. It is also interesting to note that on heat stress, rubisco, which in addition to its function in CO_2 fixation is also a member of the HSP60 family, increases nearly twofold concomitantly with the much greater increase of the low molecular weight HSPs (Waters *et al.*, 1996).

There is convincing evidence that rubisco activase, which is required for the maintenance of the catalytic activity of rubisco (involving redox regulation), also functions as a chaperone and has a role in the response of photosynthesis to temperature (Portis, 2002). These results arose from the isolation of a novel *Arabidopsis* photosynthesis mutant (Somerville *et al.*, 1982), which led to the discovery that activation of rubisco involved the heritable factor rubisco activase, an ATPase (Salvucci *et al.*, 1985; Portis and Salvucci, 2002). This also showed that light activation of rubisco was not solely governed by increases in stromal pH and Mg^{2+} as previously thought (Lorimer *et al.*, 1976; Heldt *et al.*, 1978). Even before the discovery of rubisco activase, the light activation of rubisco was reported to be one of the most labile reactions associated with the inhibition of photosynthesis by high temperatures (Weis, 1981), with subsequent work supporting this (Sharkey *et al.*, 2001). The rubisco activase is itself temperature sensitive (Feller *et al.*, 1998), although denaturation of the activase is thought to account only partially for the deactivation of rubisco at moderate temperatures. Activase levels also increase during heat shock treatment (Sánchez de Jiménez *et al.*, 1995; Law and Crafts-Brandner, 2001), probably through post-translational mechanisms (Law and Crafts-Brandner, 2001). Activase may thus be implicated in the acclimation to high temperatures and/or recovery of inactivated photosynthesis (Sharkey *et al.*, 2001).

In nature, plant response to heat, and indeed to most environmental stresses, is of course modified by many other factors to which the plant is simultaneously exposed. For example, heat may be accompanied by drought stress and high light. Drought conditions may in some cases increase tolerance of PSII to high temperatures (Havaux, 1992), demonstrating the complex interactions of different stresses. Further coupling between different stresses is also found in the underlying mechanism of action of the xanthophyll cycle pigments. The conversion of violaxanthin to zeaxanthin under high-light conditions, as a means of dissipating excess energy non-photochemically and thereby protecting PSII, has also been implicated as a protective measure under high-temperature conditions, and it has been suggested that this increased heat tolerance is due to a modified interaction between violaxanthin and LHCII (Havaux and Tardy, 1996). Thus moderately high temperatures appear to increase accessibility of violaxanthin to the membrane-localised de-epoxidase. This was independent of light since zeaxanthin accumulated during the heat stress in the dark. The increased accessibility of violaxanthin to de-epoxidase was suggested to occur as a result of energetic uncoupling of LHCII and the increased fluidity of the thylakoid membranes.

Apart from mechanisms for dealing with high temperatures at the biochemical and physiological level, phenological and morphological features also serve to protect the plant. For example, the vertical orientation of photosynthetic stems of some species from Mediterranean and desert areas enjoy a distinct advantage over leaf photosynthesis, with higher water use efficiency and retention as well as lower temperature of the inner tissues (Nilsen *et al.*, 1996). Leaf form also changes in some species, with smaller, more reflective leaves during the hot, dry season being replaced by larger leaves during cooler periods (Nilsen *et al.*, 1993). In the same way, trichomes will reduce leaf temperature and have a positive effect on the water balance, and therefore also protect the photosynthetic apparatus. In addition to the role of trichomes in radiation reflection and cooling, they also protect photosynthesis from UV–B radiation through their UV-absorptive properties (see also Section 5.1.1; Karabourniotis *et al.*, 1993; Grammatikopoulos *et al.*, 1994), an important role given that regions of high temperature and high UV–B levels tend to coincide.

5.5.2 Arid climates

Drought frequently limits photosynthetic CO_2 assimilation. In semi-arid and arid regions, drought stress is usually accompanied by salinity stress and high irradiances. Under such stress conditions, large changes in gene expression (Ingram and Bartels, 1996; Bohnert *et al.*, 1995) lead to an accumulation of sucrose, polyols like mannitol,

sorbitol and their cyclic derivatives and fructans in source leaves (Bianchi *et al.*, 1991; Bohnert *et al.*, 1995). Together with proline, amino acid derivatives and methylamines, the low molecular weight carbohydrates are effective osmoprotective substances. In addition to the genes encoding for enzymes involved in carbohydrate metabolism, the expression of the *rbcS* genes encoding for the small subunit of rubisco is also affected by drought stress and abscisic acid (Bernacchia *et al.*, 1996; Giraudat *et al.*, 1994). As a consequence, the amount of rubisco decreases (Gimenez *et al.*, 1992) reducing photosynthetic capacity. However, the photosynthetic apparatus seems to be relatively tolerant to desiccation (Cornic *et al.*, 1992). It has been shown that, at moderate leaf-water deficits, when the relative water content is reduced to 70%, photosynthetic CO_2 fixation and electron transport are impaired by stomatal closure. Up to this point, however, water loss does not affect photosynthetic capacity and leaves illuminated under saturating CO_2 concentrations show the same photosynthetic activity as control leaves (Cornic, 1994). Only when drought is extended and relative water content falls below 70% is the capacity for CO_2 fixation and photosynthetic oxygen evolution reduced (Boyer and Bowen, 1970; Cornic *et al.*, 1992). Recent investigations have re-addressed the question as to whether drought limits photosynthetic CO_2 assimilation through stomatal closure or by metabolic impairment in C_3 plants. Results support previous work whereby it would seem that stomatal closure is the earliest response under conditions of mild to moderate drought, while decreased RuBP content is the main limitation during severe drought (Flexas and Medrano, 2002). The limitation of RuBP synthesis is in turn likely to be caused by inhibition of ATP synthesis (Lawlor, 2002).

Chlorophyll fluorescence quenching analysis indicates that the loss of photosynthetic capacity is not primarily due to impairment of photosynthetic electron transport but rather to restrictions in CO_2 fixation (Cornic, 1994).

Drought, like other stress conditions, does, however, predispose plants to photoinhibition. A decrease in the relative water content of 40% affects photosynthesis at the level of PSII (Masojidek *et al.*, 1991; Giardi *et al.*, 1996) by causing considerable depletion of the PSII core as indicated by the loss of CP43 and D1 protein. As under different stress conditions, the degradation of PSII is initiated by changes in D1 protein turnover. Although synthesis is stimulated, it cannot outbalance degradation and a loss of D1 protein and PSII occurs (Giardi *et al.*, 1996). The fact that the effect of drought on PSII depends very much on growth conditions such as temperature and irradiance may explain the diverse results seen in growth chamber experiments as compared with those carried out under field conditions (Ludlow, 1987; Cornic *et al.*, 1992; Jefferies, 1994). Plant response is of course also species-dependent. For instance, under field conditions of high light and decreased water availability, photo-

inhibition in grapevines is low, indicating efficient dissipation of absorbed energy through photorespiration and thermal dissipation involving the xanthophylls cycle (Medrano *et al.*, 2002). In arid regions, salinity often accompanies drought stress and has many similar effects, affecting photosynthesis in at least three ways: by closure of stomata, by reducing photosynthetic capacity and by inhibiting electron transport (Masojidek and Hall, 1992). However, under salt stress, species specificity needs to be considered. In the plant *Suaeda salsa*, there is a high tolerance of photosynthesis to both salinity and photoinhibition during exposure to high irradiances (Lu *et al.*, 2002).

5.5.3 *Mineral deficiencies*

Mineral deficiencies are mainly caused by the quality of the soil, which is largely determined by the rocky subsoil. It has long been known that an inadequate supply of mineral nutrients leads to a decrease in net photosynthesis (Dietz and Harries, 1997) and to increased susceptibility to photoinhibition (Godde and Hefer, 1994). Plants with an inadequate supply of mineral nutrients such as magnesium, potassium and zinc are often characterised by light-enhanced chlorosis of their leaves (Marschner and Cakmak, 1989). As we saw in Section 5.2.4, chlorosis in mineral-deficient plants is caused by imbalances in D1 protein turnover, which initiates the degradation of the photosynthetic apparatus. The typical stimulation of D1 protein synthesis in the initial

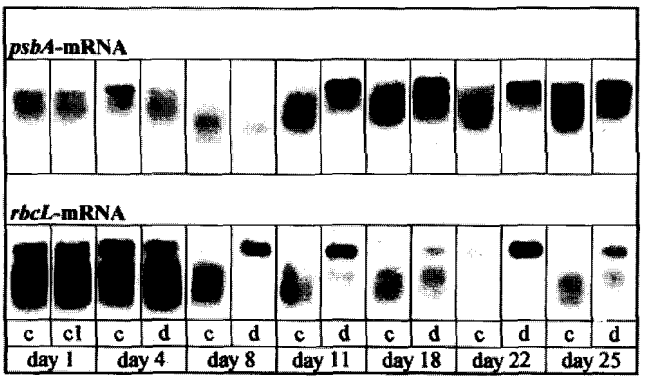


Figure 5.6 The transcript levels of the chloroplast *psbA* and *rbcL* gene are altered before chlorosis is induced in spinach kept on a Mg- and S-deficient medium. Three-week-old spinach plants, grown on a rich nutrient medium, were set on a medium deficient in Mg and S (see Dannehl *et al.*, 1995) and illuminated for 9 h per day with an irradiance of 100 μmol m^{-2} s^{-1}. A first loss of Chl (10%) was observed on day 22 after the onset of the experiment (see Fig. 5.7).

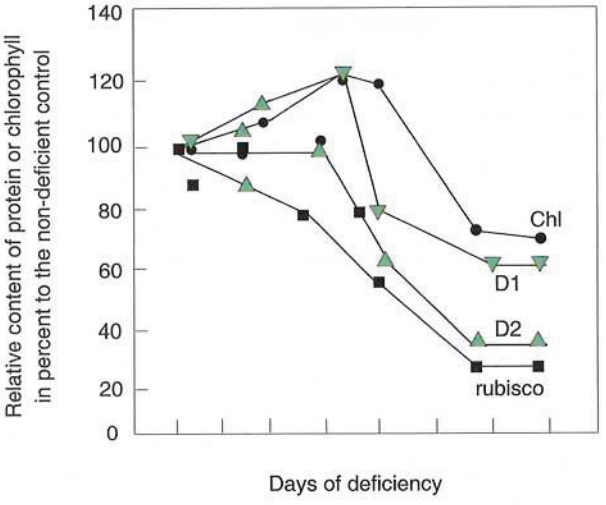


Figure 5.7 During stress-induced chlorosis, the PSII polypeptides and rubisco content are specifically reduced. As illustrated in Fig. 5.6, three-week old spinach plants were transferred to a Mg- and S-deficient medium. Under irradiation of 100 μmol $m^{-2} s^{-1}$ for 9 h per day, the first chlorotic symptoms developed on day 22 of the experiment and a 50% loss of Chl per g fresh weight was observed on day 30. The content of the D1 and D2 protein, now related to mg Chl, started to decline at day 20, and the rubisco content already on day 15 of the experiment.

phase of mineral deficiency is accompanied by an increase in the transcript levels of the *psbA* and *rbcL* genes, as shown in Fig. 5.6 (Kathol *et al.*, unpublished results).

The content of rubisco, as well as that of the PSII polypeptides, is reduced under mineral stress, for example, in outdoor spruce under Mg deficiency (Ebbert *et al.*, 1996). In Mg- and S-deficient spinach, the rubisco content decreases even sooner than that of Chl or the D1 and D2 proteins, as shown in Fig. 5.7. A reduction in rubisco content and activity has also been observed in phosphate-deficient plants (Jacob and Lawlor, 1992). This explains the reduction of photosynthetic capacity typical for mineral-deficient plants (Jacob and Lawlor, 1993) giving rise to an increased non-radiative energy dissipation and finally to photoinhibition (Godde and Hefer, 1994).

Changes in 'source-sink' relations are assumed to be responsible for the changes in D1 protein turnover and the reduction in the amount of rubisco, since plants exposed to a variety of mineral deficiencies are characterised by an increase in the content of starch and soluble sugars (Marschner and Cakmak, 1989; Lauer *et al.*, 1989; Godde and Hefer, 1994). Furthermore, Cakmak *et al.* (1994) have shown that phloem loading is decreased in Mg-deficient plants. However, it is possible that hormonal changes are also involved in the regulation of gene expression by mineral stress.

5.6 Possible plant responses to future climate changes

The increased burning of fossil fuels arising from the large increase in world population and industrialisation is likely to give rise to substantial climatic changes. It is predicted that the current atmospheric CO_2 concentration will increase from 367 ppm in 2002 to 500–1000 ppm by the year 2100 (Watson, 2002), with a consequent rise in mean surface temperatures of 1.5–6 C. Furthermore, the combustion of fossil fuels has led to large emissions of sulphur dioxide and nitrogen oxides. Atmospheric photochemistry has added tropospheric ozone, which is formed by the interaction between light, NO_2 and oxygen. On sunny days, peak tropospheric ozone concentrations in Germany may reach 200 ppb or more (Umweltbundesamt, 1991), five times the critical concentration above which direct harm to plants may result (Collins *et al.*, 2000). In North America, concentrations up to 800 ppb have been measured (Smith, 1991). It has to be expected that tropospheric ozone concentrations will increase worldwide. On the other hand, stratospheric ozone levels have been decreasing because of the emission of chlorofluorocarbons and other ozone-degrading compounds. These ozone decreases are not confined to the Antarctic but extend to mid-latitudes in both hemispheres. Apart from the effect of ozone-depleting substances, atmospheric ozone chemistry is in danger of being altered by the increasing levels of CO_2 and other greenhouses gases (Austin *et al.*, 1992; Mahlman, 1992; Shindell *et al.*, 1998), which tend to provide favourable conditions for the stratospheric loss of ozone and may also result in an increased flux of dangerous UV–B radiation at the Earth's surface (EORCU, 2001).

5.6.1 High CO_2

Elevated CO_2 concentrations influence the photosynthetic apparatus in various ways and also affect whole-plant physiology and growth (Ainsworth *et al.*, 2002). Photosynthesis, especially in C_3 plants, is limited by CO_2 through the competition of CO_2 and O_2 at the active site of rubisco. Thus an immediate increase in photosynthesis occurs in terrestrial plants on short-term exposure to atmospheric CO_2 above current ambient concentration (Kramer, 1981; Cure and Acock, 1986). This initially causes stimulated growth. However, photosynthetic rates and growth enhancement of plants that are initially stimulated by elevated CO_2 levels often diminish after days or weeks of CO_2 enrichment (Peet *et al.*, 1986; Sage *et al.*, 1989; Köner *et al.*, 1995). This acclimative process involving a decreased photosynthetic capacity is a result of a specific reduction in rubisco content (Sage *et al.*, 1989; Yelle *et al.*, 1989; Nie *et al.*,

1995b; Xu *et al.*, 1994; Moore *et al.*, 1998, 1999). It has been suggested that the amount of rubisco small subunit protein may largely control the rubisco level in plants grown at elevated CO_2 (Moore *et al.*, 1999).

The amount of LHCII or CF_0CF_1-ATPase per Chl seems to be stable (Nie *et al.*, 1995a). The activation state of rubisco is hardly affected and might even be increased under elevated CO_2 levels (Xu *et al.*, 1994; Sage *et al.*, 1989). There is evidence (Oosten *et al.*, 1994; Nie *et al.*, 1995a) that the loss of rubisco during exposure to elevated CO_2 is a result of decreased transcription of *rbcS* and *rbcL* genes. The loss of rubisco may be accompanied by a loss in leaf Chl content, so that the whole photosynthetic machinery is reduced, resulting in enhanced leaf senescence (Baxter *et al.*, 1994; Nie *et al.*, 1995b; Moore *et al.*, 1999). However, the quantum efficiency of PSII remains high at elevated CO_2, indicating that photosynthetic electron transport is not impaired. The changes in gene expression at high CO_2 levels and the loss of rubisco are associated with an increase in starch and soluble carbohydrate levels in the source leaves (Sasek *et al.*, 1985; Stitt, 1991; Nie *et al.*, 1995b; Xu *et al.*, 1994) and thus have been regarded as a special case of 'sink' control (Sheen, 1994; Stitt, 1991; Webber *et al.*, 1994; Oosten *et al.*, 1994). Hexokinase has been implicated in the reduction of photosynthetic gene expression at the transcription level (Moore *et al.*, 1999).

According to the concept of feedback inhibition, the main factor in the regulatory mechanism of the acclimation of photosynthesis to high CO_2 concentrations is the capacity of a plant to generate new sink organs. For instance, in *Opuntia ficus-indica* no down-regulation of photosynthesis is observed at increased CO_2 levels. Rather, these plants respond to elevated CO_2 concentrations by an increase in rubisco (Wang and Nobel, 1996) and in photosynthesis (Nobel and Israel, 1994), since this C_4 plant can maintain a high sink strength by generating numerous new cladodes (Wang and Nobel, 1996). However, when sink strength is limited, *e.g.* by removing the pods in soybean (Xu *et al.*, 1994), the inhibition of photosynthesis by elevated CO_2 concentrations is amplified, although leaf sugar content is only slightly stimulated. Thus, in species where sink strength is genetically limited, as in clover or sunflower, down-regulation of photosynthesis has to be expected, whereas in other plants that are more flexible in their sink capacity, like wheat or *Opuntia*, such an acclimation is less likely (Woodrow, 1994). Furthermore, maximum stimulation of growth by elevated CO_2 levels can only be expected at optimal growth conditions. Thus, an optimal supply of inorganic nutrients is essential if plants are to benefit from elevated CO_2 concentrations (Barret and Gifford, 1995; Rogers *et al.*, 1996). In contrast, stressful conditions that restrict plant growth, such as high levels of ozone, may increase feedback inhibition (Balaguer *et al.*, 1995) and stress symptoms such as chlorosis may

be amplified (see Section 5.6.2). Although hexokinase is implicated as a sugar sensor that regulates photosynthetic gene expression (Jang *et al.*, 1997; Xiao *et al.*, 2000), the link between the extent of feedback inhibition of photosynthesis and the carbohydrate content is rather diffuse, and carbohydrate signalling may also occur by pathways independent of hexokinase (Jang and Sheen, 1997). It has also been suggested that a feedback accumulation of leaf carbohydrate may play only a minor acclamatory role, since starch synthesis in leaves functions as an efficient buffer for photoassimilates (Heineke *et al.*, 1999). Feedback inhibition is not observed when assimilates accumulate in the light so long as they are transported away in the dark to support new growth. This might explain the results of Xu *et al.* (1994), where young soybean leaves, which support the growth of pods, showed no acclimation to elevated CO_2 levels, although their content of soluble sugars and starch increased substantially. It is possible that phloem loading itself is a regulatory step during acclimation to high CO_2. Furthermore, reducing the leaf carbohydrate content by shading does not restore down-regulation of photosynthesis induced under high CO_2 (Sicher and Kremer, 1994; Xu *et al.*, 1994). It should also be kept in mind that acclimation of photosynthesis to elevated CO_2 commonly does not occur in young leaves (Nie *et al.*, 1995a), although there are exceptions, *e.g.* in pea plants (Xu *et al.*, 1994).

5.6.2 High tropospheric ozone

The mechanism of ozone toxicity in plants is still a matter of debate. Ozone is one of the strongest oxidants known and it reacts with all plant surfaces. Most of the ozone is intercepted by the plasmalemma, damaging double bonds. In nature, ozone levels do not often reach concentrations that affect intracellular organelles such as chloroplasts since only certain regions have high tropospheric ozone concentrations, which generally fluctuate. However, a reduction in Chl content due to exposure to ozone has often been reported in laboratory experiments (Reich, 1983; Mikkelsen *et al.*, 1995; Nie *et al.*, 1993), showing that the photosynthetic apparatus can be affected by ozone. The resulting chlorosis is accompanied by a decrease in photosynthetic capacity (Nie *et al.*, 1993) arising from the loss of rubisco (Eckhardt and Pell, 1994; Landry and Pell, 1993; Nie *et al.*, 1993), which is caused by a repression of the nuclear *rbcS* genes encoding for the small subunits (Glick *et al.*, 1995). Further research has also shown that the decrease in CO_2 assimilation mainly results from a loss of rubisco in leaves exposed to elevated O_3 levels (Zheng *et al.*, 2002).

The chlorosis induced by ozone is again related to changes in D1 protein turnover. In ozone-sensitive plants such as maize, five hours fumigation with 240 ppb ozone is

enough to induce visible damage in the form of chlorosis and necrosis. In these plants, accumulation of the D1 protein seems to be retarded, resulting in a net loss of D1 per Chl (Pino *et al.*, 1995). A retardation of D1 protein synthesis may also occur after a long-term exposure to mineral stress (Dannehl *et al.*, 1995). In spruce, which is less ozone-sensitive than maize, even fumigation for as long as two weeks with 200 ppb ozone does not cause visible injury or changes in photosynthetic activity. However, it results in a four-fold stimulation of D1 protein synthesis (Godde and Buchhold, 1992). Since the D1 content in the thylakoid membrane stays constant, it must be assumed that D1 degradation is also stimulated under ozone fumigation but that both processes remain in equilibrium (Godde and Buchhold, 1992; Lütz *et al.*, 1992). Combined fumigation with other air pollutants such as SO_2 or NO_2 increases the detrimental effect of ozone on the photosynthetic apparatus and results in increased loss of D1 protein and leaf Chl (Lütz *et al.*, 1992).

5.6.3 Enhanced UV–B radiation

In recent years, stratospheric ozone depletion has led to increased levels of UV–B radiation reaching the Earth's surface (Kerr and McElroy, 1993; Pyle, 1997; McKenzie *et al.*, 2003). Globally there has been a ~3% loss in stratospheric ozone during the period 1997–2000 relative to 1980 (McKenzie *et al.*, 2003). UV–B radiation, although constituting less than 0.5% of the total solar radiation reaching the Earth's surface, is of biological significance not only as a natural component of sunlight influencing plant morphogenesis, but also as a modifier of increasing importance of many plant processes, and in some instances maybe even a cause of damage. This role as regulator or modifier of plant response can have important genetic, biochemical, physiological and ecological consequences. For instance, potentially greater sensitivity to certain other environmental factors (multiple stress effect) may occur. However, in some cases there may be seemingly greater tolerance to multiple stresses, possibly as a result of an enhanced induction of protective mechanisms, or that a plant already in a given stress situation can cope with increased UV–B radiation because part or all of the same defence pathways are active and can equally well take care of the additional stress.

In a research study involving a multivariate analysis of response of several white clover populations to UV–B radiation, it was mainly the plant growth attributes that were sensitive to UV–B radiation (Hofmann *et al.*, 2001). Also in a larger scale study, a meta-analysis of published work showed that overall significant changes induced by elevated UV–B were found for shoot mass, plant height and leaf area, and increased

UV–B absorbing pigments, while changes in chlorophyll and carotenoid pigments, reproductive yield, leaf mass per unit leaf area, net photosynthesis, and PSII activity were not universal in the experiments analysed (Searles *et al.*, 2001; Caldwell *et al.*, 2002). Despite this, there are many cases of quite strong UV–B effects that are dependent on species and cultivar, changing environmental conditions and previous history of the growing conditions, as well as on the interaction and duration of other environmental factors.

Perception and plant acclimation The mechanisms of UV–B perception and signal transduction are not yet well defined, although several explanations have been advanced for the stimulation of gene expression by UV–B that is reflected in many aspects of plant function, including photosynthesis, as shown in Fig. 5.8. These include direct absorption by DNA, generation of active oxygen species and the response of various photoreceptors (Jenkins *et al.*, 1997 and 2001), including the plastoquinone of the photosynthetic electron transport chain (Greenberg *et al.*, 1989a). Exposure of plants to enhanced UV–B radiation results in many acclimative and adaptive responses that in turn modify the penetration of the radiation into plant tissue. For example, enhanced UV–B radiation often changes biochemical, morphological and anatomical features such that the penetration of both UV–B radiation and photosynthetically active radiation (PAR) is modified (Bornman and Vogelmann, 1988, 1991; Ålenius *et al.*, 1995; Reuber *et al.*, 1996; Karabourniotis and Bornman, 1999; Karabourniotis *et al.*, 1999 and 2000). Increased production of UV-absorbing pigments such as flavonoids and hydroxycinnamic acids results in increased attenuation of UV–B radiation within the epidermis and upper palisade, while the structural changes, which often involve increases in leaf cell layers or cell volumes, may contribute to reducing the collimated light component of PAR, and thus alter the light environment for photosynthesis (Bornman and Vogelmann, 1991).

At the chloroplast level, UV–B radiation induces a shade-type response in *Brassica napus*, whereby the surface area of the appressed and non-appressed thylakoid membranes in the palisade tissue increases (Fagerberg and Bornman, 1997). This could conceivably increase the statistical probability of photon interception and at the same time lead to an increased energy cost to sustain the membrane surface area during exposure to UV–B radiation. This may not necessarily be seen as a direct damaging effect to the plant, but it does suggest an increased regulatory influence by UV–B radiation, although this does not appear to be a positive acclimation response.

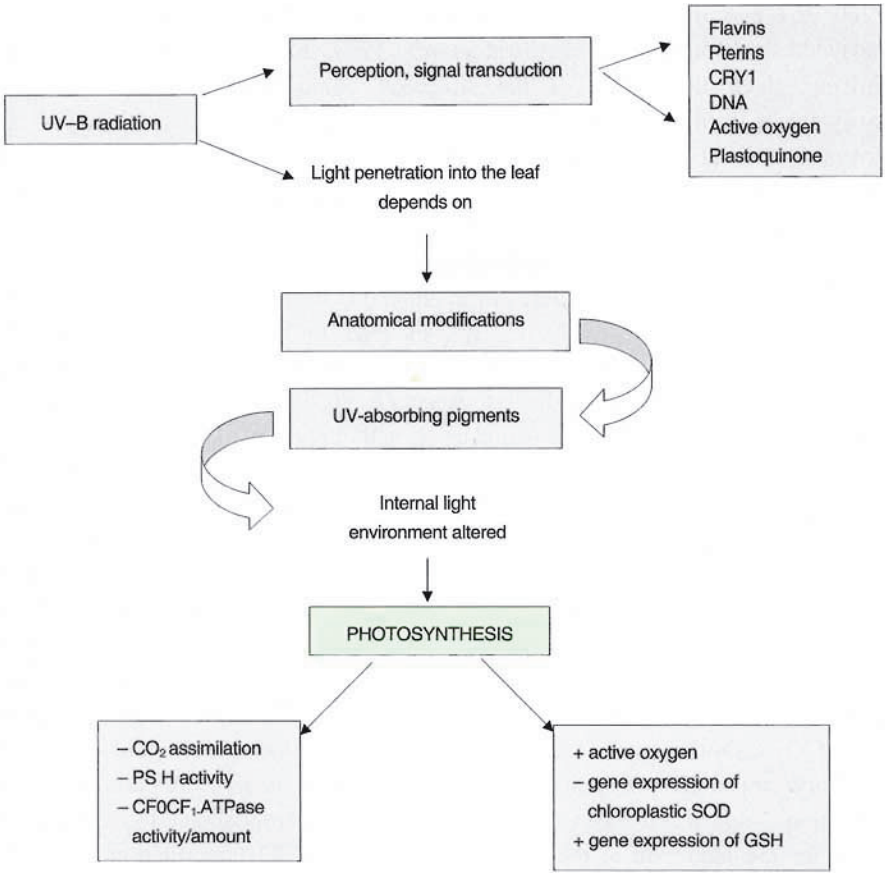


Figure 5.8 Some of the potential effects of enhanced UV-B radiation on plants, showing those processes that specifically affect the photosynthetic machinery.

Of potential interest as far as protection of photosynthesis and other vital processes is concerned, is the apparent inherent capacity of shade-acclimated leaves of several evergreen species to screen out ultraviolet radiation. Thus, although the UV-absorbing capacity of the shade leaves of these species was much lower than that of sun leaves of the same species, internal radiation measurements using a fibre optic microprobe showed in general that most of the incident UV-B radiation was screened out nearly as efficiently in shade leaves by both the adaxial and abaxial epidermis as it was in the corresponding sun leaves (Liakoura *et al.*, 2003). This may have an ecological advantage for certain plants growing initially in partially shady habitats prone to sudden sun exposure.

Targets of UV–B radiation Many different target sites have been reported for photosynthesis (Bornman, 1989; Strid *et al.*, 1990; Jordan, 2000), which is not surprising given the nature of the structural components and complexity of photosynthesis itself. Coupled with this is the intrinsic variation in sensitivity of the different functional complexes. For instance, after supplemental UV–B exposure, rubisco activity may be severely reduced with a slightly lower decline in functional PSII RCs and even less for ATP hydrolysis, whereas PSI and the cytochrome b_6/f complex may be relatively unaffected (Strid *et al.*, 1990), with some exceptions (e.g., for PSI; Krause *et al.*, 2003). There are innumerable differences in tolerance among cultivars and species. Generally, C_4 species tend to be more tolerant than C_3 (Basiouny *et al.*, 1978; Vu *et al.*, 1982). The degree of tolerance also depends greatly on prevailing environmental conditions, especially visible radiation, water potential, infection, CO_2 levels, tropospheric ozone levels and mineral availability.

CO_2 assimilation Both the light reactions of photosynthesis involving the electron transport chain and the reactions of CO_2 assimilation are affected by enhanced levels of UV–B radiation. However, according to some studies, photoinhibition of PSII occurs only after CO_2 assimilation is inhibited (Nogués and Baker, 1995). Accordingly, within the first few hours of UV–B irradiation, a reduction in the light-saturated rate of CO_2 assimilation may occur without any effect on the maximum quantum yield of PSII photochemistry. The continuing decrease in light-saturated rates of CO_2 assimilation are accompanied by reduced quantum efficiencies of CO_2 assimilation and PSII photochemistry, when measured under non-photoinhibitory illumination. Nogués and Baker (1995) also found that photodamage to PSII did not account for the reduction in the light-saturated rate of CO_2 assimilation; nor were photorespiratory effects a contributing factor to UV–B-induced reduction of CO_2 fixation in their studies, since the stimulation of CO_2 assimilation under 2% O_2 occurred in both the presence and absence of UV–B. Enhanced levels of UV-B radiation may also decrease the content of other Calvin cycle enzymes, which would explain decreases in the regeneration of RuBP, and would in turn contribute to a reduced rate of RuBP carboxylation by rubisco (Baker *et al.*, 1997).

Stomatal aperture is controlled by both visible and UV radiation, although the process is complex, whereby, depending on the metabolic state of the guard cell, high levels of UV-B either stimulate stomatal opening or closing (Teramura *et al.*, 1980; Negash and Björn, 1986; Grammatikopoulos *et al.*, 1994). In addition, the capacity for aperture readjustment under other environmental factors such as visible light, humidity or ABA is low, probably reflecting cellular damage (Jansen and van den Noort, 2000).

Reductions in the mRNA transcripts of the large and small subunits of rubisco (Jordan *et al.*, 1992) are accompanied by decreases in rubisco activity and content (Vu *et al.*, 1984; Strid *et al.*, 1990; Jordan *et al.*, 1992; He *et al.*, 1993). At present it is not quite clear whether it is the specific activity of rubisco or its content that is most influenced by UV–B radiation (Jordan *et al.*, 1992; Nogués and Baker, 1995). Jordan *et al.* (1992) suggested that the decline in enzymatic activity results from direct absorption of UV–B radiation by aromatic amino acids such as phenylalanine, tryptophan and tyrosine, which would cause ring opening or free radical production in the case of tyrosine, while the reduction in rubisco content would reflect a cessation of the biosynthesis of the enzyme or its degradation under conditions of oxidative stress. Another example where either content or activity might arguably be the more sensitive is the CF_oCF_1-ATPase protein, where the amount of protein is possibly more reduced by UV–B radiation than is its activity (Strid *et al.*, 1990; Zhang *et al.*, 1994).

Photochemistry and PSII Photoinactivation of the photosynthetic electron transport chain by visible radiation may occur at either the donor or acceptor sides of PSII, with both mechanisms leading to degradation of the D1 protein. The same can be said of the influence of UV–B radiation (Jones and Kok, 1966; Bornman *et al.*, 1984; Renger *et al.*, 1989; Vass, 1996). For example, in *in-vitro* systems, UV–B radiation affects redox components of PSII at both the donor and acceptor sides, with primary damage occurring at the water-oxidising complex. The electron donor to P680, tyrosine-D, cytochrome *b-559* and the Q_AFe^{2+} complex were all affected after the water-oxidising complex (Vass, 1996). The importance of the water-oxidising system as a UV target is also indicated by the low susceptibility of bacterial reaction centres to UV damage (Tandori *et al.*, 1996). On the other hand, the quinone electron acceptors of PSII also appear to be targets of enhanced UV–B radiation (Melis *et al.*, 1992).

Photoinhibition may not be caused in the same way by elevated levels of UV–B radiation as it is by high PAR. Several findings support this claim; for example, the recovery kinetics of inhibition are different for the two wavelength regions (Chow *et al.*, 1992) and the cleavage of the D1 protein seems to occur at different sites compared to that induced by visible radiation (Friso *et al.* 1993; Hideg *et al.*, 1993). In addition, the lack of sensitivity to temperature and protease inhibitors suggests that the breakdown of the D1 protein due to UV–B radiation may not be a proteolytic process (Melis *et al.*, 1992; Friso *et al.*, 1995). According to Spetea *et al.* (1996), it is initiated by absorption of UV–B by protein constituents such as the tyrosine residues and the manganese cluster of water oxidation. Most likely it is induced by hydroxyl radicals that are formed in the catalytic manganese cluster (Hideg and Vass, 1996).

Quantitatively, UV–B radiation appears to compound photoinhibition by PAR as reflected by increases in the rate of D1 degradation (Greenberg *et al.*, 1989b; Bornman and Sundby-Emanuelsson, 1995) and increased non-photochemical quenching of PSII (Bornman and Sundby-Emanuelsson, 1995). The non-photochemical quenching reflects protective processes such as the xanthophyll cycle and/or energy dissipation at inactive PSII reaction centres (see Sections 5.2.1 and 5.2.2), and has also been found to increase under conditions of enhanced UV–B radiation compared with PAR alone (Bornman and Sundby-Emanuelsson, 1995). However, this increase in non-photochemical quenching does not always occur (Olsson and Bornman 1999), and may reflect an increased sensitivity to UV–B radiation of the de-epoxidation reaction of violaxanthin to zeaxanthin (Pfündel *et al.*, 1992), thus weakening the protective mechanisms of the xanthophyll cycle. Apart from accelerated turnover of the D1 protein under PAR and UV–B radiation, the turnover of the D2 protein also increases, contrasting with the relative stability of the D2 protein under excessive PAR levels alone (Jansen *et al.*, 1996; Babu *et al.*, 1999).

Chlorophyll is affected in a number of ways by UV–B, both quantitatively and qualitatively, reflecting sensitive processes strongly dependent on experimental conditions and species. The biosynthetic pathway for Chl appears to be relatively stable, with decreases in Chl being due to its degradation (Strid and Porra, 1992). The mechanism of chlorophyll loss and increased lipid damage by UV–B radiation appear to be reminiscent of that induced during natural senescence, also evidenced at the gene level (John *et al.*, 2001).

Thus UV–B radiation, although only a relatively minor component of the terrestrial solar spectrum, exerts many indirect and direct influences on photosynthesis, which are observed at the molecular to the ecosystem level, where UV–B-induced changes are mirrored in altered morphology and plant competition. Added to this are the intricate processes of interaction of UV–B radiation with other environmental factors, resulting in amelioration of the UV–B effect, no apparent effect, or a synergistic or additive effect (the latter may be in the positive or negative direction). One thus might speculate whether UV–B radiation, like other stressors, first changes the growth rate and the requirements for assimilates before it affects photosynthesis.

5.7 Improving plant biomass

Improving plant biomass and crop yields has always been the major goal in agriculture and even in former times plant breeding was based on genomic changes. Until recently, such genomic changes were achieved by accident and the success of plant breeding relied

more or less on the recognition and selection of the desired varieties. Today, molecular biology offers tools for specifically changing the plant genome, as discussed by Denis Murphy in Chapter 13. The technique of reverse genetics, with its possibilities of manipulating enzyme levels,[1] offers particular opportunities in this regard.

Several strategies have been developed to increase crop yield. One way is to attempt to increase tolerance to stress. Environmental stresses such as drought (Smirnoff, 1993; Noctor *et al.*, 2002), low temperature (Foyer *et al.*, 2002) and high irradiance (Wise, 1995) or air pollution (Mehlhorn and Wellburn, 1994; Ederli *et al.*, 1997) can directly or indirectly induce oxidative stress. Under such conditions, production of highly reactive oxygen species increases significantly. Overexpression of radical-scavenging enzymes such as superoxide dismutase and gluthathione reductase have resulted in an increased tolerance to drought, ozone and low temperature combined with high light stress (Gupta *et al.*, 1993; Van Camp, 1994; McKersie *et al.*, 1996; Foyer, 2001). Even redirecting electrons to photorespiration reduces oxidative damage under high-light conditions (Kozaki and Takeba, 1996; Noctor *et al.*, 2002). Increased tolerance against damage by drought and high salinity has also been reported for plants that accumulate metabolites such as fructan (Pilon-Smits *et al.*, 1995; Kerepesi and Galiba, 2000), proline (Kishor *et al.*, 1995; van Heerden, 2002), glycine betaine (Nomura *et al.*, 1995; Chen and Murata, 2002), trehalose (Holmstrom *et al.*, 1996; Lee *et al.*, 2003) and mannitol (Tarczynski *et al.*, 1993; Prabhavathi, 2002). These compounds function primarily as osmotic adjusters. However, some of them also may be involved in scavenging active oxygen species (Shen *et al.*, 1997).

Another promising strategy involves trying to change the allocation of carbohydrates and amino acids from source to sink organs. The predominant role of carbon partitioning on crop yield is obvious in, for example, tuber dry weight in potato, which can increase from 7% of the total plant dry weight in wild cultivars up to 81% in the modern high-yield cultivars (Inoue and Tanaka, 1978). Altering the allocation of assimilates has been carried out mostly in solanaceous species such as potato (Sonnewald *et al.*, 1994; Frommer and Sonnewald, 1995). These studies have revealed that, under optimal conditions, the supply of assimilates does not limit tuber growth. Even a change from starch formation to sucrose does not result in increased tuber growth or formation. Only when phloem unloading is stimulated by the expression of yeast invertase in the cell wall of tubers is tuber yield stimulated

[1] For most genetic diseases, the normal function of the involved gene is not known. Localising a disease-causing gene without knowing anything about the disease's molecular or biochemical nature is called 'reverse genetics'.

(Frommer and Sonnewald, 1995). However, the distribution of carbohydrates is regulated in a complex way and the availability of mineral nutrients plays an important role in this process (Moore *et al.*, 1999 and references therein). This is also reflected by the work of Stitt and co-workers (Scheible *et al.*, 1997), who have emphasised the role of nitrate and phosphate in assimilate partitioning and the growth of leaves and roots. Moreover, carbohydrates cannot be regarded only as biochemical metabolites. Research has revealed that many of them function also as signals, controlling plant development and adaptation (Sheen 1990, Jang *et al.* 1994, 1997). Thus it seems as if understanding of these regulatory signal transduction pathways is one of the most important prerequisites for increasing plant growth by genetic engineering.

References

Adams III W. W., Demmig-Adams B., Verhoeven A. S. and Barker D. H. (1994), 'Photoinhibition during winter stress: involvement of sustained xanthophyll cycle-dependent energy dissipation', *Austr. J. Plant Physiol.* **22**, 261–276.

Adamska I. (1997), 'ELIPs-light-induced stress proteins', *Physiol. Plant.* **100**, 794–805.

Adir N., Shochat S. and Ohad I. (1990), 'Light-dependent D1 protein synthesis and translocation is regulated by reaction center II', *J. Biol. Chem.* **265**, 12563–1256.

Ainsworth E. A., Davey P. A., Bernacchi C. J., Dermody O. C., Heaton E. A., Moore D. J., Morgan P. B., Naidu S. L., Ra H. S. Y., Zhu X. G., Curtis P. S. and Long S. P. (2002), 'A meta-analysis of elevated [CO_2] effects on soybean (Glycine max) physiology, growth and yield', *Global Change Biol.* **8**, 695–709.

Ålenius C. M., Vogelmann T. C. and Bornman J. F. (1995), 'A three-dimensional representation of the relationship between penetration of UV–B radiation and UV-screening pigments in leaves of *Brassica napus*', *New Phytol.* **131**, 297–302.

Allen J. F. (1992), 'Protein phosphorylation in regulation of photosynthesis', *Biochim. Biophys. Acta* **1098**, 275–335.

Allen J. F. (1995), 'Thylakoid protein phosphorylation. State 1–state 2 transitions and photosystem stoichiometry adjustment: redox control at multiple levels of gene expression', *Physiol. Plant.* **93**, 196–205.

Anderson J. M. (1986), 'Photoregulation of the composition, function and structure of thylakoid membranes', *Ann. Rev. Plant Physiol.* **37**, 93–136.

Andersson B. and Aro E.-M. (1997), 'Proteolytic activities and proteases of plant chloroplasts', *Physiol. Plant.* **100**, 780–793.

Aro E.-M., Hundal T., Carlberg I. and Andersson B. (1990), '*In vitro* studies on light-induced inhibition of photosystem II and D1 protein degradation at low temperatures', *Biochim. Biophys. Acta* **1019**, 269–275.

Aro E.-M., Kettunen R. and Tyystjärvi E. (1992), 'ATP and light regulate D1 protein modification and degradation. Role of D1 in photoinhibition', *FEBS Letters* **297**, 29–33.

Aro E.-M., Virgin I. and Andersson B. (1993), 'Photoinhibition of photosystem II. inactivation, protein damage and turnover', *Biochim. Biophys. Acta* **143**, 13–34.

Aro E.-M., McCaffery S. and Anderson J. M. (1994), 'Recovery from photoinhibition in peas (*Pisum sativum* L.) acclimated to varying growth irradiances', *Plant Physiol.* **104**, 1033–1041.

Asada K. (1994), 'Mechanism for scavenging reactive molecules generated in chloroplasts under light stress', in Baker and Bowyer 1994), pp. 129–142.

Assmann S. (1993), 'Signal transduction in guard cells', *Ann. Rev. Cell Biol.* **9**, 345–375.

Austin J., Butchart N. and Shine K. P. (1992), 'Possibility of an Arctic ozone hole in a doubled-CO_2 climate', *Nature* **360**, 221–225.

Babu T. S., Jansen M. A. K., Greenberg B. M., Gaba V., Malkin S., Mattoo A. K. and Edelman M. (1999), 'Amplified degradation of photosystem II D1 and D2 proteins under a mixture of photosynthetically active radiation and UV–B radiation: dependence on redox status of photosystem II', *Photochem. Photobiol.* **69**, 553–559.

Bailey S., Silva P., Nixon P., Mullineaux C. W., Robinson C. and Mann N. (2001), 'Auxiliary functions in photosynthesis: the role of the FtsH protease,' *Biochem. Soc. Trans.* **29**, 455–459.

Baker N. R. and Bowyer J. R. (1994), eds., *Photoinhibition of Photosynthesis: From Molecular Mechanisms to the Field*, Bios Scientific Publishers, Oxford.

Baker N. R., Nogués S. and Allen D. J. (1997), 'Photosynthesis and photoinhibition', in Lumsden P., ed., *Plants and UV–B. Responses to Environmental Change*, Cambridge University Press, Cambridge, pp. 95–111.

Balaguer L., Barnes J. D., Panicucci A. and Borland A. M. (1995), 'Production and utilization of assimilates in wheat (*Triticum aestivum* L.) leaves exposed to elevated CO_2 and/or O_3', *New Phytol.* **129**, 557–568.

Barbato R., Friso G., Rigoni F. and Giacometti G. M. (1992), 'Structural changes and lateral distribution of photosystem II during donor side inhibition of thylakoids', *J. Cell Biol.* **119**, 325–335.

Barrett D. J. and Gifford R. M (1995), 'Acclimation of photosynthesis and growth to elevated CO_2: interactions with severe phosphate deficiency and restricted rooting volume', *Austr. J. Plant Physiol.* **22**, 955–963.

Basiouny F. M., Van T. K. and Biggs R. H. (1978), 'Some morphological and biochemical characteristics of C_3 and C_4 plants irradiated with UV–B', *Physiol. Plant.* **42**, 29–32.

Baxter R., Ashenden T. W., Sparks T. H. and Farrar J. F. (1994), 'Effects of elevated carbon dioxide on three montane grass species. I. Growth and dry matter partitioning', *J. Exp. Bot.* **45**, 305–315.

Bernacchia G., Salamine F. and Bartels D. (1996), 'Molecular characterization of the rehydration process in the resurrection plant *Craterostigma plantagineum*', *Plant Physiol.* **111**, 1043–1050.

Berry J. and Björkman O. (1980), 'Photosynthetic response and adaptation to temperature in higher plants', *Ann. Rev. Plant Physiol.* **31**, 491–543.

Bianchi G., Gamba A., Murelli C., Salamini F. and Bartels D. (1991), 'Novel carbohydrate metabolism in the resurrection plant *Craterostigma plantagineum*', *Plant J.* **1**, 355–359.

Björkman O. (1966), 'The effect of oxygen concentration on photosynthesis in higher plants', *Physiol. Plant.* **19**, 618–833.

Bohnert H. J., Nelson D. E. and Jensen R. G. (1995), 'Adaptations to environmental stress', *The Plant Cell* **7**, 1099–1111.

Bone R. A., Lee D. W. and Norman J. M. (1985), 'Epidermal cells functioning as lenses in leaves of tropical rain-forest shade plants', *Appl. Opt.* **24**, 1408–1412.

Bornman J. F., Björn L. O. and Åkerlund H.-E. (1984), 'Action spectrum for inhibition by ultraviolet radiation of photosystem II activity in spinach thylakoids', *Photobiochem. Photobiophys.* **8**, 305–313.

Bornman J. F. and Vogelmann T. C. (1988), 'Penetration of blue and UV radiation measured by fiber optics in spruce and fir needles', *Physiol. Plant.* **72**, 699–705.

Bornman J. F. (1989), 'Target sites of UV–B radiation in photosynthesis of higher plants. Invited Review', *J. Photochem. Photobiol.* **4**, 145–158.

Bornman J. F., Vogelmann T. C. and Martin G. (1991), 'Measurement of chlorophyll fluorescence within leaves using a fiber optic microprobe', *Plant Cell Environ.* **14**, 719–725.

Bornman J. F. and Sundby-Emanuelsson C. (1995), 'Response of plants to UV–B radiation: some biochemical and physiological effects', in *Environment and Plant Metabolism: Flexibility and Acclimation*, Smirnoff N., ed., Environmental Plant Biology Series', Bios Scientific Publishers, Oxford, pp. 245–262.

Boyer J. S. and Bowen B. L. (1970), 'Inhibition of oxygen evolution in chloroplasts isolated from leaves with low water potential', *Plant Physiol.* **45**, 612–615.

Bredenkamp G. J and Baker N. R. (1994), 'Temperature sensitivity of D1 protein metabolism in isolated *Zea mays* chloroplasts', *Plant Cell Environ.* **17**, 205–210.

Brestic M., Cornic G., Fryer M. J and Baker N. R. (1995), 'Does photorespiration protect the photosynthetic apparatus in French bean leaves from photoinhibition under drought stress?', *Planta* **196**, 450–457.

Büssis D., Heineke D., Sonnewald U., Willmitzer L., Raschke K. and Heldt H.-W. (1997), 'Solute accumulation and decreased photosynthesis in leaves of potato plants expressing yeast-derived invertase either in the apoplast, vacuole or cytosol', *Planta* **202**, 126–136.

Cakmak I., Hengeler C. and Marschner H. (1994), 'Changes in phloem export of sucrose in response to phosphorus, potassium and magnesium deficiency in bean plants', *J. Exp. Bot.* **45**, 1251–1257.

Caldwell M. M., Ballaré C. L., Bornman J. F., Flint S. D., Björn L. O., Teramura A. H., Kulandaivelu G. and Tevini M. (2002), 'Terrestrial ecosystems, increased solar ultraviolet radiation and interactions with other climatic change factors', *Photochem. Photobiol. Sci.* **2**, 29–38.

Carlberg I. and Andersson B. (1996), 'Phosphatase activities in spinach thylakoid membranes—effectors, regulation and location', *Photosynth. Res.* **47**, 145–156.

Chen Q., Lauzon L. M., DeRocher A. E. and Vierling E. (1990), 'Accumulation, stability and localization of a major chloroplast heat-shock protein', *J. Cell Biol.* **110**, 1873–1883.

Chen T. H. H. and Murata N. (2002), 'Enhancement of tolerance of abiotic stress by metabolic engineering of betaines and other compatible solutes', *Current Opin. Plant Biol.* **5**, 250–257.

Christopher D. A., Yinli L., Kim M. and Mullet J. E. (1997), 'Involvement of protein kinase and extraplastidic serine/threonine protein phosphatases in signaling pathways regulating plastid transcription and the *psbD* blue light-responsive promoter in barley', *Plant Physiol.* **113**, 1273–1282.

Clark J. B. and Lister G. R. (1975), 'Photosynthetic action spectra of trees. II. The relationship of cuticle structure to the visible and ultraviolet spectral properties of needles from four coniferous species', *Plant Physiol.* **55**, 407–413.

Coleman J. S. and McConnaughay K. (1994), 'Phenotypic plasticity', *Trends in Ecol. Evol.* **9**, 187–191.

Collins W. J., Stevenson D. S., Johnson C. E. and Derwent R. G. (2000), 'The European regional ozone distribution and its links with the global scale for the years 1992 and 2015', *Atm. Env.* **34**, 255–267.

Constant S., Perewoska I., Alfonso M. and Kirilovsky D. (1997), 'Expression of the *psbA* gene during photoinhibition and recovery in *Synechocystis* PCC 6714: inhibition and damage of transcriptional and translational machinery prevent the restoration of photosystem II activity', *Plant Mol. Biol.* **34**, 1–13.

Cornic G., Ghashghaie J., Genty B. and Briantais J.-M. (1992), 'Leaf photosynthesis is resistant to a mild drought stress', *Photosynthetica* **27**, 295–309.

Cornic G. (1994), 'Drought stress and high light effects on leaf photosynthesis', in Baker and Bowyer, pp. 297–313.

Creelman R. A. and Mullet J. E. (1995), 'Jasmonic acid distribution and action in plants: regulation during development and response to biotic and abiotic stress', *Proc. Nat. Acad. Sci. U S A* **92**, 4114–4119.

Cui M., Vogelmann T. C. and Smith W. K. (1991), 'Chlorophyll and light gradients in sun and shade leaves of *Spinacia oleracea*', *Plant Cell Environ.* **14**, 493–500.

Cure J. D. and Acock B. (1986), 'Crop responses to carbon dioxide doubling: a literature survey', *Agric. Forest Meteorol.* **38**, 127–145.

Dannehl H., Herbik A. and Godde D. (1995), 'Stress-induced degradation of the photosynthetic apparatus is accompanied by changes in thylakoid protein turnover and phosphorylation', *Physiol. Plant.* **93**, 179–186.

Dannehl H., Wietoska H., Heckmann H. and Godde D. (1996), 'Changes in D1 protein turnover and recovery of PSII activity precede accumulation of chlorophyll in plants after release from mineral stress', *Planta* **199**, 34–42.

Danon A. and Mayfield S. P. (1994a), 'ADP-dependent phosphorylation regulates RNA binding *in vitro*: implications in light-modulated translation', *EMBO J.* **13**, 2227–2235.

Danon A. and Mayfield S. P. (1994b), 'Light-regulated translation of chloroplast messenger RNAs through redox potential', *Science* **266**, 1717–1719.

Demmig-Adams B. (1990), 'Carotenoids and photoprotection in plants: a role for the xanthophyll zeaxanthin', *Biochim. Biophys. Acta* **1020**, 1–24.

Demmig-Adams B. and Adams W. W. (1992), 'Photoprotection and other responses of plants to high light stress', *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **43**, 599–626.

Dietz K. J. and Harries G. C. (1997), 'Photosynthesis under nutrient deficiency', in *Handbook of Photosynthesis*, Pessarakali M., ed., Marcel Dekker, New York, pp. 951–976.

Ebbert V. and Godde D. (1994), 'Regulation of thylakoid protein phosphorylation in intact chloroplasts by the activity of kinases and phosphatases', *Biochim. Biophys. Acta* **1187**, 335–346.

Ebbert V. and Godde D. (1996), 'Phosphorylation inhibits D1 protein degradation and increases PSII stability', *Photosynth. Res.* **50**, 257–269.

Ebbert V., Hollinderbäumer R., Wietoska H., König C. and Godde D. (1996), 'Einfluß von Meliorationsmaßnahmen auf Funktion und Reparatur des photosynthetischen Apparates einer mangelernährten Fichte am Standort Schöllkopf/Freudenstadt', in Horsch F., Bittlingmeier L., Filby W. G., Fund N., Gross S. and Kändler G., eds., 12th. Statuskolloquium des PEF, Forschungszentrum Karlsruhe GmbH, pp. 151–160.

Ederli L., Pasqualini S., Batini P. and Antonielli M. (1997), 'Photoinhibition and oxidative stress: effects on xanthophyll cycle, scavenger enzymes and abscisic acid content in tobacco plants', *J. Plant Physiol.* **151**, 422–428.

Ehleringer J. R. and Björkman O. (1978), 'Pubescence and leaf spectral characteristics of a desert shrub *Encelia farinosa*', *Oecologia* **36**, 151–162.

Ehleringer J. R., Mooney H. A., Glumon S. L. and Rundel P. W. (1981), 'Parallel evolution of leaf pubescence in *Encelia* in coastal deserts of North and South America', *Oecologia* **71**, 318–320.

Eichacker L. A., Soll J., Lauterbach P., Rüdiger W., Klein R. R. and Mullet J. E. (1990), '*In vitro* synthesis of chlorophyll *a* in the dark triggers accumulation of chlorophyll *a* apoproteins in barley etioplasts', *J. Biol. Chem.* **276**, 25908–25915.

Elich T. D., Edelman M. and Mattoo A. K. (1992), 'Identification, characterization, and resolution of the *in vivo* phosphorylated form of the photosystem II reaction center protein', *J. Biol. Chem.* **267**, 3523–3529.

Elstner E. F. and Osswald W. (1994), 'Mechanism of oxygen activation during plant stress', *Proc. Roy. Soc.* **102**, 131–154.

Enami I., Kitamura M., Tomo T., Isokawa Y., Ohta H. and Katoh S. (1994), 'Is the primary cause of thermal inactivation of oxygen evolution in spinach PSII membranes release of the extrinsic 33 kDA protein or Mn?', *Biochim. Biophys. Acta* **1186**, 52–58.

EORCU (2001), 'The Northern hemisphere stratosphere in the 2000/01 winter', European Ozone Research Coordinating Unit (*www.ozone-sec.ch.cam.ac.uk/Reports/winter_report*).

Escoubas J.-M., Lomas M., LaRoche J. and Falkowski P. G. (1995), 'Light intensity regulation of *cab* gene transcription is signaled by the redox state of the plastoquinone pool', *Proc. Nat. Acad. Sci.* **95**, 10237–10241.

Fagerberg W. R. and Bornman J. F. (1997), 'Ultraviolet–B radiation causes shade-type ultrastructural changes in *Brassica napus*', *Physiol. Plant.* **101**, 833–844.

Farrar J., Pollock C. and Gallagher J. (2000), 'Sucrose and the integration of metabolism in vascular plants', *Plant Sci.* **154**, 1–11.

Feierabend J. and Dehne S. (1996), 'Fate of the porphyrin cofactors during light-dependent turnover of catalase and of the photosystem II reaction-center protein D1 in mature rye leaves', *Planta* **198**, 413–422.

Feller U., Crafts-Brandner S. J. and Salvucci M. E. (1998), 'Moderately high temperatures inhibit ribulose–1,5–bisphosphate carboxylase/oxygenase (rubisco) activase-mediated activation of rubisco', *Plant Physiol.* **116**, 539–546.

Flexas J. and Medrano H. (2002), 'Drought-inhibition of photosynthesis in C_3 plants: stomatal and non-stomatal limitations revisited', *Ann. Bot.* **89**, 183–189.

Flügge U. I. and Heldt H. (1981), 'The phosphate translocator of the chloroplast envelope. Isolation of the carrier protein and reconstitution of transport', *Biochim. Biophys. Acta* **638**, 296–304.

Foyer C. H. (2001), 'Prospects for enhancement of the soluble antioxidants, ascorbate and glutathione', *Biofactors* **15**, 75–78.

Foyer C. H., Vanacker H., Gomez L. D. and Harbinson J. (2002), 'Regulation of photosynthesis and antioxidant metabolism in maize leaves at optimal and chilling temperatures: review', *Plant. Physiol. Biochem.* **40**, 659–668.

Frank H. A., Bautista J. A., Josue J. S. and Young A. J. (2000), 'Mechanism of nonphotochemical quenching in green plants: energies of the lowest excited singlet states of violaxanthin and zeaxanthin', *Biochem.* **39**, 2831–2837.

Friso G., Spetea C., Giacometti G. M., Vass I. and Barbarto R. (1993), 'Degradation of photosystem II reaction center D1-polypeptide induced by UV–B radiation in isolated thylakoids. Identification and characterization of C- and N-terminal breakdown products', *Biochim. Biophys. Acta* **1184**, 78–84.

Friso G., Vass I., Spetea C., Barber J. and Barbarto R. (1995), 'UV–B induced degradation of the D1 protein in isolated reaction centers of photosystem II', *Biochim. Biophys. Acta.* **1231**, 41–46.

Fromm H., Devic M., Fluhr R. and Edelman M. (1985), 'Control of *psbA* gene expression: in mature *Spirodela* chloroplasts light regulation of the 32-kD protein synthesis is independent of transcript levels', *EMBO J.* **4**, 291–295.

Frommer W. B. and Sonnewaldt U. (1995), 'Molecular analysis of carbon partitioning in solanaceous species', *J. Exp. Bot.* **46**, 587–607.

Fryer M. J., Oxborough K., Martin B., Ort D. R. and Baker N. R. (1995), 'Factors associated with depression of photosynthetic quantum efficiency in maize at low growth temperature', *Plant Physiol.* **108**, 761–767.

Furbank R. T., Chitty J. A., von Caemmerer S. and Jenkins C. J. D. (1996), 'Antisense RNA inhibition of *rbcS* gene expression reduces rubisco level and photosynthesis in the C_4 plant *Flaveria bidentis*', *Plant Physiol.* **111**, 725–734.

Giardi M. T., Cona A., Geiken B., Kucera T., Masojiidek J. and Mattoo A. K. (1996), 'Long-term drought stress induces structural and functional reorganization of photosystem II', *Planta* **199**, 118–125.

Gimenez C., Mitchell V. and Lawlor D. W. (1992), 'Regulation of photosynthetic rate of two sunflower hybrids under water stress', *Plant Physiol.* **98**, 516–524.

Giraudat J., Parcy F., Bertauche N., Gosti F. and Leung J. (1994), 'Current advances in abscisic acid action and signalling', *Plant Mol. Biol.* **26**, 1557–1577.

Glick R. E., Schlagnhaufer C. D., Arteca R. N., Pell E. J. (1995), 'Ozone-induced ethylene emission accelerates the loss of ribulose–1,5–bisphosphate carboxylase/oxygenase and nuclear-encoded mRNAs in senescing potato leaves', *Plant Physiol.* **109**, 891–898.

Godde D. and Buchhold J. (1992), 'Effect of long-term fumigation with ozone on the turnover of the D1-reaction center polypeptide in spruce (*Picea abies*)', *Physiol. Plant.* **86**, 568–574.

Godde D., Buchhold J., Ebbert V. and Oettmeier W. (1992), 'Photoinhibition in intact spinach plants: effect of high light intensities on the function of the two photosystems and on the content of the D1 protein under nitrogen', *Biochim. Biophys. Acta* **1140**, 69–77.

Godde D. and Dannehl H. (1994), 'Stress induced chlorosis and increase in D1 protein turnover precedes photoinhibition in spinach suffering under combined magnesium and sulfur deficiency', *Planta* **195**, 291–300.

Godde D. and Hefer M. (1994), 'Photoinhibition and light-dependent turnover of the D1 reaction center polypeptide of photosystem II are enhanced by mineral stress conditions', *Planta* **193**, 290–299.

Gombos Z., Wada H. and Murata N. (1994), 'The recovery of photosynthesis from low-temperature photoinhibition is accelerated by the unsaturation of membrane lipids: a mechanism of chilling tolerance', *Proc. Nat. Acad. Sci. U.S.A* **91**, 8787–8791.

Gong H. and Nilsen S. (1989), 'Effect of temperature on photoinhibition of photosynthesis, recovery and turnover of the 32-kD chloroplast protein in *Lemna gibba*', *J. Plant Physiol.* **135**, 9–14.

Gong H. and Ohad I. (1991), 'The PQ/PQH_2 ratio and occupancy of photosystem II Q_B site by plastoquinone controls the degradation of D1 protein during photoinhibition *in vivo*', *J. Biol. Chem.* **266**, 21293–21299.

Grammatikopoulos G., Karabourniotis G., Kyparissis A., Petropoulou Y. and Manetas Y. (1994), 'Leaf hairs of olive (*Olea europaea*) prevent stomatal closure by ultraviolet–B radiation', *Austr. J. Plant Physiol.* **21**, 293–301.

Gray G. R., Savitch L. V., Ivanov A. G. and Huner N. P. A. (1996), 'Photosystem II excitation pressure and development of resistance to photoinhibition', *Plant Physiol.* **110**, 61–71.

Greenberg B. M., Gaba V., Canaani O., Malkin S., Mattoo A. K. and Edelman M. (1989a), 'Separate photosensitizers mediate degradation of the 32-kDa photosystem II reaction center protein in visible and UV spectral regions', *Proc. Nat. Acad. Sci. U.S.A* **86**, 6617–6620.

Greenberg B. M., Gaba V., Mattoo A. K. and Edelman M. (1989b), 'Degradation of the 32 kDa PSII reaction center protein in UV, visible and far red light occurs through a common 23.5 kDa intermediate', *Z. Naturforschung* **44**, 450–452.

Gupta A. S., Webb R. P, Holaday A. S. and Allen R. D. (1993), 'Overexpression of superoxide dismutase protects plants from oxidative stress', *Plant Physiol.* **103**, 1067–1073.

Harari-Steinberg O., Ohad I. and Chamovitz D.A. (2001), 'Dissection of the light signal transduction pathways regulating the two early light-induced protein genes in *Arabidopsis*', *Plant. Physiol.* **127**, 986–997.

Hartl F.-U., Martin J. and Neupert W. (1992), 'Protein folding in the cell: the role of molecular chaperones *Hsp70* and *Hsp60*', *Ann. Rev. Biophys. Biophys. Chem.* **21**, 293–322.

Havaux M. (1992), 'Stress tolerance of photosystem II *in vivo*', *Plant Physiol.* **100**, 424–432.

Havaux M. and Tardy F. (1996), 'Temperature-dependent adjustment of the thermal stability of photosystem II *in vivo*: possible involvement of xanthophyll-cycle pigments', *Planta* **198**, 324–333.

Havaux M., Greppin H. and Strasser R. J. (1991), 'Functioning of photosystems I and II in pea leaves exposed to heat stress in the presence or absence of light: analysis using in-vivo fluorescence, absorbance, oxygen and photoacoustic measurements', *Planta* **186**, 88–89.

He J., Huang L.-K., Chow W. S., Whitecross M. I. and Anderson J. M. (1993), 'Effects of supplementary ultraviolet–B radiation on rice and pea plants', *Austr. J. Plant Physiol.* **20**, 129–142.

Heber U., Bligny R., Streb P. and Couce R. (1996), 'Photorespiration is essential for the protection of the photosynthetic apparatus of C_3 plants against photoinactivation under sunlight', *Bot. Acta* **109**, 307–315.

Heineke D., Kruse A., Flügge U.-I., Frommer W. B., Riesmeier J. W., Willmitzer L. and Heldt H. W. (1994), 'Effect of antisense repression of the chloroplast triose-phosphate translocator on photosynthetic metabolism in transgenic potato plants', *Planta* **193**, 174–180.

Heineke D., Kauder F., Frommer W., Kühn C., Gillissen B., Ludewig F. and Sonnewald U. (1999), 'Application of transgenic plants in understanding responses to atmospheric change', *Plant Cell Environ.* **22**, 623–628.

Heldt H. W., Chon C. J., Marande D., Herold A., Stankovich Z. S., Walker D. A., Kraminer A., Kirck M. B. and Heber U. (1977), 'Role of orthophosphate and other factors in the regulation of starch formation in leaves and isolated chloroplasts', *Plant Physiol.* **59**, 1146–1155.

Herbers K., Meuwly P., Frommer W. P., Metruax J.-P. and Sonnewald U. (1996), 'Systemic acquired resistance mediated by the ectopic expression of invertase: possible hexose sensing in the secretory pathway', *Plant Cell* **8**, 793–803.

Herrmann R. G., Westhoff P. and Link G. (1992), 'Biogenesis of plastids in higher plants', in *Cell Organelles*, Herrmann R. G., ed., Springer-Verlag, Vienna, pp. 275–349.

Hideg É., Sass L., Barbato R. and Vass I. (1993), 'Inactivation of photosynthetic oxygen evolution by UV–B radiation: a thermoluminescence study', *Photosynth. Res.* **38**, 455–462.

Hideg É. and Vass I. (1996), 'UV–B-induced free radical production in plant leaves and isolated thylakoid membranes', *Plant Science* **115**, 251–260.

Hideg É., Kálmán T., Hideg K. and Vass I. (1998), 'Photoinhibition of photosynthesis *in vivo* results in singlet oxygen production detection via nitroxide-induced fluorescence quenching in broad bean leaves', *Biochem.* **37**, 11405–11411.

Hofmann R. W., Campbell B. D., Fountain D. W., Jordan B. R., Greer D. H., Hunt D. Y. and Hunt C. L. (2001), 'Multivariate analysis of intraspecific responses to UV-B radiation in white clover (*Trifolium repens* L.)', *Plant Cell Environ.* **24**, 917–927.

Hollinderbäumer R., Ebbert V. and Godde D. (1997), 'Inhibition of CO_2 fixation and its effects on the activity of photosystem II, on D1-protein synthesis and on phosphorylation', *Photosynth. Res.* **52**, 105–116.

Holmstrom K., Mantyla E., Welin B. and Pava E. T. (1996), 'Drought tolerance in tobacco', *Nature* **379**, 683–684.

Horton P., Ruban A. V. and Walters R. G. (1996), 'Regulation of light harvesting in green plants', *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **47**, 655–684.

Huber S. C. and Huber J. L. A. (1992), 'Role of sucrose-phosphate synthase in sucrose metabolism in leaves', *Plant Physiol.* **99**, 1275–1278.

Humbeck K., Quast S. and Krupinska K. (1996), 'Functional and molecular changes in the photosynthetic apparatus during senescence of flag leaves from field-grown barley plants', *Plant Cell Environ.* **19**, 337–334.

Hurry V. M., Krol M., Öquist G. and Huner N. P. A. (1992), 'Effect of long-term photoinhibition on growth and photosynthesis of cold-hardened spring and winter wheat', *Planta* **188**, 369–375.

Hurry V. M., Malmberg G., Gardeström P. and Öquist G. (1994), 'Effects of a short-term shift to low temperatures and of long-term cold hardening on photosynthesis and ribulose–1,5–bisphosphate carboxylase/oxygenase and sucrose phosphate synthetase in leaves of winter rye (*Secale cereale* L.)', *Plant Physiol.* **106**, 983–990.

Hurry V. M., Anderson J. M., Badger M. R. and Price G. D. (1996), 'Reduced levels of cytochrome b_6/f in transgenic tobacco increases the excitation pressure on photosystem II without increasing sensitivity to photoinhibition *in vivo*', *Photosynth. Res.* **50**, 159–169.

Husic D. W., Husic H. D. and Tolbert N. E. (1987), 'The oxidative photosynthetic carbon cycle or C_2 cycle', *CRC Critical Review in Plant Sciences*, Vol. 5, CRC Press, Boca Raton, pp. 45–100.

Igamberdiev A. U., Bykova N. V., Lea P. J. and Gardestrom P. (2001), 'The role of photorespiration in redox and energy balance of photosynthetic plant cells: A study with a barley mutant deficient in glycine decarboxylase', *Physiol. Plant.* **111**, 427–438.

Ingram J. and Bartels D. (1996), 'Molecular basis of dehydration tolerance in plants', *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **47**, 377–403.

Inoue H. and Tanaka A. (1978), 'Comparison of source and sink potentials between wild and cultivated potatoes', *J. Sci. Soil Managem. Jap.* **49**, 321–327.

Inoue K., Sakurai H. and Hiyama T. (1986), 'Photoinactivation of Photosystem I in isolated chloroplasts', *Plant Cell Physiol.* **27**, 961–968.

Jacob J. and Lawlor D. W. (1992), 'Dependence of photosynthesis of sunflower and maize leaves on phosphate supply, ribulose–1,5–bisphosphate carboxylase/oxygenase and ribulose–1,5–bisphosphate pool size', *Plant Physiol* **98**, 801–807.

Jacob J. and Lawlor D. W. (1993), '*In vivo* photosynthetic electron transport does not limit photosynthetic capacity in phosphate-deficient sunflower and maize leaves', *Plant Cell Environ.* **16**, 785–795.

Jägerschöld C., Virgin I. and Styring S. (1990), 'Light-dependent degradation of the D1 protein in photosystem II is accelerated after inhibition of the water splitting reaction', *Biochem.* **29**, 6179–6186.

Jahns P. and Krause G. H. (1994), 'Xanthophyll cycle and energy-dependent fluorescence quenching in leaves from pea plants grown under intermittent light', *Planta* **192**, 176–182.

Jahns P. and Miehe B. (1996), 'Kinetic correlation of recovery from photoinhibition and zeaxanthin epoxidation', *Planta* **198**, 202–210.

Jang Y.-C. and Sheen J. (1994), 'Sugar sensing in higher plants', *The Plant Cell* **6**, 1665–1679.

Jang J.-C. and Sheen J. (1997), 'Sugar sensing in higher plants', *Trends Plant Sci.* **6**, 208–214.

Jang Y.-C., Leon P., Zhou L. and Sheen J. (1997), 'Hexokinase as a sugar sensor in higher plants', *Plant Cell* **9**, 5–19.

Jansen M. A. K., Gaba V., Greenberg B. M., Mattoo A. K. and Edelman M. (1996), 'Low threshold levels of ultraviolet–B in a background of photosynthetically active radiation trigger rapid degradation of the D2 protein of photosystem II', *Plant J.* **9**, 693–699.

Jansen M. A. K. and van den Noort R. E. (2000), 'Ultraviolet–B radiation induces complex alterations in stomatal behaviour', *Physiol. Plant.* **110**, 189–194.

Jefferies R. A. (1994), 'Drought and chlorophyll fluorescence in field grown tomato (*Solanum tuberosum*)', *Physiol. Plant.* **90**, 93–97.

Jenkins G. I., Fuglevand G. and Christie J. M. (1997), 'UV–B perception and signal transduction', in *Plants and UV–B. Responses to Environmental Change*, Lumsden P. J., ed., Cambridge University Press, Cambridge, pp. 135–156.

Jenkins G. I., Long J. C., Wade H. K., Shenton M. R. and Bibikova T. N. (2001), 'UV and blue light signalling: pathways regulating chalcone synthase gene expression in *Arabidopsis*', *New Phytol.* **151**, 121–131.

John C. F., Morris K., Jordan B. R., Thomas B. and Mackerness S. A. H. (2001), 'Ultraviolet–B exposure leads to up-regulation of senescence-associated genes in *Arabidopsis thaliana*', *J. Exp. Bot.* **52**, 1367–1373.

Jones L. W. and Kok B. (1966), 'Photoinhibition of chloroplast reactions. I. Kinetics and action spectrum', *Plant Physiol.* **41**, 1037–1043.

Jordan B. R., He J., Chow W. S. and Anderson J. M. (1992), 'Changes in mRNA levels and polypeptides of ribulose–1,5–bisphosphate carboxylase in response to supplementary ultraviolet–B radiation', *Plant Cell Environ.* **15**, 91–98.

Jordan B. R. (2000), 'Molecular response of plant cells to UV–B stress', *Funct. Plant Biol.* **29**, 909–916.

Kandler O. and Miller W. (1990), 'Dynamics of the 'acute yellowing' in spruce connected with Mg deficiency', *Water Air Soil* **54**, 21–34.

Karabourniotis G., Kyparissis A. and Manetas Y. (1993), 'Leaf hairs of *Olea Europaea* protect underlying tissues against ultraviolet–B radiation damage', *Environ. Exp. Bot.* 33, 341–345.

Karabourniotis G. and Bornman J. F. (1999), 'Penetration of UV–A, UV–B and blue light through the leaf trichome layers of two xeromorphic plants, olive and oak, measured by optical fibre microprobes', *Physiol. Plant.* **105**, 655–661.

Karabourniotis G., Bornman J. F. and Liakoura V. (1999), 'Different leaf surface characteristics of three grape cultivars affect leaf optical properties as measured with fibre optics: possible implication in stress tolerance', *Austr. J. Plant Physiol.* **26**, 47–53.

Karabourniotis G., Bornman J. F. and Nikolopoulos D. (2000), 'A possible optical role of the bundle sheath extensions of the heterobaric leaves of *Vitis vinifera* and *Quercus coccifera*', *Plant Cell Environ.* **23**, 423–430.

Kerr J. B. and McElroy C. T. (1993), 'Evidence for large upward trends of UV–B radiation linked to ozone depletion', *Science* **262**, 1032–1034.

Kilb B., Wietoska H. and Godde D. (1996), 'Changes in the expression of photosynthetic genes precede loss of photosynthetic activities and chlorophyll when glucose is supplied to mature spinach leaves', *Plant Science Lett.* **115**, 225–235.

Kim J. H., Nemson J. A. and Melis A. (1993), 'Photosystem II reaction center damage and repair in *Dunaliella salina* (green alga)', *Plant Physiol.* **103**, 181–189.

Kishor P. B. K., Hong Z., Miao G., Hu C. A. and Verma P. S. (1995), 'Overexpression of pyrroline–5–carboxylate synthetase increases proline production and confers osmotolerance in transgenic plants', *Plant Physiol.* **108**, 13287–13294.

Kloppstech K. G., Meyer G., Schuster G. and Ohad I. (1985), 'Synthesis, transport and localization of a nuclear coded 22-kDa heat-shock protein in the chloroplast membranes of peas and *Chlamydomonas reinhardi*', *Eur. Mol. Biol. Organ. J.* **4**, 1902–1909.

Knapp A. K., Vogelmann T. C. and McClean T. M. (1988), 'Light and chlorophyll gradients within *Cucurbita* cotyledons', *Plant Cell Environ.* **11**, 257–263.

Koch K. E. (1997), 'Molecular crosstalk and the regulation of C- and N-responsive genes', in *A Molecular Approach to Primary Metabolism in Higher Plants,* Foyer C. H. and. Quick W. P, eds., Taylor and Francis, London, pp. 105–124.

Köner C. H., Pelaaez-Riedl S. and van Bel A. J. E. (1995), 'CO_2 responsiveness of plants: a possible link to phloem loading', *Plant Cell Environ.* **18**, 595–600.

Komenda J. and Barber J. (1995), 'Comparison of *psbO* and *psbH* deletion mutants of *Synechocystis* PCC 6803 indicates that degradation of D1 protein is regulated by the Q_B site and dependent on protein synthesis', *Biochem.* **34**, 9625–9631.

Konopka C., Hollinderbäumer R., Ebbert V., Wietoska H. and Godde D. (1996), 'Imbalances of D1 protein turnover during stress-induced chlorosis of a declining spruce tree', *J. Plant Physiol.* **148**, 324–331.

Koroleva O. Y., Brüggemann W. and Krause G. H. (1994), 'Photoinhibition, xanthophyll cycle and *in vivo* chlorophyll fluorescence quenching-tolerant *Oxyria digyna* and chilling sensitive *Zea mays*', *Physiol. Plant.* **92**, 577–584.

Kozaki A. and Takeba G. (1996), 'Photorespiration protects C_3 plants from photooxidation', *Nature* **384**, 557–560.

Kramer P. J. (1981), 'Carbon dioxide concentration, photosynthesis, and dry matter production', *BioScience* **31**, 29–33.

Krapp A., Hofmann B., Schäfer C. and Stitt M. (1993), 'Regulation of the expression of *rbcS* and other photosynthetic genes by carbohydrates: a mechanism for the 'sink regulation' of photosynthesis', *Plant J.* **3**, 817–828.

Krause G.H., Grube E., Virgo A., Winter K. (2003), 'Sudden exposure to solar UV-B radiation reduces net CO_2 uptake and photosystem I efficiency in shade-acclimated tropical tree seedlings', *Plant Physiol.* **131**, 745–752.

Kerepesi I. and Galiba G. (2000), 'Osmotic and salt stress-induced alteration in soluble carbohydrate content in wheat seedlings', *Crop Sci.* **40**, 482–487.

Krieger A., Weis E. and Demeter S. (1993), 'Low-pH-induced Ca^{2+} ion release in the water-splitting system is accompanied by a shift in the midpoint redox potential of the primary quinone acceptor Q_A', *Biochim. Biophys. Acta* **1144**, 411–418.

Ku M. S. B, Agarie S., Nomura M., Fukayama H., Tsuchida H., Ono K., Hirose S., Toki S., Miyao M. and Matsuoka M. (1999), 'High-level expression of maize phosphoenolpyruvate carboxylase in transgenic rice plants', *Nature Biotechnol.*, **17**, 76–80.

Labate C. A. and Leegood R. C. (1987), 'Limitation of photosynthesis by changes in temperature', *Planta* **181**, 547–554.

Landry L. G. and Pell E. J. (1993), 'Modification of rubisco and altered proteolytic activity in O_3-stressed hybrid poplar (*Populus maximowizii x trichocarpa*)', *Plant Physiol.* **101**, 1355–1362.

Larcher W. (1994), *Ökophysiologie der Pflanzen*, Ulmer Verlag, Stuttgart.

Lauer M. J., Pallardy S. G., Blevins D. G. and Randall D. D. (1989), 'Whole leaf carbon exchange characteristics of phosphate-deficient soybeans (*Glycine max* L.)', *Plant Physiol.* **91**, 848–854.

Law R. D. and Crafts-Brandner S. J. (2001), 'High temperature stress increases the expression of wheat leaf ribulose–1,5–bisphosphate carboxylase/oxygenase activase protein,' *Arch. Biochem. Biophys.* **386**, 261–267.

Lawlor D. W. (2002), 'Limitation to photosynthesis in water-stressed leaves: stomata vs. metabolism and the role of ATP', *Ann. Bot.* **89**, 871–885.

Lee S. B., Kwon H. B., Kwon S. J., Park S. C., Jeong M. J., Han S. E., Byun M. O. and Daniell H. (2003), 'Accumulation of trehalose within transgenic chloroplasts

confers drought tolerance', *Mol. Breeding* **11**, 1–13.

Li X. P., Bjorkman O., Shih C., Grossman A. R., Rosenquist M., Jansson S. and Niyogi K. K. (2000), 'A pigment-binding protein essential for regulation of photosynthetic light harvesting', *Nature* **403**, 391–395.

Liakoura V., Bornman J. F. and Karabourniotis G. (2003), 'The ability of abaxial and adaxial epidermis of sun and shade leaves to attenuate UV–A and UV–B radiation in relation to the UV absorbing capacity of the whole leaf methanolic extracts', *Physiol. Plant.* **117**, 33–43.

Lindahl M., Yang D. H. and Andersson B. (1995), 'Regulatory proteolysis of the major light-harvesting chlorophyll *a/b* protein of photosystem II by a light-induced membrane-associated enzymic system', *Eur. J. Biochem.* **231**, 503–509.

Lindahl M., Spetea C., Hundal T., Oppenheim A. B., Adam Z. and Andersson B. (2000), 'The thylakoid FtsH protease plays a role in the light-induced turnover of the photosystem II D1 protein', *Plant Cell* **12**, 419–431.

Lu C. M., Qiu N. W., Lu Q. T., Wang B. S. and Kuang T. Y. (2002), 'Does salt stress lead to increased susceptibility of photosystem II to photoinhibition and changes in photosynthetic pigment composition in halophyte *Suaeda salsa* grown outdoors?', *Plant Sci.* **163**, 1063–1068.

Lucas W., Olesinski A., Hull R. J., Haudenshield J. S., Deom C. M., Beachy R. N. and Wolf S. (1993), 'Influence of the tobacco mosaic virus 30-kDa movement protein on carbon metabolism and photosynthate partitioning in transgenic potato plants', *Planta* **190**, 88–96.

Ludlow M. M. (1987), 'Light stress at high temperature', in *Photoinhibition*, Kyle D. J., Osmond C. B. and Arntzen C. J., eds., *Topics in Photosynthesis* **9**, Elsevier, Amsterdam, pp. 89–111.

Lütz C., Steiger A. and Godde D. (1992), 'The influence of air pollutants and nutrient deficiency on the content of the D1-protein and on photosynthesis in young spruce trees', *Physiol. Plant.* **85**, 611–617.

Mahlman J. D. (1992), 'A looming Arctic ozone hole?', *Nature* **360**, 209–210.

Marschner H. and Cakmak I. (1989), 'High light intensity enhances chlorosis and necrosis in leaves of zinc, potassium and magnesium-deficient bean (*Phaseolus vulgaris*) plants', *J. Plant Physiol.* **134**, 308–315.

Martin G., Vogelmann T. C. and Josserand S. (1989), 'Epidermal focussing and the light microenvironment within leaves of *Medicago sativa*', *Physiol. Plant.* **76**, 485–492.

Martin G., Myers D. A. and Vogelmann T. C. (1991), 'Characterization of plant epidermal lens effects by a surface replica technique', *J. Exp. Bot.* **42**, 581–587.

Masojiidek J., Trivedi S., Halshaw L., Alexiou A. and Hall D. O. (1991), 'The synergistic effect of drought and light stress in sorghum and pearl-millet', *Plant Physiol.* **96**, 198–207.

Masojiidek J. and Hall D. O. (1992), 'Salinity and drought stresses are amplified by high irradiances in sorghum', *Photosynthetica* **27**, 159–171.

Mattoo A. K., Hoffmann-Falk H., Marder J. B. and Edelmann M. (1984), 'Regulation of protein metabolism: coupling of photosynthetic electron transport to *in vivo* degradation of the rapidly metabolised 32-kilodalton protein of the chloroplast membrane', *Proc. Nat. Acad. Sci. U.S.A.* **81**, 1380–1384.

Mattoo A. K. and Edelman M. (1987), 'Intramembrane translocation and post-translational palmitoylation of the chloroplast 32-kDa herbicide binding protein', *Proc. Nat. Acad. Sci U.S.A.* **84**, 1497–1501.

Maxwell D. P., Laudenbach D. E. and Huner N. P. A. (1995), 'Redox regulation of light-harvesting complex II and *cab* mRNA abundance in *Dunaliella salina*', *Plant Physiol.* **109**, 787–795.

Mayfield S. P., Yohn C. B., Cohen A. and Danon A. (1995), 'Regulation of chloroplast gene expression', *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **46**, 147–166.

McKenzie R. L., Björn L. O., Bais B. and Ilyas M. (2003), 'Changes in biologically active ultraviolet radiation reaching the Earth's surface', *Photochem. Photobiol. Sci.* **2**, 5–15.

McKersie B. D., Boeley S. R., Harjanto E. and Leprince O. (1996), 'Water deficit tolerance and field performance of transgenic alfalfa overexpressing superoxide dismutase', *Plant Physiol.* **111**, 1177–1181.

Medrano H., Bota J., Abadia A., Sampol B., Escalona J. M. and Flexas J. (2002), 'Effects of drought on light-energy dissipation mechanisms in high-light-acclimated, field-grown grapevines', *Funct. Plant Biol.* **29**, 1197–1207.

Mehlhorn H. and Wellburn A. R. (1994), 'Man-induced causes of free radical damage to plants: O_3 and other gaseous pollutants', in *Causes of Oxidative Stress and Amelioration of Defense Systems in Plants*, Foyer C. H. and Mullineaux P. M., eds., CRC Press, London, pp. 155–165.

Melis A., Nemson J. A. and Harrison M. A. (1992), 'Damage to functional components and partial degradation of photosystem II reaction center proteins upon chloroplast exposure to ultraviolet–B radiation', *Biochim. Biophys. Acta* **1100**, 312–320.

Mikkelsen T. N., Dodell B. and Lütz C. (1995), 'Changes in pigment concentration and composition in Norway spruce induced by long-term exposure to low levels of ozone', *Environ. Poll.* **87**, 197–205.

Montané M.-H. and Kloppstech K. (2000), 'The family of light-harvesting-related

proteins (LHCs, ELIPs, HLIPs): was the harvesting of light their primary function?', *Gene* **258**, 1–8.

Mooney H. A., Ehleringer J. R. and Björkman O. (1977),'The energy balance of leaves of the evergreen shrub (*Atriplex hymenolytra*)', *Oecologia* **29**, 301–310.

Moore B. D., Cheng S.-H., Rice J. and Seemann J. R. (1998), 'Sucrose cycling, rubisco expression, and prediction of photosynthetic acclimation to elevated atmospheric CO_2,' *Plant Cell Environ.* **22**, 905–915.

Moore B. D., Cheng S.-H., Sims D. and Seemann J. R. (1999), 'The biochemical and molecular basis for photosynthetic acclimation to elevated atmospheric CO_2', *Plant Cell Environ.* **22**, 567–582.

Mullet J. E. (1993), 'Dynamic regulation of chloroplast transcription', *Plant Physiol.* 309–313.

Negash L. and Björn L. O. (1986), 'Stomatal closure by ultraviolet radiation', *Physiol. Plant.* **66**, 360–364.

Nie G. Y., Tomasevic M. and Baker N. R. (1993), 'Effects of ozone on the photosynthetic apparatus and leaf proteins during leaf development in wheat', *Plant Cell Environ.* **16**, 643–651.

Nie G. Y., Hendrix D. L., Webber A. N., Kimball B. A. and Long S. P. (1995a), 'Increased accumulation of carbohydrates and decreased photosynthetic gene transcript levels in wheat grown at an elevated CO_2 concentration in the field', *Plant Physiol.* **108**, 975–983.

Nie G. Y., Long S. P., Garcia R. L., Kimball B. A., Lamorte R. L., Pinter P. J. Jr., Wall G. W. and Webber A. N. (1995b), 'Effects of free-air CO_2 enrichment on the development of the photosynthetic apparatus in wheat, as indicated by changes in leaf proteins', *Plant Cell Environ.* **18**, 855–864.

Nie G. Y., Robertson E. J., Fryer M. J., Leech R. M. and Baker N. R. (1995c), 'Response of the photosynthetic apparatus in maize leaves grown at low temperature on transfer to normal growth temperature', *Plant Cell Environ.* **18**, 1–12.

Neales T. F. and Incoll L. D. (1968), 'The control of leaf photosynthesis rate by the level of assimilate concentration in the leaf—a review of the hypothesis', *Bot. Rev.* **34**, 107–124.

Nielsen E. T., Karpa D., Mooney H. A. and Field C. (1993), 'Patterns of stem assimilation in two species of invasive legumes in coastal California', *Am. J. Bot.* **80**, 1126–1136.

Nielsen E. T., Sharifi M. R. and Rundel P. W. (1996), 'Diurnal gas exchange characteristics of two stem photosynthesizing legumes in relation to the climate at two contrasting sites in the California desert', *Flora* **191**, 105–116.

Nilsson A., Stys D., Drakenberg T., Spangfort M. D., Forsén S. and Allen J. F. (1997), 'Phosphorylation controls the 3-D structure of plant light-harvesting complex II', *J. Biol. Chem.* **272**, 18350–18357.

Nishida I. and Murata N. (1996), 'Chilling sensitivity in plants and cyanobacteria: the crucial contribution of membrane lipids', *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **47**, 541–568.

Nishio J. N., Sun J. and Vogelmann T. C. (1993), 'Gradients of carbon fixation across leaves do not follow internal light gradients', *Plant Cell* **5**, 953–961.

Nobel P. S. and Israel A. A. (1994), 'Cladode development, environmental responses of CO_2 uptake, and productivity for *Opuntia ficus-indica* under elevated CO_2', *J. Exp. Bot.* **45**, 295–303.

Noctor G., Veljovic-Jovanovic S., Driscoll S., Novitskaya L. and Foyer C. H. (2002), 'Drought and oxidative load in the leaves of C3 plants: a predominant role for photorespiration?', *Ann. Bot.* (London) **89**, 841–850.

Nogués S. and Baker N. R. (1995), 'Evaluation of the role of damage to photosystem II in the inhibition of CO_2 assimilation in pea leaves on exposure to UV–B radiation', *Plant Cell Environ.* **18**, 781–787.

Nomura M., Ishitani M., Takabe T., Rai A. K. and Takabe T. (1995), '*Synechococcus* sp. PCC 7942 transformed with *Escherichia coli bet* genes produces glycine betaine from choline and requires resistance to salt stress', *Plant Physiol.* **107**, 703–708.

Niyogi K. K., Shih, C., Chow, W. S., Pogson, B. J., DellaPenna D. and Björkman O. (2001),'Photoprotection in a zeaxanthin- and lutein-deficient double mutant of *Arabidopsis*', *Photosyn. Res.* **67**, 139–145.

Ögren E. (1994), 'The significance of photoinhibition for photosynthetic productivity', in Baker and Bowyer (1994), pp. 433–447.

Öquist G., Greer D. H. and Ögren E. (1987), 'Light stress at low temperature', in *Photoinhibition*, Kyle D. J., Osmond C. B. and Arntzen C. J., eds., Elsevier, Amsterdam, pp. 67–87.

Öquist, G., Chow, W. S. and Anderson J. M. (1992), 'Photoinhibition of photosynthesis represents a mechanism for the long-term regulation of photosystem II', *Planta* **186**, 450–460.

Öquist G. and Huner N. P. A. (1993), 'Low temperature effects on photosynthesis and correlation with freezing tolerance in spring and winter cultivars of wheat and rye', *Plant Physiol.* **101**, 245–250.

Ohad I., Keren N., Zer H., Gong H., Mor T. S., Gal A., Tal S. and Domovich Y. (1994), 'Light-induced degradation of the photosystem II reaction-centre D1 protein', in Baker and Bowyer, pp. 161–178.

Olsson L. C., Fraysse L. and Bornman J. F. (2000), 'Influence of high light and UV-B radiation on photosynthesis and D1 turnover in atrazine-tolerant and -sensitive cultivars of *Brassica napus*', *J. Exp. Bot.* **51**, 265–274.

Oosten J. J. V., Wilkins D. and Besford R. T. (1994), 'Regulation of the expression of photosynthetic nuclear genes by CO_2 is mimicked by regulation by carbohydrates: a mechanism for the acclimation of photosynthesis to high CO_2', *Plant Cell Environ.* **17**, 913–923.

Ortiz-Lopez A., Nie G. Y., Ort D. R. and Baker N. R. (1990), 'The involvement of photoinhibition of PSII and impaired membrane energization in the reduced quantum yield of carbon assimilation in chilled maize', *Planta* **181**, 78–84.

Osmond C. B. and Björkman O. (1972), 'Simultaneous measurements of oxygen effects on net photosynthesis and metabolism in C_3 and C_4 species', *Carnegie Inst. Wash. Yearbk.* **71**, 141–148.

Osmond C. B. (1994), 'What is photoinhibition? Some insights from comparison of shade and sun plants', in Baker and Bowyer (1994), pp. 1–24.

Osteryoung K. W. and Vierling E. (1994), 'Dynamics of small heat-shock protein distribution within the chloroplasts of higher plants', *J. Biol. Chem.* **269**, 28676–28682.

Ottander C., Hundal T., Andersson B., Huner N. P. A. and Öquist G. (1993), 'Photosystem II reaction centres stay intact during low temperature photoinhibition', *Photosynth. Res.* **35**, 191–200.

Ottander C., Campbell D. and Öquist G. (1995), 'Seasonal changes in photosystem II organisation and pigment composition in *Pinus sylvestri*', *Planta* **197**, 176–183.

Owens T. G. (1994), 'Excitation energy transfer between chlorophylls and carotenoids. A proposed molecular mechanism for non-photochemical quenching', in Baker and Bowyer (1994), pp. 95–110.

Park Y. I., Chow W. S., Anderson J. M. and Hurry V. M. (1996), 'Differential susceptibility of photosystem II to light stress in light-acclimated pea leaves depends on the capacity for photochemical and non-radiative dissipation of light', *Plant Science* **115**, 137–149.

Paul M. J. and Pellny T. K. (2003), 'Carbon metabolite feedback regulation of leaf photosynthesis and development,' *J. Exp. Bot.* **54**, 539–547.

Peet M. M., Huber S. C. and Patterson D. T. (1986), 'Acclimation to high CO_2 in monoeious cucumbers. II. Carbon exchange rate, starch and nutrient concentrations', *Plant Physiol.* **80**, 63–67.

Pego V. J., Weisbeek P. J. and Smeekens S. C. M. (1999), 'Mannose inhibits *Arabidopsis* germination via a hexokinase-mediated step', *Plant Physiol.* **119**, 1017–1023.

Pfannschmidt T., Nilsson A. and Allen J. E. (1999), 'Photosynthetic control of chloroplast gene expression', *Nature* **397**, 625–628.

Pfündel E., Pan R.-S. and Dilley R. A. (1992), 'Inhibition of violaxanthin de-epoxidation by ultraviolet–B radiation in isolated chloroplasts and intact leaves', *Plant Physiology* **98**, 1372–1380.

Pfündel E. and Bilger W. (1994), 'Regulation and possible function of the violaxanthin cycle', *Photosynth. Res.* **42**, 89–109.

Pilon-Smits E. A. H., Ebskamp M. J. M., Paul M. J., Jeuken M. J. W., Weisbeek P. J. and Smeekens S. C. M. (1995), 'Improved performance of transgenic fructan-accumulating tobacco under drought stress', *Plant Physiol.* **107**, 125–130.

Pino M. E., Mudd J. B. and Bailey-Serres J. (1995), 'Ozone-induced alterations in the accumulation of newly synthesized proteins in leaves of maize', *Plant Physiol.* **108**, 777–785.

Poulson M. E. and Vogelmann T. C. (1990), 'Epidermal focusing and effects upon photosynthetic light-harvesting in leaves of *Oxalis*', *Plant Cell Environ.* **13**, 803–811.

Powles S. B. (1984), 'Photoinhibition of photosynthesis induced by visible light', *Ann. Rev. Plant Physiol.* **35**, 15–44.

Prabhavathi V., Yadav J. S., Kumar P. A. and Rajam M. V. (2002), 'Abiotic stress tolerance in transgenic eggplant (*Solanum melongena* L.) by introduction of bacterial mannitol phosphodehydrogenase gene', *Mol. Breeding* **9**, 137–147.

Pyle J. A. (1997), 'Global ozone depletion: observations and theory', in *Plants and UV–B. Responses to Environmental Change*, Lumsden P. J., ed., Cambridge University Press, Cambridge, pp. 3–11.

Quick W. P., Schurr U., Scheibe R., Schulze E. D., Rodermel S. R., Bogorad L. and Stitt M. (1991), 'Decreased ribulose–1,5–bisphosphate-carboxylase-oxygenase in transgenic tobacco transformed with 'antisense' *rbcS*. I. Impact on photosynthesis at ambient growth conditions', *Planta* **183**, 542–554.

Reich P. B. (1983), 'Effect of low concentrations of O_3 on net photosynthesis, dark respiration, and chlorophyll content in ageing hybrid poplar leaves', *Plant Physiol.* **73**, 291–296.

Reinbothe S., Reinbothe C., Heintzen C., Seidenbrecher C. and Parthier B. (1993), 'A methyl-jasmonate-induced shift in the length of the 5′ untranslated region impairs translation of the plastid *rbcL* transcript in barley', *EMBO J.* **12**, 1505–1512.

Renger G., Völker M., Eckert H. J., Fromme R., Hohm-Veit S. and Gräber P. (1989), 'On the mechanism of photosystem II deterioration by UV–B irradiation', *Photochem. Photobiol.* **49**, 97–105.

Reuber S., Bornman J. F. and Weissenböck G. (1996), 'A flavonoid mutant of barley (*Hordeum vulgare* L.) exhibits increased sensitivity to UV radiation in the primary leaf', *Plant Cell Environ.* **19**, 593–601.

Riesmeier J. W., Flügge U.-I., Schulz B., Heineke D., Heldt H. W., Willmitzer L. and Frommer W. (1993), 'Antisense repression of the chloroplast triose phosphate translocator affects carbon partitioning in transgenic potato plants', *Proc. Nat. Acad. Sci. U.S.A.* **90**, 6160–6164.

Rintamäki E., Salo R., Lehtonen E. and Aro E.-M. (1995), 'Regulation of D1-protein degradation during photoinhibition of photosystem II *in vivo*: phosphorylation of the D1 protein in various plant groups', *Planta* **195**, 379–386.

Rintamäki E., Kettunen R. and Aro E.-M. (1996), 'Differential D1 dephosphorylation in functional and photodamaged photosystem II centres', *J. Biol. Chem.* **271**, 14870–14875.

Rogers G. S., Milham P. J., Thibaud M.-C. and Conroy J. P. (1996), 'Interactions between rising CO_2 and nitrogen supply in cotton. I. Growth and leaf nitrogen concentration', *Austr. J. Plant Physiol.* **23**, 119–125.

Russell A. W., Critchley C., Robinson S. A. Franklin L. A., Seaton G. G. R., Chow W. S., Anderson J. A. and Osmond C. B. (1995), 'Photosystem II regulation and dynamics of the chloroplast D1 protein in *Arabidopsis* leaves during photosynthesis and photoinhibition', *Plant Physiol.* **107**, 943–952.

Sage R. F. and Sharkey T. D. (1987), 'The effect of temperature on the occurrence of O_2 and CO_2 insensitive photosynthesis in field grown plants', *Plant Physiol.* **72**, 474–480.

Sage R. F., Sharkey T. D. and Seeman J. R. (1989), 'Acclimation of photosynthesis to elevated CO_2 in five C_3 species', *Plant Physiol.* **89**, 590–596.

Sánchez de Jiménez E., Medrano L. and Martínez-Barajas E. (1995), 'Rubisco activase, a possible new member of the molecular chaperone family', *Biochem.* **34**, 2826–2831.

Sasek T. W., DeLucia E. H. and Strain B. R (1985), 'Reversibility of photosynthetic inhibition in cotton after long-term exposure to elevated CO_2 concentrations', *Plant Physiol.* **78**, 619–622.

Satoh K. (1970), 'Mechanism of photoinactivation in photosynthetic systems I. The dark reaction in photoinactivation', *Plant Cell Physiol.* **11**, 15–27.

Savitch L. V., Gray G. R. and Huner N. P. A. (1997), 'Feedback-limited photosynthesis and regulation of sucrose-starch accumulation during cold acclimation and low-temperature stress in a spring and winter wheat', *Planta* **201**, 18–26.

Schatz G. H., Brock H. and Holzwarth A. R. (1988), 'A kinetic and energetic model for the primary processes in photosystem II', *Biophys. J.* **54**, 397–405.

Scheible W. R., Lauerer M., Schulze E.-D., Caboche M. and Stitt M. (1997), 'Accumulation of nitrate in the shoots acts as a signal to regulate shoot-root allocation in tobacco', *Plant J.* **11**, 671–691.

Schmid V., Peter S. and Schäfer C. (1995), 'Prolonged high light treatment of plant cells results in changes of the amount, the localization and the electrophoretic behaviour of several thylakoid membrane proteins', *Photosyn. Res.* **44**, 287–295.

Schöner S. and Krause G. H. (1990), 'Protective systems against active oxygen species in spinach: response to cold acclimation in excess light', *Planta* **180**, 383–389.

Schreiber U. and Berry J. A. (1977), 'Heat-induced changes of chlorophyll fluorescence in intact leaves correlated with damage of the photosynthetic apparatus', *Planta* **136**, 233–238.

Schreiber U., Kühl M., Klimant I. and Reising H. (1996), 'Measurement of chlorophyll fluorescence within leaves using a modified PAM fluorometer with a fiber-optic microprobe', *Photosynth. Res.* **47**, 103–109.

Schulze E.-D. (1989), 'Air pollution and forest decline in a spruce (*Picea abies*) forest', *Science* **244**, 776–782.

Searles P. S., Flint S. D. and Caldwell M. M. (2001), 'A meta analysis of plant field studies simulating stratospheric ozone depletion', *Oecologia* **127**, 1–10.

Shapira M., Lers A., Heifetz P. B., Irihimovitz V., Osmond C. B., Gillham N. W. and Boynton J. E. (1997), 'Differential regulation of chloroplast gene expression in *Chlamydomonas reinhardtii* during photoacclimation: light stress transiently suppresses synthesis of the rubisco LSU protein while enhancing synthesis of the PSII D1 protein', *Plant Mol. Biol.* **33**, 1001–1011.

Sharkey T. D., Badger M. R., von Caemmerer S. and Andrews T. J. (2001), 'Increased heat sensitivity of photosynthesis in tobacco plants with reduced rubisco activase,' *Photosynth. Res.* **67**, 147–156.

Shindell D. T., Rind D. and Lonergan P. (1998),'Increased polar stratospheric ozone losses and delayed eventual recovery owing to increasing greenhouse-gas concentrations', *Nature* **392**, 589–592.

Sheen J. (1990), 'Metabolic repression of transcription in higher plants', *The Plant Cell* **2**, 1027–1038.

Sheen J. (1994), 'Feedback control of gene expression', *Photosynth. Res.* **39**, 427–438.

Shen B., Jensen R. G. and Bohnert H. J. (1997), 'Increased resistance to oxidative stress in transgenic plants by targeting mannitol biosynthesis to chloroplasts', *Plant Physiol.* **113**, 1177–1183.

Sicher R. C. and Kremer D. F. (1994), 'Responses of *Nicotiana tabacum* to CO_2 enrichment at low-photon flux density', *Physiol. Plant.* **92**, 383–388.

Sironvall C. and Kandler O. (1958),'Photoxidative process in normal green *Chlorella* cells: the bleaching process', *Biochim. Biophys. Acta* **29**, 359–368.

Smirnoff N. (1993), 'Tansley review no. 52: the role of active oxygen species in the response of plants to water deficit and desiccation', *New Phytol.* **125**, 27–58.

Smith W. H. (1991), 'Air pollutants and forest damage', *Chem. Eng. News* **11**, 30–43.

Sonnewald U., Lerchl J., Zrenner R and Frommer W. (1994), 'Manipulation of sink-source relations in transgenic plants', *Plant Cell Environ.* **17**, 649–658.

Sonoike K., Terashima I., Iwaki M. and Itoh S. (1995), 'Destruction of photosystem I iron-sulfur centers in leaves of *Cucumis sativus* L. by weak illumination at chilling temperatures', *FEBS Lett.* **362**, 235–238.

Sonoike K. (1996), 'Photoinhibition of photosystem I: its physiological significance in the chilling sensitivity of plants', *Plant Cell Physiol.* **37**, 239–247.

Spetea C., Hideg É. and Vass I. (1996), 'The quinone acceptors are not the main sensitizers of UV–B induced damage in isolated photosystem II reaction centre and core complexes', *Plant Sci.* **115**, 207–215.

Spetea C., Hundal T., Lohmann F. and Andersson B. (1999), 'GTP bound to chloroplast thylakoid membranes is required for light-induced, multienzyme degradation of the photosystem II D1 protein', *Proc. Nat. Acad. Sci.* **96**, 6547–6552.

Stitt M. (1986), 'Limitation of photosynthesis by carbon metabolism. I. Evidence for excess electron transport in leaves carrying out photosynthesis in saturating light and CO_2', *Plant Physiol.* **81**, 1115–1122.

Stitt M. (1991), 'Rising CO_2 levels and their potential significance for carbon flow in plant cells', *Plant Cell Environ.* **14**, 741–762.

Stitt M. and Hurry V. (2002), 'A plant for all seasons: alterations in photosynthetic carbon metabolism during cold acclimation in *Arabidopsis*', *Curr. Opin. Plant Biol.* **5**, 199–206.

Strassburger E. (1991), *Lehrbuch der Botanik*, Gustav Fischer Verlag, Stuttgart.

Strid A., Chow W. S. and Anderson J. M. (1990), 'Effects of supplementary ultraviolet–B radiation on photosynthesis in *Pisum sativum*', *Biochim. Biophys. Acta* **1020**, 260–268.

Strid A. and Porra R. J. (1992), 'Alterations in pigment content in leaves of *Pisum sativum* after exposure to supplementary UV–B', *Plant Cell Physiol.* **33**, 1015–1023

Styring S. and Jegerschöld C. (1994), 'Light-induced reactions impairing electron transfer through photosystem II', in Baker and Bowyer (1994), pp. 51–74.

Takeuchi T. S. and Thornber J. P. (1994), 'Heat-induced alterations in thylakoid membrane protein composition in barley', *Austr. J. Plant Physiol.* **21**, 759–770.

Tandori J., Máte Z., Maróti and Vass I. (1996), 'Resistance of reaction centers from *Rhodobacter sphaeroides* against UV–B radiation. Effects on protein structure and electron transport', *Photosynth. Res.* **50**, 171–179.

Tarczynski M. C., Jensen R. G. and Bohnert H. J. (1993), 'Stress protection of transgenic tobacco by production of the osmolyte mannitol', *Science* **259**, 508–510.

Teicher H. B., Lindberg Møller B. and Scheller H. V. (2000), 'Photoinhibition of Photosystem I in field-grown barley (*Hordeum vulgare* L.): induction, recovery and acclimation', *Photosyn. Res.* **64**, 53–61.

Telfer A. and Barber J. (1994), 'Elucidating the molecular mechanisms of photoinhibition by studying isolated photosystem II reaction centres', in Baker and Bowyer (1994), pp. 25–50.

Teramura A. H. (1980), 'Effects of ultraviolet–B irradiances on soybean. I. Importance of photosynthetically active radiation in evaluating ultraviolet–B irradiance effects on soybean and wheat growth', *Physiol. Plant.* **48**, 333–339.

Terashima I. and Saeki T. (1983), 'Light environment within a leaf. I. Optical properties of paradermal sections of *Camellia* leaves with special reference to differences in the optical properties of palisade and spongy tissues', *Plant Cell Physiol.* **24**, 1493–1501.

Terashima I., Funayama S. and Sonoike K. (1994), 'The site of photoinhibition in leaves of *Cucumis sativus* L. at low temperatures is photosystem I, not photosystem II', *Planta* **193**, 300–306.

The Arabidopsis Genome Initiative (2000), 'Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*', *Nature* **408**, 796–815.

Tjus S. E., Møller B. L. and Scheller H. V. (1999), 'Photoinhibition of photosystem I damages both reaction center proteins PS I-A and PS I-B and acceptor-side located small photosystem I polypeptides', *Photosyn. Res.* **60**, 75–86.

Tjus S. E., Scheller H. V., Andersson B. and Møller B. L. (2001), 'Active oxygen produced during selective excitation of photosystem I is damaging not only to photosystem I, but also to photosystem II', *Plant Physiol.* **125**, 2007–2015.

Trebst A. and Depka B. (1990), 'Degradation of the D1 protein subunit of photosystem II in isolated thylakoids by UV light', *Z. Naturforsch.* **45c**, 765–771.

Turgeon R. (1989), 'The sink–source transition in leaves', *Ann. Rev. Plant. Physiol. Plant Mol. Biol.* **40**, 119–138.

Tyystjärvi E. and Aro E.-M. (1996), 'The rate constant of photoinhibition, measured in lincomycin-treated leaves, is directly proportional to light intensity', *Proc. Nat. Acad. Sci. U.S.A.* **93**, 2213–2218.

Ulrich B. (1995), 'Der ökologische Bodenzustand—seine Veränderung in der Nacheiszeit, Ansprüche der Baumarten', *Forstarchiv* **66**, 117–127.

Umweltbundesamt (1991), Jahresbericht, 1990. Berlin.

van Bel (1993), 'Strategies of phloem loading', *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **44**, 253–281.

van Camp W., Wilekens H., Bowler C., Montagu M. and Inzé D. (1994), 'Elevated levels of superoxide dismutase protect transgenic plants against ozone damage', *Biotechnol.* **12**, 165–168.

van Heerden P. D. R. and Kruger G. H. J. (2002), 'Separately and simultaneously induced dark chilling and drought stress effects on photosynthesis, proline accumulation and antioxidant metabolism in soybean', *J. Plant Physiol.* **159**, 1077–1086.

van Wijk K. J. and van Hasselt P. R. (1993), 'Photoinhibition of photosystem II *in vivo* is preceded by down-regulation through light-induced acidification of the lumen: consequences for the mechanism of photoinhibition *in vivo*', *Planta* **189**, 359–368.

van Wijk K. J., Nilsson L. O. and Styring S. (1994), 'Synthesis of the reaction center proteins and reactivation of redox components during repair of photosystem II after light-induced inactivation', *J. Biol. Chem.* **269**, 28382–28392.

van Wijk K. J. and Eichacker L. (1996), 'Light is required for efficient translation elongation and subsequent integration of the D1 protein into photosystem II', *FEBS Lett.* **388**, 89–93.

van Wijk K. J., Andersson B. and Aro E.-M. (1996), 'Kinetic resolution of the incorporation of the D1 protein into photosystem II and localization of assembly intermediates in thylakoid membranes of spinach chloroplasts', *J. Biol. Chem.* **271**, 9627–9636.

Vass I., Styring S., Hundal T., Koivuniemi A., Aro E.-M. and Andersson B. (1992), 'Reversible and irreversible intermediates during photoinhibition of photosystem II: stable reduced Q_A species promotes chlorophyll triplet formation', *Proc. Nat. Acad. Sci. U.S.A.* **89**, 1408–1412.

Vass I. (1996), 'Adverse effects of UV-B light on the structure and function of the photosynthetic apparatus', in Pessarakli M., ed., *Handbook of Photosynthesis*, Marcel Dekker Inc., New York, pp. 931–949.

von Schaewen A., Stitt M., Schmidt R., Sonnewald U. and Willmitzer L. (1990), 'Expression of a yeast-derived invertase in the cell wall of tobacco and *Arabidopsis* plants leads to accumulation of carbohydrate and inhibition of photosynthesis and strongly influences growth and phenotype of transgenic tobacco plants', *EMBO J.* **9**, 3033–3044.

Vogelmann T. C., Bornman J. F. and Josserand S. (1989), 'Photosynthetic light gradients and spectral regime within leaves of *Medicago sativa*', *Proc. Phil. Trans. Roy. Soc. London* **323**, 411–421.

Vogelmann T. C. (1993), 'Plant tissue optics', *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **44**, 231-251.

Vogelmann T. C., Nishio J. N. and Smith W. K. (1996), 'Leaves and light capture: light propagation and gradients of carbon fixation within leaves', *Trends in Plant Sci.* **1**, 65–70.

Vu C. V., Allen L. H. and Garrard L. A. (1982), 'Effects of supplemental UV–B radiation on primary photosynthetic carboxylating enzymes and soluble proteins in leaves of C_3 and C_4 crop plants', *Physiol. Plant.* **55**, 11–16.

Vu C. V., Allen L. H. and Garrard L. A. (1984), 'Effects of ultraviolet–B radiation (280–320 nm) on ribulose–1,5–bisphosphate carboxylase in pea and soybean', *Environ. Exp. Bot.* **24**, 131–143.

Walters R. G. and Horton P. (1995), 'Acclimation of *Arabidopsis thaliana* to the light environment; regulation of chloroplast composition', *Planta* **197**, 475–481.

Wang N. and Nobel P. S. (1996), 'Doubling the CO_2 concentrations enhanced the activity of carbohydrate-metabolism enzymes, source carbohydrate production, photoassimilate transport, and sink strength for *Opuntia ficus-indica*', *Plant Physiol.* **110**, 893–902.

Waters E. R., Lee G. J. and Vierling E. (1996), 'Evolution, structure and function of the small heat-shock proteins in plants', *J. Exp. Bot.* **47**, 325–338.

Watson R. T. (2002), 'Land use, land use change and forestry', for World Bank IPCC (2000) (*www.worldbank.org/html/cgiar/publications/icw00/watson2.pdf*).

Webber A. N., Nie G. Y. and Long S. P. (1994), 'Acclimation of photosynthetic proteins to rising atmospheric CO_2', *Photosyn. Res.* **23**, 413–425.

Weis E. (1981), 'Reversible heat-inactivation of the Calvin cycle: a possible mechanism of the temperature regulation of photosynthesis', *Planta* **151**, 33–39.

Wentworth M., Ruban A. V. and Horton P. (2000), 'Chlorophyll fluorescence quenching in isolated light-harvesting complexes induced by zeaxanthin', *FEBS Lett.* **471**, 71–74.

Westhoff P. (1996), *Molekulare Entwicklungsphysiologie*, Georg Thieme Verlag, Stuttgart.

Wingler A., Lea P. J. and Leegood R. C. (1997), 'Control of photosynthesis in barley plants with reduced activities of glycine decarboxylase', *Planta* **202**, 171–178.

Wingler A., Lea P. J., Quick W. P. and Leegood R. C. (2000), 'Photorespiration—metabolic pathways and their role in stress protection', *Phil. Trans. Roy. Soc. London B* **355**, 1517–1529.

Wise R. R. (1995), 'Chilling-enhanced photooxidation: the production, action and study of reactive oxygen species produced during chilling in the light', *Photosynth. Res.* **45**, 79–97.

Woodrow I. E. (1994), 'Optimal acclimation of the C_3 photosynthetic system under enhanced CO_2', *Photosynth. Res.* **39**, 401–412.

Xiao W., Sheen J. and Jang J.-C. (2000), 'The role of hexokinase in plant sugar signal transduction and growth and development', *Plant Mol. Biol.* **44**, 451–461.

Xu D. Q., Gifford R. M. and Chow W. S. (1994), 'Photosynthetic acclimation in pea and soybean to high atmospheric CO_2 partial pressure', *Plant Physiol.* **106**, 661–671.

Yelle S., Beeson R. C., Trudel M. J. and Gosselin A. (1989), 'Acclimation of two tomato species to high CO_2 II. Ribulose–1,5–bisphosphate-carboxylase/oxygenase and phosphoenolpyruvate carboxylase', *Plant Physiol.* **90**, 1473–1477.

Zhang J., Hu X., Henkow L., Jordan B. R. and Strid Å. (1994), 'The effects of ultraviolet–B radiation on the CF_oF_1-ATPase', *Biochim. Biophys. Acta* **1185**, 295–302.

Zhang L., Paakkarinen V., van Wijk K. J. and Aro E.-M. (2000), 'Biogenesis of the chloroplast-encoded D1 protein: regulation of translation elongation, insertion, and assembly into photosystem II', *Plant Cell* **12**, 1769–1782.

Zheng Y., Shimizu H. and Barnes J. D. (2002), 'Limitations to CO_2 assimilation in ozone-exposed leaves of *Plantago major*', *New Phytol.* **155**, 67–78.

Zimmer-Rinderle U. and Lichtenthaler H. K. (1995), 'Photosyntheseaktivität und chlorophyllfluoreszenzen geschädigter Fichten (Standort Freudenstadt)', in *Waldschäden im Schwarzwald*, Bittlingmaier L., Reinhardt W. and Sierfermann-Harms D., eds., Ecomed, Landsberg, pp. 256–287.

CHAPTER 6

THE ROLE OF AQUATIC PHOTOSYNTHESIS IN SOLAR ENERGY CONVERSION: A GEOEVOLUTIONARY PERSPECTIVE

PAUL G. FALKOWSKI
Environmental Biophysics and Molecular Ecology Program, Institute of Marine and Coastal Science, Rutgers University, New Brunswick, NJ 08901, USA
falko@imcs.rutgers.edu
RICHARD GEIDER
Department of Biological Sciences, University of Essex, Colchester CO4 3SQ, UK
geider@essex.ac.uk
and
JOHN A. RAVEN
Division of Environmental and Applied Biology, School of Life Sciences, University of Dundee, Biological Sciences Institute, Dundee, UK
J.A.RAVEN@dundee.ac.uk

As the present condition of nations is the result of many antecedent changes, some extremely remote and others recent, some gradual, others sudden and violent, so the state of the natural world is the result of a long succession of events, and if we would enlarge our experience of the present economy of nature, we must investigate the effects of her operations in former epochs.

Charles Lyell, *Principles of Geology*, 1830.

6.1 Introduction

Life on Earth is inextricably linked to liquid water. Evolution of the mechanism that facilitated the photobiological oxidation of water by aquatic photoautotrophs was key to completing and sustaining the biogeochemical cycles without which life itself could not have persisted on the planet (Falkowski and Raven, 1997). The vast majority of aquatic photoautotrophs are planktonic unicellular algae, known as phytoplankton. Spanning more than four orders of magnitude in size, oxygenic aquatic photoautotrophs are comprised of approximately 20,000 species grouped in at least nine taxonomic Divisions or Phyla (Lee, 1989). Eight of these Divisions—one of which is the ancestral root of all terrestrial plants (Bhattacharya and Medlin, 1998)—

are eukaryotic (possessing a cell nucleus). Although the deeply branching phylogenetic diversity in aquatic photoautotrophs is not paralleled in terrestrial ecosystems, their photobiological energy conversion systems are highly conserved and were selected to provide reducing power for CO_2. Collectively, aquatic photoautotrophs are responsible for approximately 50% of annual global photosynthesis (Behrenfeld *et al.*, 2001; Field *et al.*, 1998). The photobiological and geological sequestration of fixed organic carbon in the lithosphere (in the form of methane and petroleum) through the photosynthetic activity of aquatic photoautotrophs is exploited in the contemporary world as a major energy source for human industrial processes. In this chapter, we examine the evolution, ecology, physiology, energy conversion efficiencies and biogeochemical roles of aquatic photoautotrophs.

6.2 From the origin of life to the evolution of oxygenic photosynthesis

In the contemporary world, photosynthetic processes provide a source of reduced carbon and energy for virtually all life on Earth, but this was not always the case. We begin with the relatively simple question, "Where did photosynthetic organisms come from and why?". We start from the premise that life originated on Earth approximately 4 billion (Ga) years ago (but do not rule out the existence of life elsewhere in the universe). Because the geological record from this (Archean) epoch is not well preserved, there is uncertainty and controversy concerning specific conditions during this early period of Earth's history (Holland, 1984). There is, however, general consensus, based on our present (imperfect) understanding of planetary accretion and solar evolution processes, that: (a) the surface of the early Earth was covered almost entirely by liquid water, and very little mafic (continental) rock had formed (Mojzsis *et al.*, 2001); (b) solar luminosity was approximately 30% lower than at present (a "faint" Sun) and the solar spectrum incident at Earth's surface contained high amounts of short-wave radiation (Kasting, 1990); (c) the atmosphere was neutral or mildly reducing and contained significant amounts of N_2, but probably only trace amounts of NH_3 (Holland, 1984); (d) meteorite bombardment and vulcanism were significantly more pronounced;[1] (e) radiogenic heat production and the accretion process itself acted in concert to elevate surface temperatures, but never to the extent that surface water could boil off and potentially escape gravitational forces and be lost to space (a situation that may have occurred on Earth's neighbouring planet, Mars: Kasting, 1993).

[1] Indeed, these processes were jointly responsible for the formation of oceans (Robert, 2001).

Phylogenetic trees, constructed from comparing the nucleotide sequences of ribosomal RNA molecules in prokaryotes (Pace, 1997; Woese, 1987), suggest that the earliest organisms were non-photosynthetic, thermophilic chemoautotrophs[2] that are placed at the root branch between the Kingdoms of the Archea and the Eubacteria, as shown in Fig. 6.1. These early organisms could have used inorganic substrates such as H_2, or H_2S and FeS to reduce CO_2 to carbohydrate (Wächtershauser, 1990). Indeed, such organisms persist and thrive today in deep-sea vents, volcanic hot springs, deep in Earth's crust, and in other extreme environments where liquid water and suitable oxidisable inorganic substrates are available. Chemoautotrophs are almost certainly the precursors of photosynthetic cells (Blankenship, 1992). Why and how a photosynthetic process evolved in chemoautotrophs requires consideration of the selective forces responsible (the 'why') and the mechanism of the evolution (the 'how').

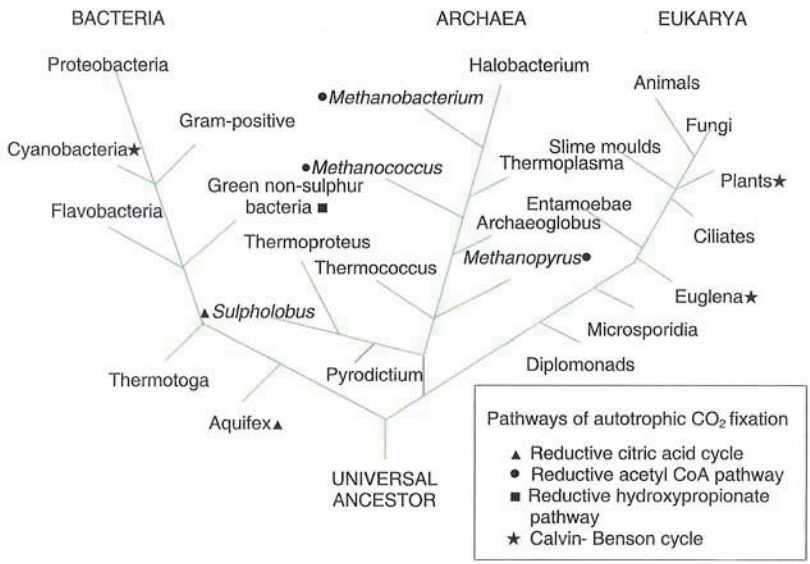


Figure 6.1 Universal phylogenetic tree, showing the distribution of autotrophic carbon fixation pathways. Cyanobacteria, all eukaryotic algae and all higher plants fix carbon using a photosynthetic electron transport system coupled to the Calvin–Benson (rubisco-based) cycle. There are no photosynthetic Archea; however several groups within that Kingdom can fix carbon chemoautotrophically.

[2] A *thermophilic* (heat-loving) organism is usually defined as having an optimum growth temperature above 45 C; *chemoautotrophs* are prokaryotic organisms that obtain their energy through the oxidation of inorganic compounds such as H_2S or Fe^{2+}.

The reductants that are nutrients for chemoautotrophs are generally deep within Earth's crust. However, vent fluids rich in these nutrients are produced in magma chambers connected to the athenosphere (the Earth's middle mantle, made of hot, soft, malleable rock) and these flow abundantly from vents in the ocean floor. In the contemporary ocean, the chemical disequilibria between vent fluids (which are highly reduced) and bulk seawater (which is highly oxidised) provide a sufficient thermodynamic gradient continuously to support chemoautotrophic metabolism. However, before the evolution of a sustained biologically or chemically mediated reaction that produced a strong oxidant (*e.g.* oxygenic photosynthesis), the redox gradient in early Earth's oceans would not have had a sufficiently large thermodynamic potential to support a pandemic outbreak of chemoautotrophy in the ocean basins. Moreover, magma chambers, vulcanism and vent fluid fluxes are tied to tectonic subduction regions, which are transient features of Earth's crust and hence only temporary habitats for chemoautotrophs. To colonise new vent regions, the chemoautotrophs would have had to have been dispersed throughout the oceans by physical mixing. This same dispersion process would have helped ancestral chemoautotrophs exploit solar energy near the ocean surface. Indeed, phylogenetic analyses suggest that contemporary deep-sea-vent communities are colonised by organisms living on continental margins or coastal regions (Robert Vrijenhoek, personal communication).

Although the processes that selected photosynthetic reactions as the major energy transduction pathway remain obscure, central hypotheses based on our understanding of photosynthesis, biophysics and molecular phylogeny have emerged. The metabolic pathway for the synthesis of porphyrins and chlorins is one of the oldest in biological evolution, and is found in all chemoautotrophs. Muladjanian and Junge (1997) proposed that the chlorin-based photosynthetic energy conversion apparatus originally arose from the need to screen UV radiation and prevent it from damaging essential macromolecules such as nucleic acids and proteins. The UV excitation energy could be transferred from the aromatic amino acid residues in proteins or the purines and pyrines in the nucleic acids to the Soret band of membrane-bound chlorins to produce a second excited state, which subsequently decayed to the lowest energy excited singlet. While non-photochemical dissipation of energy from the lowest energy singlet excited state can occur, photochemical energy transduction involving electron transport inward across a membrane is also an energy-dissipating process. This energy dissipation pathway can be harnessed to metabolism if the photochemically produced charge-separated primary products are prevented from undergoing charge recombination on the time scale necessary for the reaction pair to interact with the redox catalysts of the chemoautotrophic machinery. In this way, light energy could be used to energise the reduction of CO_2 to carbohydrates using reductants such as S^{2-} or

Fe^{2+}, which have redox potentials that are too positive to reduce CO_2 directly except under very constrained environments.

There are sufficient nucleotide sequence differences (Xiong *et al.*, 1998) between the two types of photosynthetic reaction centre (the ancestral reaction centre 1, from which descend Chlorobiaceae and Heliobacteriaceae, and the ancestral reaction centre 2, from which descend Chloroflexaceae and proteobacterial phototrophs) to reasonably conclude that the two reaction centres evolved *independently* from an ancestral UV-screening protein–chlorin complex in the plasma membrane (Blankenship, 2001; Olson, 2001). However, there are some fundamentally similar structural features of the two reaction centres that are highly conserved: both are heterodimers, both have five transmembrane spanning domains, and both bind chlorophyll *a* or bacterial analogues. Reaction centre 1 is capable of directly photoreducing a reductant that can reduce CO_2, while reaction centre 2 requires additional photochemical energy input transformed *via* a transmembrane H^+-concentration gradient to generate a strong (low-potential) reductant.

The geochemical consequences of photoautotrophy using either reaction centre 1 or reaction centre 2 mechanisms are the reduction of carbon and the oxidation of such reductants as Fe^{2+} (to Fe^{3+}) and S^{2-} (to S^0 or SO_4^{2-}). This synthesis of reduced (*i.e.* organic) carbon and the oxidised form of the electron donor permits the phototroph to develop 'respiratory' systems that effectively operate as the reverse of photosynthesis. Thus, the exergonic conversion of photosynthetically reduced carbon to CO_2, coupled to the reduction of Fe^{3+}, S^0 or SO_4^{2-} to Fe^{2+}, S^{2-} or S^0, can support growth and maintenance processes in the dark. However, not all of the reduced carbon and oxidants remain accessible to aquatic photoautotrophs: cells sink, carrying with them organic carbon; Fe^{3+} precipitates. The sedimentation and subsequent burial of carbon and Fe^{3+} can remove these components from the water column. Without replenishment, the essential reductants for anoxygenic photosynthesis would eventually become depleted in the surface waters. Thus the necessity to regenerate reductants potentially prevented anoxygenic photoautotrophs from permanently providing the major source of fixed carbon on Earth. Major *net* accumulation of reduced organic C in sediments from the Proterozoic implies at least *local* depletion of reductants such as S^{2-} and Fe^{2+} from the photic zone of the ocean and sets the scene for the evolutionary selection pressure to find an alternative electron donor. Such a geochemical limitation of photosynthesis is consistent with $^{13}C/^{12}C$ measurements on representative slightly metamorphosed sedimentary rocks dated from 3.7 Ga bp, which reveal depletion in ^{13}C relative to ^{12}C in the preserved organic matter (Rosing, 1999). This isotopic fractionation is consistent with ribulose–1,5–bisphosphate carboxylase–oxygenase (rubisco) catalysed CO_2 fixation early in Earth's history.

H_2O is a reductant in effectively unlimited supply on Earth relative to any redox-active solute dissolved in it. Liquid water contains ~55 kmol H_2O per cubic metre and there are 10^{18} m^3 of water in the hydrosphere and cryosphere. However, use of H_2O as a reductant for CO_2 requires a larger energy input than does the use of Fe^{2+} or S^{2-}. Indeed, for oxygenic photosynthesis to occur, the Q_y band of the chlorin had to become blue-shifted to provide sufficient energy to extract an electron from water by means of energy transfer from the lowest excited singlet state. Stabilisation of the primary electron acceptor to prevent back reaction necessitated thermodynamic inefficiency that prevents the direct reduction of NADP in a single photochemical reaction. Hence the overall energy input is ~180 $kJmol^{-1}$ for each electron transferred from $½H_2O$ to $½NADP^+$. This sequential action of two photochemical reactions is unique to oxygenic photoautotrophs and presumably involved lateral gene transfer by some symbiotic event or perhaps as a virally-mediated gene infection (Blankenship, 1992). A prerequisite for O_2 evolution is that the primary oxidant of reaction centre 2 (Photosystem II) shall have a higher redox potential (*ca.* +0.8 V, E_{m7}) than is found in any extant anoxygenic photoautotroph (the highest is *ca.* +0.4 V, E_{m7}). This increase in the oxidising power of the primary oxidant could have been either gradual, driven by a need to oxidise reductants of increasing redox potential as reductants of lower redox potential became locally depleted, or "instantaneous" via the wholesale incorporation of a preformed Mn-containing prosthetic group cannibalised from another enzyme (*e.g.* superoxide dismutase) to operate as the water oxidase (Debus, 1992; Dismukes *et al.*, 2001).

The timing of the origin of O_2-evolving photosynthesis is still incompletely resolved. While presumptive microfossils resembling cyanobacteria have been found in cherts that date from 3.45 Ga bp (Schopf, 1993), more compelling evidence of the origins of the first oxygenic photoautotrophs is based on the analyses of sterols (Summons *et al.*, 1999). These results place the origin at about 2.8 Ga. This later origin is further supported by macroscopic stromatolites. These are laminated sediment accumulations, generally of biological (oxygenic phototrophic) origin, in which organic-rich layers alternate with organic-poor layers. Although photo-dissociation of H_2O vapour could have provided a source of atmospheric O_2, the UV absorption cross section of O_2 constrains the reaction, and theoretical calculations supported by geochemical evidence suggests that prior to about 2.2 Ga bp, atmospheric O_2 was less than 10^{-3} of the present level (Holland and Rye, 1998; Ohmoto and Goldhaber, 1997). It seems that there was a lag between the first occurrence of oxygenic photosynthesis and a global build-up of atmospheric O_2 because of the presence of alternative electron acceptors, especially Fe^{2+} and S^{2-}, in the ocean. Indeed, the dating of the oxidation of Earth's oceans and atmosphere is in

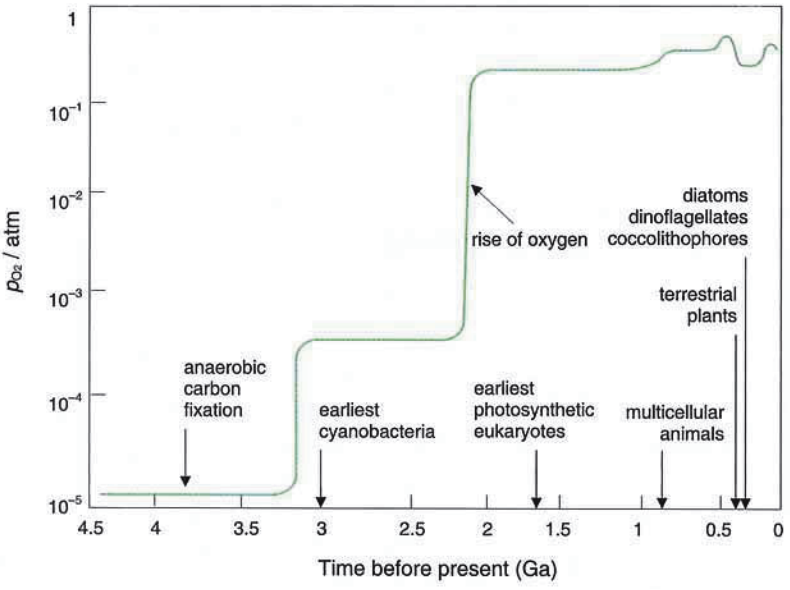


Figure 6.2 A historical reconstruction of the pressure p_{O_2} of oxygen in Earth's atmosphere since the formation of the planet (modified from Holland and Rye, 1998). Note the great 'rust event' that coincided with the rise of oxygen pressure about 2.1 Ga bp.

large measure based on analysis of the chemical precipitation of oxidised Fe in sedimentary rocks (the "Great Rust Event": Holland, 1984). Such paleosol analyses indicate that atmospheric oxygen rose sharply from virtually insignificant levels to between 1 and 10% of the present atmospheric concentration over a 100 million year period beginning 2.2 Ga bp. As shown in Fig. 6.2, there is a ~600 Ma delay between the oxygenation of Earth's oceans and atmosphere and the occurrence of the first oxygenic photoautotrophs in the fossil record (Des Marais, 2000).

6.2.1 The cyanobacteria

Cyanobacteria are the only oxygenic photoautotrophs known to have existed earlier than about 2 Ga bp (Lipps, 1993). These prokaryotes numerically dominate the photoautotrophic community in contemporary marine ecosystems, and clearly their continued success bespeaks an extraordinary adaptive capacity.

Most cyanobacteria contain phycobilins, usually located in major light-harvesting complexes known as *phycobilisomes*, as their primary light-harvesting pigments. (It is the colouration of these pigments that has led to the older vernacular appellation of

blue-green algae for cyanobacteria.) However, three classes of oxygenic prokaryotes use chlorophylls *a* and *b* (or divinyl chlorophylls *a* + *b*) as the primary pigments in their light-harvesting complexes. These organisms, the *prochlorophytes*, seem to have evolved independently on three occasions from three different taxa of cyanobacteria. The prochlorophytes also contain a chlorophyll *c*-like pigment (Falkowski and Raven, 1997).

Between 3 and 1.6 billion years ago, cyanobacteria were probably the major primary producers,[3] with small contributions from anoxygenic phototrophs and chemoautotrophs. In the contemporary ocean, the cyanobacteria fix approximately 60% of the 45 Pg C assimilated annually by aquatic phototrophs (Falkowski and Raven, 1997). Their proportional contribution to 'local' marine primary productivity is greatest in the oligotrophic central ocean gyres that form about 70% of the surface waters of the seas. Two major groups of marine cyanobacteria can be distinguished. The phycobilin-containing *Synechococcus* are more abundant nearer the surface and the (divinyl) chlorophyll *b*-containing *Prochlorococcus* are generally more abundant at depth (Chisholm, 1991; Falkowski and Raven, 1997).

The marine filamentous cyanobacterium *Trichodesmium* spp. is the major contributor to the biological fixation of dinitrogen in the oligotrophic central gyres (Capone *et al.*, 1997). Biological reduction of N_2 to NH_3 is catalysed by nitrogenase, a heterodimeric enzyme that is irreversibly inhibited by O_2. Molecular phylogenetic trees suggest that N_2 fixation evolved in eubacteria prior to the evolution of oxygenic photosynthesis (Ben-Porath *et al.*, 1993). The early evolution of nitrogenase is also indicated by the very large Fe requirement for the enzyme; this transition metal was much more available in the Archean and early Proterozoic epochs than it is today (Berman-Frank *et al.*, 2001a).

The co-existence of oxygen evolution and nitrogen fixation within the same organism led to mechanisms designed to isolate the two processes either temporally or spatially. In more advanced cyanobacteria, specialised nitrogen-fixing cells (heterocysts) that are morphologically differentiated from vegetative cells evolved. Heterocysts have no PSII reaction centres but retain the capability of supplying ATP for cell metabolism via cyclic electron flow around PSI. This strategy is ubiquitous in some filamentous freshwater and marine benthic cyanobacteria. In marine filamentous cyanobacteria such as *Trichodesmium*, heterocysts are lacking; cells appear to have the ability to isolate nitrogenase within individual cells within the filaments (Berman-Frank *et al.*, 2001b). In some unicellular marine cyanobacteria, nitrogen fixation is temporally separated from oxygen evolution; the cells fix N_2 at

[3] A primary producer is an organism that supplies organic carbon to heterotrophic organisms.

night. There is a relative paucity of N_2-fixing cyanobacteria in marine environments. This paucity has been suggested to be a consequence of Fe limitation in many parts of the contemporary ocean (Falkowski, 1997).

6.2.2 *The eukaryotes*

Eukaryotes are (unicellular or multicellular) organisms whose cells possess nuclei that contain their DNA. Evidence that eukaryotes evolved about 2 Ga bp comes largely from palaeontological studies, especially the presence of Grypanea (Knoll, 1992). This organism is thought to have been a photosynthetic eukaryote at the algal level of organisation, in that it was too large to have been a prokaryote and did not have identifiable features suggesting a mode of nutrition other than phototrophy (Falkowski and Raven, 1997).

It is generally believed that all eukaryotes evolved a nuclear and endomembrane (including endocytotic[4]) apparatus by direct descent from an Archean ancestor, but became capable of oxidative phosphorylation and photosynthesis by endosymbiosis of a proteobacterium and a cyanobacterium (both of which are eubacteria) respectively (Delwiche, 1999). The endosymbionts became incapable of independent existence by loss from their genomes of genes essential for life outside the symbiotic association. Many endosymbiont genes were lost entirely from the evolving symbiosis, and most of the rest were transferred to the host nucleus. Some of the transferred genes are essential for the operation of the energy-transducing organelles, so that the product of (cytoplasmic) translocation of these genes must be targeted to the appropriate intra-organellar sites. However, other transferred genes usurped the host genes coding for extra-organellar functions, *e.g.* many of the enzymes of cytosolic glycolysis (Feng and Doolittle, 1997). The phylogeny of the eukaryotic photoautotrophs can be inferred from analysis of ("host" nuclear) 18S rDNA nucleotide sequences. Such sequences reveal the ancestry of the nucleus in extant organisms. The evolutionary history of the plastids can be inferred by studies of plastid 16S rDNA nucleotide sequences. The last possible date of the origin of the various higher taxa can be found by the date of first occurrence of fossils attributable unequivocally to that taxon. Such palaeontological evidence can in turn be used to calibrate the 'molecular clock' for that higher taxon, and thus permit estimates of the timing of origin of related higher

[4] Endocytosis is the inward bulging of the cell envelope so that portions of the external medium can be engulfed. Endomembranes are membranes inside the cell (the cell envelope constitutes <10% of the membrane of a typical eukaryotic cell).

taxa without a good fossil record (Falkowski and Raven, 1997). This analysis assumes that lateral gene transfer is minimal and that the rate of mutation of ribosomal genes is constant (Feng and Doolittle, 1997).

Molecular genetic studies, combined with ultrastructural and biochemical considerations, allow distinction of some eight Divisions (Phyla) of eukaryotic algae, namely Chlorarachniophyta, Chlorophyta, Cryptophyta, Dinophyta, Euglenophyta, Haptophyta, Heterokontophyta and Rhodophyta (Lewin, 1993). Of these, the Chlorophyta and Rhodophyta appear to have originated by a single *primary* endosymbiosis involving a phagotrophic eukaryote flagellate (which already had mitochondria) and a cyanobacterium. Loss of flagella gave rise to the Rhodophyta (red algae), while evolution of the chlorophytan (green algal) line involved the loss of phycobilins as light-harvesting pigments and their replacement by chlorophyll *a/b* pigment protein complexes. The evolution of a chlorophyll *a/b* pigment protein complex in the Chlorophyta was a separate and independent process from the evolution of chlorophyll *a/b* complexes in the prokaryotic prochlorophytes (Urbach *et al.*, 1992). Sequence analysis of the genes encoding the apoproteins in the three prochlorophyte lines and in the eukaryotic Chlorophyta indicates that, in the former, the pigment–protein complex is derived from a core light-harvesting complex, CP43, while in the latter, a precursor of the modern LHCb pigment-binding family of proteins (with a distinctly different primary structure from CP43) provides a scaffold for the chromophores (La Roche *et al.*, 1997). Thus, it would appear that chlorophyll *b* evolved at least four times during the evolution of oxygenic photoautrotrophs (three times in prokaryotes and once in the Chlorophyta).

The remaining six Divisions of eukaryotic algae arose by *secondary* endosymbiosis, with different eukaryotic phagotrophic flagellates ingesting eukaryotic phototrophs, as shown in Fig. 6.3. These secondary endosymbioses are characterised by the presence of one (Dinophyta, Euglenophyta) or two (the four other Divisions) additional membranes around the plastids (in addition to the two 'normal' envelope membranes found in the primary symbiont, *i.e.* the Chlorophyta- and Rhodophyta-derived plastids). In the case of the Chlorarachniophyta and Euglenophyta, the cell ingested to become the plastid was a member of the Chlorophyta, while a member of the Rhodophyta served as the source for the Cryptophyta, Haptophyta, Heterokontophyta and (probably some of the) Dinophyta (Delwiche, 1999).

The primary and secondary endosymbioses that gave rise to plastids presumably occurred with unicellular host cells; these may not be readily recognisable in the fossil record as belonging to a particular Division unless there are very distinctive secondary features. Such features include cysts of some members of the

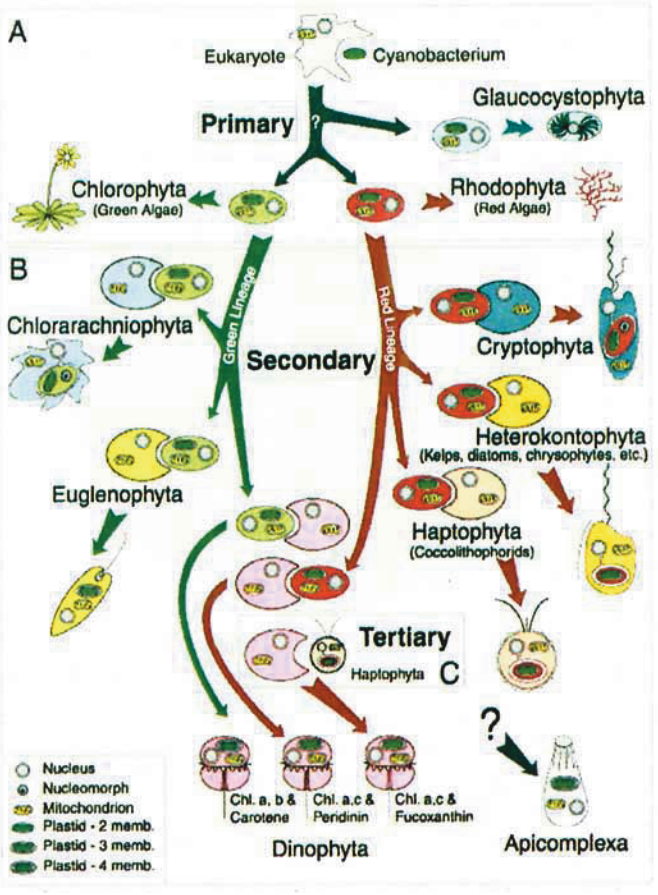


Figure 6.3 A schematic representation of the primary and secondary endosymbiotic events that gave rise to the major taxonomic divisions of oxygenic photosynthetic organisms. All terrestrial plants are derived from a primary green eukaryote. Most of the dominant aquatic photoautotrophs are in the "red" plastid lineage and are secondary symbionts. After Delwiche, 1999.

Prasinophyceae (Chlorophyta) and Dinophyta, the siliceous scales and cysts of the Chrysophyceae and Synurophyceae and the siliceous walls of the Bacillariophyceae (all three classes in the Heterokontophyta) and calcified (calcite) coccoliths of the coccolithophorids (Haptophyta). There is fossil evidence that a (macrophyte) red alga existed 1.2 Ga bp, with unicellular (cysts) and macrophytic green algae from 1 and 0.6 Ga bp. respectively. While fossils referable to heterokonts are known from the Proterozoic, it is possible that these are not photosynthetic, granted the much later

(280 Ma bp) origin of *photosynthetic* heterokonts suggested by molecular clock data calibrated from the molecular phylogeny and fossil record of diatoms (Bhattacharya and Medlin, 1995). The precursors of modern dinoflagellates probably originated more than 250 million years ago (Fensome *et al.*, 1996), while at least the coccolithophorid variants of the haptophytes are first known from fossils of the Triassic. There is no significant fossil record of the Chlorarachniophyta, Cryptophyta or Euglenophyta.

The heterokonts may have become photosynthetic in response to the environmental factors that precipitated the mass extinctions at the end of the Permian. This division largely emerged in the Mesozoic and rapidly diversified to produce the highly successful diatoms as well as the brown algae (Phaeophyceae), which contain the largest (in terms of size) extant marine primary producers. The coccolithophorids (Haptophyta) had great geochemical importance in the Cretaceous (and later) as major catalysts of marine $CaCO_3$ precipitation. Also involved in marine $CaCO_3$ precipitation are benthic photosynthetic precipitators of $CaCO_3$ such as green and red macroalgae (which have been active in this geochemical role for 550 million years) and symbiotically photosynthetic foraminifera, corals and giant clams. Such symbioses involve potentially free-living dinoflagellate endosymbionts, and represent an alternative to the evolution of a unicellular microalgae into a macroalgae as means of producing a large photosynthetic organism. These symbioses awaited the evolution of photosynthetic dinoflagellates (Falkowski and Raven, 1997).

This brief account of the evolution and diversification of aquatic photoautrophs reveals that there is much more higher-taxon diversity among aquatic than terrestrial primary producers; 95% or more of species involved in terrestrial primary production are embryophytes derived from a single class (Charophyceae) of a single Division (Chlorophyta) among the eight Divisions of eukaryotic aquatic photoautotrophs. By contrast, there is no comparable taxonomic dominance of primary producers in aquatic ecosystems. Let us now examine the processes responsible for the selection of these deeply branching taxa in marine photosynthetic organisms.

6.3 Photophysiological adaptations to aquatic environments

The distribution of photosynthetic organisms in the sea is largely dictated by the distribution of nutrients, especially fixed nitrogen (Eppley and Peterson, 1979; Ryther, 1969). The distribution of nutrients is, in turn, determined to a first order by ocean circulation. Ocean circulation is dictated by three fundamental physical processes: gravity, friction and topographically modified momentum.

The influence of gravity is translated to ocean circulation largely as a consequence of inhomogeneities in the density of water. The density of water is determined by its temperature and dissolved constituents (*i.e.* salts). Denser water, formed when water cools at high latitudes or evaporates at low latitudes, sinks into the ocean interior where it is advected through the system. This *thermohaline circulation* connects all of the world oceans via a large sub-surface 'conveyor belt' of waters that, in the contemporary ocean, originates in the North Atlantic, proceeds southward to the South Atlantic, transits to the Indian Ocean and eastward to the Pacific, to return again to the North Atlantic. The time required for the complete cycle is on the order of 1000 years. Thus, waters in the deep Pacific are, on average, several hundred years "older" (*i.e.* have been isolated from the atmosphere longer) than waters in the North Atlantic. Older waters are generally richer in nutrients and more depleted in oxygen.

Throughout the temperate and tropical regions, the upper ocean is isolated from the deep ocean by a density discontinuity (generally due to a thermal gradient). The atmosphere directly affects ocean circulation in this upper mixed layer via the frictional coupling of winds to the ocean surface. Wind 'stress' vectors are modified by Earth's rotation (the Coriolis effect) and topological features of the continents to produce current systems. On a macroscopic level, upper ocean currents form major gyres, the borders of which circulate in a clockwise fashion in the Northern Hemisphere and in an anticlockwise fashion in the Southern Hemisphere. Simultaneously, rotation of the currents as they move facilitates an upward (divergent) flux of deep waters along the edges of western and eastern boundaries of the gyres. In the core of the ocean gyres, vertical fluxes of deep waters into the upper ocean are relatively small and dominated by turbulent diffusion. The net effect of these physical processes is that nutrient fluxes are relatively large along the borders of ocean gyres, and especially along eastern and western ocean margins (*e.g.* continental shelves), while in the central ocean gyres, nutrient fluxes are relatively small.

Four nutrients have been identified as primary factors that limit phytoplankton biomass and growth rate in the sea on seasonal and ocean basin scales (Falkowski *et al.*, 1998). These nutrients are silicate, nitrogen, phosphorus and iron. Silicate limitation is one of the factors that determines the distribution of diatoms (Brzezinski *et al.*, 1998), although it does not directly influence that of other phytoplankton. Inorganic nitrogen (ammonium, nitrate) is the limiting nutrient in many coastal and continental shelf waters and in the subtropical gyres (Codispoti, 1989). Phosphorus may be a limiting nutrient regionally, such as in the eastern Mediterranean Sea (Thingstad *et al.*, 1998) or western North Atlantic (Wu *et al.*, 2000). Iron has been shown to be a limiting factor in the Equatorial and Northeast Pacific Oceans (Coale *et al.*, 1996; Martin, 1991), and may, together with light, control photosynthesis in the

Southern Ocean (Boyd, 2000). It is likely that the limiting nutrient varies both in space and time (Falkowski *et al.*, 1998).

Phytoplankton blooms occur when and where high concentrations of inorganic nutrients are introduced to stable (*i.e.*, stratified) surface waters. Over most of the ocean, phytoplankton typically face severe competition for nutrients. Nitrate, phosphate and ammonium may be present at concentrations as low as 10^{-8} to 10^{-7} M in oligotrophic waters (Harrison *et al.*, 1996; Karl and Tien, 1997), whereas the half-saturation constants for uptake of these nutrients lies in the range of 10^{-9} to 10^{-5} M (Harrison *et al.*, 1996). Physiological acclimations to nutrient limitation include reduction of cellular nutrient content (Morel *et al.*, 1991), increases in the abundance of cell surface nutrient transporters (Morel *et al.*, 1991), and induction of high-affinity transport systems (Scanlan *et al.*, 1993). Many phytoplankton species can access organic phosphorus via cell surface phosphatases (Graziano *et al.*, 1996), and organic nitrogen via cell surface amino acid oxidases (Mulholland *et al.*, 1998). In addition, organic nitrogen may be taken up directly and deaminated intracellularly (Palenik and Hensen, 1997).

Iron may present a special case. Most of the iron in seawater appears to be tightly bound to organic chelates that are not well characterised (Rue and Bruland, 1995) or is in colloidal forms (Wu *et al.*, 2000). In either case, the complexed metals or colloids are not directly accessible to phytoplankton; indeed, most algae appear to transport inorganic iron, designated Fe' (Hudson and Morel, 1993; Sunda and Huntsman, 1997). Thus phytoplankton growth depends on the replenishment of the very small Fe' pool via dissociation from organic chelates or extraction from colloids. The Fe' concentration in equilibrium with the organic chelates probably limits plankton growth in many ocean regions (Sunda and Huntsman, 1995). However, phytoplankton may not depend exclusively on Fe'. Some oceanic cyanobacteria produce siderophores[5] that facilitate iron acquisition (Wilhelm, 1995; Butler, 1998). Iron may also be obtained from organic chelates via cell surface-mediated reduction of Fe^{III} to Fe^{II} followed by dissociation and assimilation of the inorganic Fe^{II} (Anderson and Morel, 1980; Soria-Dengg and Horstmann, 1995). Some phytoplankton, including many haptophytes and dinoflagellates, are phagotrophic and can derive nutrients from particulate matter including bacteria (Maranger *et al.*, 1998).

Yet another response to nutrient scarcity may be symbiosis. Pelagic foraminifera and radiolaria are hosts to symbiotic zooxanthellae. The zooxanthellae may benefit from nutrient release by their cnidarian hosts and the hosts benefit by harvesting some of the organic carbon from their garden of zooxanthellae (Falkowski *et al.*, 1993).

[5] Siderophores are organic chelating agents that are use to acquire Fe by prokaryotes (Butler, 1998).

In summary, the mechanisms of nutrient acquisition by marine phytoplankton are varied. Inorganic nutrients are the preferred source for many bloom-forming species. However, other species, including the bloom-forming coccolithophorid *Emiliania huxleyi*, are able to grow perfectly well on dissolved organic nutrients, and some phagotrophic species are capable of accessing nutrients from particulate organic matter.

The fluxes of nutrients into the euphotic zone and between functional groups of organisms within the euphotic zone constrain the biomass of photoautotrophs that can be supported at any one time in a specific location. This profoundly influences the size of cells and has been a major selection pressure on aquatic photoautotrophs.

6.3.1 Cell size

Marine phytoplankton range in size from $<10^{-6}$ m diameter of cyanobacteria within the genera *Synechococcus* and *Prochlorococcus* to $>10^{-3}$ m equivalent spherical diameter in large diatoms. Colony formation can extend the size range to 10^{-2} m, and the largest macroalgae exceed 10 m in length. Size constrains the rate at which dissolved nutrients can be assimilated (Hudson and Morel, 1993; Raven, 1986; Sunda and Huntsman, 1997), the movement of phytoplankton within the water column (Raven, 1986) the efficiency of light absorption (Finkel, 2000; Raven, 1986) and the susceptibility to grazing (Chisholm, 1991).

Phytoplankton assemblages are sometimes characterised by cell size. Small cells ($<5 \times 10^{-6}$ m) dominate in stable, nutrient-poor waters. However, small cells do not attain high biomass, probably due to ecological control by grazers (Chisholm, 1991). The cells that form blooms are of intermediate size (5×10^{-6} m to 10^{-4} m). This size range includes organisms with high intrinsic growth rates. Cells within this size range are also less susceptible to grazing by protozoa, and may possess the ability to store relatively large amounts of potentially limiting nutrients. Large, fragile cells are often found in stable water columns where turbulent shear is low.

One response to the chronically low nutrient concentrations in the oligotrophic regions has been reduced cell size. Picoplankton (cells of $<2 \times 10^{-6}$ m diameter) dominate the biomass of nitrogen-limited oligotrophic open ocean regions and the iron-limited equatorial Pacific Ocean. Small size provides at least two competitive advantages: (a) a high surface-area-to-volume ratio that minimises diffusion limitation of nutrient assimilation (Chisholm, 1991; Hudson and Morel, 1993); and (b) increased efficiency of light absorption by photosynthetic pigments due to minimal self-shading (Raven, 1986).

Some phytoplankton have literally escaped from diffusive limitation of nutrient assimilation by employing vertical migration to move between high-light/low-nutrient surface waters to low-light/high-nutrient waters at depths of 100 m or more (Villareal *et al.*, 1993). Photosynthesis occurs in the surface mixed layer, whereas nutrients are assimilated at depth. Vertical migration is found in diatoms, dinoflagellates and the colony-forming cyanobacterium *Trichodesmium*. Vertically migrating phytoplankton are typically >50 μm in diameter and may form colonies in order to control their movement through stratified waters by buoyancy regulation (Raven, 1986) and, in the case of dinoflagellates, by directional swimming.

Although extremely small size may reduce or eliminate diffusion limitation of nutrient uptake, small size does not come without a price. Small size prevents the picoplankton from controlling their position in the water column (Raven, 1986), and thus growth of picoplankton is constrained by local conditions. Non-scaleable components of a cell, including the genetic material, cell wall and membranes, set a lower limit of about 0.25μm to cell size (Raven, 1986). As cell size approaches this lower limit, essential catalysts, including Calvin cycle enzymes and light-harvesting components, become a smaller proportion of cell mass, with consequent reductions in the maximum potential resource-saturated growth rate (Raven, 1986). Thus reductions in maximum resource-saturated growth rates are expected to accompany reductions of size in cells that are less than about 1 μm in diameter (Raven, 1986). A decrease in maximum specific growth rate with increasing cell or organism size is universal in biological systems.

Although cell size can account for variability in the maximum (resource-saturated) growth rate within an algal class, there exist statistically significant differences amongst algal classes in the growth rate that can be achieved at any given cell size (Chisholm, 1991). For a given cell size, diatoms typically possess higher-than-average growth rates, whereas dinoflagellates possess lower-than-average growth rates. Genome size appears to be a better predictor of maximum growth rates than cell size, suggesting that the smaller the genome size, the faster the growth rate (Shuter *et al.*, 1983). Interestingly, within a given cell type, DNA content is tightly correlated with cell volume. However, the proportion of cell volume that is occupied by the nucleus differs between cell types. For cells of a given size, cell DNA content appears to be correlated with the metabolic complexity (Shuter *et al.*, 1983).

A physico-chemical explanation may exist for the size dependence of maximum growth rate. The explanation rests on the observations that (a) the solubility of iron in seawater places an upper limit on the Fe' concentration of 10^{-9} M; (b) at this Fe' concentration, diffusion limits iron assimilation; (c) the diffusive supply of iron scales with the surface area of a cell; (d) the iron content scales with the volume of a cell;

and (e) photosynthesis rate scales with cell iron content (Sunda and Huntsman, 1997). Thus, the maximum Fe' concentration permitted by the physical chemistry of iron in seawater places an upper limit on the rate of iron supply to a phytoplankton cell, which in turn affects the maximum growth rate. Cells cannot increase growth rate at a given Fe' by reducing cellular iron content because light-saturated photosynthetic electron transfer depends on cell iron content (Raven, 1990).

6.3.2 Light and its utilisation

Whereas the distribution of photoautotrophic biomass in the oceans is primarily controlled by the fluxes of nutrients into the upper ocean, light is the primary factor regulating the biomass-specific rate of photosynthetic electron transport (Behrenfeld and Falkowski, 1997a). In aquatic ecosystems, spectral irradiance is highly modified by water itself, which, as a consequence of fluctuation density scattering and selective molecular absorption, increasingly isolates blue light deeper in the water column (Kirk, 1992). Hence, the attenuation of total irradiance through the water column is inevitably accompanied by a modification of the spectral quality of the irradiance (Fig. 6.6). Moreover, because spectral irradiance at any given position in the water column is a function of solar zenith angle, dissolved organics and the concentration and taxonomic composition of the photoautotrophs themselves, light is constantly changing underwater. Not surprisingly perhaps, aquatic photoautotrophs have evolved numerous strategies for optimising the harvesting and utilisation of light, the supply of which, on a community level, always limits photosynthesis.

The photosynthetic apparatus can be considered to consist of light-harvesting pigment–proteins, the electron transfer chain and the Calvin cycle enzymes. In eukaryotes, the light-harvesting apparatus is contained within the chloroplasts, which can occupy up to 50% of cell volume and a corresponding proportion of cell mass. Light-harvesting pigments and associated proteins may account for over 20% of cell mass in light-limited algae. Depending on the ratio of light-harvesting pigments to electron-transfer chain components (the so-called photosynthetic unit size, PSU), up to 40% of cell mass may be associated with the thylakoid membranes. Rubisco accounts for about 3% of cell mass in nutrient-replete cultures of the diatom *Thalassiosira weisflogii* (Hobson *et al.*, 1985) and the chlorophyte *Dunaliella tertiolecta* (Sukenik *et al.*, 1987).

Algae possess at least four major classes of light-harvesting systems. These include the phycobiliproteins of cyanobacteria and rhodophytes, the chlorophyll *a/c* fucoxanthin light-harvesting complexes of haptophytes, pelagiophytes, diatoms and

cryptomonads, and the chlorophyll *a/b* complexes of *Prochlorococcus*, chlorophytes and prasinophytes (Falkowski and Raven, 1997). Within the dinoflagellates are representatives of all three of the above-mentioned light-harvesting systems. However, most photosynthetic dinoflagellates contain a water-soluble light-harvesting complex containing peridinin that is not found in other algal classes.

The light-harvesting complexes funnel excitation energy to the reaction centres, which are linked in a photosynthetic electron transfer (PET) chain. The PET chain includes three large membrane-spanning complexes within the thylakoid membranes; these are the Photosystem II, cytochrome b_6/f and Photosystem I complexes. Electron flow amongst these complexes is via small electron and hydrogen carriers. There is wide variability in the relative abundance of PET components and the ratio of chlorophyll *a* to PET complexes. These functional processes and structural elements are largely conserved in all oxygenic photoautotrophs, including higher plants, with the primary variations occurring in the light-harvesting systems (Larkum and Barrett, 1983).

Control of light-saturated photosynthesis may reside in the photosynthetic electron transfer, in carboxylation, or downstream of the photosynthetic apparatus in protein synthesis or cell division. In the green alga of the *Dunaliella tertiolecta*, light-saturated photosynthesis in *Dunaliella tertiolecta* is the ratio photosynthetic electron transport chain component to the rubisco content (Sukenik *et al.*, 1987). The reduction of rubisco contents in nitrogen-limited microalgae is accompanied by declines of light-saturated photosynthesis (Falkowski *et al.*, 1989). However, the decline in light-saturated photosynthesis rates may be greater than the decline in abundance of large rubisco subunits, indicating that catalytic activity declines as well (Falkowski *et al.*, 1989; Geider *et al.*, 1998). Finally, there is frequently an afternoon depression of photosynthesis in nature. This depression can arise from photodamage to reaction centres or may be due to a metabolic feedback between cell division and photosynthesis.

Light-limited photosynthesis is regulated by the rate of light absorption and the efficiency of photochemistry in the reaction centres. Changes in light-limited photosynthesis typically parallel changes in cell chlorophyll *a* content, although allowance must be made for variations in pigment complement and in the extent of intracellular self-shading (Berner and Canfield, 1989). Although the efficiencies of excitation energy transfer to the reaction centres and of photochemistry within the reaction centres can vary, reductions from maximum efficiency are typically observed in response to supraoptimal light and are not evident in chronically light-limited conditions.

Acclimation to variations in irradiance, the spectral quality of light, nutrient limitation and temperature involves changes in the absolute and relative abundances of photosynthetic pigments and proteins. With the exception of a few mutants whose habitat is the laboratory culture room, all algae and cyanobacteria studied to date acclimate to increased irradiance by decreasing the ratio of light-harvesting pigments to cell mass (Geider *et al.*, 1998). The change in pigment content may be accompanied by changes in the ratio of pigments to reaction-centre proteins (Falkowski and Raven, 1997). Variations in the ratio of chlorophyll *a* to biomass of nutrient-replete *Dunaliella tertiolecta* in response to changes in growth irradiance can be accounted for by the redox state of the plastoquinone pool (Escoubas *et al.*, 1995). As the proportion of reduced plastoquinone increases, the rate of light-harvesting complex synthesis declines. The redox state of plastoquinone can be quantified as an "excitation pressure" using pulse amplitude modulated and fast repetition rate fluorescence (Maxwell *et al.*, 1994) or by the degree of light saturation of photosynthesis under ambient light (Geider *et al.*, 1998). Regulating excitation pressure by reducing pigment content at moderate to high irradiances appears to have been a major selective pressure shaping photoacclimation in phytoplankton (Durnford and Falkowski, 1997).

A decline in the cellular contents of photosynthetic electron transfer chain components at supraoptimal irradiances may lead to a shift in control of light-saturated photosynthesis from the Calvin cycle to the photosynthetic electron-transfer chain. Although a shift is not evident in *Dunaliella tertiolecta* grown at irradiances up to 1900 μmol photons m^{-2} s^{-1} (Sukenik *et al.*, 1987), this alga possesses a very low rubisco activity of only 12 mole CO_2 [mole rubisco s]$^{-1}$ at 15 C. A shift in control of light-saturated photosynthesis to electron transfer is more likely to be observed in diatoms like *Thalassiosira weisflogii*, which possess much higher rubisco activity *in vivo* (78 mole CO_2 [mole rubisco s]$^{-1}$, calculated from the data of Hobson *et al.*, 1985. In order to support this rubisco activity, the photosynthetic units of *T. weisflogii* must turn over at a rate of about 500 s^{-1} (assuming a photosynthetic unit size of 500 chlorophyll *a* molecules per Photosystem II, a measured carbon: chlorophyll *a* ratio of 43 g C : [g chlorophyll *a*]$^{-1}$, and a measured rubisco : C ratio of 0.03). If correct, this calculated electron transfer rate is 2–3 times greater than previously reported for *in vivo* electron-transfer rates (Raven, 1990).

6.3.3 *Temperature selection*

Temperature affects both the catalytic activity of enzymes and the fluidity and lipid composition of membranes. Typically, a metabolic rate or an enzyme activity increases with temperature up to an optimum temperature, beyond which there is a marked decline (Li, 1980). The optimum temperature varies widely, from 0 C for photosynthesis in some psychrophilic diatoms isolated from Antarctic sea ice to 70 C in thermophilic cyanobacteria isolated from hot springs (Li, 1980). Within the increasing region (*i.e.*, below the optimum temperature), the temperature dependence of enzyme activity can be described by an Arrhenius equation. Although rubisco carboxylation and other enzymatic reactions have a Q_{10}[6] of about 2–3, the rates of light absorption and light-limited electron transfer are largely independent of temperature (Raven and Geider, 1988). Thus a step reduction in temperature will lead to an increase in excitation pressure since the light reactions will be unaffected but the rates of the enzymatic dark reactions will be reduced (Davison, 1991). Interestingly, there is reciprocity between the effects of a decrease in temperature and an increase in irradiance on the pigment contents of microalgae. At a given irradiance, pigment content typically increases with increasing temperature (Geider *et al.*, 1998). As with the light dependence of pigment content described above, the temperature dependence can be described in terms of variations in excitation pressure (Maxwell *et al.*, 1994).

6.4 Quantum yields of photosynthesis in the ocean

The maximum quantum yield of photosynthesis can be defined as the ratio of the maximum rate at which a product is formed (*e.g.* carbon is fixed) per unit light absorbed. In phytoplankton, determination of the maximum quantum yield is technically challenging because measurement of light absorption is especially difficult. A basic approach is to determine the chlorophyll-normalised *in-vivo* optical absorption cross section over the visible spectrum, and simultaneously to determine the incident spectral irradiance. The integrated product of these two parameters gives the rate of photon absorption per unit chlorophyll. The yield is calculated from a non-linear regression of a photosynthesis–irradiance curve (also normalised to chlorophyll) to derive the initial slope, and the rate of photon absorption.[7] In natural aquatic

[6] Q_{10} is the change in activity of a reaction per 10 C change in temperature.

[7] Unlike in higher plant leaves, the initial slope of the photosynthesis–irradiance curve in unicellular algae is *not* directly proportional to the maximum quantum yield of photosynthesis. Because algal cells are

ecosystems, the values of the maximum yields are almost always significantly less than 0.10, often by up to an order of magnitude.

An alternative approach to assessing changes in quantum yield is based on the measurement of variable chlorophyll fluorescence (Kolber and Falkowski, 1993). Variable fluorescence measures the quantum yield of photochemistry and effective absorption cross section of Photosystem II, from where photosynthetic electron transport can be calculated. This approach is exceptionally attractive because it is highly sensitive, rapid, and can be determined continuously across large swaths of ocean (*e.g.*, Behrenfeld *et al.*, 1996). Measurements of the quantum yield of photochemistry of PSII should *not* be taken as direct reflections of the maximum quantum yield of carbon fixation, because the latter is strongly affected by non-photosynthetic pigment absorption, the photosynthetic quotient, photorespiration and cyclic electron flow around PSI and PSII, while the former is not. Nonetheless, changes in variable fluorescence, normalised to either the initial or maximal value, provide a diagnostic correlation that, together with physical and chemical environmental information, can be used to assess the factors controlling the maximum quantum yield of photochemistry in natural aquatic ecosystems. The results of several large-scale surveys (*e.g.* from North Africa to Canada, from Portsmouth, UK to the Falkland Islands, and from Tahiti to Hawaii) invariably reveal strong variations in the quantum yield of photochemistry. To a first order the variations are correlated with nutrient fluxes, suggesting that nutrient supplies not only limit photosynthetic biomass but also the photosynthetic energy conversion efficiency of that biomass (Falkowski and Kolber, 1995). The limitations appear to be manifested within the photosynthetic apparatus as reductions in specific proteins that are essential for energy transduction within the reaction centres, or, in the case of iron limitation, a loss of key prosthetic groups that are essential for energy transduction (*e.g.* cytochrome b_{559}: Vassiliev *et al.*, 1994).

6.5 Net primary production in the contemporary ocean

Net primary production (NPP) is defined as that fraction of photosynthetically produced carbon that is retained following all respiratory costs of the photoautotrophs

optically thin, they absorb relatively little of the incident light. Moreover, the fraction of light absorbed is highly variable as a consequence of pigment packaging effects (*e.g.* thylakoid stacking, plastid orientation, cell size, *etc.*). Hence, unravelling the optical absorption cross section in unicellular algae requires a significant investment in time and equipment.

and consequently is available to the next trophic level in the ecosystem. In aquatic ecosystems, NPP is generally estimated from rates of incorporation of ^{14}C-labelled inorganic carbon (supplied as bicarbonate) into organic matter. Alternative approaches include oxygen exchange in an enclosed volume (a technique which is far less sensitive and much more time-consuming) and variable fluorescence (which embraces a variety of approaches). Extrapolation of photosynthetic rates from any technique to the water column requires a mathematical model. The commonly used strategy is to derive an empirical relationship between photosynthesis and irradiance for a given phytoplankton or macrophyte community, and to integrate that (instantaneous) relationship over time and depth. Assuming some respiratory costs during the photoperiod and for the dark, this approach gives an estimate of NPP for the water column.

Analysis of thousands of vertical profiles of measured photosynthetic rates permits development of global carbon models based on statistical distribution rate measurements. By scaling the profiles to optical (as opposed to physical) depth, chlorophyll *a* as a proxy for photosynthetic biomass (as opposed to water mass volume), and accounting for day length, the shapes of the profiles converge to a common form that is readily described mathematically. The scaling factor for the curves is given by the time-integrated maximum photosynthetic rate, which is closely tied to, but is not precisely, the light-saturated rate (Behrenfeld and Falkowski, 1997a). Variance in the time-integrated maximum rates of photosynthesis in the water column is primarily influenced by temperature and nutrient supply as they affect the quantum yield of photosynthesis.

Using satellite-based images of ocean-water leaving radiances, it is possible to construct maps of the global distributions of phytoplankton chlorophyll based on the ratio of green to blue light. Briefly, in the absence of photosynthetic pigments in the upper ocean, radiances leaving water would appear blue to an observer in space. In the presence of chlorophylls, some of the blue radiation is absorbed in the upwelling stream of photons, thereby reducing the overall radiance. The fraction of blue light that is absorbed is proportional to the chlorophyll concentration. After accounting for scattering and absorption by the atmosphere, satellite-based maps of ocean colour can be used to infer the spatial and temporal variations of photosynthetic biomass, sea surface temperature and many other physical processes (*e.g.* wind stress on the sea surface, gravitational anomalies from sea height, cloud cover and incident solar radiation *etc.*).

Monthly, seasonal and annual maps of global ocean net primary production have been developed that incorporate a primary production model into global ocean colour images of phytoplankton chlorophyll. Figure 6.4 shows oceanic NPP for average

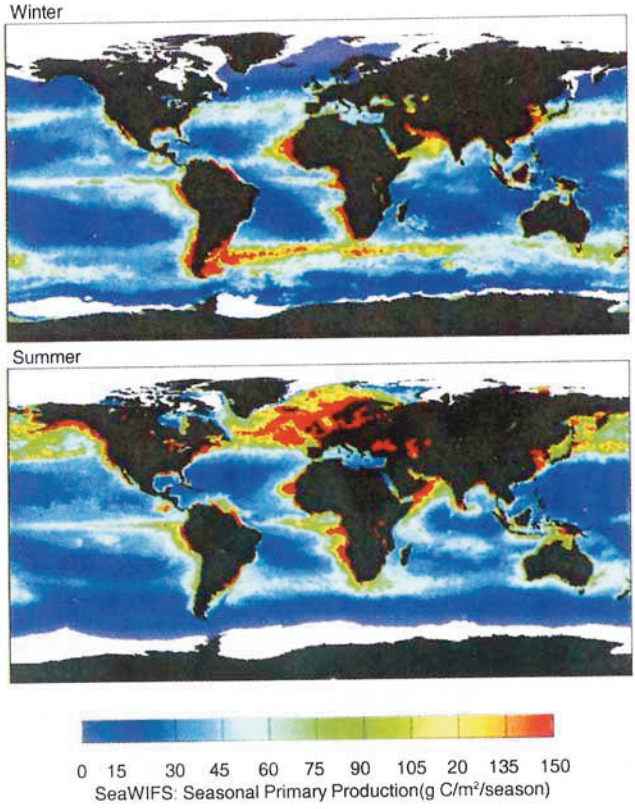


Figure 6.4 Average oceanic net primary production (NPP) in Northern Hemisphere winter and summer. SeaWIFs (*http://seawifs.gsfc.nasa.gov/SEAWIFS.html*) map generated at Rutgers University and Goddard Space Flight Center, using both an oceanic and terrestrial algorithm to calculate NPP.

(Northern Hemisphere) winter and summer. Such models suggest that annual net ocean photosynthetic carbon fixation is 45 +/- *ca.* 5 GtC per annum (Behrenfeld and Falkowski, 1997b). This productivity is driven by a photosynthetic biomass that amounts to ~1 GtC. Hence phytoplankton biomass in the oceans turns over on the order of once per week. In contrast, net primary production in terrestrial ecosystems accounts for approximately 52 GtC per annum, and is supported by a biomass of ~500 GtC (Behrenfeld and Falkowski, 1997a; Field *et al.*, 1998). Thus the turnover time of terrestrial plant biomass is on the order of a decade. Simply put, less than 1% of the photosynthetic biomass on Earth accounts for about 50% of the photosynthetic activity. Table 6.1 shows the NPP of the major components of the land and oceans.

Table 6.1 Annual and seasonal net primary production (NPP) of the major units of the biosphere (after Field *et al.*, 1998).

Ocean NPP		Land NPP	
Seasonal		**Seasonal**	
April–June	10.9	April–June	15.7
July–September	13.0	July–September	18.0
October–December	12.3	October–December	11.5
January–March	11.3	January–March	11.2
TOTAL	47.5	TOTAL	56.4
Biogeographic		**Biogeographic**	
Oligotrophic	11.0	Tropical rainforests	17.8
Mesotrophic	27.4	Broadleaf deciduous forests	1.5
Eutrophic	9.1	Broadleaf and needleleaf forests	3.1
Macrophytes	1.0	Needleleaf evergreen forests	3.1
		Needleleaf deciduous forest	1.4
		Savannas	16.8
		Perennial grasslands	2.4
		Broadleaf shrubs with bare soil	1.0
		Tundra	0.8
		Desert	0.5
		Cultivation	8.0
TOTAL	48.5	TOTAL	56.4

All values in GtC. Ocean colour data are averages from 1978 to 1983. The land vegetation index is from 1982 to 1990. Ocean NPP estimates are binned into three biogeographic categories on the basis of annual average C_{sat} for each satellite pixel, such that oligotrophic = $C_{sat} < 0.1$ mg m^{-3}, mesotrophic = $0.1 < C_{sat} < 1$ mg m^{-3}, and eutrophic = $C_{sat} > 1$ mg m^{-3} (Antoine *et al.*, 1996). This estimate includes a 1 GtC contribution from macroalgae (Smith, 1981). Differences in ocean NPP estimates between Behrenfeld and Falkowski (1997b) and those in the global annual NPP for the biosphere and this table result from (i) addition of Arctic and Antarctic monthly ice masks; (ii) correction of a rounding error in previous calculations of pixel area; and (iii) changes in the designation of the seasons to correspond with Falkowski *et al.* (1998). The macrophyte contribution to ocean production from the aforementioned is not included in the seasonal totals. The vegetation classes are those defined by DeFries and Townshend (1994).

6.6 Biogeochemical controls and consequences

From a purely chemical perspective, oxidation of Earth's atmosphere and ocean by photoautotrophs requires a net sink for the electron acceptor other than the carbon fixed by photosynthesis. Were this not so, the fixed carbon would be reoxidised in a respiratory pathway, leading to no net gain of oxygen. Hence, the very presence of oxygen in the atmosphere implies a sequestration of organic carbon, and it follows that the photosynthetic fixation of carbon cannot have been in a steady state on geological time scales. Calculations of the net organic carbon sequestered on geological time scales account not only for the net flux of oxygen to the atmosphere, but for the oxidation of lithospheric iron and sulphur. These calculations suggest that the lithosphere contains approximately 15×10^6 GtC, of which approximately 5×10^3 Gt is exploitable in the form of fossil fuel as organic carbon (Table 6.2). The vast majority of the sequestered organic carbon, which is locked up in sedimentary rocks (mainly shale), was produced as a result of marine photosynthesis. The accumulation of the dead, decaying bodies of these single-celled photoautotrophs over hundreds of millions of years led to the production of fossil oxygen in the Precambrian era, which permitted the extraordinary evolution and radiation of multicellular animals that followed.

The movement of organic carbon from the surface layers of the ocean to the deep sea and ocean sediments is called *export production*. Empirically, the fraction of primary production exported is a non-linear function of total production. The oxidation of organic carbon in the ocean interior leads to an increase in total inorganic carbon, such that the gradient between the atmosphere and oceans is inverted; that is, the concentration of inorganic carbon at depth is much higher than its equilibrium saturation value with the atmosphere. The sinking and oxidation of organic matter in the oceans is called the *biological pump* (Volk and Hoffert, 1985). The strength of the biological pump is primarily a function of nutrient fluxes: the higher the flux, the higher the export production. The export of cells is a function of cell size and motility (as these processes affect sinking rates), and some groups of marine photoautotrophs, such as diatoms, are exported much more efficiently than other groups, such as cyanobacteria.

The deposition of skeletal remains of marine photoautotrophs also plays a significant role in the physical geology of the lithosphere. Siliceous-rich sediments of the deep ocean basins are a consequence of the export flux of diatoms and radiolarians, whereas, carbonate-dominated sediments often represent the accumulation of coccolithophores and other carbonate-producing taxa. The global inventory of inorganic carbon (in the form of carbonates) in the lithosphere amounts

to about 60×10^6 GtC, of which virtually all was a by-product of marine photosynthetic organisms. Although this is by far the largest sink of carbon on Earth, locally carbonate deposition leads to an increased partial pressure of CO_2, due simply to mass balance of the reaction

$$2\,HCO_3^- + Ca^{2+} \leftrightarrow CaCO_3 + H_2O + CO_2$$

Over geological time, primary productivity and export fluxes in the oceans have waxed and waned, largely driven by those climatological processes that affect ocean circulation and nutrient availability. In recent geological time, the drawdown of atmospheric CO_2 during glacial periods appears to have been strongly influenced by enhancement of both total net primary production and the biological pump that takes carbon from the surface to the deep waters. The underlying causes for enhanced productivity and export fluxes remain contentious. However, a major contributing factor was probably an increased flux of iron to the oceans (Falkowski, 1997; Martin *et al.*, 1990). A major source of iron to the oceans is the aeolian (wind-blown) delivery of continental dust containing the element. Analyses of ice cores from both polar regions indicate that aeolian iron fluxes were approximately an order of magnitude greater during glacial periods than during interglacials. The enhanced iron would have directly stimulated primary production in high-nutrient, low-chlorophyll regions, especially the polar regions. In the Southern (Antarctic) Ocean, for example, the sediment record clearly shows an increased flux of diatoms during glacial periods. In low-latitude ocean gyres, enhanced iron fluxes would have stimulated N_2 fixation by supplying the limiting element for cyanobacteria in those regions that are presently limited by iron (*e.g.*, the South Pacific). In both cases, the effect of iron, on a geochemical level, would be to enhance the uptake of atmospheric CO_2 by enhancement of photosynthesis (Falkowski, 1997). The feedback between iron fluxes and carbon fixation in the oceans plays a major role in regulating CO_2 in the atmosphere.

The rich taxonomic diversity and geochemical role of marine photoautotrophs is the product of four billion years of co-evolution in the biological and physical realms of Earth, which have interacted to modify Earth's atmosphere, climate and physical geology. The oceans are a major sink for atmospheric CO_2; the mass of total inorganic carbon in the oceans is 3500 GtC, which is 50 times that in the atmosphere (Table 6.2). On time scales of decades to millennia, the oceans largely determine the atmospheric levels of CO_2, not *vice versa*. In the present geological epoch, human extraction and combustion of fossil fuels have replenished the atmosphere with CO_2 at rates that are unprecedented in the geological record. One can only speculate as to

Table 6.2 Carbon pools in the major reservoirs on Earth

Pools	Quantity ($\times 10^{15}$ g)
Atmosphere	720
Oceans	38,400
Total inorganic	37,000
Surface layer	670
Deep layer	36,730
Total inorganic	1,000
Dissolved organic carbon	600
Lithosphere	
Sedimentary carbonates	>60,000,000
Kerogens	15,000,000
Terrestrial biosphere (total)	2,000
Living biomass	600–1,000
Dead biomass	1,200
Aquatic biosphere	1–2
Fossil fuels	4130
Coal	3510
Oil	230
Gas	140
Other (peat)	250

Revised from Falkowski and Raven, 1997.

the consequences of these activities. Ultimately virtually all this carbon will be absorbed by the oceans. However, whatever the climatological effects, the enhanced atmospheric concentration of CO_2 will certainly lower the pH of the upper ocean by *ca.* 0.3 units by the middle of the 21st century, and probably reduce nutrient inputs to the upper ocean by increasing stratification (Sarmiento *et al.*, 1998). This analysis suggests that the anthropogenically enhanced CO_2 levels in the atmosphere will lead to a positive feedback in the ocean carbon cycle. In other words, less carbon will be absorbed by marine photoautotrophs and net primary production and export fluxes will decline. Such an effect has not been recorded in the geological record since the mid-Cretaceous, about 75 million years ago.

The potential for retrieving a fraction of the anthropogenically emitted CO_2 from the atmosphere by fertilising the oceans with nutrients has been examined. The purposeful addition of iron to both high-nutrient, low-chlorophyll regions and to iron-

limited regions of the oligotrophic oceans such as the South Pacific (Behrenfeld and Kolber, 1999) and the Southern Ocean (Boyd, 2000) suggests that iron addition leads to increased net primary production, but does not necessarily lead to an increase in carbon export. In the latter, stimulation of phytoplankton photosynthesis would be indirect, resulting from an enhancement of N_2 fixation (Falkowski, 1997). Simple box models suggest that iron fertilisation could reduce the atmospheric inventory of CO_2 by about 100 ppm over a century. Such a drawdown is a natural consequence of biogeochemical interactions on glacial-interglacial time scales. Both global climate change and purposeful manipulation of the carbon cycle by the deliberate addition of iron to the ocean will alter phytoplankton community structure and marine food webs in ways about which we can only speculate (Chisholm *et al.*, 2001; Falkowski *et al.*, 1998). The option of ocean fertilisation should be explored as one approach to sequestration and as a potential "emergency escape" from runaway CO_2 emissions in the coming millennia but wholesale experiments on the Earth's waters should not be viewed as a first kind of defence.

References

Anderson M. A. and Morel F. M. (1980), 'Uptake of Fe(II) by a diatom in oxic culture medium', *Marine Biol. Lett.* **1**, 263–268.

Antoine D., Andre J. M. and Morel A. (1996), 'Oceanic primary production. 2. Estimation at global scale from satellite (coastal zone color scanner) chlorophyll', *Global Biogeochem. Cycles* **10**, 57–69.

Behrenfeld M., Bale A., Kolber Z., Aiken J. and Falkowski P. (1996), 'Confirmation of iron limitation of phytoplankton photosynthesis in the equatorial Pacific', *Nature* **383**, 508–511.

Behrenfeld M. and Falkowski P. (1997a), 'A consumer's guide to phytoplankton productivity models', *Limnol. Oceanogr.* **42**, 1479–1491.

Behrenfeld M. J. and Falkowski P. G. (1997b), 'Photosynthetic rates derived from satellite-based chlorophyll concentration', *Limnol. Oceanogr.* **42**, 1–20.

Behrenfeld M. and Kolber Z. (1999), 'Widespread iron limitation of phytoplankton in the South Pacific Ocean', *Science* **283**, 840–843.

Behrenfeld M. J., Randerson J. T., McClain C. R., Feldman G. C., Los S. O., Tucker C. J., Falkowski P. G., Field C. B., Frouin R., Esaias W. E., Kolber D. D. and Pollack N. H. (2001), 'Biospheric primary production during an ENSO transition', *Science* **291**, 2594–2597.

Ben-Porath J., Carpenter E. J. and Zehr J. P. (1993), 'Genotypic relationships in Trichodesmium (Cyanophyceae) based on nifH sequence comparisons', *J. Phycol.* **29**, 806–810.

Berman-Frank I., Cullen J. T., Shaked Y., Sherrell R. M. and Falkowski P. G. (2001a), 'Iron availability, cellular iron quotas, and nitrogen fixation in Trichodesmium', *Limnol. Oceanogr.* **46**, 1249–1260.

Berman-Frank I., Lundgren P., Chen Y.B., Küpper H., Kolber Z., Bergman B. and Falkowski P. G. (2001b), 'Segregation of nitrogen fixation and oxygenic photosynthesis in the marine cyanobacterium *Trichodesmium*', *Science* **294**, 1534–1537.

Berner R. and Canfield D. (1989), 'A model for atmospheric oxygen over Phanerozoic time', *Am. J. Sci.* **289**, 333–361.

Bhattacharya D. and Medlin L. (1995), 'The phylogeny of plastids: a review based on comparison of small-subunit ribosomal RNA coding regions', *J. Phycol.* **31**, 489–498.

Bhattacharya D. and Medlin L. (1998), 'Algal phylogeny and the origin of land plants', *Plant Physiol.* **116**, 9–15.

Blankenship R. E. (1992), 'Origin and early evolution of photosynthesis', *Photosynth, Res.* **33**, 91–111.

Blankenship R. E. (2001), 'Molecular evidence for the evolution of photosynthesis', *Trends in Plant Sci.* **6**, 4–6.

Boyd P. W., Watson A. J., Law C. S. *et al.* (2000), 'A mesoscale phytoplankton bloom in the polar Southern Ocean stimulated by iron fertilization', *Nature* **407**, 695–702.

Brzezinski M. A., Villareal T. A. and Lipschultz F. (1998), 'Silica production and the contribution of diatoms to new and primary production in the central North Pacific', *Mar. Ecol. Prog. Ser.* **167**, 89–104.

Butler A. (1998), 'Acquisition and utilization of transition metal ions by marine organisms', *Science* **281**, 207–209.

Capone D., Zehr J., Paerl H., Bergman B. and Carpenter E. (1997), '*Trichodesmium*, a globally significant marine cyanobacterium', *Science* **276**, 1221–1229.

Chisholm S. W. (1992), 'Primary productivity and plankton size', in Falkowski P. G. and Woodhead A. D., eds., *Biogeochemical Cycles in the Sea*, Plenum. N.Y., pp. 213–237.

Chisholm S. W., Falkowski P. G. and Cullen J. J. (2001), 'Discrediting ocean fertilization', *Science* **294**, 309–310.

Coale K. H., Johnson K. S., Fitzwater S. E., Gordon R. M., Tanner S., Chavez F. P., Ferioli L., Sakamoto C., Rogers P., Millero F., Steinberg P., Nightingale P.,

Cooper D., Cochlan W. P., Landry M. R., Constantinou J., Rollwagen G., Trasvina A. and Kudela R. (1996), 'A massive phytoplankton bloom induced by an ecosystem-scale iron fertilization experiment in the equatorial Pacific Ocean', *Nature* **383**, 495–501.

Codispoti L. (1989), 'Phosphorus *vs.* nitrogen limitation of new and export production', in Berger W., Smetacek V., and Wefer G., eds., *Productivity of the Ocean: Present and Past*, John Wiley and Sons, New York, pp. 377–394.

Davison I. R. (1991), 'Environmental effects on algal photosynthesis: temperature', *J. Phycol.* **27**, 2–8.

Debus R. J. (1992), 'The manganese and calcium ions of photosynthetic oxygen evolution', *Biochim. Biophys. Acta* **1102**, 269–352.

DeFries R. S. and Townshend J. R. G. (1994), 'NDVI-derived land-cover classifications at a global scale', *Int. J. Remote Sens.* **15**, 3567.

Delwiche C. (1999), 'Tracing the thread of plastid diversity through the tapestry of life', *Am. Naturalist* **154**, S164–S177.

Des Marais D. J. (2000), 'When did photosynthesis emerge on Earth?', *Science* **289**,1703–1705.

Dismukes G., Klimov V., Baranov S., Kozolov Y. N., DasGupta J. and Tyrushkin A. (2001), 'The origin of atmospheric oxygen on Earth: the innovation of oxygenic photosynthesis', *Proc. Nat. Acad. Sci. USA* **98**, 2170–2175.

Durnford D. G. and Falkowski P. G. (1997), 'Chloroplast redox regulation of nuclear gene transcription during photoacclimation', *Photosynth. Res.* **53**, 229–241.

Eppley R. W. and Peterson B. J. (1979), 'Particulate organic matter flux and planktonic new production in the deep ocean', *Nature* **282**, 677–680.

Escoubas J.-M., Lomas M., LaRoche J. and Falkowski P. G. (1995), 'Light intensity regulation of *cab* gene transcription is signaled by the redox state of the plastoquinone pool', *Proc. Nat. Acad. Sci. USA* **92**, 10237–10241.

Falkowski P. G., Sukenik A. and Herzig R. (1989), 'Nitrogen limitation in *Isochrysis galbana* (Haptophyceae). II. Relative abundance of chloroplast proteins', *J. Phycol.* **25**, 471–478.

Falkowski P. G., Dubinsky Z., Muscatine L. and McCloskey L. (1993), 'Population control in symbiotic corals', *BioScience* **43**, 606–611.

Falkowski P. G. and Kolber Z. (1995), 'Variations in chlorophyll fluorescence yields in phytoplankton in the world oceans', *Austr. J. Plant Physiol.* **22**, 341–355.

Falkowski P. G. (1997), 'Evolution of the nitrogen cycle and its influence on the biological sequestration of CO_2 in the ocean', *Nature* **387**, 272–275.

Falkowski P. G. and Raven J. A. (1997), *Aquatic Photosynthesis,* Blackwell, Oxford, pp. 1–256.

Falkowski P., Barber R. and Smetacek V. (1998), 'Biogeochemical controls and feedbacks on ocean primary production', *Science* **281**, 200–206.

Feng D. G. C. and Doolittle R. (1997), 'Determining divergence times with a protein clock: update and reevaluation', *Proc. Nat. Acad. Sci. USA* **94**, 13028–13033.

Fensome R., Macrae R. A., Moldowan J. M., Taylor F. J. R., and Williams G. L. (1996),'The early Mesozoic radiation of dinoflagellates', *Paleobiol.* **22**, 329–338.

Field C., Behrenfeld M., Randerson J. and Falkowski P. (1998), 'Primary production of the biosphere: integrating terrestrial and oceanic components', *Science* **281**, 237–240.

Finkel Z. V. (2000), 'Light absorption and size scaling of light-limited metabolism in marine diatoms', *Limnol. Oceanogr.* **46**, 86–94.

Geider R. J., MacIntyre H. L., Graziano L. M. and McKay R. M. (1998), 'Responses of the photosynthetic apparatus of *Duniella tertiolecta* to nitrogen- and phosphorus limitation', *Euro. Phycol. J.* **33**, 315–322.

Graziano L., LaRoche J. and Geider R. (1996), 'Physiological responses to phosphorus limitation in batch and steady-state cultures of *Dunaliella tertiolecta* (Chlorophyta): a unique stress protein as an indicator of phosphate deficiency', *J. Phycol.* **32**, 825–838.

Harrison W. G., Harris L. R. and Irwin B. D. (1996), 'The kinetics of nitrogen utilization in the oceanic mixed layer: nitrate and ammonium interactions at nanomolar concentrations', *Limnol. Oceanogr.* **41**, 16–32.

Hobson L. A., Morris W. J. and Guest K. P. (1985), 'Varying photoperiod, ribulose 1,5-bisphosphate carboxylase/oxygenase and CO_2 uptake in *Thalassiosira fluviatilis* (Bacillariophyceae)', *Plant Physiol.* **79**, 833–837.

Holland H. D. (1984), *The Chemical Evolution of the Atmosphere and Oceans,* Princeton University Press, Princeton, N. J.

Holland H. and Rye R. (1998), 'Paleosols and the evolution of atmospheric oxygen: a critical review', *Am. J. Sci.* **298**, 621–672.

Hudson R. J. M. and Morel F. M. M. (1993), 'Trace metal transport by marine micro-organisms: implications of metal coordination kinetics', *Deep-Sea Research* **40**, 129–150.

Karl D. M. and Tien G. (1997), 'Temporal variability in dissolved phosphorus concentrations in the subtropical North Pacific Ocean', *Marine Chem.* **56**, 77–96.

Kasting J. F. (1990), 'Bolide impacts and the oxidation state of carbon in the Earth's early atmosphere', *Origins Life Evol. Biosphere* **20**, 199–231.

Kasting J. F. (1993), 'Earth's early atmosphere', *Science* **259**, 920–926.

Kirk J. T. O. (1992), 'The nature and measurement of the light environment in the ocean', in Falkowski P. G., ed., *Primary Productivity and Biogeochemical Cycles in the Sea*, Plenum Press, New York and London, pp. 9–29.

Knoll A. H. (1992), 'The early evolution of eukaryotes: a geological perspective', *Science* **256**, 622–627.

Kolber Z. and Falkowski P. G. (1993), 'Use of active fluorescence to estimate phytoplankton photosynthesis *in situ*', *Limnol. Oceanogr.* **38**, 1646–1665.

La Roche J., van der Staay G. W. M., Partensky F., Ducret F., Aebersold R., Li R., Golden S. S., Hiller R. G., Wrench P. M., Larkum A. W. D. and Green B. R. (1997), 'Independent evolution of the prochlorophyte and green plant chlorophyll *a/b* light-harvesting proteins', *Proc. Nat. Acad. Sci.* **9**, 1–16.

Larkum A. W. D. and Barrett J. (1983), 'Light-harvesting processes in algae', *Adv. Bot. Res.* **10**, 3–219.

Lee R. E. (1989), *Phycology*, Cambridge University Press, Cambridge.

Lewin R. A. (1993), *Origins of Plastids*, Chapman and Hall, New York.

Li W. K. W. (1980), 'Temperature adaptation in phytoplankton: cellular and photosynthetic characteristics', in Falkowski P. G., ed., *Primary Productivity in the Sea*, Plenum Press, New York, pp. 259–279.

Lipps J. H. (1993), *Fossil Prokaryotes and Protists*, Blackwell, Oxford.

Maranger R., Bird D. F. and Price N. M. (1998), 'Iron acquisition by photosynthetic marine phytoplankton from ingested bacteri', *Nature* **396**, 248–251.

Martin J. H. (1991), 'Iron, Liebig's Law and the greenhouse', *Oceanogr.* **4**, 52–55.

Martin J. H., Gordon R. M. and Fitzwater S. E. (1990), 'Iron in Antarctic waters, *Nature* **345**, 156–158.

Maxwell D. P., Falk S., Trick C. G. and Huner N. P. A. (1994), 'Growth at low temperature mimics high-light acclimation in *Chlorella vulgaris*', *Plant Physiol.* **105**, 535–543.

Mojzsis S., Harrison T. and Pidgeon R. (2001), 'Oxygen-isotope evidence from ancient zircons for liquid water at the Earth's surface 4,300 Myr ago', *Nature* **409**, 178–181.

Morel F. M. M., Hudson R. J. M. and Price N. M. (1991), 'Limitation of productivity by trace metals in the sea', *Limnol. Oceanogr.* **36**, 1742–1755.

Muladjanian A. Y. and Junge W. (1997), 'On the origin of photosynthesis as inferred from sequence analysis. A primordial UV-protector as common ancestor of reaction centres and antenna proteins', *Photosyn. Res.* **51**, 27–42.

Mulholland M. R., Glibert P. M., Berg G. M., van Heukelem L., Pantoja S. and Lee C. (1998), 'Extracellular amino acid oxidation by microplankton: a cross-ecosystem comparison', *Aq. Microbial. Ecol.* **15**, 141–152.

Ohmoto H. and Goldhaber M. (1997), 'Sulfur and carbon isotopes', in Barnes H. L., ed., *Geochemistry of Hydrothermal Ore Deposits*, John Wiley and Sons, New York, pp. 517–611.

Olson J. M. (2001), '*Evolution of Photosynthesis* (1970)', re-examined thirty years later', *Photosyn. Res.* **68**, 95–112.

Pace N. R. (1997), 'A molecular view of microbial diversity and the biosphere', *Science* **276**, 734–740.

Palenik B. and Hensen S. E. (1997), 'The use of amides and organic nitrogen sources by the phytoplankton *Emiliana huxeyi*', *Limnol. Oceanogr.* **42**, 1544–1551.

Raven J. A. (1986), 'Physiological consequences of extremely small size for autotrophic organisms in the sea', *Can. Bull. Fish. Aq. Sci.* **214**, 1–70.

Raven J. A. (1990), 'Predictions of Mn and Fe use efficiencies of phototrophic growth as a function of light availability for growth and of C assimilation pathway', *New Phytol.* **116**, 1–18.

Raven J. A. and Geider R. J. (1988), 'Temperature and algal growth', *New Phytol.* **110**, 441–461.

Robert F. (2001), 'The origin of water on Earth', *Science* **293**, 1056–1058.

Rosing M. T. (1999), '^{13}C-depleted carbon microparticles in >3700-MA sea-floor sedimentary rocks from West Greenland', *Science* **283**, 674–676.

Rue E. and Bruland K. W. (1995), 'Complexation of iron(III) by natural organic ligands in the central North Pacific determined by a new competitive equilibrium/absorptive cathodic stripping voltammetric method', *Mar. Chem.* **50**, 117–138.

Ryther J. H. (1969), 'Photosynthesis and fish production in the sea', *Science* **166**, 72–77.

Sarmiento J. L., Hughes T. M. C., Stouffer R. J. and Manabe S. (1998), 'Simulated response of the ocean carbon cycles to anthropogenic climate warming', *Nature* **393**, 245–249.

Scanlan D. J., Mann N. H. and Carr N. G. (1993), 'The response of the picoplanktonic marine cyanobacterium synechococcus species *WH7803* to phosphate starvation involves a protein homologous to the periplasmic phosphate-binding protein of *Escherichia coli*', *Mol. Microbiol.* **10**, 181–191.

Schopf J. (1993), 'Microfossils of the early Archean Apex Chert: new evidence of the antiquity of life', *Science* **260**, 640–646.

Shuter B. J., Thomas J. E., Taylor W. D. and Zimmerman A. M. (1983), 'Phenotypic correlates of genomic DNA content in unicellular eukaryotes and other cells', *Am. Naturalist* **122**, 26–44.

Smith S. V. (1981), 'Marine macrophytes as a global carbon sink', *Science* **211**, 838–840.

Soria-Dengg S. and Horstmann U. (1995), 'Ferrioxamines B and E as iron sources for the marine diatom *Phaeodactylum tricornutum*', *Mar. Ecol. Prog. Ser.* **127**, 269–277.

Sukenik A., Bennett J. and Falkowski P. G. (1987), 'Light-saturated photosystem limitation by electron transport or carbon fixation?', *Biochim. Biophys. Acta* **891**, 205–215.

Summons R., Jahnke L., Hope J. and Logan G. (1999), '2-Methylhopanoids as biomarkers for cyanobacterial oxygenic photosynthesis', *Nature* **400**, 55–557.

Sunda W. G. and Huntsman S. A. (1995), 'Iron uptake and growth limitation in oceanic and coastal phytoplankton', *Mar. Chem.* **50**, 189–206.

Sunda W. G. and Huntsman S. A. (1997), 'Interrelated influence of iron, light and cell size on marine phytoplankton growth', *Nature* **390**, 389–392.

Thingstad T. F., Zweifel U. and Rassoulzadegan F. (1998), 'P limitation of heterotrophic bacteria and phytoplankton in the northwest Mediterranean', *Limnol. Oceanogr.* **43**, 88–94.

Urbach E., Robertson D. C. and Chisholm S. W. (1992), 'Multiple evolutionary origins of prochlorophytes within the cyanobacterial radiation', *Nature* **355**, 267–270.

Vassiliev I. R., Prasil O., Wyman K. D., Kolber Z., Hanson Jr. A. K., Prentice J. E. and Falkowski P. G. (1994), 'Inhibition of PSII photochemistry by PAR and UV radiation in natural phytoplankton communities', *Photosyn. Res.* **42**, 51–64.

Villareal T. A., Altabet M. A. and Culver-Rymsza K. (1993), 'Nitrogen transport by vertically migrating diatom mats in the North Pacific Ocean', *Nature* **363**, 709–712.

Volk T. and Hoffert M. I. (1985), 'Ocean carbon pumps: analysis of relative strengths and efficiencies in ocean-driven atmospheric CO_2 exchanges', in Sunquist E. T. and Broeker W. S., eds., *The Carbon Cycle and Atmospheric CO_2: Natural Variations Archean to Present*, Vol. 32, American Geophysical Union, Washington D. C., pp. 99–110.

Wächtershauser G. (1990), 'Evolution of the first metabolic cycles', *Proc. Nat. Acad. Sci. USA* **87**, 200–204.

Wilhelm S. W. (1995), 'Ecology of iron-limited cyanobacteria—a review of physiological responses and implications for aquatic systems', *Aq. Microb. Ecol.* **9**, 295–303.

Woese C. R. (1987), 'Bacterial evolution', *Microbiol. Rev.* **51**, 221–271.

Wu J., Sunda W., Boyle E. A. and Karl D. M. (2000), 'Phosphate depletion in the western north Atlantic Ocean', *Science* **289**, 759–762.

Xiong J., Inoue K. and Bauer C. E. (1998), 'Tracking molecular evolution of photosynthesis by characterization of a major photosynthesis gene cluster from *Heliobacter mobilis*', *Proc. Nat. Acad. Sci. USA* **95**, 14851–14856.

This page intentionally left blank

CHAPTER 7

USEFUL PRODUCTS FROM ALGAL PHOTOSYNTHESIS

ROSA MARTÍNEZ
Departamento de Ciencias y Técnicas del Agua
and Departamento de Biología Molecular,
Universidad de Cantabria, 39005-Santander, Spain
martiner@unican.es
and
ZVY DUBINSKY
Department of Life Sciences,
Bar-Ilan University, 52900 Ramat Gan, Israel
dubinz@mail.biu.ac.il

"With what materials may the Sabbath lamp be lighted, and with what may it not be lighted? It may not be lighted with cedar-bast, nor with uncombed flax, nor with floss-silk, nor with willow-fibre, nor with nettle fibre, nor with water-weeds."

Talmud, Treatise Shabbath, Ch. ii:

7.1 Introduction

The conversion of virtually unlimited solar energy into valuable products such as chemicals, fuels or energy-rich food has attracted the attention of scientists and entrepreneurs for some time. Although photosynthetic conversion has been the cornerstone of agriculture since the chalcolithic material cultures of some ten thousand years ago, marine and freshwater algae have only rarely been considered in this context. Historically, algae have been harvested from the wild, and attempts to cultivate them have lagged considerably behind the farming of terrestrial plants (van der Meer, 1988).

Since the 1940s, however, the vastness of the oceans and the recognition of their productive potential have led to extensive basic and applied research into algal culture on both the laboratory and the mass scale. These efforts have resulted in many reports, symposia and books (Burlew, 1953; Shelef and Soeder, 1980; Richmond,

1986; Stadler *et al.*, 1988; Cresswell *et al.*, 1989; Akatsuka, 1990; Lee, 1994; Phang *et al.*, 1994). Moreover, algae have fired popular myth, cult and fancy, as amply demonstrated by the titles of some of the books devoted to their promotion: *Miracle Superfood ...*; *Algae to the Rescue ...*; *Chlorella, Jewel of the Far East ...*; *The Genesis Effect: Spearheading Regeneration with Wild Blue-Green Algae*; *Fifty Algae Stories of Hope, Health and Freedom* (Henrikson, 1989; McKeith, 1996; Abrahams, 1996, 1997; Cribbs, 1997; Jensen, 1992; Apsley, 1996; Moore, 1997).

Algae, like all plants, depend on photosynthesis, the process whereby light is captured and its energy transformed into chemical energy stored in reduced carbon compounds (Dubinsky *et al.*, 1995). Algae are responsible for about half of the total photosynthesis on Earth, and are therefore crucial in supporting the biosphere. They do not form a genetically homogeneous group of organisms—some are related to bacteria, but others are closer to higher plants (Radmer, 1996). The algal kingdom is as yet little explored. New species are still being identified at the rate of about one per week (Radmer and Parker, 1994). Approximately 36,000 currently known algal species comprise only 17% of the species that actually exist, and are therefore instrumental in supporting the biosphere (John 1994; Field *et al.*, 1998).

The use of algae as food and fertiliser by humans, although limited, goes back to antiquity. The harvesting of seaweeds for food and medicines and as a source of the useful gelatinous substance agar was common practice in ancient Chinese and Japanese cultures (Nisizawa and Oofusa, 1990). According to Nisizawa *et al.* (1987), the earliest archaeological record of seaweed phycophagy dates from the Jomon culture of Japan (10,000–400 BC). Kelp has been found in middens on the coast of Peru at a site dated around 2500 BC. The cyanobacterium *Spirulina maxima* found in Lake Texcoco was eaten by Mexican people in Aztec and probably earlier times as well, and *Spirulina platensis* from Lake Chad in Africa was also gathered as a native food (Aaronson, personal communication). Cyanobacteria have traditionally been used as agricultural fertilisers in rice paddies, mainly because of their ability to fix nitrogen, and macroalgae have been used as growth enhancers and nutrients (Dubey and Rai, 1995) and can also play an important role as soil conditioners in arid lands (Isichei, 1990).

In the last two decades, the algal culture industry has been growing alongside the aquaculture of other aquatic organisms. Aquaculture is presently one of the fastest growing food production systems in the world, with a growth rate of 9% per year. World trade in seafood is valued at more than US$ 100 billion per year. The major markets are in Japan (40 kg per capita per year), the USA (7 kg per capita per year) and the EU (17 kg per capita per year) (Aquaculture Production Technology, 2000). The increase in world demand is estimated at 0.5–3% per year, which means

additional annual requirements of 650×10^3 t in the EU and 250×10^3 t in the USA. This increasing demand can only be satisfied by aquaculture. Between 1998 and 1999, world fishery production by aquaculture increased from 30.8 to 33.3 million t, accounting for 26% of total fishery production. In addition, world fisheries produced 10.7 million tonnes of seaweeds (wet weight) in 1999, the largest part of which came from cultures (FAO, 2000). Algal production amounted to one-quarter of total aquaculture production by the end of the last decade, and its value was estimated as US $ 6 billion (FAO, 2000).

Figure 7.1 summarises the ways in which algae are grown and used commercially. Techniques for the production of commercial compounds range in sophistication, from the direct harvesting of algae from the wild with only a little low-technology post-harvest processing at one end of the spectrum, to the use of intensive-culture closed bioreactors with high-technology extraction of valuable products at the other (Radmer, 1996). Most algal aquaculture is aimed either at producing macroalgae for human food and polysaccharides, or at producing microalgae for feeding farmed molluscs, crustaceans and fish, or for the health food and pharmaceutical markets.

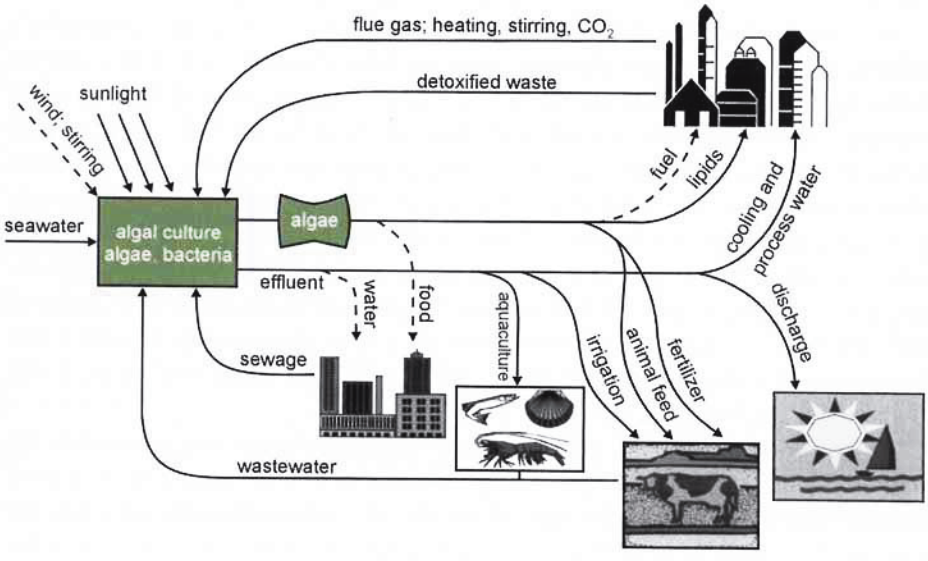


Figure 7.1 Diagram showing the main uses of algae. Algae convert the Sun's energy into biomass, through the uptake of nutrients present in the aquatic medium in which they live. This biomass can be used directly, or its many components can be extracted and used in the ways shown. Integrated systems combine the growth of algae for food or biologically active compounds with wastewater treatment.

7.2 Microalgae

Interest in microalgal mass production developed towards the end of the last century, after Beijerinck (1890) was able to obtain pure cultures of the green microalga *Chlorella vulgaris* on agar plates. Warburg (1910) started dense *Chlorella* cultures and used these in the study of algal physiology.

Microalgae lack the non-photosynthetic structures such as roots, flowers, bark, xylem and phloem that are inherent in terrestrial plants. This leaves them with little other than the photosynthetic apparatus, which results in efficient utilisation of solar energy and fast growth. They have a nutritionally rich cell composition, with protein levels in some species comparable to that of high-value foods like eggs, meat and fish. All these features make them promising candidates for overcoming any food shortage. Photosynthetic mass production of microalgae was first developed in Germany during World War II, when the lipids produced by diatoms under nitrogen-starved conditions were considered as a potential replacement for fossil oil (Harder and von Witsch, 1942).

In the late 1940s and 1950s, there was a boom in the study of microalgal mass culture, especially *Chlorella.* This was summarised in the Carnegie Institution's volume *Algal Culture from the Laboratory to Pilot Plant* (Burlew, 1953) and by Tamiya (1957): for reviews, see Shifrin (1984) and Soeder (1986). However, early optimism about extensive use of microalgae as food for humans was not well founded—it turned out that microalgae were mostly incompatible with traditional diets, especially in western countries, and production costs were not competitive with those of soybean protein (Tamiya, 1956). However, from the many physiological studies conducted with that end in view came a wealth of knowledge about the nutritional requirements, optimal light and temperature regimes and life cycles of microalgae, and this has subsequently been applied to improvement in culture and harvest systems (Richmond 1988, 1996; Hu *et al.*, 1996; Laing and Ayala, 1990; Gudin 1988; Poelman *et al.*, 1997).

In the period 1950–1960, mass cultures of microalgae were directed towards the commercial production of low-value substances (food, feed or energy) in open-pond systems. The limitations of this type of culture have been addressed, although not entirely solved, by the use of enclosed photobioreactors. In spite of these limitations, a trend towards improved productivity is evident. For instance, the global annual production of food-grade *Chlorella* in the last years is at least 1000 t, with retail prices often exceeding \$100 kg^{-1}, and the total market value is probably in excess of \$100m (Radmer, 1996).

Trends in microalgal technology changed in the 1970s and 1980s, with the focus shifting from the production of relatively cheap, readily grown biomass to that of cells rich in specific, high-value chemicals and pharmaceuticals (Dubinsky and Aaronson, 1982). Since the 1980s, high-quality food supplements and fine chemicals have become the most attractive large-scale microalgal products (Borowitzka, 1986; Cohen, 1986). Some of these products are already manufactured on a commercial scale, for example *Chlorella* and *Spirulina* pills, β-carotene from *Dunaliella*, phycobiliproteins from red algae and cyanobacteria, and compounds with stable or radio-labelled isotopes. Other products in an advanced experimental phase, at pilot-plant stage or under limited production are: antibiotics, toxins, astaxanthin and poly-unsaturated fatty acids (PUFA). The high cost of microalgal mass production is still the bottleneck for the establishment of competitive industrial plants. The problem stems mainly from our incomplete understanding of the physiology of algae in mass culture, since most of our knowledge stems from laboratory-scale cultures, usually grown under limiting light conditions. However, knowledge of algal physiology and molecular biology and understanding of biosynthetic pathways are constantly increasing, allowing researchers to aim at developing unique strains endowed with important properties such as overexpression of desired molecules, or expression of foreign genes through mutagenesis and other genetic manipulations (López-Alonso *et al.*, 1996; Semenenko, 1996; Grossman, 2000; Dent *et al.* 2001).

Microalgal aquaculture is much smaller in economic impact than seaweed cultivation but is the subject of much research. Presently, the main uses of microalgae are based either on the utilisation of whole algal cells or the extraction of cellular products. Table 7.1 lists the main products, uses, algal genera or species grown, together with information on product, culture system and market growth estimates. Four main types of use can be identified, and these are described below.

7.2.1 Aquaculture and animal feed

The main developments in the mass culture of microalgae have come about because of their role in the culture of commercially valuable fish and shellfish. Much research effort has been dedicated to the improvement of techniques for microalgal mass culture in this context (Laing and Ayala, 1990). Different algal species have different proportions of nutritional compounds such as protein, carbohydrate, lipids, vitamins and oligoelements. Marine microalgae are the only food for rearing bivalve molluscs such as mussels, and they are also used to feed rotifers and brine shrimp, which constitute the food of larval and juvenile fish and prawns (Mitchell, 1986).

Table 7.1 Sources and prices of the main microalgal products

Product	Uses	Source	Culture	Price ($/kg)	Market	Market value ($million)	Total production (tons/year)	Ref.
β-carotene	dietary supplement	*Dunaliella*	open pond	500	small			1
	pigment (chicken feed)	*Dunaliella*	open pond	300	medium			1
β-carotene	nutritional supplement	*Dunaliella,* synthetic	open pond	1,400 600		25 75		2
β-carotene	nutritional supplement	*Dunaliella*		1,100–2,500		15		3
xanthophylls	pigment (chicken feed)	greens, diatoms	open pond	200–500	medium			1
vitamin C	pharmaceutical	green algae	fermentor	>10	medium			1
vitamin C	pharmaceutical	*Chorella pyrenoidosa*	fermentor	no commercial				2
vitamin E	pharmaceutical	green algae	fermentor	>50	medium			1
algal biomass	health food	*Chlorella* *Spirulina*	lined pond	10–20	medium large			1
polysaccharides	gelling agents	*Porphyridium*	lined pond	5–10	large			1
algal biomass for aquaculture	seed rising feeding	diatoms *Chrysophyta*	open pond open pond	20–200 4–10	small large			1
SCP	animal feed	greens, other	open pond	0.3–0.5	very large			1
PUFA	food, feed health supplement	greens diatoms	open pond lined pond	0.4–0.6 3–30	very large small			1
PUFA	nutritional supplement	diatoms, *Chrysophyta*	closed reactor	1,000		25		3

Table 7.1 cont'd.

Product	**Uses**	**Source**	**Culture**	**Price ($/kg)**	**Market**	**Market value (million $)**	**Total production (tons year^{-1})**	**Ref.**
aminoacids: proline arginine aspartic acid	 pharmaceutical food supplement food supplement			 5–50 50–100 2–5				1
soil inoculum	conditioner fertiliser	*Chlamydomonas* N_2-fixing cyanobacteria	indoors lined pond	>100				
biomass (SCP)	health food	*Spirulina* *Chlorella*	open pond fermentor	100 100		80 100	>800 >1,000	2
isotopic compounds	growth media	several spp.				5		2
astaxanthin	pigment	mostly synthetic		1,600		25		3
antioxidants	food preservatives *etc.*	mostly synthetic		1,500		250		3

SCP = single-cell protein, PUFA = polyunsaturated fatty acids.

References: 1. Dubinsky (1980), unpublished report; 2. Benemann *et al.* (1987); 3. Radmer (1996).

The nutritional value of microalgal species as food for cultured animals is determined by their cell size and digestibility, toxicity and biochemical composition, as well as by the feeding behaviour of the animals (Yúfera and Lubián, 1990). (See de Pauw, 1981 for a review of the nutritive value of 43 microalgal species.) Riboflavin is an essential vitamin for farmed marine animals. Most microalgae used in mariculture are good sources of riboflavin, especially during their stationary growth phase (Brown and Farmer, 1994). *Chaetoceros gracilis* contains more riboflavin than any other diatom or Prymnesiophyta species tested (106 μg g^{-1}, versus 48 to 61 μg g^{-1} in other species). *Chlorella* and *Chlamydomonas* have a high protein content (Renaud *et al.*, 1994) and are an excellent food source for cladocerans (DeBiase *et al.*, 1990). Dry, protein-rich algal meal can be obtained as a main or secondary product of algal culture and used to feed fish or poultry. The dry algal product of *Dunaliella*, after extraction of the most valuable components (β-carotene and glycerol), is rich in protein (Ben-Amotz and Avron, 1980).

Cell content in fatty acids (FA), and especially the quality of these FA, is the most important nutritional factor in rearing molluscs (de Pauw *et al.*, 1984; Yúfera and Lubián, 1990). *Monochrysis lutheri* has a high food value due to its content in PUFA (poly-unsaturated fatty acids). It has been cultured at high density and fed to rotifers used in aquaculture, and to oysters (Spektorova *et al.*, 1986). However, several diatom species are better food for commercially important bivalves. Cultures of the PUFA-rich diatom *Skeletonema costatum,* grown at pH 8, had the maximum growth rates and biomass productivity, as well as the best nutritive quality (Sanchez *et al.*, 1995) with a protein content of 35%, and 17% total lipids, of which 9.2% were PUFA. Laing and Millican (1992) used *S. costatum* to supplement diets of dried *Tetraselmis suecica* powder as feed for these molluscs. Cultivation of *Isochrysis galbana* in open 100 m^2 ponds produced an average output rate of 23 g m^{-2} day^{-1} of biomass and 5.6 g m^{-2} day^{-1} of lipid (Boussiba *et al.*, 1988). The genus *Isochrysis* is rich in sterols, which have high nutritional value for cultured bivalve molluscs (Patterson *et al.*, 1994).

Outdoor mass cultures of *Nannochloropsis* sp. contained between 1.6 and 3.8% of the PUFA eicosapentaenoic acid (EPA) (Sukenik *et al.*, 1993). When large outdoor ponds (6000 m^2) were used, *Nannochloropsis* yielded between 7 and 20 g dw biomass m^{-2} day^{-1}, with a w/w EPA content of between 2% (summer) and 4% (winter) (Sukenik, 1992). The total content of FA, lipids and carbohydrate in *Nannochloropsis sp.* increases under light saturation as compared with light-limiting conditions, but the proportion of PUFA in the total FA decreases under high irradiance (Sukenik *et al.*, 1989). During the batch-culture growth cycle PUFA content decreases once the culture reaches the stationary phase (Hodgson *et al.*, 1991). Since cultured molluscs

have different nutritional requirements, and the nutritional value of different microalgal species is variable, mixed microalgal food is likely to provide more complete nutrition. New production systems for microalgae used in aquaculture are under way, including continuously run and closed loop reactors (Muller-Feuga, 2000).

7.2.2 Wastewater treatment systems

Algal waste ponds, also called oxidation or stabilisation ponds, are an effective mean of treating sewage effluent at the lowest possible cost. They perform best in climates with high solar irradiance and no persistent frost. Oxygen generated by microalgae supports and accelerates the aerobic catabolism of organic wastes by aerobic bacteria. The bacteria break down the incoming sewage and make its constituent nutrients available for algal growth. The microalgal biomass itself can be used as feed. The two main types of ponds currently in use are high-rate ponds (HRPs) and facultative ponds. HRPs employ mechanical stirring, are shallow (0.2–0.9 m) and remain aerobic throughout their depth. Facultative ponds are deeper (1.5–4 m) and have an aerobic and an anaerobic zone.

Developing countries often suffer from a shortage of high-quality food and poor sanitary conditions. The combination of wastewater treatment with microalgal mass culture and the use of algal biomass as feed, mostly in aquaculture of fish or other seafood would contribute to alleviate these problems (Oswald, 1980). In such multipurpose systems, the production costs are partly offset by the sewage treatment and partly by sale of effluent for restricted agricultural use. Human, plant and animal waste is treated in a closed digester and then fed to open *Spirulina* ponds where the algae take up the nutrients, and the quality of the effluent improves up to clean water level. The gas mixture from the digester contains methane that is used as a source of energy (biogas) and CO_2, which is added to the algal cultures, while the *Spirulina* can be used for feed and food (Fox, 1988). Integrated systems have been developed by Shelef *et al.* (1980), Edwards (1980), Lincoln and Hill (1980) and others, in which the dried algae are harvested and used to feed commercially grown freshwater fish or poultry. Reactor types and choices of algal species for wastewater treatment are reviewed in de la Noue *et al.* (1992). A treatment for recycling swine manure on a small scale (Sevrin-Reyssac, 1998) includes mass production of chlorococcal algae, zooplankton and fish, in a total area of 2100 m^2, at low cost and easy management.

Intensive semi-continuous cultures of *Scenedesmus* through biomass recirculation have been used in tertiary water treatment, with simultaneous production of usable biomass (de la Noue and Eidhin, 1987). *Scenedesmus* immobilised in polymeric

foams is used for nitrate removal from water (Urrutia *et al.*, 1995). Other species such as the cyanobacteria *Phormidium* sp. are also used for wastewater treatment, both in ponds (Talbot and de la Noue, 1988) and immobilised on chitosan or foam (de la Noue and Proulx, 1988; Garbisu *et al.*, 1991). Nutrient removal from sewage in open ponds is affected by algal cell density and also by their associated bacteria (Lau *et al.*, 1995; Baumgarten *et al.*, 1999). Obligate aerobic *Pseudomonas* spp. has a beneficial effect on the growth of *Scenedesmus* and *Chlorella* spp. growth, and this is attributed to both CO_2 supply to the algae and consumption of excess photosynthetic oxygen by the bacteria, thus preventing high oxygen concentrations that could inhibit photosynthesis (Muget *et al.*, 1995).

Recent research describes a combination of *Chlorella* and *Eichornia* (water hyacinth) to remove nitrogen from wastewater (Bich *et al.*, 1999). Treatment of recalcitrant anaerobic industrial effluent (from ethanol and citric acid production) using first the microalga *Chlorella vulgaris* followed by the macrophyte *Lemna minuscula* demonstrates the feasibility of combining microalgae and macrophytes for bioremediation of recalcitrant industrial wastewater (Valderrama *et al.*, in press). Co-immobilisation of *Chlorella vulgaris* in alginate beads with the microalgae growth-promoting bacterium *Azospirillum brasilense* under semi-continuous synthetic wastewater culture conditions significantly increased the removal of ammonium and soluble phosphorus ions compared with immobilisation of the microalgae alone (Bashan *et al.*, in press)

Many microalgal species are resistant to toxic metals and possess mechanisms for their accumulation and detoxification (Wood and Wang, 1983). These properties can be applied to the biological detoxification of these metals in natural waters and industrial effluents (Maeda and Sakaguchi, 1990). Cyanobacterial mats are grown in columns packed with glass wool, and used to remove zinc and manganese from contaminated waters (Bender *et al.*, 1994). Efficacy of metal bioremoval varies according to algal species and metal (Radway *et al.*, 2001). Organic pollutants, including chlorinated hydrocarbons, are also degraded by filamentous cyanobacteria (Kuritz and Wolk, 1994). Photosynthesis-enhanced biodegradation of toxic aromatic pollutants has been achieved in algal-bacterial microcosms (*Chlorella sorokiniana* and several bacterial species) in a one-stage treatment (Borde *et al.*, in press). Aromatic aldehydes are transformed into the corresponding primary alcohols by several microalgal species (Hook *et al.*, 1999).

The use of microalgal mass cultures in photobioreactors has been suggested as a key element in life-support systems designed to regenerate oxygen, absorb carbon dioxide and treat wastes in long-term manned space missions (Oswald, 1980; Lee and Palson, 1995; Morist *et al.*, 2001).

7.2.3 Health food for human consumption

The production of microalgal biomass, with its high content of macromolecules of high nutritious and health value, for the health food market, is increasing. Microalgae production plants operate mainly in Japan, Taiwan, Mexico, Israel, Thailand and the USA. Overall production of algal health food was about 2000 t yr^{-1} in the 1980s (Benemann *et al.*, 1987). The main species grown at present for that purpose are *Chlorella, Spirulina* and *Dunaliella*. Their production costs are high, ranging from \$10 to 20 kg^{-1}, with retail prices of over \$100 kg^{-1}. The high cost is due to the strict controls as to cell composition and absence of toxicity, which make it necessary to culture the algae under sterile conditions. Vonshak (1992) calculated and compared the costs of high-quality *Spirulina* cultures for human consumption versus cultures produced for animal feed. Table 7.2 shows his data.

Table 7.2 Comparison of major items of investment and production costs for high-value food-grade *Spirulina* (US site) and quality-feed *Spirulina* (Thailand site)

Item	**Cost (thousand US$)**	
	USA site	**Thailand site**
Investment costs		
Land preparation and development; site operation	138	112
Water and power network	257	58
Buildings (labs, offices, shops)	79	105
Nutrients: storage and stock	50	
Pond: including lining, pump, mixing	493	120
Harvesting: including filtering, drying, packing	541	76
TOTAL	1558	471
Annual production costs		
Manpower	320	24
Repair and maintenance	42	20
Fixed operating costs	163	44
Variable operating costs (gas, nutrients, power *etc.*)	123	70
Administration, capital and depreciation	390	90
TOTAL	1038	248

Source: Vohnshak, 1992.

Chlorella has been produced and consumed in significant amounts since the 1960s, mostly in the Far East. The *Chlorella* industry in Japan and Taiwan has developed successfully through advanced bioengineering and ingenious marketing. An annual production of 8.6 g dw m^{-2} day^{-1} was reported in the 1950s. In the 1980s, it amounted to 18–21 g dw m^{-2} day^{-1}, peaking to 27 g dw m^{-2} day^{-1} in the six most productive months (Borowitzka, 1986). World production capacity was estimated as 5150 t in 1999 (Lim, 1999). *Chlorella* is sold as manufactured health food items, such as pills and extracts, noodles, yoghurt and even honey, for which there is a well-established market, with mean retail prices of \$100 kg^{-1}. *Chlorella* for the health-food market is mostly produced mixotrophically. This process consists of two stages: first, the biomass is generated in a closed fermenter fed with glucose or acetate in the dark, and in a second stage, the algal cells are greened in open ponds or transparent plastic tubes exposed to light (Soong, 1980).

Spirulina is a filamentous cyanobacterium with a high protein content (60–70%) (Vonshak, 1992). This concentration is much higher than in other vegetable protein sources used as food, such as dry soybeans (35%). *Spirulina* also has a high content of β-carotene, vitamin B_{12}, thiamin and riboflavin, iron and other important oligo-elements, as well as essential fatty acids (Henrickson, 1989). This organism has a soft cell wall made of complex sugars and proteins, which makes it easily digestible, in contrast to most algae. Belay *et al.* (1993) summarised the potential health benefits of *Spirulina* as a food supplement, in a study that includes pre-clinical and clinical studies, in which the absence of toxicity was established. A recent study has shown that hot-water extracts of *Spirulina platensis* administered to humans activate the immune system by acting directly on myeloid lineages and either directly or indirectly on NK (natural killer) cells (Hirahashi *et al.*, 2002). Other therapeutic uses can be derived from the free radical scavengin capacity of *Spirulina* phycocyanobilin (Bhat and Madyastha, 2001).

Spirulina thrives under alkaline conditions that inhibit growth of other, undesired, organisms, making it possible to culture it by simple methods, in open ponds in a relatively pure form. Moreover, the tendency of the cells to float and clump together makes the harvest easy. Wu *et al.* (1993) cultured *S. platensis* on a large scale in seawater enriched with a commercial fertiliser containing $NaHCO_3$ and $FeSO_4$, producing a high-quality biomass rich in protein and amino acids, and at lower cost than the usual fresh-water cultures.

The USA leads world production of *Spirulina*, followed by Thailand, India and China. World production of *Spirulina* in 1998 was estimated as more than 2500 t (Borowitzka, 1999), mostly for human consumption, with retail prices of \$100 kg^{-1} or higher (Earthrise Co., 2000).

The green biflagellate *Dunaliella* (Volvocales) is characterised by a lack of a rigid polysaccharide cell wall, only having an elastic plasma membrane (Borowitzka and Borowitzka, 1988a). It thrives in hypersaline media and accumulates large amounts of β–carotene and glycerol. It is cultured in open ponds in which the extreme saline conditions prevent contamination by competing organisms. The two main β–carotene-accumulating species are *D. bardawil* and *D. salina*. Under appropriate cultivation, more than 10% of the dry weight of *D. bardawil* is β–carotene (Ben-Amotz and Avron, 1990), whereas the content in higher plant leaves and in other algae is around 0.3%. The annual world market for β–carotene is evaluated at US$150–250m per year, corresponding to a production of 300–400 t, of which the natural products account for about 10 %.

β–carotene protects the algal cell against photooxidative damage and plays a role in photosynthesis. High irradiance, combined with high salinity, low temperature and nitrogen or sulphur deficiency, induces its synthesis (Ben-Amotz and Avron, 1983). The β–carotene of *Dunaliella* is mainly a mixture of two stereoisomers: 9–*cis* and all-*trans*. The two stereoisomers have different physico-chemical as well as nutritional properties. All-*trans* β–carotene is water–soluble and crystallises easily, whereas 9–*cis* β–carotene is lipid-soluble and accumulates in the chloroplastic fat globules of *Dunaliella*. The synthetic β–carotene used in most commercial vitamin supplements is mostly the all-*trans* isomer. The commercialised product for the health food market is the dried alga, containing 3–5% β–carotene. It has found a lucrative market in the Far East and in some European countries.

The harvest of *Dunaliella* cells from the dilute culture medium is difficult and therefore expensive. They must be separated from large volumes of hypersaline solution without breaking the fragile wall-less cells. After harvest, the high-β-carotene biomass is processed by several different methods to obtain several products, ranging from crude algal biomass rich in carotene to concentrated purified carotene dissolved in vegetable oil. The high costs of the process make the algal β-carotene considerably more expensive than the synthetic product (Borowitzka, 1992). However, if the advantages of natural versus synthetic β–carotene are confirmed, and if the improvement already seen in the harvest techniques continues, this could be reversed in the near future.

Figure 7.2 shows a photograph of a commercial culture plant of the green alga *Dunaliella* in Eilat, Israel.

Figure 7.2 Production-scale ponds for the culture of the halophilic green alga *Dunaliella*. This organism is grown by the Japanese company NBT (Nature Beta Carotene) on the shores of the Gulf of Eilat, Northern Red Sea in Israel. The algae are harvested by centrifugation and dried, and then sold as a food supplement rich in β-carotene, mostly the essential 9–*cis* isomer.

7.2.4 *Specific products from microalgae*

There is an increasing interest in microalgae as producers of biochemical compounds for the medical, pharmaceutical and food industries. Large-scale algal cultures can be used for the production of both biomass and biochemical compounds (Dubinsky *et al.*, 1978; Aaronson *et al.*, 1980). The main products commercially obtained from microalgae, or in an advanced phase of research prior to their commercial exploitation, are carotenoids, phycobiliproteins, PUFA, polysaccharides, some new pharmaceuticals (such as anticancer drugs) and fuels. Less than ten microalgal species had been commercially exploited by the early 1990s (Tredici *et al.*, 1991).

The many examples found in the literature on environmentally-inducible changes in pigments, protein, carbohydrate, lipids *etc.* of microalgae indicate their plasticity and fast response to the environment, which open the way to optimisation of culture conditions to give maximal yields of these valuable products (Dubinsky, 1989).

Carotenoids The crucial role of carotenoids and their metabolites in protection against photoxidative damage and in photosynthesis in green plants, as well as in nutrition, vision and cellular differentiation in animals, make them an important class of biological pigments. Significant advances have been made in the last decade in the understanding of the genetics and molecular biology of carotenoid biosynthesis Armstrong and Hearst (1996). However, oxidising oxy-radicals (*e.g.* those associated with smoking or environmental pollution) convert carotenoids into their radical cations, with deleterious consequences. These risks are also related to high carotenoid dose intake (for a review, see Mortensen *et al.*, 2001).

One of the most advanced processes for the production of fine chemicals from algae is that of β–carotene from *Dunaliella bardawil* (see Section 7.2.3). Nutritional studies carried out in the 1980s (Ben-Amotz *et al.*, 1989) showed that animals absorb the *Dunaliella* stereoisomer mixture better than the synthetic (all-*trans*) β–carotene. Since β–carotene has been suggested to play a role in cancer prevention (Ziegler, 1989), the natural β–carotene from *Dunaliella* seems to be a better choice than the synthetic product. Studies on blood serum absorption suggest that 9–*cis* β–carotene acts as an *in-vivo* antioxidant more efficiently than does all-*trans* β–carotene (Ben-Amotz and Levy, 1996). Using the photoprotective activity of β–carotene as a selection criterion, several over-producing mutants have been obtained (Shaish *et al.*, 1991). The final product is an extract of β–carotene in edible oil, mostly used for colouring margarine and other food products and to replace synthetic β–carotene in 'all-natural' food items.

In the study of the mechanisms and kinetics of dietary carotenoid uptake, a labelled tracer is of great use, since the measurement of tracer enrichment allows the determination of absorption and clearance kinetics in the organism. Stable isotopes offer a safe means of labelling compounds for use in human metabolic studies. Labelled β–carotene by *Dunaliella salina* is easily produced by feeding the cultures with ^{13}C-labelled $NaHCO_3$ (Wilson *et al.*, 1997). Similar production of additional labelled dietary or pharmaceutical compounds from algae, for use in human metabolic studies, can be foreseen in the near future.

Pigments are important in the food industry as dyes for meat, fish and eggs. For instance, a rich egg yolk colour is regarded adding to quality and market value. Some algal species are rich in xanthophylls, the yellow pigments that can be added to poultry feed to give this colour to eggs. The green microalgae *Neospongiococcum* (Gellenbeck and Burrascano, 1988) and *Chlorella vulgaris* (Gouveia and Veloso, 1996) have been investigated for their ability to produce the xanthophyll lutein for poultry feed.

Astaxanthin is a ketocarotenoid found in *Chlamydomonas nivalis, Haematococcus pluvialis, Euglena rubida* and *Acetabularia mediterranaea*. It is the principal pigment responsible for the characteristic pink colour of salmon and is also used for colouring egg yolks. The worldwide increase in salmon farming has created an important market for astaxanthin. It has also been shown to be a more active antioxidant than β–carotene (Kobayashi *et al.*, 1991). Astaxanthin strongly inhibits lipoperoxidation, both *in vitro* and *in vivo*. This fact is probably due to the combination of its radical-scavenging capacity and its rigidifying effect on cell membrane (Barros *et al.*, 2001).

The biflagellate freshwater green microalga *Haematococcus pluvialis* is among the main biological sources of astaxanthin, owing to its ability to accumulate this pigment in large amounts in its aplanospores.[1] The alga grows best at high nitrate concentrations (0.5–1 g KNO_3 l^{-1}), intermediate phosphate concentration (0.1 g l^{-1}) and over a wide range of Fe concentrations. It can grow in the light (Boussiba and Vohnshak, 1991; Lee and Soh, 1991) or heterotrophically in the dark (Kobayashi *et al.*, 1992). Low nitrate or high phosphate induces the formation of the reddish palmella stage cells, and subsequently hard-walled aplanospores (Borowitzka *et al.*, 1991). Aplanospores of *H. pluvialis* contain 0.7% total carotenoids, of which 46% is (3S,3'S) astaxanthin monoester and 34% is (3S,3'S) astaxanthin diester (Grung *et al.*, 1992). These show an increase of respiration rate and a reduction in photosynthetic rate as compared with the flagellate, mobile cells (Zlotnik *et al.*, 1993) and impairment of the linear electron flow from photosystem II (PSII) to PSI (Tan *et al.*, 1995). Increasing partial pressure of dissolved oxygen contributes to astaxanthin accumulation (Lee and Ding, 1995).

Natural astaxanthin is expensive because outdoor cultures grow slowly and the the aplanospore wall must be disrupted to obtain the pigment (Johnson and An, 1991; Mendes-Pinto *et al.*, 2001). However, manipulation of *Haematococcus* cultures to create stress significantly increases the amount of astaxanthin produced. Phosphate or nitrate starvation, together with high irradiance, are the methods of choice (Fan *et al.*, 1994), by which the pigment forms up to 3% (w/w) of the algal biomass. The culture conditions required for optimal cell growth and astaxanthin accumulation in the alga are very different. Of particular importance is the photon flux: the optimum for growth is 50–60 μmol photons m^{-2} s^{-1}, whereas the optimum for astaxanthin synthesis is much higher, 1600 μmol photons m^{-2} s^{-1}. This suggests that these secondary carotenoids play a role in protecting chlorophyll and other photosynthetic pigments from oxidative damage (Rise *et al.*, 1994). The optimum temperature for astaxanthin

[1] An aplanospore is a nonmotile, asexual spore formed within a cell, the cell wall of which is distinct from that of the parent cell.

accumulation is 14–15 C (Harker *et al.*, 1995). The biochemical pathways of astaxanthin synthesis have been investigated (Fan *et al.*, 1995). The enzymes *β*–carotene ketolase and *β*–carotene hydroxylase, involved in astaxanthin biosynthesis of *H. Pluvialis*, have been isolated and identified (Kajiwara *et al.*, 1995; Linden, 1999).

Recent developments in bioreactor technology have increased *Hematococcus pluvialis* biomass and astaxanthin production, reaching 2.5% astaxanthin per dry weight of the alga (Olaizola, 2000). Astaxanthin can also accumulate in the flagellated state, which permits easier extraction (Hagen *et al.*, 2001). Further developments have been made through sequential heterotrophic-photoautotropic culture of *Chlorococcum* with a yield of 32 mg astaxanthin l^{-1} (Zhang and Lee, 2001). Other algal groups have been studied for their capacity of synthesising a whole array of commercially valuable pigments. Two *Nannochloropsis* species are able to produce chlorophyll *a*, zeaxanthin, cantaxanthin and astaxanthin, each with production levels that depended on salinity, temperature and irradiance, attaining 0.6–0.7% dry weight (Lubián *et al.*, 2000). The technological improvements of the last decade make natural astaxanthin production commercially viable and able to compete with the synthetic product.

Phycobiliproteins Phycobiliproteins are naturally occurring, pigmented proteins that have unique fluorescent characteristics. They are major components of the light-harvesting antennae of cyanobacteria and some algal taxa, such as cyanobacteria (Bryant, 1981), rhodophyta (Gant, 1989) and cryptomonad algae (MacColl and Guard-Friar, 1987; Rowan, 1989). In cyanobacteria and rhodophyta, phycobiliproteins are incorporated into supramolecular complexes called phycobilisomes, whose function is to extend the wavelength range of light that can be used to drive photosynthesis beyond that absorbed by chlorophyll *a*.

Phycobilisomes consist of many subunits, both pigmented and colourless. Their molecular organisation and internal pigment ratio varies with both the species and its exposure to different light quality and intensity (Falkowski and LaRoche, 1991). The photons absorbed by phycobiliproteins are transferred to PSII, which then provides energy for the production of ATP and reducing equivalents. Efficient energy transfer breaks down when the pigments are removed from the phycobilisome. The isolated pigments then absorb light and fluoresce at characteristic wavelengths, which makes them valuable as fluorescent markers. Conjugation to antibodies, biotin, latex spheres and other proteins make phycobiliproteins very useful for such applications as immunohistochemistry and flow cytometry (Stryer *et al.*, 1985; Stryer and Glazer, 1989). Phycoerythrin, for instance, is used in cancer diagnosis by flow cytometry (Corver *et al.*, 1994; Simpson *et al.*, 1995).

Some algal species are an excellent source of one or more phycobiliproteins. Phycocyanin and allophycocyanin have been isolated from *Spirulina platensis* (Kageyama *et al.*, 1994; Brejc *et al.*, 1995). C-Phycocyanin (from *Spirulina platensis*) effectively inhibits lipid peroxidation in rat liver *in vivo* (Bhat *et al.*, 2000). The covalently linked chromophore phycocyanobilin is involved in the phycocyanin antioxidant and radical scavenging activity. In several heterocystous nitrogen-fixing bacteria of the genera *Anabaena* and *Nostoc*, phycobiliproteins typically account for about 50% of total cell protein. The prevalent pigment is phycocyanin, but in some strains of *Nostoc,* phycoerythrin is the major pigment (Moreno *et al.*, 1994). The marine unicellular cyanobacterium *Synechococcus* found in oligotrophic parts of the open ocean in great abundance (Olson *et al.*, 1991) is an important producer of phycoerythrins, but it has not yet started to be cultured on a large scale.

Phycobiliproteins are presently produced on a commercial level from several microalgal species. R-phycocyanin and allophycocyanin and B–phycoerythrin are extracted from the unicellular red alga *Porphyridium cruentum*, allophycocianin and C–phycocyanin from *Spirulina platensis* (Martek Biosci. Corp., 1997). B-phycoerythrin and R-phycocyanin can be obtained in the native state from *P. cruentum* and purified by an inexpensive process, which can be scaled up by a factor of 13 to a large preparative level by column chromatography, with an overall yield 32.7% of B-PE and 11.9% of R-PC (Bermejo *et al.*, 2002).

Polyunsaturated Fatty Acids (PUFA) Algal lipids and their constituent fatty acids are compounds of special commercial interest (further discussed in Section 7.2.1). Many microalgal species have a high content of PUFA, which are of pharmaceutical and nutritional value. The most important PUFA for human nutrition are eicosapentanoic acid (EPA), arachidonic acid (AA) and docosahexanoic acid (DHA). EPA is effective in blood cholesterol reduction (Kromhaut *et al.*, 1986). DHA and AA are important structural fatty acids in the grey matter of the brain and are recommended for infant and maternal supplementation due to their link to neurological development (Maurage *et al.*, 1998). Humans synthesise only small amounts of these PUFA and therefore have to obtain them primarily from their diet.

There is a high content of PUFA in fish oil, the traditional dietary source for these compounds. However, fish do not synthesise PUFA, but obtain them directly or indirectly through the food web, from algae. Growth of cod larvae fed the copepod *Acartia* was directly related to the content of DHA in the microalgae eaten by the *Acartia* parental generation (St John *et al.*, 2001). An alternative strategy for extracting PUFA from fish is therefore to culture these marine algae and extract the fatty acids directly from them. Many microalgal species, especially diatoms, are rich

in PUFA (see also Section 7.2.1). PUFA comprises 23% of total FA in *Nitzschia frustulum* (Renaud *et al.*, 1994), 21% in *Tetraselmis suecica*, 17% in *Porphyridium cruentum* and 17% in *Isochrysis galbana* (Servel *et al.*, 1994). EPA content in *Phaeodactylum tricornutum* is 40% of total FA. The nitrogen source affects the content and composition of the PUFA. In *I. Galban*, PUFAs reached 62.3% of total fatty acids in the early stationary phase of cells grown in urea, as opposed to 48.6% and 51.5%, obtained in nitrate and nitrite media, respectively, in the same phase. The highest content of EPA (55.5 mg per g dw and 27.7% of total fatty acids) and DHA (28.4 mg per g dw and 14.1% of total fatty acids) was obtained in urea cultures at the early stationary phase (Fidalgo *et al.*, 1998). DHA is the predominant PUFA of the marine heterotrophic dinoflagellate *Crypthecodinium cohnii*, comprising 30–50% of its constituent fatty acids. It is easy to separate DHA from the fatty acid mixtures. For this reason, *C. cohnii* represents a promising microalga for the commercial production of DHA (Jiang *et al.*, 1999). Heterotrophic cultures of *C. cohnii* on acetic acid, in a pH-auxostat, are one of the most productive DHA sources, resulting in up to 4.4 g l^{-1}, which represents 26% of algal dry weight (Ratledge *et al.*, 2001).

Table 7.3 shows the FA content of some microalgal species. Enhanced production of EPA or AA by *Porphyridium* cultures is achieved by varying the culture conditions (Cohen, 1990; Klyachko *et al.*, 1994). High EPA content is obtained at high growth rate, whereas AA content is maximal near the stationary phase. The freshwater chlorophyta *Parietochloris incisa* is the richest known plant source of AA (Bigogno *et al.*, 2002). EPA production of the diatom *Phaedactylum tricornutum*, grown semicontinuously in a helical tubular photobioreactor, increased with irradiance (peaking at 1700 μmol photons m^{-2} s^{-1}) and high cell density, with addition of CO_2 (Chrismadha and Borowitzka, 1994). Nannochloropsis grown in outdoor closed reactors produced 0.5–0.76 g dw l^{-1} day^{-1} with an EPA content of 18–32 mg l^{-1} day^{-1} (Chini Zitelli *et al.*, 1999). PUFAs may be extracted and separated by solvent partition (Yongmanitchai and Ward, 1993).

Temperature is an important factor in PUFA content. The ratio of PUFA to total FA in tropical *Isochrysis* and *Nitschia* species is higher if they are cultured at 10–20 C rather than their normal ambient temperatures (Renaud *et al.*, 1995). Low temperature also increases the DHA content of *I. Galbana* (Burgess *et al.*, 1993), by increasing membrane fluidity and lowering the freezing point. In general, the fraction of PUFA in the total FA is higher at lower temperatures.

In order to reach high enough contents of PUFA to allow economic production from algae in the future, we have to rely on combining the choice of best species and selection for the most productive strains with the optimisation of light, temperature and nutrient regimes in the cultures. Closed photobioreactors ensure that cultures are

Table 7.3 EPA and AA content of several microalgal species

Species	PUFA content (% dry weight)		Comments	Reference
	EPA	AA		
Porphyridium cruentum	1.22	0.87	batch with CO_2, 25 C, log phase	Klyachko *et al.* (1994)
P. cruentum	0.22	1.19	*idem*, stationary phase	*idem*
P. aerugineum	3.08	0.75	*idem*, log phase	*idem*
P. aerugineum	1.68	0.96	*idem*, stationary phase	*idem*
P. cruentum 1380–1c	2.10	0.90	*idem*, log phase	Cohen (1990)
P. cruentum 1380–1c	1.50	1.30	*idem*, stationary phase	*idem*
P.cruentum 1380–1e	2.10	1.00	*idem*, log phase	*idem*
P.cruentum 1380–1e	0.90	1.90	*idem*, stationary phase	*idem*
P. cruentum	2.20–2.30	0.73–0.76	open pond, winter	Cohen *et al.* (1988)
P. cruentum	1.00–1.28	0.70–1.30	open pond, summer	*idem*
P. cruentum	5.30	5.40	batch, young cells	Thepenier *et al.* (1988)
Chlorella minutissima	3.18		*idem*	Seto *et al.* (1984)
Isochrysis galbana ALII4	4.65		indoor, continuous CO_2	Molina *et al.* (1994b)
Phaeodactylum tricornutum WT	1.87		*idem*	Molina *et al* (1994a)
P. tricornutum WT	1.73		batch culture	Lopez-Alonso *et al.* (1996)
P. tricornutum II242	3.86		mutated, batch culture	*idem*
Nannochloropsis sp.	1.60		open pond, summer	Sukenik *et al.* (1993)
Nannochloropsis sp.	3.80		open pond, winter	*idem*

free from undesired parasites or predators. Improved understanding of biosynthetic pathways (intermediates and enzymes) and enhancement by genetic engineering are under way. The synthesis of FA in *Nannochloropsis* has been investigated by labelling cells *in vivo* with ^{14}C bicarbonate or acetate (Schneider and Roessler, 1994). Shiran *et al.* (1996) fed algal cultures with exogenously supplied fatty acid intermediates to find the pathways of EPA biosynthesis in *Porphyridium cruentum*. Bigogno *et al.* (2002) labelled *Parietochloris incisa* with ^{14}C-AA to investigate the

role of this PUFA in cold-stress response. Induced mutagenesis has proved an aditional useful way to increase the concentration of EPA in *Phaeodactylum tricornutum* (Lopez-Alonso *et al.*, 1996).

Polysaccharides Red unicellular algae such as *Porphyridium cruentum* are rich in sulphated polysaccharides (Arad, 1988; Dubinsky *et al.*, 1988). The cells are encapsulated in an envelope consisting of amorphous sulphated polysaccharides, the external part of which dissolves in the medium. Polysaccharide content per cell increases during the stationary growth phase, especially in its cell-wall-bound fraction. Polysaccharide production increases with increasing irradiance (Friedman *et al.*, 1991). Sulphated polysaccharides from *P. cruentum* possess antiviral activity (Huleihel *et al.*, 2001).

Continuous cultures of *P. cruentum* grown in tubular bioreactors can be harvested by centrifugation or filtration; the supernatant or the filtrate contains the extracellular polysaccharides (Chaumont *et al.*, 1988). With this system, a biomass production of 20 g m^{-2} day^{-1} has been achieved, giving an energy conversion yield of 2% and a polysaccharide production of 0.6 to 0.9 g l^{-1}.

The halophilic cyanobacterium *Aphanocapsa halophytia* produces large amounts of sulphated exopolysaccharide (Sudo *et al.*, 1995). Research towards commercial production of sulphated polysaccharides from *Porphyridium cruentum* has been summarised by Arad (1994). Exopolysaccharides from cyanobacteria show promise as thickening, emulsifying or cation-chelating compounds, and the residual biomass is effective in the recovery of heavy metals (de Philippis *et al.*, 2001).

Other fine chemicals and pharmaceuticals In recent years, there has been a great surge of interest in microalgae as culturable sources of biomedically useful compounds, other than those already mentioned above, in recognition of the fact that microalgae are producers of important compounds, albeit often in minute concentrations. In spite of this potential, culturing the desired species and/or obtaining a sufficient amount of the target product have often proved to be difficult.

The assimilation of atmospheric nitrogen into organic cell constituents by nitrogen-fixing cyanobacteria provides the basis for the production of various high value N-containing metabolites by these organisms. A range of amino acid-releasing strains has been obtained by mutation (Kerby *et al.*, 1988). The amino acids are released from the free-living or immobilised cyanobacteria following osmotic stress. Immobilisation in Ca-alginate beads and continuous amino acid production is sustained for over 500 hours (Kerby *et al.*, 1987).

Large-scale semicontinuous monocultures of *Euglena gracilis* have been successfully used for the production of pure specific proteins (Schwartz *et al.*, 1995). Tryptophan synthase was obtained by this method, although with low yield (20%).

The *Spirulina platensis* strain *pacifica* has been shown to be a source of restriction–modification enzymes. Four of these enzymes have been identified in the soluble-protein fraction of this cyanobacterium (Tragut *et al.*, 1995). Their advantage over bacterial restriction enzymes is that they are highly active at 37 C, compared with 65 C for the bacterial isoschisomer. In the last decade, the screening of microalgae, and especially cyanobacteria, for antibiotics and pharmacologically active compounds has received increasing interest (Borowitzka, 1994). Microalgae are attractive as natural sources of bioactive molecules since their cultures can produce structurally complex molecules that are difficult or impossible to synthesise chemically. Chrysophyta species, especially *Chaetoceros, Nitzschia* and *Thalassiosira*, are known for their antibacterial activity (Pesando, 1990). Methanol extracts of *Chlorococcum* strain HS–101 showed a strong antibiotic effect on a *Staphylococcus aureus* strain that is resistant to other antibiotics. The effect is due to alpha-linolenic acid (Ohta *et al.*, 1995). Immobilised cultures of the cyanobacterium *Scytonema sp.* in a photobioreactor produce a broad-spectrum antibiotic (Chetsumon *et al.*, 1994). Recent research on cyanobacteria has resulted in the discovery of more than thirty antibiotic substances (Skulberg, 2000).

Several microalgal species have been studied *in vitro* or *in vivo* as possible inhibitors of carcinogenesis. The cyanobacterium *Spirulina* has an inhibitory effect on oral carcinogenesis, as shown by studies in India with pan tobacco chewers (Mathew *et al.*, 1995). This action was not associated with increased serum concentration of either retinol or β-carotene. Inhibition of tumour cells *in vitro*, or cytotoxicity, is shown by novel cytotoxins (scytophycines) extracted from cyanobacteria (Patterson *et al.*, 1994a), mostly in renal and central nervous system cancers. Welwitindolinones are a family of novel alkaloids recently isolated from the cyanobacterium *Hapalosiphon weltwitschii*. Welwitindolinone C isothiocyanate, termed welwistatine, has been shown to be an antimitotic in human ovarian carcinoma cells. Its mode of action is through inhibition of tubulin polymerisation, which circumvents multiple drug resistance (Zhang and Smith, 1996). Some microalgal lipids act by interfering with enzymes involved in signal transduction (Gerwick *et al.*, 1993; Hasui *et al.*, 1995). Extracellular sulphated polysaccharides of some marine microalgae are active against HIV and other enveloped viruses. Other compounds that constitute good candidates in this field are some cyanobacterial sulpholipids, indolocarbazoles and β-carbolines (Patterson *et al.*, 1994).

Algal toxins are extracted and purified to make pure standards for assays of toxins in toxic algal blooms and seafood, as well in physiological studies. To cite only a few examples, the neurotoxin saxitoxin and its derivatives from some dinoflagellates are inhibitors of cell sodium channels, and are used in studies of the function and regulation of these channels (Yanagita *et al.*, 2000, Yotsu-Yamashita *et al.*, 2000). Domoic acid from some diatoms produces excitotoxic effects on animals and is used in studies of epilepsy (Fujita, 1996) and neuroprotection (Azcoitia *et al.*, 2001).

Fuels *Botryococcus braunii* is a species known to be rich in hydrocarbons (Metzger, 1985), especially in the resting stage, in which hydrocarbons can reach up to 90% of its dry weight (Largeau *et al.*, 1980). Immobilised culture techniques have been applied to extract hydrocarbons from this species. Largeau *et al.* (1988) achieved a recovery of 32% of the total cell hydrocarbon content, using hexane as extractant. The advantage of this method is that it is non-toxic for the cells, so these remain viable and retain their photosynthetic activity. The optimum yield was obtained in batch cultures, with 0.4% CO_2-enriched aeration, at 26 C and 16 W m^{-2} white fluorescent light (Singh and Kumar, 1994).

Newly isolated Japanese strains of *Botryococcus braunii* showed a botryococcene composition that differed from that formerly known from the Darwin and Berkeley strains (Okada *et al.*, 1995). *B. Braunii* can be grown on secondarily treated sewage in a continuous bioreactor system (Sawayama *et al.*, 1994), giving a hydrocarbon yield of 49% of the dry weight.

Heterotrophycally cultured *Chlorella protothecoides* exhibits yellowing of cells, chlorophyll disappearance, decreasing protein and lipid accumulation. When such cells were thermally degraded at 300 C, the yield of aliphatic hydrocarbons was 32 times higher than that obtained from green cells (Wu *et al.*, 1994). A maximum yiled of 52% was achieved at 500 C for 5 minutes (Peng *et al.*, 2000).

Glycerol is an important commercial organic chemical, mostly produced from tallow and petrochemical sources. The secondary metabolite glycerol is the osmo-regulatory-compatible solute in the halotolerant green alga *Dunaliella*. Depending on the salt concentration in the medium, more than 50% of the alga dry weight can be glycerol (Ben-Amotz and Avron, 1990). A mutant of *Dunaliella parva* has been found to release high amounts of glycerol into the medium (Gilmour and Hard, 1990; Hard and Gilmour, 1991). Extraction of glycerol from *Dunaliella terctiolecta* has been achieved by direct thermochemical liquefaction at around 300 C and 10 MPa (Minowa *et al.*, 1995), with a 75% yield. The calorific value of this glycerol is similar to that of fuel oil.

Hydrogen production has been a subject of applied research on and off for the three last decades, prompted by the oil crisis in 1973. Hydrogen gas can be sustainably generated, does not evolve the greenhouse gas CO_2 in combustion, liberates large amounts of energy per unit weight in combustion and is easily converted to electricity by fuel cells. Hydrogenase and nitrogenase enzymes are both capable of hydrogen production, enabling a few groups of microalgae and cyanobacteria under certain growth conditions to consume biochemical or light energy and produce molecular hydrogen instead of reducing CO_2. Greenbaum *et al.* (1995) reported very high (10–20%) efficiencies of light conversion to hydrogen, based on PAR (photosynthetically active radiation). For a review, see Ghirardi *et al.* (2000). Hydrogenases have been purified and partially characterised in a few cyanobacteria and microalgae (Schulz, 1996). Biological hydrogen production is now receiving much attention worldwide as an environmentally acceptable technology, and Boichenko, Greenbaum and Seibert discuss this in detail in the next chapter.

Production through genetic techniques The genetic tools useful for improving the production of target molecules from microalgae range from the classical induction of mutagenesis to gene manipulation to obtain overexpression or expression of foreign genes. Mutagenesis, induced by chemicals or UV radiation, has been used to obtain mutants that overproduce ammonium (Thomas *et al.*, 1991), β-carotene (Pick, 1992) and EPA (Lopez-Alonso *et al.*, 1996). Expression of foreign genes in microalgae and cyanobacteria, or overexpression of their own genes, are attractive possibilities for obtaining useful products.

Cyanobacteria are very appropriate candidates for biotechnological exploitation. In evolutionary terms, they are conventional bacteria, to which most genetic tools devised for manipulating conventional laboratory bacteria can be applied. However, there is currently little biotechnological exploitation of the knowledge of cyanobacterial genetics on a mass production level, as compared with biotechnological achievements on bacterial products (for example amino acid-excreting *Bacillus*: see Elhai, 1994). To give just a few examples, these include site-directed mutagenesis (Williams, 1988), mutations tagged with transposons, and fusion of the regulatory region of one gene with an open reading frame of another gene (Elhai and Wolk, 1990; Wolk *et al.*, 1991; Semenenko, 1996). Manipulation of cyanobacterial genomes is well established; for a review, see Thiel (1994) and references therein. A dechlorination operon from *Arthrobacter* has been expressed in an *Anabaena* and a *Nostoc* strain (Kuritz and Wolk, 1994). These transformants can be useful for decomposing chlorinated hydrocarbons in natural aquatic ecosystems. Several transformant strains of *Anacystis nidulans* overexpress a human gene encoding CuZn

superoxide dismutase (Takeshima *et al.*, 1993) and strain R2SPC overexpresses desaturase of 18:1 fatty acid, which increases chilling tolerance (Wada *et al.*, 1990). *Synechococcus* transformants overexpressing heat-shock proteins increase their thermal resistance (Nakamoto *et al.*, 2000). Foreign genes can be expressed in *Spirulina* subjected to electroporation (Toyomizu *et al.*, 2001).

Progress has also been made in transforming the DNA of eukaryotic algae. Several methods are in use for delivering cloned DNA into algal cells: high velocity bombardment of tissues or cells with DNA-coated microparticles, microinjection, electroporation of cells or protoplast suspensions and agitation of wall-deficient cells with DNA and silicon carbide whiskers (Stevens and Purton, 1997). Transformation of the nuclear, chloroplast and mitochondrial genomes can now be accomplished in *Chlamydomonas reinhardii* (Sodeinde and Kindle, 1994), and the *Chlamydomonas* chloroplast has become a study model for gene function and expression in photosynthesis (for reviews, see Hippler *et al.*, 1998; Dent *et al.*, 2001). It is a goal of the *Chlamydomonas* genome project to sequence every gene involved in photosynthesis (Grossman, 2000).

The *Chlamydomonas reinhardtii* HSP70A promoter, induced by both heat shock and light, appears to be a useful tool for improved transgene expression in this alga. Its fusion to reporter genes HSP70B or ARS yields high levels of transgene product (Schroda *et al.*, 2000). A new marker developed for *Chlamydomonas* makes possible the serial transformation of its chloroplast genome (Bateman and Purton, 2000). Transformations for other algae, such as *Volvox* (Hallmann and Rappel, 1999), are being developed, and it can be forecast that genetic manipulation of these algae will lead to a better commercial knowledge of their biology and the development of new strains that can overexpress products of value in the near future.

7.2.5 Culture systems

The productivity of a microalgal culture depends mainly on the culture system used. One of the main causes of the high costs of microalgal mass on the industrial scale is the inefficient utilisation of solar energy (Richmond, 1996). Very sophisticated systems can enhance productivity, but may not be economically feasible for large-scale production. Existing commercial microalgae culture systems range in volume from about 10^2 l (used for the production of organic compounds labelled with stable isotopes) to more than 10^{10} l (used for the culture of *D. salina*) (Borowitzka, 1999). The culture systems currently in use belong to two basic types: open ponds and closed photobioreactors.

Open-pond versus closed bioreactors Open-pond cultures are usually exposed to natural sunlight. They can be neither unialgal nor axenic (bacteria-free) and should be managed to maintain a continuous bloom state of the desired alga and give a good yield of the target product. However, this is only adequate for culturing either extremophilic algae (*e.g. Spirulina* and *Dunaliella*), whose growth conditions prevent contamination by other species, or for waste treatment systems, in which the biomass concentration is more important than its quality. Borowitzka (1999) has reviewed the main types of open and closed culture systems.

Open-pond systems are cheaper than closed bioreactors are easier to scale up to industrial size and can use arid or saline lands. They can be extensive or intensive. Extensive ponds are large and do not have any special modification. Intensive ponds are smaller and are modified to improve algal biomass productivity, for example by CO_2 addition and stirring. They achieve better control of contamination and light utilisation, and sufficient CO_2 for photosynthesis. However, they have some important drawbacks, such as contamination with other undesired algae ("weeds"), grazers and parasites, and lack of control of light, nutrients and temperature regimes, which, in turn, interact in a complex manner (Richmond, 1986).

In closed photobioreactors, almost absolute control over the light regime can be reached, using either sunlight or artificial light sources. The geometry of the vessel and the light source, intensity and quality of light can be chosen. In outdoor ponds, the choices are restricted by the local solar regime; however, there is a choice of latitude, position and pond depth. In both cases, the best way to control light intensity is the algal concentration (Dubinsky *et al.*, 1995). Figure 7.3 illustrates the interplay of latitude, depth and cell density on the amount of light reaching the algal cells in an open pond. Biomass concentration affects production rates. At low concentrations, production is suboptimal because of photoinhibition. At high concentrations, production is sub-optimal because self-shading reduces the growth rate. At some intermediate concentration, production reaches a maximum, but this optimum is a function of environmental conditions and algal species (Laws *et al.*, 1988). Automated methods have been developed to reach and maintain the desired culture conditions. Biomass and pigment concentration of *Spirulina platensis* outdoor cultures can be estimated through remote sensing (Gitelson *et al.*, 1995). Automated methods allow the online monitoring of open pond cultures and facilitate the rapid xestimation of the effect of different parameters on the culture growth (Guterman *et al.*, 1989).

Microalgal productivity reaching yields of 20 to 30 g (dw) m^{-2} day^{-1} for 100 to 300 days (depending on the site) has been often achieved (Shelef *et al.*, 1980) by maintaining pilot-scale cultures in the exponential growth phase. The construction of

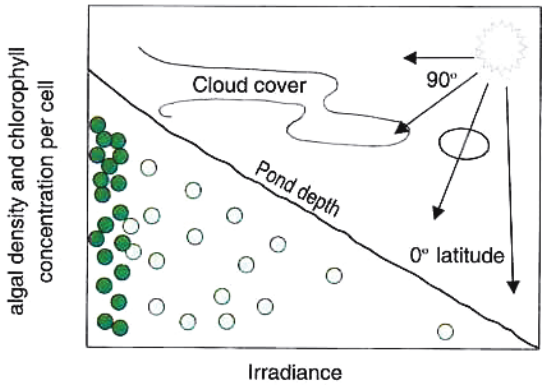


Figure 7.3 Interplay of solar irradiance, algal cell density, latitude and pond depth in open microalgal culture ponds. High cell concentration, high latitude and increasing pond depth decrease algal productivity. algal production is optimised at intermediate concentrations, but this, in turn, is a function of environmental conditions and algal species.

mathematical models (Guterman *et al.*, 1990; Sukenik *et al.*, 1991) and computer controlled systems (Bernard *et al.*, 1996) permit the optimisation of yield and lowering of biomass losses in open pond cultures.

Many commercially desired algal species are unlikely to grow in outdoor systems, and large-scale culture of microalgae for valuable products is unlikely to be sustainable or economic in such systems. Algae for human consumption or pharmaceuticals have to be clean and pure, and this is better achieved in closed reactors (Oswald, 1980). The advantage of these is that they can maintain axenic and strictly controlled growth conditions when defined chemical compounds have to be produced from algal biomass (Pohl *et al.*, 1988).

Richmond (1988) designed closed outdoor photobioreactors with low surface-to-volume ratios and with mixing to ensure more efficient utilisation of solar energy at optimal algal density. This systems permit cell density to be increased, while maintaining axenic cultures and taking advantage of sunlight. It has been used succesfully in mass cultivation of *Spirulina* at cell densities above 10 g (dw) l^{-1} (Hu *et al.*, 1996). *Nodularia* grown in tubular reactors for biomass and protein produced 13.2 g (dw) m^{-2} day^{-1}, versus 11 g m^{-2} day^{-1} in open ponds, with a protein and amino acid composition very similar to *Spirulina* (Pushparaj *et al.*, 1995). Tubular systems can be scaled-up to surface areas of the order of 1 ha (10,000 m^2). Tubular photobioreactors are used for producing EPA from *Phaeodactylum tricornutum* under a semicontinuous or continuous regime. Maximum yield is obtained at the highest irradiances (1,700 μmol photons m^{-2} s^{-1}) and high cell density, with addition of CO_2

(Chrismadha and Borowitzka, 1994). EPA yield reaches 48 mg l^{-1} day^{-1} (Molina-Grima *et al.*, 1994a). Photoinhibition can be a problem under outdoor conditions and strains that exhibit the greater tolerance to high photon flux should be selected (Vonshak *et al.*, 1996).

The geometry of the reactor and its materials, as well as the circulation flow rate, can be chosen to avoid sedimentation or biofouling caused by cell aggregation. The productivity of *Spirulina platensis* is increased by turbulent mixing when the culture has high density and high irradiance (1800 μmol photons m^{-2} s^{-1}) (Hu and Richmond, 1996). High temperatures are usually reached, on account of the high irradiance. This may harm several cell functions, increase respiration and reduce yields. Whenever possible, thermotolerant strains should be selected. Studies on a thermotolerant strain of *Spirulina platensis* (strain M2, from Lake Chad) showed that temperatures above 40 C decreased the specific growth rate, protein content and polyunsaturated/saturated FA ratio (Tomaselli *et al.*, 1988).

Richmond (1996, 2000) has reviewed basic principles of photobioreactor design and culture maintenance. These include optimum temperature, short light pathways (*ca.* 2 cm), ultra-high cell density, very turbulent flow, short oxygen-diffusion paths, highest absorbance of photons averaged over the year and avoidance of accumulation of inhibitory substances. Tubular photobioreactors with an airlift device to circulate the culture are especially attractive for several reasons: circulation is achieved without moving parts and this provides a robust culture system with less risk of contamination. The airlift device combines the function of a pump and a gas exchanger, removing the oxygen produced by photosynthesis and avoiding the cell damage associated with mechanical pumping. *Phaeodactylum tricornutum* yielded a biomass of 1.90 g l^{-1} d^{-1} in one of these reactors (Molina *et al.*, 2001).

Culture medium The culture medium has to be adequate for the particular algae, reflect the cell elemental chemical composition and it must also be cost-effective. The biochemical profile of microalgae changes with the medium composition (Lourenço *et al.*, 1997). Spaargaren (1996) designed media formulations based on the elemental composition of organisms by using linear programming algorithms. In order to minimise the invasion of cultures by competing 'weed' or pest organisms, the media must be sterilised. Filtration of the culture medium through 0.2 μm membrane filters proved to be a better method than pasteurisation for eliminating bacteria (Lewis *et al.*, 1988b). However, the presence of bacteria in the medium has in some cases been found to be beneficial for algal growth, for example in *Chlorella* and *Scenedesmus* cultures (Mouget *et al.*, 1995). It may be that bacteria reduce the harmful build-up of

photosynthetic oxygen pressure within the microenvironment of microalgae, as well as enhancing CO_2 supply.

The recycling of medium to save water and nutrients in outdoor cultures of *Scenedesmus obliquus* has been shown to cause a decrease in algal productivity (Livansky *et al.*, 1996), owing to accumulation of algal metabolites. Conversion of urea into ammonia has been observed, causing a loss of free NH_3 to the ambient atmosphere and nitrogen deficiency in the medium. However, the influence of extracellular carbohydrates produced by *Dyctyosphaerium pulchellum* and released into the culture medium significantly increased the growth rate of *Scenedesmus armatus*, where they acted as growth factors (Burkiewicz and Synak, 1996).

Harvesting Harvesting methods depend on several factors, such as the algal species, cell size, physiological state, viscosity of the medium and the product sought. The main methods are filtration, centrifugation and natural or induced sedimentation or flotation (Gudin, 1988). For most products, centrifugation is prohibitively expensive. Filtration is efficient only for filamentous algae. For cells that tend to float and clump together, like Spirulina, flotation is the easiest and cheapest method. Flocculation seems to be the most efficient method, although some flocculants can be toxic. Autoflocculation can be attained by increasing the pH of the culture, which promotes quick settling of 80% of the biomass (Vohnshak *et al.*, 1985). The most recent development in this methodology is electrolytic flocculation, which involves no chemical flocculants and reaches efficiencies of 95% or higher in the recovery of algal biomass (Poelman *et al.*, 1997).

Immobilised systems Algal systems immobilised on a solid matrix of alginate or polyurethane foam can be used instead of liquid-medium systems. The culture medium circulates through the support, supplying the algae with nutrients and carrying away the soluble algal metabolites. This technology is being developed for the production of extracellular compounds and products readily released by osmotic shock: extracellular polysaccharides of *Porphyridium* (Thepenier *et al.*, 1988), fuels, pharmaceuticals and fertilisers, as well as for wastewater treatment and for extraction of heavy metals from aqueous solutions.

Nitrogen-fixing cyanobacteria yield more ammonium in immobilised matrices than as free-living cells (Brouers *et al.*, 1988), since the matrix–microalga systems seem to mimic the symbiosis in cyanobacteria–fern associations, triggering enhanced extracellular ammonium production. This offers advantages such as increased stability and easier manipulation of the bioreactor (Hall and Rao, 1990). Greene and Bedell (1990) reviewed the use of immobilised algae for metal recovery. Nishida *et*

al. (1996) described the use of immobilised lipoxygenase enzyme for screening microalgal extracts for potential antioxidant activity.

Application of stress to algal cultures Stress conditions can be createded by several procedures, mainly by imposing an extreme in any of the factors implicated in algal growth regulation, or by creating a shock with conditions never met by the cells. Stress may be caused by too low or too high irradiance, UV–B radiation, nutrients, temperature, or by the addition of chemicals. All of these methods are presently being tested and used to achieve overproduction of a substance that the cells would under normal conditions synthesise in smaller amounts.

In general, algae increase the production of extracellular organic substances under stress. Saline stress enhances the release of carbohydrates, whereas organic substances and heavy metals induce the release of proteins (Marsalek and Rojickova, 1996). Both stresses induce changes in the patterns of protein synthesis. Hypo-osmotic stress produces low molecular weight proteins whereas hyperosmotic stress produces high molecular weight (~170 kDa) proteins, the expression being induced very early after the shock (Golldack *et al.*, 1995). Light and salt stress play a major role in the regulation of the xanthophyll-cycle pigments. *Dunaliella salina*, when exposed to high light stress, accumulates β–carotene plus zeaxanthin, whereas under hypersaline stress it accumulates β–carotene plus violaxanthin, the last being formed at the expense of zeaxanthin (Cowan *et al.*, 1995).

Dunaliella salina can grow in up to saturating NaCl concentrations. The mechanisms involved are not fully known. Fisher *et al.* (1996) found a plasma membrane protein, p60, and identified it as a structurally novel carbonic anhydrase, transcriptionally regulated by CO_2 availability and induced by salt. *Dunaliella bardawil* is the richest natural source of β–carotene, and accumulates it under special stress conditions (Pick, 1992). Decreasing the temperature of *D. bardawil* cultures from 30 C to 10 C increased the concentration of β–carotene twofold, and the ratio of 9–*cis* to all-*trans* β–carotene fourfold (Ben-Amotz, 1996). Rapid carotenoid accumulation occurs at 20–24% NaCl, and low nitrogen concentrations (Borowitzka and Borowitzka, 1988). UV–A radiation causes massive increases in carotenoid levels without slowing the growth of *D. bardawil* (Jahnke, 1999).

Haematococcus pluvialis accumulates astaxanthin under phosphate starvation and high salinity (Boussiba and Vohnshak, 1991). Free *trans*-astaxanthin accumulated in mixotrophic cultures of *Chlorococcum* sp. was enhanced from 3.6 mg g^{-1} (cell dw) to 5.7 mg g^{-1} when the culture was subjected to oxidative stress by hydrogen peroxide addition (Yin-Nin and Chen, 2001). Oxidative stress generally causes accumulation of carotenoids, since they constitute antioxidant cell defences.

Heterotrophic culture Heterotrophic dark culture of microalgae with addition of energy-rich organic compounds to the medium might be the future method of choice for some commercial products of microalgae, especially some of the highly valuable ones. Although there was some interest in the 1960s in heterotrophic microalgal cultures for the production of pigments, commercial research on this technology did not start until the 1980s. Chen (1996) reviewed the work done on this mode of cultivation, which has mostly been conducted on the laboratory scale. However, some attempts have been made to exploit it on an industrial scale (Hilaly *et al.*, 1994). Spray-dried heterotrophic *Schizochytrium* fed to *Artemia nauplii* and the rotifer *Brachionus* enriches them in EPA and DHA (Barclay and Zeller, 1996). *Crypthecodinium cohnii* (Kyle, 1992; Ratledge, 2001) and *Nitzschia* (Kyle and Gladue, 1991) are successfully cultured for producing PUFAs. Other microbial systems (fungal and bacterial) that have been used for omega–3 FA give lower yield, making heterotrophic microalgal culture the system of choice for these important products (Barclay *et al.*, 1994). Extracellular production of L-ascorbic acid (vitamin C) on an industrial scale has been achieved through heterotrophic culture of *Chlorella pyrenoidosa* (Running *et al.*, 1994). There is a limited number of microalgae that can grow heterotrophically on organic substrates, which makes extensive screening programmes for suitable species necessary. Some problems inherent in the heterotrophic mode, such as the need of light for efficient production of some metabolites, or the inhibition of growth by some soluble organic substances, can be overcome by the use of mixotrophic (usually, cyclic heterotrophic/photoautotrophic cultivation in dark-light cycles) (Cohen, 1996; Ogbona and Tanaka, 2000).

7.3 Macroalgae

Seaweeds are macroscopic algae growing in marine habitats ranging from open seas to coastal zones. Their size is very variable, from a few centimetres to 60–70 m in length. They belong to three main classes, defined by their pigmentation: red algae (*Rhodophyta*), green algae (*Chlorophyta*) and brown algae (*Phaeophyta*). 107 genera and 493 species of macroalgae are used as food or as sources of phycocolloids (Critchley, 1993), mostly in Asian countries. Lobban and Harrison (1994) have summarised the knowledge of seaweed ecology and physiology. Research and development is broadening the scope for their utilisation. The commercial exploitation of macroalgae for the extraction of gelling agents and as food is well established. The main products extracted from them are agar, agarose, carrageenan, alginates and mannitol. Powdered, dried, pickled and processed macroalgae (*e.g.* nori

from *Porphyra*, wakame from *Undaria* and kombu from *Laminaria*) are also directly used as food.

The major algal products presently commercialised are those produced by the seaweed industry. The annual market value of these products is more than $3500 million, compared with about $200 million for microalgal products. The main share of the macroalgal product value, accounting for $3000 million, is for food, which needs a minimum amount of processing after harvest (Radmer, 1996).

The first uses of macrophytes were limited to harvesting from the wild, which was supplemented or replaced by farming in the field and culture in tanks. Farmers in Japan started farming *Porphyra* in the early 1950s, soon after the discovery of the conchocelis phase. Jensen (1993) reviewed market needs for macroalgae, which are mainly for the production of phycocolloids and food condiments. Worldwide shortage of agarophytes, due to their overexploitation, contrasts with huge, unexploited beds of alginate-producing brown seaweeds. Growth of human population, pollution, overexploitation of land and lack of freshwater will encourage the use of seaweeds and improve their culture methods. The main present uses of macrophyta are described below and summarised in Table 7.4. Altogether about 60 seaweed species are commonly included in the Japanese diet.

7.3.1 Food products and animal feed

The consumption of seaweeds as food is high in the Far East and the Pacific region but limited in western countries. Global consumption in the last decade reached 453,730 t yr^{-1} dry weight (Critchley, 1993), the highest per capita consumption occurring in Korea followed by Japan and China. Seaweeds are rich in minerals, amino acids, vitamins, PUFA and dietary fibre.

An important food from algae, called laver in the British Isles and nori in Japan, comes from the red alga *Porphyra*. Its preparation is a major industry in Japan. Laver is also eaten in coastal areas of Ireland, Britain and New Zealand (Chapman and Chapman, 1980). Its cultivation in the USA started in the 1980s. All the stages of its growth, reproduction, harvesting and processing have now been elucidated, and this knowledge is being applied to its production, in addition to research on strain selection (Merrill and Waaland, 1988). The dried alga contains large amounts of protein, ash, vitamins and carbohydrates. Many of its ingredients, for example EPA, B–vitamins, taurine and inositol, as well as porphyosines that prevent gastric ulcers and betaines that lower blood cholesterol (Noda, 1992), are beneficial to health.

Table 7.4 Sources and prices of the main products from macroalgae

Product	**Uses**	**Source**	**Price ($/kg)**	**Market value ($ m)**	**Ref.**
Nori	Food	*Porphyra*		1,800	3
Wakame	Food	*Undaria*		600	3
Kombu	Food	*Laminaria*		600	3
Seaweed meal	Animal feed	Several		5	3
Manure	Agriculture	Several		10	3
Liquid fertiliser	Agriculture	Several		5	3
Agar	Food industry Pharmaceuticals	Red algae	20 135	132 405	1
Carrageenans	Food products Cosmetics Pharmaceuticals	Red algae		100	3
Alginates	Food products Paper products Biomed. appl.	Brown algae		230	3
Agarose	Research	Red algae	1,000	100	1
Phycobiliproteins	Research	Red algae	10,000		2
R-phycoerythrin	Research	Red algae	5×10^6	2	3
Carrageenans	Food products Cosmetics Pharmaceuticals	Red algae		100	3

References: 1. Dubinsky (1980) unpublished report; 2. Benemann *et al.* (1987); 3. Radmer (1996).

Nori is a primary constituent of the Japanese food sushi, which is becoming increasingly popular in the West. Modern farming methods introduced in the 1960s, which were further improved in the 1970s, resulted in increased yields of nori that were accompanied by the development of mechanised methods of processing (Oohusa, 1993). With a retail price of about \$140 kg^{-1} (Pacific Rim Goumet, 2002), a market value of \$2000 million yr^{-1} and a product volume of 40,000 t yr^{-1}, nori is by far the most commercially successful product of seaweed farming (Jensen, 1993).

The brown alga *Undaria pinnatifida* is the source for the food product wakame. It has been cultivated commercially since the 1950s. Wakame needs more extensive

processing than other macroalgal foods. It is mostly produced in Japan, Korea and China, and its primary market is Japan. It is used as an ingredient in soups, salads and noodles (Yamanaka and Akiyama, 1993). The retail price of dried wakame is about \$70 kg^{-1} (Pacific Rim Gourmet, 2002). Kombu is the third major algal product, derived from the brown alga *Laminaria japonica* and related species. Its annual market value is aproximately \$600 million (Radmer, 1996) and the retail price of the dried alga is about \$125 kg^{-1} (Mendocino Sea Vegetable Company, 2002). *Laminaria* is extensively cultivated in China, with an annual production of about 250,00 t (van der Meer, 1988). Half the harvest is consumed as food, and it constitutes an important souce of iodine in the Chinese diet (Yamanaka and Akiyama, 1993). In 1990, about 20,000 t, with a market value of \$600 million, were sold (Radmer, 1996).

Some macroalgae are being used or assayed as feed for aquaculture. *Ulva pertusa* is a nutritious food for mariculture since it has a high level of PUFA (36–54% of total FA) (Floreto *et al.*, 1993). In *Sargassum* species there is a high content of PUFA (34–53% of total FA), of which the predominant one is arachidonic acid (Khotimchenko, 1991). Other macroalgae have been tested as food for the highly valued abalone, but few of them are eaten and digested by molluscs. The preferred ones belong to the genera *Gracilaria, Lobospira, Pterocladia, Laurencia* and *Asparogopsis* (Westphalen and Cheshire, 1995).

7.3.2 Wastewater treatment and integrated systems

Seaweed cultures can be used in recirculating integrated systems for fish and seaweed culture for the tertiary wastewater treatment of fish-pond effluents (Neori *et al.*, 1991; Neori, 1996). Seaweeds are produced as valuable products at the same time as the main nitrogenous nutrient, ammonium, is removed. *Gracilaria tenuistipitata* has been successfully cultivated in Baltic brackish seawater and outdoor ponds, using rainbow-trout farm effluent as the nutrient source (Haglund and Pedersen, 1993). *Gracilaria parvispora* is able to grow in shrimp-farm effluent in extensive production systems in Hawaii (Nelson *et al.*, 2001).

Macrophyte biofilters are especially suitable for incorporation into recirculating mariculture systems. They have the advantage over bacteria that they can be easily harvested and generate income as human or animal food. *Ulva lactuca* has a protein content comparable to that of cereal grain, and is used as a ruminant feed and for the mariculture of abalone (Neori, 1996). A fully integrated system for culture of abalone, fish and seaweed has adjusted water and nutrient fluxes between the three modules to

optimise water use, nutrient recycling and economically sustainable production (Neori *et al*, 2000).

The ability of seaweeds to accumulate heavy metals makes them a good tool for monitoring heavy metals and removing them from wastewaters. A study in a tropical coastal area (Filho *et al.*, 1997) showed that species of *Padina* and *Sargassum* had the highest accumulation capacity. Heavy-metal binding capacity in brown macroalgae is directly proportional to the total carboxyl group content of their alginates and related to the electronegativity of the metal (Fourest and Volesky, 1997). Alginate fibre from *Ecklonia maxima* adsorbed nickel faster than the intact alga (Williams and Edyvean, 1997). Full understanding of the mechanisms of biosorption and associated chemical interactions will allow the development of full-scale wastewater treatment systems.

7.3.3 Agricultural uses

Seaweed meal applied to the soil serves two main functions. As a fertiliser it promotes plant growth through slow release of mineral nutrients, and as a soil conditioner it improves aeration and aggregate stability, and enhances water retention by poor-quality soils (Metting *et al.*, 1990). Most seaweeds employed in agriculture are *Pheophyceae.* Liquid extracts include the breakdown of the algal cells and release of intracellular components, among which there are plant growth regulators such as cytokinins (Tay *et al.*, 1986; Taylor *et al.*, 1990). Metting *et al.* (1990) reviewed the use of algae in agriculture. The application of algal extract to potato cultivars gave higher protein content and assimilable fibre than other commercial plant-growth enhancers (Lozano *et al.*, 1999), while applications of seaweed extract to pasture increased the activity of the antioxidant superoxide dismutase (SOD) and specific vitamin precursors (Allen *et al.*, 1999) Crude extracts of green and red seaweeds increased protein, total soluble sugars and chlorophyll content of *Vicia faba*, the cytokinin content of the green algae being higher than that of the red algae (el-Sheek and el-Saied, 2000).

7.3.4 Specific products from macroalgae

Polysaccharides Seaweed polysaccharides have unique gelling properties that have led to a variety of applications. These gels, which are formed in water solutions, result from peculiar regular chemical structures, specific ordered molecular conformations and aggregations (Lahaye, 2001). Heyraud *et al.* (1990) reviewed the physical and

chemical properties of phycocolloids, the general behaviour of ionic polymers, and the mechanisms and properties of the gels. Guist (*1990*) reviewed the use of phycocolloids in the food industry. The different compounds show diversity in their ways of gel formation and liquefaction, thermal and chemical stability and crosslinking capacity (Guiseley, 1989). Algal polysaccharides (also called hydrocolloids or phycocolloids) constitute the major industrial non-food products derived from macroalgae. Global production of phycocolloids amounts to 145,000 tonnes, with an annual market value exceeding US$500 million (Indegaard and Ostgaard, 1991). Agars and carrageenans can be obtained from red algae, and alginates from brown algae through extensive extraction and purification of the raw materials. Table 7.5 shows the polysaccharide composition of several commercially important seaweed species.

Agars and carrageenans are galactans extracted from red algae. Agars are mostly extracted from the Rhodophyta Gelidiellaceae and Gracilariaceae: *Gracilaria, Gelidium, Pterocladia*. Their common feature is that they are all composed of the galactose-related monomers D-galactose and 3,6–anhydro–L–galactose. The agars also contain different, species-specific amounts of sulphate, pyruvate and methoxy groups. Agars are usually extracted with hot water and concentrated through several steps, the concentrate being allowed to gel and then dehydrated and ground. The structural strength of agar gels depends mainly on molecular size during initial gelation process (Lai and Lii, 1997) and is negatively correlated with sulphate content (de Castro, 1996). Since the generic term 'agar' has been generally used to refer to a family of related polysaccharides, with important variations that affect their gelling properties, Craigie (1990) proposed that the generic name 'agar' be replaced by 'agarcolloids' and that 'agar' be used only in the original sense of gelling polysaccharides, the non-gelling ones being named 'agaroids'.

High-gel strength is required for commercial agar uses. The major agar-producing countries are Japan, Spain, Chile, Mexico, China and Korea. *Gelidium* gives the best quality agar, but it is not as widely distributed and easy to culture as *Gracilaria* (de Castro, 1996). The largest *Gracilaria* farms are in Chile, which produces 13,000 t dry weight of raw material annually (Critchley, 1993). Close to 5,000 t of agar were processed from 25,000 t to 30,000 t of *Gracilaria* in 1989, mostly harvested from the wild in Chile, Argentina, Brazil and South Africa, or from fishpond culture in Taiwan and China. In Chile, the development of *Gracilaria* farming was made possible by the existing basic knowledge and an increasing market demand. Buschmann *et al.* (1995) reviewed the cultivation of *Gracilaria* on the sea-bottom off southern Chile.

Table 7.5 Properties of polysaccharides from macroalgae

Species	Source	Product	Yield (%)	Gel strength (g cm^{-2})	Melting T (C)	Gelling T (C)	Ref.
Gracilaria sp.	cultured natural	agar	10.1 18.3	113.7 122.2	79.7 78.3	27.3 25.5	1
Pterocladia capillacea	cultured natural	agar	13.2 23.5	392.8 526.0	95.3 90	27.4 27	1
Hypnea musciformis	cultured natural	carra-geenan	27.7 34.9	12.5 32.1	27 27	9 9.1	1
Hypnea cornuta	cultured natural	carra-geenan	33.7 38.4	19.4 16.4	26 28.7	8.6 7.7	1
Gracilari-opsis heteroclada	cultured	agar	16.9	400	86	38	2
G. gracilis	natural	agar	17.1	859		45–50	3

References: 1. Frielander and Zelikovitch (1984); 2. Hurtado-Ponce (1994); 3. Rebello *et al.* (1996).

Natural seaweed growth rates have been shown to be highly variable, owing to seasonal fluctuations of natural factors. These also affect the quantity of phycocolloid extracts such as agar and carrageenan and their physical properties (Ohno *et al.*, 1994). The higher agar strength of *Gracilaria* in the tropics is obtained during the dry season (Luhan, 1992). Less attention has been paid to the influence of algal life cycle on agar properties. Vegetative *Gracilaria bursa-pastori* plants gave a significantly higher gel strength than reproductive plants, which suggests that harvesting should be undertaken outside reproductive periods (Marinho-Soriano *et al.*, 1999).

Agaroses are highly purified products obtained from agar as the highest gelling fraction by isolation of the less ionic fractions under controlled conditions. *Gelidium sesquipedale* can give a yield of 43% (dry weight) of agarose (Vignon *et al.*, 1994). The products obtained have properties that serve various applications, mostly in biotechnology, and they play a key role in molecular biology (Upcroft and Upcroft, 1993; Van *et al.*, 2002). Market data about agaroses are difficult to obtain because of the highly specialised and competitive nature of this commercial area, but the market has been estimated to be worth $50 million per year. Some products can reach a price of $25,000 kg^{-1}.

Carrageenans are complex galactans produced by Rhodophyta of the genera *Chondrus, Euchema, Furcellaria, Gigartina* and *Kappaphycus*. They are composed of galactose-related monomers α–1,3–D–galactose and β–1,4–3,6–anhydro–D–galactose, to which sulphate groups are bound. Three main types of carrageenans, named κ (kappa), λ (lambda) and ι (iota), are used commercially. Native carrageenans often contain combinations of these idealised units, with variations in structure occurring not only between different species of seaweed but also within the different life-stages of a single species. The general extraction method is by treatment of the macroalgal biomass with hot water. Subsequent treatment depends on the desired final product (Lewis *et al.*, 1988a). The main uses of the carrageenans are in the food industry as gelling and thickening agents (Guist, 1990). The annual market for carrageenans has been growing exponentially for the last 25 years. About 15,500 t were sold annually in the early 1990s, with a market value of $100 million (Jensen, 1993), and in the 1990s the annual market value was worth over $200 million (Bixler, 1996), at prices varying from US$ 0.06 to 0.26 per kg. The largest carrageenan factories are found in Europe and the USA. However, in the past decade, factories have also been established in Chile and the Philippines (Critchley, 1993). Hurtado *et al.* (2001) discuss the seasonality and economic viability of *Kappaphycus* cultures in the Philippines.

Galactans with disaccharide sequences in which the galactose has one or more sulphate radicals possess lower gelling strength, and are considered as biological precursors since these structures are mostly present in actively growing young tissues and are converted by specialised enzymes into their 3,6-anhydride-containing disaccharide structures (Zinoun *et al.*, 1997).

Lahaye (2001) has reviewed the current knowledge of galactan primary structure. The precise determination of chemical composition and primary structure of algal gelling galactans has progressed in the last decade using the many available chemical, physical and physico-chemical methods. Little information is available as yet on the genes involved in galactan biosynthesis or their seconday and tertiary (gel) structures. Advances in these two fields will permit the design of new methods of biosynthesis and/or extraction.

Alginates are salts of alginic acid. They are polymers of D–mannuronic acid and L–glucuronic acid, the proportions and sequences of these monomers changing with the algal species. The major alginate producers are the brown macroalgae *Laminaria, Macrocystis, Ascophyllum, Sargassum, Ecklonia* and *Turbinaria*. Water-insoluble alginates are extracted with hot sodium carbonate. The resultant sodium alginate is separated from the biomass residue by filtration and further purified. Alginate production is limited to a few companies in the USA, Norway, UK and France.

Alginates form viscous solutions when dissolved in cold water, and are used in the food industry as thickening, gelling, emulsifying and water-retaining agents, in the textile and paper industries and in biotechnological applications such as tissue engineering (Bak *et al.*, 2002; Suzuki *et al.*, 2002). Alginate beads remove heavy metal ions from dilute aqueous solutions (Ibáñez and Umetsu, 2002). Approximately 27,000 tonnes of alginates, worth $230 million, were sold annually as of 1990 (Jensen, 1993). The market price varies with the purity grade. The pharmaceutical grade product reaches US$13–15 per kg, and the food grade US$6.5–11 per kg (Critchley, 1993).

Pharmaceuticals and medical products Seaweeds are sources of valuable products in health diets and in medical treatment and diagnosis. Rhodophyta show antibiotic activity against multi-antibiotic resistant bacteria (Mahasneh *et al.*, 1995). Ethanol extracts from 56 southern African seaweeds showed antibacterial activity (Vlachos *et al.*, 1997). The highest amount and broadest spectrum of activity was shown by the brown alga *Zonaria subarticulata*.

Sulphated polysaccharides from marine macroalgae have been reported to possess anticoagulant, antithrombic fibrinolytic and anti-platelet aggregation (Siddhanta *et al.*, 1999) and are free from the disadvantages associated with heparin. A major anti-coagulant has been purified from a commercial fucoidan fraction extracted from the brown seaweed *Fucus vesiculosus* and subjected to chemical sulphation. The anti-thrombin activity increases with the sulphate content (Nishino *et al.*, 1995). Fucoidan is thought to act like heparin (Mauray *et al.*, 1998). Lectins from several macroalgae have been found to inhibit platelet aggregation (Matsubara *et al.*, 1996).

Macroalgal species are currently screened for anti-tumour and anti-viral activity. Anti-mutagenic activity has been observed in some species, and the active substances are presently under identification. Shiomi and Hori (1990) reviewed the use of haemaglutinins from seaweeds in immunology, cell biology and cancer research. *Porphyra tenera* (the Japanese Asakusa-nori) was shown to have a suppressive effect on experimentally induced mutagenesis, and this action has been linked to β-carotene, chlorophyll *a* and lutein. The combined pigment treatment showed synergistic effect compared with either alone (Okai *et al.*, 1996).

Significant anti-tumour activity has been found in some seaweeds of the brown, red and green algae classes (Noda *et al.*, 1990; Fischel *et al.*, 1995; Egorin *et al.*, 1996; Pec *et al.*, 1999; Hiroishi *et al.*, 2001). Their activity is mostly linked to fucoidan, carrageenan, glycolipids, phospholipids and terpenoids. Seaweed extracts stimulated activity on B cells and macrophages (Liu *et al.*, 1997), which could be used clinically to modulate immune responses. Fucans have been found to inhibit

vascular smooth muscle cell proliferation (Logeart *et al.*, 1997). Some polysaccharides also exhibited significant inhibitory action against several types of carcinoma and sarcoma tumours (Renn *et al.*, 1995). Two sesquiterpene hydroquinones from the red alga *Peyssonelia* are potent inhibitors of the RNA-directed synthesis of the reverse transcriptase of HIV–1 and HIV–2 (Loya *et al.*, 1995).

Polysaccharides from some brown and red macroalgae have been found to be active as anti-hypertensive and anti-hyperlipidemic agents. These are: sulphated glucuronoxylorhamnan, fucoidan, sodium alginate, porphyran, funoran and agar. All of these depressed total and free cholesterol, triglycerides and low-density lipoprotein in rats fed a high-salt, high-cholesterol diet (Renn *et al.*, 1994). The mechanism of action could be to bind the sodium ions and the cholesterol, leading to their excretion. Dietary use of *Undaria pinnatifida* prevents hyperlipidemia in rats (Murata *et al.*, 1999). Xilo-mannans from red seaweeds showed anti-viral activity against four types of herpes virus (Kolender *et al.*, 1997).

Lipid peroxidation causes various pathologies and is involved in both arteriosclerosis and degenerative brain disease (Stocker, 1994; Inselmann *et al.*, 1998), as well as in the spoilage of stored foods. Drying in the form of thin films preserves most edible algae in Japan. These algae contain high amounts of PUFA, known to be very prone to oxidation. However, no oxidative spoilage happens during storage, which suggests the presence of antioxidant substances. Seaweeds screened for antioxidant activity reduced the activities of lipoxygenase and tyrosinase by up to 50% (Matsukawa *et al.*, 1997). Yan *et al.* (1999) found the antioxidant activity of the Japanese edible seaweed *Hijikia* to be due to fucoxanthin. The antioxidant action is more effective *in vivo* than for the purified fractions.

Pigments Phycoerythrins from red algae are important compounds in the biopharmaceutical industry. The main uses of phycoerythrins are as fluorescent markers for flow cytometry, fluorescence-activated cell sorting, histochemistry (Glazer, 1994) diagnosis and research, and as food colouring (see Section 7.2.4). The major product is R-phycoerythrin, currently derived from *Porphyra* species. Sophisticated and costly separation and purification procedures are needed to obtain the pure product. The high cost of the process is balanced by the value of the product as a biomedical reagent. The purified phycobiliprotein has a current value of \$5000 kg^{-1}, with an annual market of \$2m (Radmer, 1996). R-phycoerythrin has also been isolated from another red alga, *Ceramium isogonum* (Kaixian *et al.*, 1993).

7.3.5 Culture systems

Macroalgae have traditionally been harvested from the wild in their native habitats. A steady increase in market demand, together with lack of crop management, has led to overharvesting and, in some cases, disappearance of some populations. This, in turn, has made the adoption of farming methods necessary. This has especially been the case for *Gracilaria*. Phycological studies during the past century have provided the critical information on the life histories of seaweeds needed to initiate improvement of marine crops. The life cycles of most macroalgae are complicated, and this information has made it possible to predict the timing of reproduction, which has permitted genetic selection of the better plants as 'seed stock' for the next crop (van der Meer, 1988).

Farming systems Approaches to the mariculture of seaweeds have been numerous, and can be classified into four basic methods: 1) in tanks or in raceways; 2) in excavated ponds; 3) in the sea; 4) in suspended, spray or drip culture. Tanks are small and made of concrete or other building materials. They require energy inputs such as air bubbling, agitation with paddles, light, nutrients and temperature control. Ponds are larger areas, dug in the ground, and sometimes lined with plastic sheets to prevent seepage and minimise contamination, with nutrient addition and water pumping. Pond cultivation is commonly practised for *Gracilaria* species. Nitrate and ammonium enrichment enhance both the phycobilin content of *Gracilaria* and its agar quality (Hemalatha and Rengasamy, 1993). About half of the seaweed used as raw material for agar comes from pond cultivation (Santelices and Dotty, 1989). Intensive cultivation is carried out in tanks with aeration, water motion and CO_2 addition (Friedlander *et al.*, 1990). Cultivation in the sea makes use of natural bays, estuaries, *etc.* Spray or drip systems are made by growing the plants out of the water, attached to ropes or nets, and irrigating them with aerosols or showers that also provide the necessary nutrients. For general reviews on seaweed culture techniques worldwide, see Mathieson (1992) and Robledo-Ramirez (1993). Successful cultivation depends on a good knowledge of the algal life history in the field (Destombe *et al.*, 1988). Following the depletion of wild populations due to overexploitation in Taiwan (Shang, 1976), Brazil (Oliveira, 1981) and Chile (Alveal, 1986), the most successful method has been cultivation from spores taken from reproductive plants. The microthalli are then attached to ropes, and grown to harvestable size in the sea (Alveal *et al.*, 1996).

The key in the development of *Porphyra* farming was the discovery, about 50 years ago (Drew-Baker, 1949), that the genus *Conchocelis* was actually the

sporophyte phase of *Porphyra*. Several succeeding studies followed the development of *Conchocelis*, its morphology and ultrastructure (Conway and Cole, 1981) and growth conditions (Chiang and Wang, 1980; Waaland *et al.*, 1987). Progress in the knowledge of *Porphyra* life history permitted selection of strains adapted to local growing conditions. In Japan, there are more than 30 recognised cultivars, most of them derived from *P. yezoensis* and *P. tenera*. Several aquaculture techniques used in Japan are described in Pena (1981).

Laminaria is extensively cultivated in China, where it was introduced for experimental culture in the 1930s, and has been successfully cultivated in rafts since the 1950s. Integrated seafarming of *Laminaria* with mussels and scallops is common (Suo and Wang, 1992) and its production has increased steadily up to more than 250,000 t of dry weight annually. Plant-breeding studies conducted in the 1960s showed that iodine content and other important characteristics are under quantitative genetic control (Fang, 1983). This provided the basis for a selection and breeding programme to obtain enhanced lines for aquaculture. With increasing knowledge of the complicated reproductive biology of *Laminaria,* further strain selection and hybridisation programs have been established.

Production ecology studies on macroalgae have been important for development of new farming methods (Santelices and Doty, 1989; Santelices and Ugarte, 1990). Strain selection has been a very important step in the improvement of macroalgal cultures, and has shown that some foreign strains might have higher potential for outdoor cultivation than the local wild type populations. A strain selection procedure using *Gracilaria verrucosa* sporelings was an adequate method for the improvement of *Gracilaria* strains (Levy and Friedlander, 1990; Levy *et al.*, 1990). The improved strains showed higher growth rate and photosynthesis, and produced 40% more agar than the wild type.

Cultivation of *Gracilaria* in unattached form in outdoor tanks and ponds has been reviewed by Friedlander and Levy (1995). This may be done in intensive or non-intensive ways, the main difference being in the agitation of water in the intensive cultures. The maximum annual yield of non-intensive cultures ranged between 20 t dw ha^{-1} in Florida and 40 t dw ha^{-1} in Taiwan. Intensive tank cultivation of free-floating seaweeds has been developed mainly in the two last decades. Intensive cultivation of *G. conferta* in Israel has been developed during the last 15 years, and in 1995 it reached an annual yield of 6 t dw 1,000 m^{-2} (or 16.5 g dw m^{-2} day^{-1}) in a pilot-scale plant. The main physical variables affecting the cultures, as reviewed by Friedlander and Levy (1995), are: tank and pond structure, seawater motion, light and temperature. The main chemical factors are: nutrients, inorganic carbon and pH. The main biological factors are: seaweed density, epiphyte competition, grazer damage

and bacterial attack. Strain selection and genetic improvement are necessary tools to obtain economically attractive macroalgal cultures. In raceway cultures, it is also necessary to improve the raceway operation system to obtain uniform seaweed dispersal. Biological control of competing epiphytes and copepods is best done by introducing fish. Efficient harvesting, sun drying and packaging of the seaweeds requires mechanical devices. An optimised integration of fish and algal cultures reduces nutrient and water pumping costs (Friedlander and Levy, 1995). The ecophysiology, life-history, geographic distribution and cultivation technology of the carrageenan-producing *Euchema* and *Kappaphycus* has been recently reviewed in depth by Ask and Azanza (2002), who stress the need for further advances in the field. The farming and harvest of seaweeds is complicated by the existence of two kinds of thalli, unitary and clonal, in all seaweed, plus a third class, coalescent, only described at present for Rhodophyta (Santelices, 2001). Unitary individuals exhibit differentiation and propagation is by zygotes or spores. Clonal indiciduals propagate by self-replication and do not show differentiation. Coalescence of genetically different plants originates chimeric seaweeds (Santelices *et al.*, 1996). Each kind of individual has different ecophysiology and needs different farming methods (Santelices *et al.*, 1999).

Cell and tissue culture Recent trends in the culture of macroalgae involve protoplast and tissue culture, as well as strain selection. Plant cell and tissue culture may be defined as the axenic growth of parts isolated from plants in a nutrient medium (Butler and Evans, 1990). This can be done at different organisational levels, for example as protoplasts, single cells, cell suspensions, callus or organs. Early attempts at tissue culture of seaweeds were hampered mostly by the difficulty of preparing axenic tissues and lack of knowledge of the substances controlling growth and differentiation. Once this knowledge was gained, protoplasts and single cells could be obtained. Cheney *et al.* (1986) isolated protoplasts and managed to obtain protoplast fusion between *Gracilaria tikvaiae* and *G. lemaneiformis*. Protoplasts and single cells were prepared from pieces of sterilised tissue by enzymatic treatment with snail extracts (Polne-Fuller, 1988; Rusig and Cosson, 2001). For a review of protoplast isolation, see Butler *et al.* (1990).

Tissue and callus cultures of several commercially important seaweeds of varying levels of success in regeneration and differentiation have been reported (Polne-Fuller and Gibor, 1987; Cheney *et al.*, 1987). The main principles and techniques for tissue culture of higher plants have proved successful with certain seaweeds. However, several problems concerning the initiation and maintenance of callus, cells and protoplasts in axenic culture have to be solved for seaweeds before the processes can

be performed routinely and on a large scale. Lawlor *et al.* (1989, 1991) reported the successful tissue culture of *Ecklonia radiata*, and described the light and nutrient regime used and the sterilisation method applied. Butler and Evans (1990) reviewed the techniques and state-of-the-art in cell and tissue culture of seaweeds.

Efforts to farm the Japanese *Porphyra yezoensis* (nori) on the northeastern coast of the USA have not succeeded, because of the different ecological conditions. This problem is being circumvented by creating hybrids with local species, through protoplast isolation and fusion (Watson *et al.*, 1996). Protoplast fusion (somatic hybridisation) overcomes sexual incompatibility barriers to hybridisation (Evans, 1983).

Isolated protoplasts are living plant cells from which the walls have been removed. Protoplasts from several *Ulva* species have been isolated using commercial enzymes, together with crude abalone preparation, producing a high yield (Reddy *et al.*, 1989). Protoplasts isolated from thalli of four *Porphyra* species regenerated successfully into differentiated plantlets. The culture medium was very important for the development pattern. Sorbitol, nitrogen and agar concentration in the medium were the factors controlling cell division rate, wall thickening, rhizoid development and callus formation (Polne-Fuller and Gibor, 1990). This technology allows vegetative cloning of selected *Porphyra* plants.

Genetic engineering has started to be applied to macroalgae, although it is confronted with more difficulties than in the case of microalgae. It has been applied to polysaccharide production from several algae (Renn, 1997, and references therein). The development of adequate transformation systems for macroalgae has proved difficult, mostly due to the need to establish tissue cultures. The new techniques for delivering DNA-coated microparticles into cells, protoplasts and tissues (Stevens and Purton, 1997; see also Section 7.2.1) also seem to be promising for species improvement and production of commercially important seaweeds. Hybrid marine algae are produced by preparing protoplasts from spores of parental algal plants, fusing the spore-protoplast to form heterokaryon fusion products. Transgenic marine algae are produced by preparing protoplasts from spores of a marine algal plant, introducing foreign DNA into the resulting spore-protoplasts to form transformed spore-protoplasts. After either procedure, selected fusion products are isolated and cultured to produce multicellular material (Cheney and Duke, 1996).

7.4 Concluding remarks

The heterogeneity of algae in their phylogenetic relationships and the large number of unexploited species make them a vast, untapped resource pool of valuable compounds

important for such diverse uses as nutrition, industrial feedstocks and advanced biotechnology. They resemble bacteria on one end and higher plants on the other (Gibbs, 1992) and show high structural, ecological and metabolic diversity (Radmer, 1996). These characteristics allow them to adopt very different life forms and adapt to virtually any environment. Algal metabolites are varied and include unusual biochemical compounds. Presently, the uses of algae, including their direct consumption as food or feed, are extremely limited, considering their potential. In the past, the main difficulty in developing valuable products has been the lack of suitable mass-culture systems. This problem, which has largely been overcome with macroalgae (which constitute the most commercially successful group), is still impeding commercial mass culture of microalgae, although some important species (*Spirulina, Chlorella, Dunaliella*) are produced on a commercial basis.

The production of fine chemicals is generally an expensive process and must often compete with similar, cheaper synthetic products, although some important compounds cannot be synthetically obtained. Improvements in technology, such as the use of enclosed, thoroughly controlled photobioreactors, or heterotrophic culture of some species, are giving promising results. The tools of molecular biology are starting to be applied to both microalgae and macroalgae, although they lag behind the development of bacteria and yeast biotechnology. The progress made in the last decade on the basic knowledge of algal genetics and molecular biology makes it reasonable to expect an expansion in the range and quantity of valuable products that will become commercially available from algae in the near future.

Acknowledgements

Rosa Martínez was the recipient of Grant No. PR95–586 from the Spanish Comision Interministerial de Ciencia y Tecnologia (CICYT, Ministry of Science) for a sabbatical stay at Bar-Ilan University in 1997.

References

Aaronson S., Berner T. and Dubinsky Z. (1980), 'Microalgae as a source of chemicals and natural products', in Shelef G. and Soeder C. J. (1980, eds.), *Algal Biomass. Production and Use*, Elsevier, Amsterdam, pp. 575–601.

Abrahams K. J. (1996), *Algae to the Rescue! Everything you Need to Know about Nutritional Blue-Green Algae*, Logan House Publications, Studio City, CA.

Abrahams K. J. (1997), *All A's with Algae. Vol. 1: Energize your Mind with Nutritional Blue-Green Algae*, Logan House Publications, Studio City CA, 70 pp.

Akatsuka I. (1990, ed.), *Introduction to Applied Phycology*, SPB, The Hague.

Alveal V. K. (1986), 'Fragilidad y estrategia de perduración de *Gracilaria*', *Est. Oceanol.* **5**, 27–58.

Alveal V. K., Romo H., Werlinger C. and Oliveira E. C. (1996), 'Mass cultivation of the agar-producing alga *Gracilaria chilensis* (Rhodophyta) from spores', *Aquacult.* **148**, 77–83.

Apsley J. W. (1996), *The Genesis Effect: Spearheading Regeneration with Wild Blue-Green Algae*, Genesis Communications, Tuscaloosa, AL.

Aquaculture Production Technology (2000) (*www.aquaculture.co.il*).

Arad S. (1988), 'Production of sulfated polysaccharides from red unicellular algae', in Stadler T., Mollion J., Verduis M. C., Karamanos Y., Morvan H. and Christiaen D. (1988, eds.), *Algal Biotechnology*, Elsevier, London, pp. 65–87.

Arad S. (1994), 'Sulfated polysaccharides from red microalgae: towards production', *3rd. Int. Mar. Biotechnol. Conf.* (abstracts), p. 51.

Armstrong G. A. and Hearst J. E. (1996), 'Genetics and molecular biology of carotenoid pigment biosynthesis', *FASEB J.* **10,** 228–237.

Ask E. I. and Azanza R. V. (2002), 'Advances in cultivation technology of commercial euchematoid species: a review with suggestions for future research', *Aquacult.* **206**, 257–277.

Azcoitia I., Sierra A., Veiga S., Honda S., Harada N. and Garcia-Segura L M. (2001), 'Brain aromatase is neuroprotective', *J. Neurobiol.* **47**, 318–329.

Bak H., Afokeb A., McLeodc A. J., Brown R., Shamlou P. A.and. Dunnill P. (2002), 'The impact of rheology of human fibronectin–fibrinogen solutions on fibre extrusion for tissue engineering', *Chem. Engin. Sci.* **57**, 913–920.

Barclay W. R., Meager K. M. and Abril J. R. (1994), 'Heterotrophic production of long chain omega–3 fatty acids utilizing algae and algae-like microorganisms', *J. Appl. Phycol* **6**, 123–129.

Barclay W. R. and Zeller S. (1996), 'Nutritional enhancement of n–3 and n–6 fatty acids in rotifers and *Artemia* nauplii by feeding spray-dried *Schizochytrium* sp', *J. World Aquacult. Soc.* **27**, 314–322.

Barros M.P., Pinto E., Colepicolo P., Pedersén M. (2001), 'Astaxanthin and peridinin inhibit oxidative damage in Fe^{2+}-loaded liposomes: scavenging oxyradicals or changing membrane permeability?', *Biochem. Biophys. Res. Comm.* **288**, 225–232.

Bashan L. E., Moreno M., Hernandez J.-P. and Bashan Y., 'Removal of ammonium and phosphorus ions from synthetic wastewater by the microalgae *Chlorella*

vulgaris co-immobilized in alginate beads with the microalgae growth-promoting bacterium *Azospirillum brasilense*', *Water Research*, in press.

Bateman J. M. and Purton S. (2000), 'Tools for chloroplast transformation in Chlamydomonas: expression vectors and a new dominant selectable marker', *Mol. Gen. Genet.* **263**, 404–410.

Baumgarten E., Nagel M. and Tischner R. (1999), 'Reduction of the nitrogen and carbon content in swine waste with algae and bacteria', *Appl. Microbiol. Biotechnol.* **52**, 281–284.

Beijerinck M. W. (1890), 'Kulturversuche mit Zoochloren, Lichenengonidien und anderen niederen Algen', *Bot. Ztg.* **48**, 725–778.

Belay A., Ota Y., Miyakawa K. and Shimamatsu H. (1993), 'Current knowledge on potential health benefits of *Spirulina*', *J. Appl. Phycol.* **5**, 235–241.

Ben-Amotz A. and Avron M. (1980), 'Glycerol, beta-carotene and dry algal meal production by commercial cultivation of *Dunaliella*', in Shelef G. and Soeder C. J. (1980, eds.), *Algae Biomass*, Elsevier, The Hague, pp. 603–610.

Ben-Amotz A. and Avron M. (1983), 'On the factors which determine massive beta-carotene accumulation in the halotolerant alga *Dunaliella bardawil*', *Plant Physiol.* **72**, 593–597.

Ben-Amotz A., Mokady S. and Edelstein S. (1989), 'Bioavailability of a natural isomer mixture as compared with synthetic all-*trans* beta-carotene in rats and chicken', *J. Nutr.* 119, 1013–1118.

Ben-Amotz A. and Avron M. (1990), 'The biotechnology of cultivating the halotolerant alga *Dunaliella*', *Trends Biotech.* **8**, 121–126.

Ben-Amotz A. (1996), 'Effect of low temperature on the stereoisomer composition of β-carotene in the halotolerant alga *Dunaliella bardawil*', *J. Phycol.* **32**, 272–275.

Ben-Amotz A. and Levy Y. (1996), 'Bioavailability of a natural isomer mixture compared with synthetic all-*trans* beta-carotene in human serum', *Am. J. Clin. Nutr.* **63**, 729–734.

Bender J., Gould J. P., Vatcharapijarn Y., Young J. S. and Phillips P. (1994), 'Removal of zinc and manganese from contaminated water with cyanobacteria mats', *Water Environ. Res.* **66**, 679–683.

Benemann J. R., Tillett D. M. and Weissman J. C. (1987), 'Microalgae biotechnology', *Trends in Biotechnol.* **5**, 47–53.

Benemann J. R. (1992), 'An overview of microalgae industrial phycology', *J. Phycol.* **28**/3 (suppl.), p. 11.

Bermejo R., Alvarez-Pez J. M., Acién F. J., Molina Grima E. (2002), 'Recovery of pure B-phycoerythrin from the microalga *Porphyridium cruentum*', *J. Biotechnol.* **93**, 73–85.

Bernard O., Malara G. and Sciandra A. (1996), 'The effects of a controlled fluctuating nutrient environment on continuous cultures of phytoplankton monitored by computers', *J. Exp. Mar. Biol. Ecol.* **197,** 263–278.

Bhat, V. B., Madyastha K M. (2001), 'Scavenging of peroxynitrite by phycocyanin and phycocyanobilin from *Spirulina platensis*: protection against oxidative damage to DNA', *Biochem. Biophys. Res. Comm.* **285**, 262–266.

Bich N. N., Yaziz M. I. and Bakti N. A. K. (1999), 'Combination of *Chlorella vulgaris* and *Eichornia crassipes* for wastewater nitrogen removal', *Water Res.* **33,** 2357–2362.

Bigogno C., Khozin-Goldberg I. and Cohen Z. (2002), 'Accumulation of arachidonic-acid rich triacylglycerols in the microalga *Parietochloris incisa* (Trebuxiophyceae, Chlorophyta)', *Phytochem.* **60**, 135–143.

Bixler H. J. (1996), 'Recent developments in manufacturing and marketing carrageenan', *Hydrobiologia* **326/327**, 35–37.

Borde X., Guieysse B., Delgado O., Muñoz R., Hatti-Kaul R., Nugier-Chauvin C., Patin H. and Mattiasson B., 'Synergistic relationships in algal-bacterial microcosms for the treatment of aromatic pollutants', *Bioresource Technol.*, in press.

Borowitzka M. A. (1986), 'Micro-algae as sources of fine chemicals', *Microbiol. Sci.* **3**, 372–375.

Borowitzka M. A. and Borowitzka L. J. (1988a), '*Dunaliella*', in Borowitzka M. A. and Borowitzka L. J. (1988, eds.), *Microalgal Biotechnology*, Cambridge University Press, Cambridge, pp. 27–58.

Borowitzka M. A. and Borowitzka L. J. (1988b, eds.), *Microalgal Biotechnology*, Cambridge University Press, Cambridge.

Borowitzka M. A., Huisman J. M. and Osborn A. (1991), 'Culture of the astaxanthin-producing green alga *Haematococcus pluvialis*. 1. Effects of nutrients on growth and cell type', *J. Appl. Phycol.* **3**, 295–304.

Borowitzka M. A. (1992), 'Algal biotechnology products and processes—matching science and economics', *J. Appl. Phycol.* **4**, 267–279.

Borowitzka M. A. (1994), 'Microalgae as sources of pharmaceuticals and other boilogically active compounds', in Lee Y. K. (1994, ed.), *Algal Biotechnology: Trends and Opportunities* (*J. Appl. Phycol* **7/1–2**), pp. 3–5.

Borowitzka M. A. (1999), 'Commercial production of microalgae: ponds, tanks, tubes and fermenters', *J. Biotechnol.* **70**, 313–321.

Boussiba S. and Vonshak A. (1991), 'Astaxanthin accumulation in the green alga *Haematococcus pluvialis*', *Plant Cell Physiol.* **32,** 1077–1082.

Boussiba S., Sandbank E., Shelef G., Cohen Z., Vohnshak A., Ben-Amotz A., Arad S. and Richmond A. (1988), 'Outdoor cultivation of the marine alga *Isochrysis galbana* in open reactors', *Aquacult.* **72**, 247–253.

Brejc K., Ficner R., Huber R. and Steinbacher S. (1995), 'Isolation, crystallization, crystal structure analysis and refinement of allophycocyanin from the cyanobacterium *Spirulina platensis* at 2.3 Å resolution'. *J. Mol. Biol.* **249**, 424–440.

Brouers M., de Jong H., Shi D. J. and Hall D. O. (1988), 'Immobilized cyanobacteria for sustained ammonium production', in Stadler T., Mollion J., Verduis M. C., Karamanos Y., Morvan H. and Christiaen D. (1988, eds.), *Algal Biotechnology*, Elsevier, London, pp. 265–275.

Brown M. R. and Farmer C. L. (1994), 'Riboflavin content of six species of microalgae used in mariculture', *J. Appl. Phycol.* **6**, 61–65.

Bryant D. A. (1981), 'The photoregulated expression of multiple phycocyanin species. A general mechanism for control of phycocyanin synthesis in chromatically adapting cyanobacteria', *Eur. J. Biochem.* **119**, 425–429.

Burgess J. G., Iwamoto K., Miura Y., Takano H. and Matsunaga T. (1993), 'An optical fibre photobioreactor for enhanced production of the marine unicellular alga *Isochrysis aff galbana T-Iso* (UTEX LB 2307) rich in docosahexanoic acid', *Appl. Microbiol. Biotechnol.* **39**, 456–459.

Burkiewicz K. and Synak R. (1996), 'Biological activity of the media after algal cultures can result from extracellular carbohydrates', *J. Plant Physiol.* **148**, 662–666.

Burlew J. S. (1953, ed.), *Algal Culture from Laboratory to Pilot Plant*, Pub. 600, Carnegie Institution of Washington, Washington D. C.

Buschmann A. H., Westmeier R. and Retamales C. A. (1995), 'Cultivation of *Gracilaria* on the sea-bottom in southern Chile: a review', *J. Appl. Phycol.* **7**, 291–301.

Butler D. M. and Evans L. V. (1990), 'Cell and tissue culture of macroalgae', in Akatsuka I. (1990, ed.), *Introduction to Applied Phycology,* Academic Publishing, The Hague, pp. 629–645.

Butler D. M., Evans L. V. and Kloareg B. (1990), 'Isolation of protoplasts from marine macroalgae', in Akatsuka I. (1990, ed.), *Introduction to Applied Phycology*, Academic Publishing, The Hague, pp. 647–668.

Carlucci M. J., Scolaro L. A., Errea M. I., Matulewicz M. C. and Damonte E. B. (1997), 'Antiviral activity of natural sulphated galactans on herpes virus multiplication in cell culture', *Planta Medica* **63,** 429–432.

Chapman V. J. and Chapman D. J. (1980, 3rd. ed.), *Seaweeds and Their Uses,* Chapman and Hall, London.

Chaumont D., Thepenier C., Gudin C. and Junjas C. (1988), 'Scaling up a tubular photoreactor for continuous culture of *Porphyridium cruentum* from laboratory to

pilot plant', in Stadler T., Mollion J., Verduis M. C., Karamanos Y., Morvan H. and Christiaen D. (1988, eds.), *Algal Biotechnology*, Elsevier, London, pp. 199–208.

Chen F. (1996), 'High cell density culture of microalgae in heterotrophic growth', *Trends in Biotechnology* **14**, 421–426.

Cheney D. P., Mar E., Saga N. and van der Meer J. P. (1986), 'Protoplast isolation and cell division in the agar-producing seaweed *Gracilaria* (Rhodophyta)', *J. Phycol.* **22**, 238–243.

Cheney D. P., Listro A. H. and Bradley P. M. (1987), 'Carrageenan analysis of tissue culture and whole plants of *Agardhiella subulata*', *Hydrobiologia* **151/152**, 161–166.

Cheney D. P. and Duke C. (1996), 'Methods for producing improved strains of seaweed by fusion of spore-protoplasts, and resultant seaweeds and phycocolloids, *Biotechnol. Advances* **14**, 310–311.

Chetsumon A., Maeda I., Umeda F. and Yagi K. (1994), 'Antibiotic production by the immobilized cyanobacterium, *Scytonema sp.* TISTR 8208, in a seaweed-type photobioreactor', *J. Appl. Phycol.* **6**, 539–543.

Chiang Y. M. and Wang J. C. (1980), 'A study on the production of conchosporangia in the conchocelis phase of *Porphyra angusta*', *Phycologia* **19**, 20–24.

Chini Zitelli G., Lavista F., Bastianini A., Rodolfi L., Vincenzini M. and Tredici M. R. (1999), 'Production of eicosapentaenoic acid by Nannochloropsis sp. Cultures in outdoor tubular bioreactors', *J. Biotechnol.* **70**, 299–312.

Chrismadha T. and Borowitzka M. A. (1994), 'Effect of cell density and irradiance on growth, proximate composition and eicosapentanoic production of *Phaodactylum tricornutum* grown in a tubular photobioreactor', *J. Appl. Phycol.* **6**, 67–74.

Cohen Z. (1986), 'Products from microalgae', in Richmond A. (1986, ed.), *CRC Handbook of Microalgal Mass Culture*, CRC Press, Boca Ratón, FL, pp. 421–454.

Cohen Z. (1990), 'The production potential of eicosapentanoic and arachidonic acids by the red alga *Porphyridium cruentum*', *J. Am. Oil Chem. Soc.* **67**, 916–920.

Conway E. and Cole K. (1981), 'Some cytological considerations of life-histories in the genus *Porphyra*', *Proc. Int. Seaweed Symp.* **8**, 83–86.

Corver W. E., Cornelisse C. J. and Fleuren C. J. (1995), 'Simultaneous measurement of two cellular antigens and DNA using fluorescein-isothiocyanate, R-phycoerythrin and propidium iodide on a standard FACScan', *Cytometry* **15**, 117–128.

Cowan A. K., Logie M. R., Rose P. D. and Phillips L. G. (1995), 'Stress induction of zeaxanthin formation in the beta-carotene-accumulating alga *Dunaliella salina*', *Teod. J. Plant Physiol.* **146**, 554–562.

Cresswell R. C., Rees T. A. V. and Shah N. (1989, eds.), *Algal and Cyanobacterial Biotechnology*, Longman, Harlow.

Cribbs G. (1997), *Nature's Superfood: The Blue-Green Algae Revolution*, Gill and Macmillan, Dublin, Ireland.

Critchley A. T. (1993), 'Introduction: seaweed resources', in Ohno M. and Critchley A. (1993, eds.), *Seaweed Cultivation and Marine Ranching*, JICA, Yokosuka, pp. 1–6.

Davidson B. S. (1995), 'New dimensions in natural products research: cultured marine microorganisms', *Curr. Opinion Biotechnol.* **6**, 284–291.

DeBiase A. E., Sanders R. W. and Porter K. G. (1990), 'Relative nutritional value of ciliate protozoa and algae as food for *Daphnia*', *Microb. Ecol.* **19**, 199–210.

de Castro T. (1996), 'Agar yield, gel strength and sulfate content in *Gracilariopsis heteroclada* farmed in brackish water canals', *Israeli J. Aquacult.—Bamidgeh* **48**, 94–98.

de la Noue J. and Proulx D. (1988), 'Tertiary treatment of urban wastewaters by chitosan-immobilized *Phormidium sp.*', in Stadler T., Mollion J., Verduis M. C., Karamanos Y., Morvan H. and Christiaen D. (1988, eds.), *Algal Biotechnology*, Elsevier, London, pp. 159–168.

de la Noue J., Laliberte G. and Proulx D. (1994), 'Algae and wastewater', *J. Appl. Phycol.* **4**, 247–254.

de Pauw N., Morales J. and Persoone G. (1984), 'Mass culture of microalgae in aquaculture systems: progress and constraints', *Hydrobiologia* **116/117**, 121–134.

de Philippis R., Sili C., Paperi R. and Vincenzini M. (2001), 'Exopolysaccharide-producing cyanobacteria and their possible exploitation: a review', *J. Appl. Phycol.* **13**, 293–299.

Degen J., Uebele A., Retze A., Schmid-Staiger U. and Trösch W. (2001), 'A novel airlift photobioreactor with baffles for improved light utilization through the flashing light effect', *J. Biotechnol.* **92**, 89–94.

Destombe C., Godin J. and Bodard M. (1988), 'The decay phase in the life history of *Gracilaria verrucosa*: the consequences of intensive cultivation', in Stadler T., Mollion J., Verduis M. C., Karamanos Y., Morvan H. and Christiaen D. (1988, eds.), *Algal Biotechnology*, Elsevier, London, pp. 287–304.

Drew-Baker K. M. (1949), 'Conchocelis-phase in the life-history of *Porphyra umbilicalis* (L.) Kutz', *Nature* **164**, 748–749.

Dubey A. K. and Rai A. K. (1995), 'Application of algal biofertilizers (*Aulosira fertilissima tenuis* and *Anabaena doliolum Bhardawaja*) for sustained paddy cultivation in northern India', *Isr. J. Plant Sci.* **43**, 41–51.

Dubinsky Z., Berner T. and Aaronson S. (1978), 'Potential of large-scale algal culture for biomass and lipid production in arid lands', *Biotechnol. Bioeng.* **8**, 51–68.

Dubinsky Z. and Aaronson S. (1982), 'Review of the potential uses of microalgae', in San Pietro A. (1982, ed.), *Biosaline Research: A Look To The Future*, Plenum, New York, pp. 181–206.

Dubinsky O., Lerental Y., Christiaen D., Glaser R., Barak Z. and Arad S. (1988), 'Production and characterization of polysaccharides in the unicellular red alga *Rhodella reticulata*', in Stadler T., Mollion J., Verduis M. C., Karamanos Y., Morvan H. and Christiaen D. (1988, eds.), *Algal Biotechnology*, Elsevier, London, pp. 451–462.

Dubinsky Z. (1989), 'The application of environmental stress in the biotechnological exploitation of microalgae', *J. Chem. Technol. Biotechnol.* **42**, 306–308.

Dubinsky Z., Matsukawa R. and Karube I. (1995), 'Photobiological aspects of algal mass culture', *J. Mar. Biotechnol.* **2**, 261–265.

Earthrise Company (2000), *Spirulina Health Library: World Spirulina Production and Consumption* (*www.earthrise.com*).

Edwards P. (1980), 'The production of microalgae on human wastes and their harvest by herbivorous fish', in Shelef G. and Soeder C. J. (1980, eds.), *Algae Biomass—Production and Use*, Elsevier, Amsterdam, pp. 191–203.

Egorin M. J., Sentz D. L., Rosen D. M., Ballesteros M. F., Kearns C. M., Callery P. S. and Eiseman J. L. (1996), 'Plasma pharmacokinetics, bioavailability, and tissue distribution in CD2F1 mice of halomon, an antitumor halogenated monoterpene isolated from the red alga *Portieria hornemannii*', *Cancer Chemoter. Pharmacol.* **39**, 51–60.

El-Sheek M. M. and el-Saied A. D. (2000), 'Effect of crude seaweed extracts on seed germination, seedling growth and some metabolic processes of Vicia faba L.', *Cytobios* **101**, 23–35.

Elhai J. and Wolk C. P. (1990), 'Developmental regulation and spatial pattern of expression of the structural genes for nitrogenase in the cyanobacterium *Anabaena*', *EMBO J.* **9**, 3379–3388.

Elhai J. (1994), 'Genetic techniques appropriate for the biotechnological exploitation of cyanobacteria', *J. Appl. Phycol.* **6**, 177–186.

Evans D. A. (1983), 'Agricultural applications of plant protoplast fusion', *Biotechnology.* **1**, 253–261.

Falkowski P. G. and LaRoche J. (1991), 'Acclimation to spectral irradiance in algae', *J. Phycol.* **27**, 8–14.

Fan L., Vonshak A. and Boussiba S. (1994), 'Effect of temperature and irradiance on growth of *Haematococcus pluvialis* (Chlorophyceae)', *J. Phycol.* **30**, 829–833.

Fan L., Vohnshak A., Gabbay R., Hirshberg J., Cohen Z. and Boussiba S. (1995), 'The biosynthetic pathway of astaxanthin in a green alga *Haematococcus pluvialis* as indicated by inhibition with diphenylamine', *Plant Cell Physiol.* **36**, 1519–1524.

Fang Z. X. (1983), 'A summary of the genetic studies of *Laminaria japonica* in China', in Tseng C. K. (1983, ed.) *Proc. Joint China–U.S. Phycol. Symp.*, Science Press, Beijing, pp. 123–136.

FAO (2000), *The state of world fisheries and aquaculture*. FAO Information Division (*www.fao.org*).

Fidalgo J. P., Cid A., Torres E., Sukenik A. and Herrero C. (1998), 'Effects of nitrogen source and growth phase on proximate biochemical composition, lipid classes and fatty acid profile of the marine microalga Isochrysis galbana', *Aquacult.* **166**, 105–116.

Field C. B., Behrenfield M. J., Randerson J. T. and Falkowski P. (1998), 'Primary production of the biosphere: integrating terrestrial and oceanic components'. *Science* **281**, 237–240.

Fike J. H., Allen V. G., Schmidt R. E., Zhang X., Fontenot J. P., Bagley C. P., Ivy R. L., Evans R. R., Coelho R. W. and Wester D. B. (2001), 'Tasco-forage: I. Influence of a seaweed extract on antioxidant activity in tall fescue and in ruminants', *J. Animal Sci.* **79**, 1011–1021.

Filho G. M. A., Karez C. S., Andrade L. R., Yoneshiguevalentin Y. and Pfeiffer W. C. (1997), 'Effects on growth and accumulation of zinc in six seaweeds species', *Ecotoxicol. Environ. Safety* **37**, 223–228.

Fischel L., Lemee R., Formento O., Caldani C., Moll J. L., Pesando D., Meinesz A., Grelier P., Pietra P., Guerriero A. and Milano G. (1995), 'Cell growth inhibitory effects of caulerpenyne, a sesquiterpenoid from the marine alga *Caulerpa taxifolia*', *Anticancer Res.* **5**, 2155–2160.

Fisher M., Gokhman Y., Pick U. and Zamir A. (1996), 'A salt-resistant plasma membrane carbonic anhydrase is induced by salt in *Dunaliella salina*', *J. Biol. Chem.* **271**, 17718–17723.

Floreto E. A. T., Hirata H. and Yamasaki S. (1993), 'Fatty acid composition of *Ulva pertusa* Kjellman (Clorophyta) and *Gracilaria incurvata* Okamura (Rhodophyta) in Japanese coastal waters', *Botanica Mar.* **36**, 217–222.

Fourest E. and Volesky B. (1997), 'Alginate properties and heavy metal biosorption by marine algae', *Appl. Biochem. Biotechnol.* **67**, 215–226.

Fox R. D. (1988), 'Nutrient preparation and low cost basin construction for village production of *Spirulina*', in Stadler T., Mollion J., Verduis M. C., Karamanos Y.,

Morvan H. and Christiaen D. (1988, eds.), *Algal Biotechnology*, Elsevier, London, pp. 355–364.

Friedlander M. and Zelikovitch N. (1984), 'Growth rate, phycocolloid yield and quality of the red seaweeds *Gracilaria* sp., *Pterocladia capillacea, Hypnea musciformis* and *Hypnea cornuta*, in field studies in Israel', *Aquacult.* **40**, 57–66.

Friedlander M., Galai N and Farbstein H. (1990), 'A model seaweed growth in an outdoor culture in Israel', *Hydrobiologia* **204/205**, 367–373.

Friedlander M. and Levy I. (1995), 'Cultivation of *Gracilaria* in outdoor tanks and ponds', *J. Appl. Phycol.* **7**, 315–324.

Friedman O., Dubinsky Z. and Arad (Malis) S. (1991), 'Effect of light intensity on growth and polysaccharide production in red and blue-green rhodophyta unicells', *Bioresource Technol.* **38**, 105–110.

Fujita T., Tanaka T., Yonemasu Y., Cendes F., Cashman N. R. and Andermann F. (1996), 'Electroclinical and pathological studies after parenteral administration of domoic acid in freely moving nonanesthetized rats: an animal model of excitotoxicity', *J. Epilepsy* **9**, 87–93.

Gantt E. (1989), '*Porphyridium* as a red algal model for photosynthesis studies', in Liss A. R. (1989, ed.), *Algae as Experimental Systems*, pp. 249–268.

Garbisu C, Gil J. M., Bazin M. J., Hall D. O. and Serra J. L. (1991), 'Removal of nitrate from water by foam-immobilized *Phormidium laminosum* in batch and continuous-flow bioreactors', *J. Appl. Phycol.* **3**, 1–14.

Gellenbeck K. W. and Burrascano C. G. (1988), 'A re-evaluation of the green algae *Neospongiococcum* for production of the xanthophyll, lutein', *J. Phycol.* **24** (suppl.), p. 19.

Ghirardi M. L., Zhang L., Lee J. W., Flynn T., Seibert M., Greenbaum E. and Melis A. (2000), 'Microalgae: a green source of renewable H_2', *Trends Biotechnol.* **18**, 506–511.

Gibbs S. P. (1992), 'The evolution of algal chloroplasts', in Levin R. A. (1992, ed.), *Origins of Plastids*, Chapman and Hall, New York, pp. 107–117.

Gilmour D. J. and Hard B. C. (1990), 'Commercial exploitation of *Dunaliella*: current situation and future potential', *Br. Phycol. J.* **5**, 88–89.

Gitelson A. A., Laorawat S., Keydan G. P. and Vohnshak A. (1995), 'Optical properties of dense algal cultures outdoors and their application to remote estimation of biomass and pigment concentration in *Spirulina platensis* (cyanobacteria)', *J. Phycol.* **31**, 828–834.

Glazer A. N. (1994), 'Phycobiliproteins—a family of valuable, widely used fluorophores', *J. Appl. Phycol.* **6**, 131–141.

Golldack D., Dietz K. J. and Gimmler H. (1995), 'The effect of sudden salt stress on protein synthesis in *Dunaliella parva*', *J. Plant Physiol.* **146**, 508–514.

Gouveia L., Veloso V., Reis A., Fernandes H., Novais J. and Empis J. (1996), '*Chlorella vulgaris* used to color egg yolk', *Sci. Food Agric.* **70**, 17–172.

Greenbaum E., Lee J. W., Tebault C. V., Blankinship S. L. and Mets L. J. (1995), 'CO_2 fixation and photoevolution of H_2 and O_2 in a mutant of Chlamydomonas lacking photosystem I', Nature 376, 438–441.(1995), *Nature* **376**, 438–441.

Greene B. and Bedell G. W. (1990), 'Algal gels or immobilized algae for metal recovery', in Akatsuka I. (1990, ed.), *Introduction to Applied Phycology*, SPB Academic Publishing, The Hague.

Grung M., D'Souza F. M. L., Borowitzka M. and Liaaen-Jensen S. (1992), 'Algal carotenoids. 51. Secondary carotenoids. 2. *Haematococcus pluvialis* aplanospores as a source of (3S, 3S′) astaxanthin esters'. *J. Appl. Phycol.* **4**, 165–171.

Gudin C. (1988), 'Why bother with microalgae?', in Stadler T., Mollion J., Verduis M. C., Karamanos Y., Morvan H. and Christiaen D. (1988, eds.), *Algal Biotechnology*, Elsevier, London, pp. 33–40.

Guterman H., Ben-Yaakov S. and Vohnshak A. (1989), 'Automated on-line growth estimation method for outdoor algal biomass production', *Biotechnol. Bioeng.* **34**, 143–152.

Guiseley K. B. (1989), 'Chemical and physical properties of algal polysaccharides used for cell immobilization', *Enzyme Microbiol. Technol.* **11**, 706–716.

Guist G. G. (1990), 'Application for seaweed hydrocolloids in prepared foods', in Akatsuka I. (1990), *Introduction to Applied Phycology*, SPB Academic Publishing, The Hague, pp. 391–400.

Guterman H., Vonshak A. and Ben-Yaakov S. (1990), 'A macromodel for outdoor algal mass production', *Biotechnol. Bioeng.* **35**, 809–819.

Hagen C., Grünewald K., Xyländer M. and Rothe E. (2001), 'Effect of cultivation parameters on growth and pigment biosynthesis in flagellated cells of *Haematococcus pluvialis*', *J. Appl. Phycol.* **13**, 79–87.

Haglund K. and Pedersen M. (1993), 'Outdoor pond cultivation of the subtropical marine red alga *Gracilaria tenuistipitata* in brackish water in Sweden. Growth, nutrient uptake, co-cultivation with trout and epiphyte control', *J. Appl. Phycol.* **5**, 271–284.

Hall D. O. and Rao K. K. (1990), 'Immobilized microalgal systems', *Br. Phycol J.* **25**, 1–89.

Hallmann A. and Rappel A. (1999), 'Genetic engineering of the multicellular green alga Volvox: a modified and multiplied bacterial antibiotic resistance gene as a dominant selectable marker', *Plant J.: Cell. Mol. Biol.* **17**, 99–109.

Hard B. C. and Gilmour D. J. (1991), 'A mutant of *Dunaliella parva* CCAP 19/9 leaking large amounts of glycerol into the medium', *J. Appl. Phycol.* **3**, 367–372.

Harder R. and von Witsch H. (1942), *Ver. Dtsch. Bo.t Ges.* **60**, 146–152.

Harker M., Tsavalos A. J. and Young A. J. (1995), 'Use of response surface methodology to optimise carotenogenesis in the microalga, *Hematococcus pluvialis'*, *J. Appl. Phycol.* **7**, 399–406.

Hasui M., Matsuda M., Okutani K. and Shigeta S. (1995), 'In vitro antiviral activities of sulphated polysaccharides from a marine microalga (*Cochlodinium polycricoides*) against human immunodeficiency virus and other enveloped viruses', *Int. J. Biol. Molecules* **17**, 293–297.

Hemalatha R. and Rengasamy R. (1993), 'Studies on *Gracilaria edulis* (Gmelin) Silva', *Phycos* **32**, 65–75.

Henrikson R. (1989), *Earth Food Spirulina : How This Remarkable Blue-Green Alga Can Transform Your Health and Our Planet*, Ronore Enterprises, Kenwood, CA.

Heyraud A., Rinaudo M. and Rochas C. (1990), 'Physical and chemical properties of phycocolloids', in Akatsuka I. (1990, ed.), *Introduction to Applied Phycology*, SPB Academic Publishing, The Hague.

Hilaly A. K., Karim M. N and Guyre D. (1994), 'Optimization of an industrial microalgae fermentation', *Biotechnol. Bioeng.* **43**, 314–320.

Hirahashi T., Matsumoto M., Hazeki K., Saeki Y., Ui M. and Seya T. (2002), 'Activation of the human innate immune system by Spirulina: augmentation of interferon production and NK cytotoxicity by oral administration of hot water extract of *Spirulina platensis*', *Internat. Immunopharmacology* **2**, 423–434.

Hiroishi S., Sugie K., Yoshida T., Morimoto J., Taniguchi Y., Imai S. and Kurebayashi J. (2001), 'Antitumor effects of *Marginisporum crassissimum* (Rhodophyceae), a marine red alga', *Cancer Lett.* **167**, 145–150.

Hodgson P. A., Henderson R. J., Sargent J. R. and Leftley J. W. (1991), 'Patterns of variation in the lipid class and fatty acid composition of *Nannochloropsis oculata* (Eustigmatophyceae) during batch culture. 1. The growth cycle', *J. Appl. Phycol.* **3**, 19–181.

Hook I. L., Ryan S. and Sheridan H. (1999), 'Biotransformation of aromativc aldehydes by five species of marine microalgae', *Phytochem.* **51**, 621–627.

Hu Q. and Richmond A. (1996), 'Productivity and photosynthetic efficiency of *Spirulina platensis* as affected by light intensity, algal density and rate of mixing in a flat plate bioreactor', *J. Appl. Phycol.* **8**, 139–145.

Hu Q., Guterman H. and Richmond A. (1996), 'Physiological characteristics of *Spirulina platensis* (cyanobacteria) cultured at ultrahigh cell density', *J. Phycol.* **32**, 1066–1073.

Huleihel M., Ishanu V., Tal J. and Arad S. (Malis) (2001) 'Antiviral effect of red microalga polysaccharides on *Herpes simplex* and *Varicella zoster* viruses', *J. Appl. Phycol.* **13**, 127–134.

Hurtado A. Q., Agbayani R. F., Sanares R. and Castro-Mayare T. R. (2001), 'The seasonality and economic feasibility of cultivating *Kappaphycus alvarezii* in Panagatan Cays, Caluya, Antique, Philippines', *Aquacult.* **199**, 295–310

Hurtado-Ponce A. Q. (1994), 'Agar production from *Gracilariopsis heteroclada* (Gracilariales, Rhodophyta) grown at different salinity levels', *Botanica Marina* **37**, 97–100.

Ibáñez J. P. and Umetsu Y. (2002), 'Potential of protonated alginate beads for heavy metals uptake', *Hydrometallurgy* **64**, 89–99.

Indegaard M. and Ostgaard K. (1991), 'Polysaccharides for food and pharmaceutical uses', in Guiry M. D. and Blunden G. (1991, eds.), *Seaweed Resources in Europe: Uses and Potential*, Wiley and Sons, Chichester, pp. 169–183.

Inselman G., Köhler K., Lange V., Silber R. and Nellesen U. (1998), 'Lipid peroxidation and cardiac troponin T release during routine cardiac surgery', *Cardiology* **89**, 124–129.

Isichei A. O. (1990), 'The role of algae and cyanobacteria in arid lands: a review', *Arid Soil Res. Rehabilitation* **4**, 1–17.

Jahnke L. S. (1999), 'Massive carotenoid accumulation in *Dunaliella bardawil* induced by ultraviolet-A radiation', *J. Photochem. Photobiol. B.* **48**, 68–74.

Jensen A. (1993), 'Present and future needs for algae and algal products', *Hydrobiologia* **260/261**, 15–23.

Jensen B. (1992), *Chlorella, Jewel of the Far East: a Food Algae for a Nutritionally Hungry World*, Bernard Jensen Publisher, Escondido, CA.

Jiang Y., Chen F. and Liang S.-Z. (1999), 'Production potential of docosahexaenoic acid by the heterotrophic marine dinoflagellate *Crypthecodinium cohnii*', *Process Biochem.* **34**, 633–637.

Johnson E. A. and An G. H. (1991), 'Astaxanthin from microbial sources', *CRC-CRCIT Rev. Biotechnol.* **11**, 297–326.

John D. M. (1994), 'Biodiversity and conservation: an algal perspective', *The Phycologist,* **38**, 3–5.

Jones W. K. and Dunstan R. H. (1997), 'Effect of irradiance on fatty acid, carotenoid, total protein composition and growth of *Microcystis aeruginosa*', *Phytochem.* **44**, 817–824.

Kageyama H., Ishii A., Matsuoka T., Kodera Y., Hiroto M., Matsushima A. and Inada Y. (1994), 'Simple isolation of phycocyanin from *Spirulina platensis* and phycocyanobilin–protein interaction', *J. Mar. Biotechnol.* **1**, 185–188.

Kaixian Q., Franklin M. and Borowitzka M. A. (1993), 'The study for isolation and purification of R-phycoerythrin from a red alga', *Appl. Biochem. Biotechnol.* **43**, 133–140.

Kajiwara S., Takizono T., Saito T., Kondo K., Ohtani T., Nishio N., Nagai S. and Misawa N. (1995), 'Isolation and functional identificationof a novel cDNA for astaxanthin biosynthesis from *Haematococcus pluvialis*, and astaxanthin sysnthesis in *Escherichia coli*', *Plant Mol. Biol.* **29**, 343–352.

Kerby N. W., Niven G. W., Rowell P. and Stewart W. D. P. (1987), 'Photoproduction of amino acids by mutant strains of nitrogen-fixing cyanobacteria', *Appl. Microbiol. Biotechnol.* **25**, 547–552.

Kerby N. W., Niven G. W., Rowell P. and Stewart W. D. P. (1988), 'Ammonia and aminoacid production from cyanobacteria', in Stadler T., Mollion J., Verduis M. C., Karamanos Y., Morvan H. and Christiaen D. (1988, eds.), *Algal Biotechnology*, Elsevier, London, pp. 277–286.

Khotimchenko S. V. (1991), 'Fatty acid composition of seven *Sargassum* species', *Phytochem.* **30**, 2639–2641.

Klyachko G. L., Doucha J., Kopezkii J., Ryabykh I. E. and Semenenko V. E. (1994), 'Comparative investigation of fatty acid composition in lipids of various strains of *Porphyridium cruentum* and *Porphyridium aerugineum*', *Russian J. Plant Physiol.* **41**, 248–255.

Kobayashi M., Kazizono T. and Nagai S. (1991), 'Astaxanthin production by a green alga, *Haematococus pluvialis*, accompanied with morphological changes in acetate media'. *J. Ferment. Bioeng.* **71**, 335–339.

Kobayashi M., Kakizono T., Yamaguchi K., Nishio N. and Nagai S. (1992), 'Growth and astaxanthin formation of *Haematococcus pluvialis* in heterotrophic and mixotrophic conditions', *J. Ferment. Bioeng.* **74**, 17–20.

Kolender A. A., Pujol C. A., Damonte E. B., Matulewicz M. C. and Cerezo A. S. (1997), 'The system of sulphated alpha-(1-]3)-linked *d*-mannans from the red seaweed *Nothogenia fastigiata*—structures, antiherpetic and anticoagulant', *Carbohydrate Res.* **304**, 53–60.

Kromhout D., Bosschieter B. E. and de Lezene Coulander C. (1986), 'The inverse relation between fish consumption and 20-year mortality from coronary heart disease', *New England J. Med.* **312**, 1205–1209.

Kumazawa S. and Mitsui A. (1994), 'Efficient hydrogen production by synchronously grown cells of a marine cyanobacterium, *Synechococcus sp.* Miami BG 043511, under high cell density conditions', *Biotechnol. Bioeng.* **44**, 854–858.

Kuritz T. and Wolk C. P. (1995), 'Use of filamentous cyanobacteria for biodegradation of organic pollutants', *Appl. Environ. Microbiol.* **61**, 234–238.

Kyle D. J. and Gladue R. M. (1991), 'Eicosaenopentaenoic acid and methods for their production', Int. Patent Appl., Patent Cooperation Treaty Publication WO 91/14427.

Kyle D. J. (1992), 'Microbial oil mixtures and uses thereof', Int. Patent Appl., Patent Cooperation Treaty Publication WO 92/12711.

Lahaye M. (2001), 'Developments on gelling algal galactans, their structure and physico-chemistry', *J. Appl. Phycol.* **13**, 173–184.

Lai M. F. and Lii C. Y. (1997), 'Rheological and thermal characteristics of gel structures from various agar fractions', *Int. J. Biol. Macromol.* **21**, 123–130.

Laing I. and Ayala F. (1990), 'Commercial mass culture techniques for producing microalgae', in Akatsuka I. (1990, ed.), *Introduction to Applied Phycology*, SPB, The Hague, pp. 447–477.

Laing I. and Millican P. F. (1992), ' Indoor nursery cultivation of juvenile bivalve molluscs using diets of dried algae', *Aquacult.* **102**, 231–243.

Largeau C., Casadevall E., Berkaloff C. and Dhamelincourt D. (1980), 'Sites of accumulation and composition of hydrocarbons in *Botryococcus braunii*', *Phytochem.* **19**, 1043–1051.

Largeau C., Bailliez C., Yang L. W., Frenz J. and Casadevall E. (1988), 'Immobilization of the microalga *Botryococcus braunii* in calcium alginate gels in polyurethane foams. Effect of the immobilization on the alga metabolism-metabolite recovery by solvent extraction', in Stadler T., Mollion J., Verduis M. C., Karamanos Y., Morvan H. and Christiaen D. (1988, eds.), *Algal Biotechnology*, Elsevier, London.

Lau P. S., Tam N. F. Y. and Wong Y. S. (1995), 'Effect of algal density on nutrient removal from primarily settled wastewater', *Environ. Pollution* **89**, 59–66.

Lawlor H. J., McComb J. A. and Borowitzka M. A. (1989), 'Tissue culture of *Ecklonia radiata* (Phaeophyceae, Laminariales): effects on growth of light, organic carbon sources and vitamins', *J. Appl. Phycol.* **1**, 105–112.

Lawlor H. J., Borowitzka M. A. and McComb J. A. (1991), 'A rapid and inexpensive method for surface sterilization of *Ecklonia radiata* (Phaeophyta) for tissue culture', *Bot. Mar.* **34**, 261–264.

Laws E. A., Taguchi S., Hirata J. and Pang L. (1988), 'Mass culture optimization studies with four marine microalgae', *Biomass* **16**, 19–32.

Lee Y. K. (1994), 'Algal biotechnology: trends and opportunities' (Proc. 2nd. Asia Pacific Conference on Algal Biotechnology), *J. Appl . Phycol.* **7** (1–2), 100 pp.

Lee Y. K. and Ding S. Y. (1995), 'Effect of dissolved oxygen partial pressure on the accumulation of astaxanthin in chemostat cultures of *Haematococcus lacustris* (Cholorophyta)', *J. Phycol.* **31**, 922–924.

Lee Y. K. and Soh C.-W. (1991), 'Accumulation of astaxanthin in *Hematococcus lacustris* (Chlorophyta)', *J. Phycol.* **27**, 575–577.

Lee C. G. and Palsson B. O. (1995), 'Light-emitting diode-based algal photobioreactor with external gas exchange', *J. Ferment. Bioeng.* **79**, 257–263.

Levy I. and Friedlander M. (1990), 'Strain selection in *Gracilaria* spp. I. Growth, pigment, and carbohydrates characterization of strains of *G. Conferta* and *G. Verrucosa* (Rhodophyta, Gigartinales)', *Bot. Mar.* **33**, 4–11.

Levy I., Beer S. and Friedlander M. (1990), 'Strain selection in *Gracilaria spp.* 2. Selection for high and low temperature resistance in *G. verrucosa* sporelings', *J. Appl. Phycol.* **2**, 163–171.

Lewis J. G., Stanley N. F. and Guist G. G. (1988a), 'Commercial production and applications of algal hydrocolloids', in Lembi C. A. and Waaland J. R. (1988, eds.), *Algae and Human Affairs*, Cambridge University Press, Cambridge, pp. 205–236.

Lewis T. E., Garland C. D., O'Brien T. D., Frase M. I., Tong P. A., Ward C., Dix T. G. and McMeekin T. A. (1988b), 'The use of 0.2 μm membrane-filtered seawater for improved control of bacterial levels in microalgal cultures fed to larval pacific oysters (*Crassostrea gigas*)', *Aquacult.* **69**, 241–251.

Lim B. S. (1999), 'Present status and prospect of Korean bioindustry', *Symp. 3rd. Inauguration Anniversary Inst. Bioscience Biotechnol.*, Korea University.

Lincoln E. P. and Hill D. T. (1980), 'An integrated microalgae system', in Shelef G. T. and Soeder C. J. (1980, eds.), *Algae Biomass—Production and Use*, Elsevier, Amsterdam, pp. 229–244.

Liu J. N., Yoshida Y., Wang M. Q., Okay Y. and Yamashita U. (1997), 'B cell stimulating activity of seaweed extracts', *Int. J. Immunopharmacol.* **19**, 135–142.

Livansky K., Dedic K., Binova J., Tichy V., Novotny P. and Doucha J. (1996), 'Influence of the nutrient solution recycling on the productivity of *Scenedesmus obliquus*, utilization of nutrients and water in outdoor cultures', *Archiv Hydrobiol. Suppl.* **114**, 105–113.

Lobban C. S. and Harrison P. J. (1994, eds.), *Seaweed Ecology and Physiology*, Cambridge University Press, Cambridge.

Logeart D., Prigentrichard S., Jozefonvicz J. and Letourneur D. (1997), 'Fucans, sulfates polysaccharides extracted from brown seaweeds, inhibit vascular smooth muscle cell proliferation. 1. Comparison with heparin for antiproliferative activity, binding and internalization', *Eur. J. Biol.* **74**, 367–384.

Lopez-Alonso D., Molina-Grima, E., Sanchez J. A. and García F. (1992), 'Isolation of clones of *Isochrysis galbana* rich in eicosapentaenoic acid', *Aquacult.* **102**, 363–371.

Lopez-Alonso D., Segura C. I., Molina-Grima E. and Cohen Z. (1996), 'First insights into improvement of eicosapentanoic acid content in *Phaeodactylum tricornutum* (bacillariophyceae) by induced mutagenesis', *J. Phycol.* **32**, 339–345.

Lourenzo S. O., Marquez U. M. L., Mancini-Filho J., Barbarino E. and Aidar E. (1997), 'Changes in biochemical profile of *Tetraselmis gracilis*. Y. Comparison of two culture media', *Aquacult.* **148**, 153–168.

Loya S., Bakhanashvili M., Kashman Y. and Hizi A. (1995), 'Peyssonols A and B, two novel inhibitors of the reverse transcription of human immunodeficiency virus types 1 and 2', *Arch. Biochem. Biophys.* **316**, 789–796.

Lubián L. M., Montero O., Moreno-Garrido I., Huertas I. E., Sobrino C., González M. and Parés G. (2000), '*Nannochloropsis* (Eustigmatophyceae) as a source of commercially valuable pigments', *J. Appl. Phycol.* **12**, 249–255.

Luhan M. R. J. (1992), 'Agar yield and gel strength of *Gracilaria heteroclada* collected from Iloilo, Central Philippines', *Bot. Mar.* **35**, 169–172.

MacColl R. and Guard-Frair D. (1987), *Phycobiliproteins*, CRC Press, Boca Ratón.

Maeda S. and Sakaguchi T. (1990), 'Accumulation and detoxification of toxic metal elements by algae', in Akatsuka I. (1990, ed.), *Introduction to Applied Phycology*, SPB, The Hague, pp. 109–136.

Mahasneh I., Jamal M., Kahasneh M. and Zibdeh M. (1995), 'Antibiotic activity of marine algae against multi-antibiotic resistant bacteria', *Microbios* **83**, 23–26.

Marinho-Soriano E., Bourret E., de Casabianca M. L. and Maury L. (1999), 'Agar from the reproductive and vegetative stages of *Gracilaria bursa-pastoris*', *Biores. Technol.* **67**, 1–5.

Marsalek B. and Rojickova R. (1996), 'Stress factors enhancing production of algal exudates—a potential self-protective mechanism', *Zeit. Naturforsh C* **51**, 646–650.

Martek Biosci (1997), *Product Catalogue.*

Mathew B., Sankaranarayanan R., Nair P. P., Varghese C., Somanathan T., Amma B. P., Amma N. S. and Nair M. K. (1995), 'Evaluation of chemoprevention of oral cancer with *Spirulina fusiformis*', *Nutr. Cancer* **24**, 197–202.

Mathieson A. C. and Guo Z. (1992), 'Patterns of fucoid reproductive biomass allocation', *British Phycol. J.* **27**, 271–292.

Matsubara K., Sumi H. and Hori K. (1996), 'Platelet aggregation is inhibited by phycolectins', *Experientia* **52**, 540–543.

Matsukawa R., Dubinsky Z., Masaki K., Takeuchi T. and Karube Y. (1997), 'Enzymatic screening of microalgae as a potential source of natural oxidants', *Appl. Biol. Biochem.* **66**, 239–247.

Maurage C., Guesnet P., Pinault M., Rochette de Lepdes J., Durand G., Antoine G. and Couet C. (1998), 'Effect of two types of fish oil supplementation on plasma and erythrocyte phospholipides in formula-fed term infants', *Biol. Neonate* **74**, 1746–1748.

Mauray S., Deraucourt E., Chaubet F., Maigarevel O., Sternberg C. and Fischer A. M. (1998), 'Comparative anticoagulant activity and influence of thrombin generation of dextran derivatives and of a fucoidan fraction', *J. Biomat. Sci., Polymer Ed.* **9**, 373–387.

McKeith G. (1996), *Miracle Superfood: Wild Blue-Green Algae*, Keats Publishing, New Canaan, CT.

Merrill J. E. and Waaland J. R. (1988)', *Porphyra* aquaculture in Washington State', *J. Phycol.* **24** (suppl.), 5 (abstract).

Mendocino Sea Vegetable Company (2002), *On-Line Catalogue.*

Metting B., Zimmerman W. J., Crouch I and van Staden J. (1990), 'Agronomic uses of seaweed and microalgae', in Akatsuka I. (1990, ed.), *Introduction to Applied Phycology*, pp. 589–627.

Metzger P., Merkaloff C., Casadevall C. and Coute A. (1985), 'Alkadiene and botryococcene producing yields of wild strains of *Botryococcus braunii*', *Phytochem.* **24**, 2305–2312.

Minowa T., Yokoyama S., Kishimoto M. and Okamura T. (1995), 'Oil production from algal cells of *Dunaliella tertiolecta* by direct thermochemical liquefaction', *Fuel* **74**, 1735–1739.

Mitchell S. A. (1986), 'Experiences with outdoor semi-continuous mass culture of *Brachionus calyciflorus* Pallas (Rotifera)', *Aquacult.* **51**, 155–168.

Molina-Grima E., García F., Sánchez J. A., Urda J., Acien F. and Fernández J. M. (1994a), 'Outdoor chemostat culture of *Phaeodactylum tricornutum* UTEX 640 in a tubular photobioreactor for the production of eicosapentaenoic acid', *Biotechnol. Appl. Biochem.* **20**, 279–290.

Molina-Grima E., Sánchez J. A., García F., Fernández F. J. and Acien F. G. (1994b), 'Effect of growth rate on the eicosapentaenoic acid and docosahexaenoic acid content of *Isochrysis galbana* in chemostat culture', *Appl. Microbiol. Biotechnol.* **41**, 23–27.

Molina E., Fernández J., Acién F. G. and Chisti Y. (2001), 'Tubular photobioreactor design for algal cultures', *J. Biotechnol.* **92**, 113–131.

Moore L. (1997), *Fifty Algae Stories of Hope, Health and Freedom*, Ransom Hill Press, Ramona CA.

Moreno J., Rodríguez H., Vargas M. A., Rivas J. and Guerrero M. G. (1992), 'Nitrogen-fixing cyanobacteria as a source of phycobiliprotein pigments.

Composition and growth performance of ten filamentous heterocystous strains', in Lee Y. K. (1992, ed.), *Algal Biotechnology: Trends and Opportunities* (*J. Appl. Phycol.* **7**, Vol. 1–2), pp. 17–23.

Morist A., Montesinos J. L., Cusidó J. A. and Gòdia F. (2001), 'Recovery and treatment of *Spirulina platensis* cells cultured in a continuous photobioreactor to be used as food', *Process Biochem.* **37**, 535–547.

Mortensen A., Skibsted L. H. and Truscott T. G. (2001), 'The interaction of dietary carotenoids with radical species', *Arch. Biochem. Biophys.* **385**, 13–19.

Mouget J. L., Dakhama A., Lavoie M. C. and de la Noue J. (1995), 'Algal growth enhancement by bacteria: is consumption of photosynthetic oxygen involved?', *FEMS Microbiol. Ecol.* **18**, 35–43.

Muller-Feuga A. (2000), 'The role of microalgae in aquaculture: situation and trends', *J. Appl. Phycol.* **12**, 527–534.

Murata M., Ishihara K. and Saito H. (1999), 'Hepatic fatty acid oxidation enzyme activiies are stimulated in rats fed the brown seaweed, *Undaria pinnatifida* (wakame)', *J. Nutr.* **129**, 146–151.

Neori A., Cohen Y. and Gordin H. (1991), '*Ulva lactuca* biofilters for marine fishpond effluent', *Aquacult.* **4**, 69–79.

Neori A. (1996), 'The type of N-supply (ammonia or nitrate) determines the performance of seaweed biofilters integrated with intensive fish culture', *Israel. J. Aquacult.* **48**, 19–27.

Neori A., Schpigel M. and Ben-Ezra D. (2000), 'A sustainable integrated system for culture of fish, seaweed and abalone', *Aquacult.* **186**, 279–291.

Nishida T., Matsukawa R., Masaki K., Dubinsky Z. and Karube I. (1996), 'A method for screening potential antioxidant activity', *J. Biotechnol.* **51**, 149–155.

Nishino T., Ura H. and Ngumo T. (1995), 'The relationship between the sulfate content and the antithrombin activity of an alfa–2–fucoidan purified from a commercial fucoidan fraction', *Bot. Mar.* **38**, 187–193.

Nisizawa K., Noda H., Kikochi R. and Watanabe T. (1987), 'The main seaweeds foods in Japan', *Hydrobiologia* **151–152**, 5–29.

Nisizawa K. and Oofusa T. (1990), 'Aroma and taste of dried purple laver (Hosi-Nori)', in Akatsuka I. (1990, ed.), *Introduction to Applied Phycology*, SPB, The Hague, pp. 191–198.

Noda H., Amano H., Arashima K. and Nisizawa K. (1990), 'Antitumor activity of marine algae', *Hydrobiologia* **204/205**, 577–584.

Noda H. (1992), 'Health benefits and nutritional properties of nori', *J. Appl. Phycol.* **5**, 255–258.

Ogbona J. C. and Tanaka H. (2000), 'Light requirements and photosynthetic cell cultivation—development of processes for efficient light utilization in photobioreactors', *J. Appl. Phycol.* **12**, 207–218.

Ohno M., Largo D. B. and Ikumoto T. (1994), 'Growth rate, carrageenan yield and gel properties of cultured kappacarrageenan producing red alga *Kappaphycus alvarezii* in the subtropical waters of Shikoku, Japan', *J. Appl. Phycol.* **6**, 1–5.

Ohta S., Chang T., Kawashima A., Nagate T. and Murase M. (1995), 'Anti-methicillin resistant *Staphylococcus aureus* (MRSA) activity by linolenic acid isolated from the marine alga *Chlorococcum* HS–01', *Bull. Environ. Contam. Toxicol.* **52**, 673–680.

Okada S., Murakami M. and Yamaguchi K. (1995), 'Hydrocarbon composition of newly isolated strains of the green microalga *Botryococcus braunii*', *J. Appl. Phycol.* **7**, 555–559.

Okai Y., Higashi-Okai K., Yano Y. and Otani S. (1996), 'Identification of anti-mutagenic substances in an extract of the edible red alga, *Porphyra tenera* (asakusa-nori)', *Cancer Lett.* **100**, 233–240.

Oliveira E. C. (1981), 'Marine phycology and exploitation of seaweeds in South America', *Proc. Int. Seaweed Symp.* **9**, 97–112.

Olson R. J., Chisholm S. W., Zettler E. R. and Ambrust E. V. (1991), 'Pigments, size and distribution of *Synechococcus* in the North Atlantic and Pacific Oceans', *Limnol. Oceanogr.* **35**, 45–58.

Oohusa T. (1993), 'Recent trends in nori products and markets in Asia', *J. Appl. Phycol.* **5**, 155–159.

Oswald W. J. (1980), 'Algal production—problems, achievements and potential', in Shelef G. and Soeder C. J. (1980, eds.), *Algae Biomass*, Elsevier, pp. 1–8.

Pacific Rim Goumet (2002), *On-line Catalogue.*

Parker B. C. (1994, ed.), *Microalgal Biotechnology and Commercial Applications (J. Appl. Phycol.* **6**).

Patterson G. M. L., Larsen L. K. and Moore R. E. (1994), 'Bioactive natural products from blue-green algae', *J. Appl. Phycol.* **6**, 151–157.

Patterson G. W, Tsitsa-Tsardis E., Wickfors G. H., Gladu P. K., Chitwood D. J. and Harrison D. (1994), 'Sterols and alkenones of *Isochrysis*', *Phytochem.* **35**, 1233–1236.

Pec M. K., Moser-Thier K., Fernández J. J., Souto M. L. and Kubista E. (1999), 'Growth inhibition by dehydrothyrsiferol—a non-Pgp modulator, derived from a marine red alga—in human breast cancer cell lines', *Int. J. Oncol.* **14**, 739–743.

Pena J. B. (1981), 'La acuicultura en Japón. 1. Técnicas de cultivo de algas superiores y de crustáceos', *Inf. Tec. Inst. Invest. Pesq. Barcelona*, **87**, 20 pp.

Pesando D. (1990), 'Antibacterial and antifungal activities of marine algae', in Akatsuka I. (1990, ed.), *Introduction to Applied Phycology*, SPB Academic Publishing, The Hague, pp. 3–26.

Phang S. M., Lee Y. K., Borowitzka M. A. and Whitton M. A. (1994, eds.), *Algal Biotechnology in the Asia-Pacific region*, Univ. of Malaya, Kuala Lumpur.

Pick U. (1992), 'Regulation of massive beta-carotene production in *Dunaliella bardawil*', *Isr. J. Aquacult. Bamidgeh.* **44**, 149.

Poelman E., de Pauw N. and Jeurissen B. (1997), 'Potential of electrolytic flocculation for recovery of microalgae', *Resources Cons. Rec.* **19**, 1–10.

Pohl P., Kohlhase M. and Martin M. (1988), 'Photobioreactors for the axenic mass cultivation of algae', in Stadler T., Mollion J., Verduis M. C., Karamanos Y., Morvan H. and Christiaen D. (1988, eds.), *Algal Biotechnology*, Elsevier, London, pp. 209–217.

Polne-Fuller M. and Gibor A. (1987),' Callus and callus-like growth in seaweeds: induction and culture', *Hydrobiologia* **151/152**, 131–138.

Polne-Fuller M. (1988), 'The past, present and future of tissue culture and biotechnology of seaweeds', in Stadler T., Mollion J., Verduis M. C., Karamanos Y., Morvan H. and Christiaen D. (1988, eds.), *Algal Biotechnology*, pp. 17–32.

Polne-Fuller M. and Gibor A. (1990), 'Developmental studies in *Porphyra* (Rhodophyceae). 3. Effect of culture conditions on wall regeneration and differentiation of protoplasts', *J. Phycol.* **26**, 674–682.

Pushparaj B., Pelosi E., Carlozzi P. and Torzillo G. (1995), 'Yield and biochemical composition of a marine cyanobacteria (*Nodularia sp.*) in outdoors culture', *Aquat. Microb. Ecol.* **9**, 13–16.

Radmer R. J. (1996), 'Algal diversity and commercial algal products', *Bioscience* **46**, 263–270.

Radmer R. J. and Parker B. C. (1994), 'Commercial opportunities of algae: opportunities and constraints', *J. Appl Phycol.* **6**, 93–98.

Ramus J. (1986), 'Rodophyte unicells: biopolymer physiology and production', in Barclay W. R. and McIntoch R. P. (1986, eds.), *Algal Biomass Technologies*, J. Cramer, Berlin.

Ratledge C., Kanagachandran K., Anderson A. J., Grantham D. J. and Stephenson J. C. (2001), 'Production of docosahexaenoic acid by *Crypthecodinium cohnii* grown in a pH-auxostat culture with acetic acid as principal carbon source', *Lipids* **36**, 1241–1246.

Rebello J., Ohno M., Critchley A. T. and Sawamura M. (1996), 'Growth rates and agar quality of *Gracilaria gracilis* (Stackhouse) Steentoft from Namibia, Southern Africa', *Bot. Mar.* **39**, 273–279.

Reddy C. R. K., Migita S. and Fujita Y. (1989), 'Protoplast isolation and regeneration of three species of *Ulva* in axenic culture', *Bot. Mar.* **32**, 483–490.

Renaud S. M., Parry D. L. and Thin L. V. (1994), 'Microalgae for use in tropical aquaculture 1: Gross chemical and fatty acid composition of twelve species of microalgae from the Northern Territory, Australia', *J. Appl. Phycol.* **6**, 337–345.

Renaud S. M., Zhou H. C., Parry D. L., Thinh L. V. and Woo K. C. (1995), 'Effect of temperature on the growth, total lipid content and fatty acid composition of recently isolated tropical microalgae *Isochrysis sp., Nitzschia closterium, Nitzschia paleacea* and commercial species of *Isochrysis sp.* (clone T. ISO)', *J. Appl. Phycol.* **7**, 595–602.

Renn D. W. (1997), 'Biotechnology and the red seaweed polysaccharide industry: status, needs and prospects', *Trends Biotechnol.* **15**, 9–14.

Renn D. W., Hiroyuki N., Hydeomi A., Takashiro N. and Kazutoshi N. (1994), 'Study on antihypertensive and antihyperlipidemic effects of marine algae', *Fish. Sci.* **60**, 83–88.

Richmond A. (1986, ed.), *CRC Handbook of Microalgal Mass Culture*, CRC Press, Boca Ratón, FL.

Richmond A. (1988), 'A prerequisite for industrial microalgaculture: efficient utilization of solar irradiance', in Stadtler T., Mollion J., Verduis M. C., Karamanos Y., Morvan H. and Christiaen D. (1988, eds.), *Algal Biotechnology*, Elsevier, Essex, UK, pp. 237–244.

Richmond A. (1996), 'Efficient utilization of high irradiance for production of photoautotrophic cell mass: a survey', *J. Appl. Phycol.* **8**, 381–387.

Richmond A. (2000), 'Microalgal biotechnology at the turn of the millennium: a personal view', *J. Appl. Phycol.* **12**, 441–451.

Richmond A., Boussiba S. and Vohnshak A. (1993), 'A new tubular reactor for mass production of microalgae outdoors', *J. Appl. Phycol.* **5**, 327–332.

Rise M., Cohen E., Vishkautsan M. and Cojokaru M. (1994), 'Accumulation of secondary carotenoids in *Chlorella zofingiensis*', *J. Plant Physiol.* **144**, 287–292.

Robledo-Ramirez D. (1993), 'Biotecnología vegetal marina', *Cienc. Desarr.* **19**, 46–54.

Rowan S. R. (1989), *Photosynthetic Pigments of Algae*, Cambridge University Press, Cambridge.

Running J. A., Huss R. J. and Olson P. T. (1994), 'Heterotrophic production of ascorbic acid by microalgae', *J. Appl. Phycol.* **6**, 99–104.

Rusig A.-M. and Cosson J. (2001), 'Plant regeneration from protoplasts of *Enteromorpha intestinalis* (Chlorophyta, Ulvophyceae) as seedstock for macroalgal culture', *J. Appl. Phycol.* **13**, 103–108.

Sanchez S., Martínez M. E., Molina-Grima E. and Casa J. A. (1995), '*Skeletonema costatum* as a potential source of fatty acids and single cell protein: the effect of pH on growth rate and biomass composition', *J. Mar. Biotechnol.* **2**, 23–26.

Santelices B. (2001), 'Implications of clonal and chimeric-type thallus organization on seaweed harvest and harvesting', *J. Appl. Phycol.* **13**, 153–160

Santelices B. and Doty M. S. (1989), 'A review of *Gracilaria* farming', *Aquacult.* **78**, 95–133.

Santelices B. and Ugarte R. (1990), 'Ecological differences among Chilean populations of commercial *Gracilaria*', *J. Appl. Phycol.* **2**, 17–26.

Santelices B., Correa J. A., Meneses I., Aedo D. and Varela D. (1996), 'Sporeling coalescence and intraclonal variation in *Gracilaria chilensis* (Gracilariales, Rhodophyta)', *J. Phycol.* **32**, 313–322.

Santelices B., Correa J. A., Meneses I., Hormazábal M. and Sánchez P. A. (1999), 'Convergent biological processes in coalescent Rhodophyta', *J. Phycol.* **35**, 1127–1149.

Sawayama S., Inoue S. and Yokohama S. (1994), 'Continuous culture of hydrocarbon-rich microalga *Botryococcus braunii* in secondarily treated sewage', *Appl. Microbiol. Biotechnol.* **41**, 729–731.

Schneider J. C. and Roessler P. (1994), 'Radiolabeling studies of lipids and fatty acids in *Nannochloropsis* (Eustigmatophyceae), an oleaginous marine alga' *J. Phycol.* **30**, 594–598.

Schroda M., Blöcker D. and Beck C. F. (2000), 'The HSP70A promoter as a tool for the improved expression of transgenes in *Chlamydomonas*', *Plant J. Cell Mol. Biol.* **21**, 121–131.

Schwarz T., Bartholomes P. and Kauffmann M. (1995), 'Large-scale production of algal biomass for protein purification: tryptophan synthase from *Euglena gracilis*', *Biotechnol. Appl. Biochem.* **22**, 179–190.

Semenenko V. E. (1996), 'Physiological and genetic potentials of microalgae and molecular biology aspects of photoautotrophic biosynthesis technology'. *ITIT Symposium: Microalgal Biotechnology—Basics and Applications*, pp. 1–8.

Servel M. O., Claire C., Derrien A. and Ciffard L. (1994), 'Fatty acid composition of some marine microalgae', *Phytochem.* **36**, 691–693.

Seto A., Wang H. L. and Hesseltine C. W. (1984), 'Culture conditions affect eicosapentaenoic acid content of *Chlorella minutissima*', *J. Am. Oil Chem. Soc.* **61**, 892–894.

Seto A. (1990), 'Overview of marine technology in Japan and its commercial application for aquaculture', Voigt M. N. and Botta J. R. (1990, eds.), *Advances in Fisheries Technology and Biotechnology for Increased Profitability*, pp. 524–541.

Sevrin-Reyssac J. (1998), 'Biotreatment of swine manure by production of aquatic valuable biomasses', *Agric. Ecosyst. Environ.* **68**, 177–186.

Shaish A., Ben-Amotz A. and Avron M. (1991), 'Production and selection of high β-carotene mutants of *Dunaliella bardawil* (Chlorophyta)', *J. Phycol.* **27**, 652–656.

Shang Y. C. (1976), 'Economic aspects of *Gracilaria* culture in Taiwan', *Aquacult.* **8**, 1–7.

Shelef G. and Soeder C. J. (1980, eds.), *Algal Biomass—Production and Use*, Elsevier, Amsterdam.

Shelef G., Azov Y., Moraine R. and Oron G. (1980), 'Algal mass production as an integral part of a wastewater treatment and reclamation system', in Shelef G. and Soeder C. J. (1980, eds.), *Algal Biomass—Production and Use*, Elsevier, Amsterdam, pp. 163–189.

Shifrin N. S. (1984), 'Oils from microalgae', in Ratledge C., Dawson P. S. S. and Rattray J. (1984, eds.), *Biotechnology for the Oils and Fats Industry*, Am. Oil Chem. Soc., pp. 146–161.

Schulz R. (1996), 'Hydrogenases and hydrogen production in eukaryotic organisms and cyanobacteria', *J. Mar. Biotechnol.* **4**, 16–22.

Shiomi K. and Hori K. (1990), 'Hemaglutinins from algae', in Akatsuka I. (1990, ed.), *Introduction to Applied Phycology*, SPB Academic Publishing, pp. 93–108.

Shiran D., Khozin I., Heimer Y. M. and Cohen Z. (1996), 'Biosynthesis of eicosapentaenoic acid in the microalga *Porphyridium cruentum*. 1. The use of externally supplied fatty acids', *Lipids* **12**, 1277–1282.

Simpson S. J., Vachula M., Kennedy M. J., Kaizer H., Coon S. J., Ghalie R., Williams S. and van Epps D. (1995), 'Detection of tumor cells in the bone marrow, peripheral blood and apheresis products of breast cancer patients using flow cytometry', *Exp. Hematol.* **23**, 1062–1068.

Singh Y. and Kumar H. D. (1994), 'Growth of *Botryococcus ssp.* in improved culture medium', *Phykos* **33**, 77–87.

Siddhanta A. K., Shanmugam M., Mody K. H., Goswani A. M. and Ramawat B. K. (1999), 'Sulphate polysaccharides from *Codium dwarkense* Boergs from the west coast of India: chemical composition and blood anticoagulant activity', *Int. J. Biol. MacroMol.* **26**, 151–154.

Skulberg O. M. (2000), 'Microalgae as a source of bioactive molecules—experience from cyanophyte research', *J. Appl. Phycol.* **12**, 341–348.

Sodeinde O. A. and Kindle K. L. (1993), 'Homologous recombination in the nuclear genome of *Chlamydomonas*', *Proc. Nat. Acad. Sci. USA* **90**, 9199–9203.

Soeder C. J. (1986), 'An historical outline of applied algology', in Richmond A. (1986, ed.), *Handbook of Microalgal Mass Culture*', CRC Press, Boca Ratón, FL, pp. 147–198.

Soong P. (1980), 'Production and development of *Chlorella* and *Spirulina* in Taiwan', in Shelef G. and Soeder C. J. (1980, ed.), *Algal Biomass*, Elsevier, Amsterdam, pp. 97–113.

Spaargaren D. H. (1996), 'The design of culture media based on the elemental composition of biological material', *J. Biotechnol.* **45**, 97–102.

Spectorova L. V., Goronkova O. I., Nosova L. P., Albitskaya O. N. and Danilova G. Y. (1986), 'High-density culture of marine microalgae—promising items for mariculture', *Aquacult.* **55**, 231–240.

St. John M. A., Clemmesen c., Lund T. and Köster T. (2001), 'Diatom production in the marine environment: implications for larval fish growth and condition', *ICES J. Mar. Sci.* **58**, 1106–1113.

Stadler T., Mollion J., Verduis M. C., Karamanos Y., Morvan H. and Christiaen D. (1988, eds.), *Algal Biotechnology*, Elsevier, London.

Stevens D. R. and Purton S. (1997), 'Genetic engineering of eukaryotic algae: progress and prospects', *J. Phycol.* **33**, 713–722.

Stocker R. (1994), 'Lipoprotein oxidation: mechanistic aspects, methodological approaches and clinical relevance', *Curr. Opinion in Lipidology* **5**, 422–433.

Stryer L., Glazer A. N. and Oi V. T. (1985), 'Fluorescent conjugates for analysis of molecules and cells', Canadian Patent No. 841227.

Stryer L. and Glazer A. N. (1989), 'Fluorescent conjugates for analysis of molecules and cells', U.S. Patent No. 4,859,582.

Sudo H., Burgess J. G., Takemasa H., Nakamura N. and Matsunaga T. (1995), 'Sulfated polysaccharide production by the halophilic cyanobacterium *Aphanocapsa halophytia*', *Curr. Microbiol.* **30**, 219–222.

Sukenik A., Carmeli Y. and Berner T. (1989), 'Regulation of fatty acid composition by irradiance level in the eustigmatophyceae *Nannochloropsis sp.*', *J. Phycol.* **25**, 8–92.

Sukenik A., Levy R., Falkowski P. G. and Dubinsky Z. (1991), 'Optimizing biomass production in an outdoor pond: a simulation model', *J. Appl. Phycol.* **3**, 191–201.

Sukenik A. (1992), 'Production of omega–3 fatty acids by marine microalgae—from a laboratory to a production scale', *Isr. J. Aquacult. Bamidgeh.* **44**, 150.

Sukenik A., Zmora O. and Carmeli Y. (1993), 'Biochemical quality of marine unicellular algae with special emphasis on lipid composition. 2. *Nanochloropsis* sp.', *Aquacult.* **117**, 313–326.

Suo R. and Wang Q. (1992), '*Laminaria* culture in China', *Infofish Int.* **1**, 40–42.

Suzuki, Y., Kitaura M., Wu S., Kataoka K, Suzuki K., Endo K., Nishimura, Y. and Ide C. (2002), ' Electrophysiological and horseradish peroxidase-tracing studies of nerve regeneration through alginate-filled gap in adult rat spinal cord', *Neurosci. Lett.* **318**, 121–124.

Takeshima Y., Takatsugu N., Sugiura M. and Hagiwara H. (1994), 'High-level expression of human superoxide dismutase in the cyanobacterium *Anacystis nidulans* 6301', *Proc. Nat. Acad. Sci. USA* **91**, 9685–9689.

Talbot P. and de la Noue J. (1988), 'Evaluation of *Phormidium bohneri* for solar biotechnology', in Stadler T., Mollion J., Verduis M. C., Karamanos Y., Morvan H. and Christiaen D. (1988, eds.), *Algal Biotechnology*, Elsevier, London, pp. 403–412.

Tamiya H. (1956), in *World Summ. Appl. Solar Energy* (Phoenix Arizona, November 1955), Stanford Res. Inst., Menlo Park, CA, pp. 231–241.

Tamiya H. (1957), 'Mass culture of algae', *Stud. Tokugawa Inst.* **8**, 309–334.

Tay S. A. B., MacLeod J. K., Palni L. M. S. and Letham D. S. (1986), 'Detection of cytokinins in a seaweed extract', *Proc. Ann. Meet. Plant Growth Reg. Soc. Am.*, **13**, 184–187.

Taylor J. S., Harker K. N., Robertson J. M. and Foster K. R. (1990), 'The effect of seaweed extract containing cytokinin on the growth and yield of barley', *Can. J. Plant Sci.* **70**, 1163–1167.

Thepenier C., Chaumont D. and Gudin C. (1988), 'Mass culture of *Porphyridium cruentum*: a multiproduct strategy for the biomass valorisation', in Stadler T., Mollion J., Verduis M. C., Karamanos Y., Morvan H. and Christiaen D. (1988, eds.), *Algal Biotechnology*, Elsevier, London, pp. 413–420.

Thiel T. (1994), 'Genetic analyses of cyanobacteria', in Bryant D. A. (1994, ed.), *The Molecular Biology of Cyanobacteria*, Kluwer, Dordrecht, pp. 581–611.

Thomas S., Zaritsky A. and Boussiba S. (1991), 'Ammonium excretion by a mutant of the nitrogen-fixing cyanobacterium *Anabaena siamensis*', *Bioresource Biotechnol.* **38**, 161–166.

Tomaselli L., Giovannetti L., Sacchi A. and Bocci F. (1988), 'Effects of temperature on growth and biochemical composition in *Spirulina platensis* strain M2', in Stadler T., Mollion J., Verduis M. C., Karamanos Y., Morvan H. and Christiaen D. (1988, eds.), *Algal Biotechnology*, Elsevier, London, pp. 305–314.

Toyomizu M., Suzuki Y., Kojima H. and Akiba Y. (2001), 'Effective transformation of the cyanobacterium *Spirulina platensis* using electroporation', *J. Appl. Phycol.* **13**, 209–214.

Tragut V., Xiao J., Bylina E. J. and Borthakur D. (1995), 'Characterization of DNA restriction–modification systems in *Spirulina platensis* strain pacifica', *J. Appl. Phycol.* **7**, 561–564.

Tredici M. R., Carlocci P., Chini Zittelli G. and Materasi R. (1991), 'A vertical alveolar panel (VAP) for outdoor mass cultivation of microalgae and cyanobacteria', *Bioresource Technol.* **38**, 153–159.

Upcroft P. and Upcroft J. A. (1993) 'Comparison of properties of agarose for electrophoresis of DNA', *J. Chromatogr. Biomed. Appl.* **618**, 79–93

Urrutia I., Serra J. L. and Llama M. J. (1995) 'Nitrate removal from water by *Scenedesmus obliquus* immobilized in polymeric foams', *Enzyme Microbiol. Technol.* **17**, 200–205.

Valderrama L. T., Del Campo C. M., Rodriguez C. M., Bashan L. E., and Basham Y. 'Treatment of recalcitrant wastewater from ethanol and citric acid production using the microalga *Chlorella vulgaris* and the macrophyte *Lemna minuscula*', *Water Res.*, in press.

Van W. D., Beheshti A. and Rill R. L. (2002), 'DNA electrophoresis in agarose gels: a simple relation describing the length dependence of mobility', *Electrophoresis* **23**, 15–19.

van der Meer J. P. (1988), 'The development and genetic improvenment of marine crops', in Stadler T., Mollion J., Verduis M. C., Karamanos Y., Morvan H. and Christiaen D. (1988, eds.), *Algal Biotechnology*, Elsevier, London, pp. 1–15.

Vignon M. R., Morgan E. and Rochas C. (1994), '*Gelidium sesquipedale* (Gelidiales, Rhodophyta), 1. Soluble polymers', *Bot. Mar.* **37**, 325–329.

Vlachos V., Critchley A. T. and Vonholy A. (1997), 'Antimicrobial activity of extracts from selected Southern African marine macroalgae', *South African J. Sci.* **93**, 328–332.

Vohnshak A., Cohen Z. and Richmond A. (1985), 'The feasibility of mass cultivation of *Porphyridium*', *Biomass* **8**, 13–25.

Vonshak A. (1992), 'Microalgal technology: is it an economic success?', in da Silva E. J., Ratledge C. and Sasson A. (1992, eds.), *Biotechnology: Economic and Social Aspects: Issues for Developing Countries*, Cambridge University Press, Cambridge, pp. 70–80.

Vonshak A., Chanawongse L., Bunnang B. and Tanticharoen M. (1996), 'Light acclimation and photoinhibition in three *Spirulina platensis* (cyanobacteria) isolates', *J. Appl. Phycol.* **8**, 35–40.

Waaland J. R., Dickson L. G. and Carrier J. E. (1987), 'Conchocelis growth and photoperiodic control of conchospore release in *Porphyra torta* (Rhodophyta)', *J. Phycol.* **23**, 399–406.

Wada H., Gombos Z. and Murata N. (1990), 'Enhancement of chilling tolerance of a cyanobacterium by genetic manipulation of fatty acid desaturation', *Nature* **347,** 200–203.

Warburg O. (1919), 'Ueber die Geschwindigkeit der Kohlen sauerezussamensetzung in lebenden Zellen', *Biochem Zeitschr.* **100**, 230–270.

Watson K. L., Roberts K. M. and Cheney D. P. (1996), 'Protoplast isolation and fusion of North Atlantic *Porphyra* species and *P. yezoensis*', *J. Phycol.* **32,** 50–56.

Westphalen G. and Cheshire A. C. (1995), 'Macro-algal digestion survival in two commercial species of abalone (*Haliotis laevigata Donovan* and *Haliotis rubra Leach*)', *Botanica Marina* **38**, 53–59.

Williams C. J. and Edyvean R. G. J. (1997), 'On exchange in nickel biosorption by seaweed materials', *Biotechnol. Prog.* **13**, 424–428.

Williams J. G. K. (1988), 'Construction of specific mutations in photosystem II photosynthetic reaction center by genetic engineering methods in *Synechocystis* 6803', *Meth. Enzymol.* **167**, 766–778.

Wilson P. D. G., Hilton M. G., Waspe C. R., Steer D. C. and Wilson D. R. (1997), 'Production of ^{13}C-labelled beta-carotene from *Dunaliella salina*', *Biotechnology Lett.* **19**, 401–405.

Wolk C. P., Cai Y. and Panoff J. M. (1991), 'Use of a transposon with luciferase as a reporter to identify environmentally responsive genes in a cyanobacterium', *Proc. Nat. Acad. Sci. USA* **88**, 5355–5359.

Wood J. M. and Wang H. (1983), 'Microbial resistance to heavy metals', *Envir. Sci. Technol.* **17,** 582A–590A.

Wu B., Tseng C. K. and Xiang W. (1993), 'Large-scale cultivation of *Spirulina* in seawater-based culture medium', *Bot. Mar.* **36**, 99–102.

Wu Q. Y., Yin S., Sheng G. Y. and Fu J. M. (1994), 'New discoveries in the study of hydrocarbons from thermal degradation of heterotrophically yellowing algae', *Sci. China Ser. B* **37**, 326–335.

Yamanaka R. and Akiyama K. (1993), 'Cultivation and utilization of *Undaria pinnatifida* (wakame) as food', *J. Appl. Phycol.* **5**, 249–253.

Yan X., Chuda Y. Suzuki M. and Nagata T. (1999), 'Fucoxanthin as the major antioxidant in *Hijikia fusiformis*, a common edible seaweed', *Biosci. Biotechnol. Biochem.* **63**, 605–607.

Yanagita T., Kobayashi H., Yamamoto R., Kataoka H., Yokoo H., Shiraishi S., Minami S., Koono M. and Wada A. (2000), 'Protein kinase C-alpha and -epsilon down-regulate cell surface sodium channels via differential mechanisms in adrenal chromaffin cells', *J. Neurochem.* **74**, 1674–1684.

Yin-Nin Ma R. and Chen F. (2001), 'Enhanced production of free *trans*-astaxanthin by oxidative stress in the cultures of the green microalga *Chlorococcum sp*', *Process Biocem.* **36**, 1175–1179.

Yongmanitchai W. and Ward O. P. (1992), 'Growth and eicosapentaenoic acid production by *Phaeodactylum tricornutum* in batch and continuous culture systems, *J. Am. Chem. Soc.* **69**, 584–590.

Yongmanitchai W. and Ward O. P. (1993), 'Positional distribution of fatty acids, and molecular species of polar lipids, in the diatom *Phaeodactylum tricornutum*', *J. Gen. Microbiol.* **139**, 465–472.

Yotsu-Yamashita M., Nishimori K., Nitanai Y., Isemura M., Sugimoto A. and Yasumoto T. (2000), 'Binding properties of (3)H-PbTx-3 and(3)H-saxitoxin to brain membranes and to skeletal muscle membranes of puffer fish Fugu pardalis and the primary structure of a voltage-gated Na^+ channel alpha-subunit (fMNa1) from skeletal muscle of *F. pardalis*', *Biochem. Biophys. Res. Comm.* **267**, 403–412.

Yúfera M. and Lubián L. M. (1990), 'Effects of microalgal diet on growth and development of invertebrates in marine aquaculture', in Akatsuka I. (1990, ed.), *Introduction to Applied Phycology*, SPB, The Hague, pp. 208–227.

Zhang X. and Smith C. D. (1996), 'Microtubule effects of welwistatine, a cyanobacterial indolinone that circumvents multiple drug resistance', *Mol. Pharmacol.* **49**, 288–294.

Ziegler R. G. (1989), 'A review of epidemiologic evidence that carotenoids reduce the risk of cancer', *J. Nutr.* **119**, 116–122.

Zlotnik Y., Sukenik A. and Dubinsky Z. (1993), 'Physiological and photosynthetical changes during the formation of red aplanospores in the chlorophyte *Haematococcus pluvialis*', *J. Phycol.* **29**, 463–469.

This page intentionally left blank

CHAPTER 8

HYDROGEN PRODUCTION BY PHOTOSYNTHETIC MICROORGANISMS

VLADIMIR A. BOICHENKO
Institute of Basic Biological Problems, Russian Academy of Sciences, 142290 Pushchino Moscow Region, Russia
vboichenko@scientist.com
ELIAS GREENBAUM
Oak Ridge National Laboratory, Oak Ridge, TN 37831, USA
greenbaum@ornl.gov
and
MICHAEL SEIBERT
National Renewable Energy Laboratory, Golden, CO 80401, USA
mike_seibert@nrel.gov

I believe that water will one day be used as a fuel, because the hydrogen and oxygen that constitute it, used separately or together, will furnish an inexhaustible source of heat and light.

Jules Verne, *l'Île Mystérieuse*, 1874.

8.1 Photobiological hydrogen production—a useful evolutionary oddity

Oxygenic photosynthesis may well be the most significant development in the evolution of life on Earth. Ecologically, the process encompasses the turnover of carbon in the biosphere powered by a continuous supply of renewable energy. This energy is stored as reduced carbon compounds in plant cells and is coupled to the production of O_2 from water. Both result from the efficient conversion of harvested photons from solar energy. Oxygen availability in turn allows for effective recycling of the stored energy *via* cellular respiration. This is the perpetual and indispensable role of global photosynthesis in the biosphere. Moreover, geologically stored products of ancient photosynthesis, fossil fuels, remain the main energy resources in contemporary civilisation. These resources will, however, be depleted in the foreseeable future. Worldwide oil production, for example, is expected to peak somewhere in the period 2010–2020 (Kerr, 1998), and the world's energy economics

will then require fundamental revision. Both the oil crises in the 1970s and present concern within scientific and governmental circles about the possible greenhouse effect of CO_2 emissions that may result from continued fossil fuel use have generated interest in alternative energy resources and technologies, including solar hydrogen production (Bockris, 1976; Benemann, 1996).

Solar-driven biophotolytic systems for H_2 production from water are considered to be especially promising for future ecologically clean technologies (Hollaender *et al.*, 1972; Gibbs *et al.*, 1973; Weaver *et al.*, 1980; Greenbaum, 1988b; Hall *et al.*, 1995). The idea was based on the seminal discovery of Gaffron and Rubin (1942) that some hydrogenase-containing green algae are capable of H_2 photoproduction as well as H_2 uptake in the absence of atmospheric oxygen. At the time, this fundamental discovery was important evidence in support of van Niel's general scheme relating plant photosynthesis and more primitive bacterial photosynthesis, as in eq. 8.1

$$CO_2 + 2H_2A \rightarrow [CH_2O] + 2A + H_2O \qquad (8.1)$$

where $[CH_2O]$ represents carbohydrate, H_2A is an oxidisable molecule (*e.g.* an organic acid or water) and A is the oxidised product. Note that Gaffron's research demonstrated that $2H_2$ could substitute for $2H_2A$ in some algae.

Figure 8.1 further illustrates the relations between the light reactions of plant and bacterial photosynthesis. Both are driven by charge-separation acts occurring within pigment–protein complexes called reaction centres. The key difference is that plants can use water as the source of reductant (electrons) for carbon reduction (or H_2 evolution), whereas bacteria cannot. This is an important distinction since the simultaneous photoevolution of O_2 and fixation of carbon dioxide (or H_2 evolution) using water as the substrate is an energetically uphill reaction, whereas the dehydrogenation of energy-rich organic substrates by bacteria is not. The central part of Fig. 8.1 shows the so-called Z-scheme, the mainly linear electron transport scheme in which the two photosystems of green-plant photosynthesis, Photosystem I and II (PSI and PSII), cooperate to span the potential difference between water oxidation and H^+ reduction. This normally results in the photoevolution of molecular O_2 (PSII) and the reduction of CO_2 (PSI) by electrons from reduced ferredoxin (Fd) and NADPH in the Calvin cycle. Figure 8.1 also shows the two types of bacterial photosynthetic reaction centre that convert light energy into chemical potential: the pheophytin–quinone and the iron–sulphur types. Interestingly, the Z-scheme has one centre of each type, suggesting an evolutionary relationship between plants and bacteria. Low-potential reductant (reduced Fd) in purple photosynthetic bacteria is probably generated by proton-motive force, Δp, coupled to reverse electron transfer (Nicholls and Ferguson, 1992). Electrons from Fd can then reduce NAD^+ and CO_2.

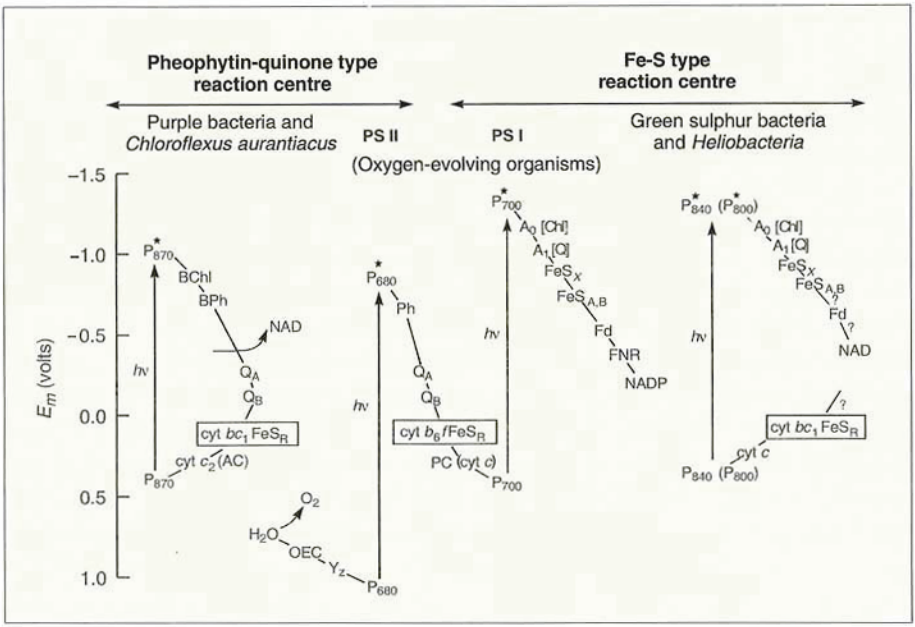


Figure 8.1 A comparison of bacterial and plant photosynthetic electron-transport pathways (Blankenship, 1992; modified and reproduced with permission of Kluwer Academic Publishers) plotted on an electrochemical scale (*vs.* SHE) where the top is lower redox potential (more reducing). On an evolutionary scale, the two reaction centres PSI and PSII are closely related in algae and plants, but each is closely related to different types of more primitive bacterial reaction centres, as shown by the horizontal arrows. The abbreviations represent electron transport carriers, and electrons enter the Benson–Calvin cycle (where CO_2 is fixed) at the level of NADH or NADPH. Electron-transfer-coupled proton transport across the membrane (from top to bottom in the schematic) is not shown, but the resultant H^+ gradient drives ATP production. Reduction of NAD^+ in the case of purple bacteria is coupled to reversed electron transfer. See the text for more details.

The mechanism of H_2 photoproduction by algae has evoked some controversy, and it now appears that there are at least two different pathways for this process. Both pathways are traditionally thought of as terminating in PSI. One pathway involves water splitting and electron transport through both PSII and PSI with simultaneous O_2 and H_2 evolution. The direct source of electrons for H_2 production is reduced ferredoxin. The second pathway involves electron donation from products of fermentation and oxidative carbon metabolism to PSI through the plastoquinone pool (see Section 8.4 and Fig. 8.3 for details), leading to the simultaneous production of H_2 (also *via* ferredoxin) and CO_2. In some respects, the second mode of H_2 photoproduction in algae is somewhat analogous to nitrogenase-dependent H_2 photoproduction in photosynthetic bacteria and cyanobacteria, which normally use organic

compounds as intermediate electron donors to photochemical reaction centres. However, in oxygenic microorganisms, irrespective of the mode of H_2 photoproduction, water is the ultimate electron source for biosynthesis of stored organic compounds, unlike anoxygenic photosynthetic bacteria, which require energy-rich organic or inorganic substrates as sacrificial donors.

The physiological significance and usefulness of H_2 evolution in phototrophs are still not well known (Kessler, 1974; Boichenko and Hoffmann, 1994), but the process is probably a regulatory phenomenon associated with the survival of the organisms under extreme conditions. Since it is a strictly anaerobic process, it was extremely important in the distant geological past as a mechanism of eliminating excess cellular reductant. However, H_2 metabolism still occurs today in restricted water and soil habitats protected from the Earth's atmosphere or in organisms that have developed cellular strategies to protect H_2-evolving enzymes from O_2. Although the by-product of anaerobiosis, H_2, constitutes a negligible part of the current atmosphere (500 ppb; *www.c-f-c.com/charts/atmosph.htm*), this does not indicate a minor role for this metabolism in the global biosphere cycles that have resulted from the evolution of life and the environment. Rather, H_2 production is immediately followed by H_2 consumption involving scavenging methanogens, sulphate reducers and some aerobes. What H_2 does actually enter the atmosphere quickly escapes into outer space. Nevertheless, the unique ability of some algae to photoproduce H_2 from water may have significant biotechnological applications in the future. Progress along these lines using intact cells (Reeves and Greenbaum, 1985; Markov *et al.*, 1995a; Markov *et al.*, 1997; Ghirardi *et al.*, 1997; Melis *et al.*, 2000; Ghirardi *et al.*, 2000b) has been made recently. Moreover, new proposals on the design of photobioreactors for the production of fuels and chemicals using biomimetic systems incorporating bio-electronics and biometallocatalysis have appeared (Lee and Greenbaum, 1995, 1997).

8.2 Distribution and activity of H_2 photoproducers

Since the discovery of H_2 photoproduction in the green alga *Scenedesmus obliquus* (Gaffron and Rubin, 1942) and in the purple bacterium *Rhodospirillum rubrum* (Gest and Kamen, 1949), numerous phototrophs have been found capable of this reaction (Kessler, 1974; Weaver *et al.*, 1980; Lambert and Smith, 1981; Vignais *et al.*, 1985; Brand *et al.*, 1989; Sasikala *et al.*, 1993; Boichenko and Hoffmann, 1994). With a few exceptions (Boichenko and Hoffmann, 1994), all such species belong either to anoxygenic photosynthetic bacteria or to oxygenic cyanobacteria and green algae; Tables 8.1 and 8.2 give lists of some of these species and their hydrogen-evolving

activity. A complete list of the H_2 photoproducers includes several hundreds of species from at least 50 genera of prokaryotes and 33 genera of eukaryotes. The website *www.aist.go.jp/NIBH/ourpages/iea/index.html* lists many H_2-producing organisms of interest to the International Energy Agency (IEA) cooperative program in biohydrogen (now Annex 15, Photobiological H_2 Production).

8.2.1 Photosynthetic bacteria

With few exceptions, purple and some green photosynthetic bacteria related to gram-negative prokaryotes, as well as heliobacteria related to the group of gram-positive *Clostridia*, are active diazotrophs that synthesise nitrogenase for N_2 fixation under anoxic nitrogen-limiting conditions (Madigan *et al.*, 1984; Kimble and Madigan, 1992; Warthmann *et al.*, 1992). Although these bacteria contain hydrogenases (Adams *et al.*, 1981; Kovács and Bagyinka, 1990; Wu and Mandrand, 1993), they normally photoproduce H_2 using nitrogenase (Sasikala *et al.*, 1993) when both ATP and low-potential electrons from ferredoxin are available.

Purple photosynthetic bacteria contain a PSII-like reaction centre (see Fig. 8.1) that is incapable of direct photochemical reduction of ferredoxin but can generate ATP *via* cyclic electron flow. In these bacteria, ferredoxin reduction (hence CO_2 fixation and H_2 evolution) is coupled to organic substrate-driven reverse electron transport. In contrast, the photogeneration potential of the 'PSI–like' reaction centres in green bacteria and heliobacteria is sufficiently low for direct reduction of ferredoxin. Table 8.1 shows that the maximum sustainable rate of nitrogenase-mediated H_2 photoproduction reported in purple and green bacteria for a period of days or more (rates depend on the strain, growth conditions, and assay conditions) is 4–12 mmol H_2 (g dry mass)$^{-1}$ h^{-1} (Weaver *et al.*, 1980; Vignais *et al.*, 1985; Warthmann *et al.*, 1992; Sasikala *et al.*, 1993; Klemme, 1993), when an organic acid or sulphide is the electron donor. These rates vary more widely among species of bacteria when expressed on the basis of pigment content, as a result of differences in organisation of light-harvesting antenna complexes and light-adaptation changes. Although a theoretical ratio of 1:1 should exist for nitrogenase activities in terms of H_2 evolution and acetylene reduction to ethylene (a specific assay for N_2 fixation), the net H_2 production rates in many species and strains are lower than 1:1. This is due to the H_2 recycling activity of membrane-bound 'uptake hydrogenase' (Kovács and Bagyinka, 1990; Sasikala *et al.*, 1993). The function of uptake hydrogenases is to improve the efficiency of nitrogen fixation by preventing the loss of cellular reductant in the form of H_2, produced as a by-product of normal nitrogenase function. Thus,

Table 8.1 Maximal rates of H_2 photoproduction on a time scale of hours to days in photosynthetic organisms

Species	Electron donor	Maximum photoproduction rate mmol H_2 (C_2H_4) (g dry mass)$^{-1}$ h^{-1}		mmol H_2 (g [B]Chl)$^{-1}$ h^{-1}	References
Purple bacteria					
Chromatium sp. PBS 1071	succinate	6			Vignais *et al.*, 1985
Ectothiorhodospira shaposhnikovii	sulphide	0.4			Vignais *et al.*, 1985
Thiocapsa roseopersicina	pyruvate	0.9			Vignais *et al.*, 1985
Rhodobacter capsulatus B10	lactate	11.6			Vignais *et al.*, 1985
Rb. capsulatus Z1	malate	5.8			Hillmer and Gest, 1977a
Rb. capsulatus J2	lactate	4	(1.5)	800	Hillmer and Gest, 1977b
Rb. capsulatus 37b4	malate			270	Steinborn and Oelze, 1989
Rb. sphaeroides 8703	lactate	11.7			Miyake and Kawamura, 1987
Rhodocyclus gelatinosus	malate		(2.5)		Madigan *et al.*, 1984
Rhodomicrobium vanniellii	malate		(1.6)		Madigan *et al.*, 1984
Rhodopila globiformis	malate		(0.5)		Madigan *et al.*, 1984
Rhodopseudomonas palustris	lactate	2.7	(0.7)		Vignais *et al.*, 1985; Madigan *et al.*, 1984
Rps. Viridis	lactate	0.2	(2.5)		Vignais *et al.*, 1985; Madigan *et al.*, 1984
Rhodospirillum rubrum S1	lactate	5.5	(5.0)	2500	Klemme, 1993; Madigan *et al.*, 1984
Green bacteria					
Chlorobium vibrioforme	acetate	3.8		32	Warthmann *et al.*, 1992
Heliobacteria					
Heliobacillus mobilis	pyruvate		(4.4)		Kimble and Madigan, 1992
Heliobacterium chlorum	pyruvate		(2.9)		Kimble and Madigan, 1992
H. gestii	pyruvate		(1.8)		Kimble and Madigan, 1992

Table 8.1 cont'd.

Species	Electron donor	Maximum photoproduction rate mmol H_2 (C_2H_4) (g dry mass)$^{-1}$ h^{-1}		mmol H_2 (g [B]Chl)$^{-1}$ h^{-1}	References
Cyanobacteria					
Anabaena cylindrica 1403	water			335	Berchtold and Bachofen, 1979
A. variabilis ATCC29413	fructose	2			Reddy *et al.*, 1996
Lyngbya sp.	glycogen	0.2		226	Kuwada and Ohta, 1987
Mastigocladus laminosus	glycogen	1.0	(2.6)		Miyamoto *et al.*, 1979a
Oscillatoria sp. B97	glycogen	0.4	(0.4)	260	Kumazawa and Mitsui, 1981
O. limnetica	sulphide			50	Belkin and Padan, 1978
Synechococcus sp. BGO43511	pyruvate	1.3	(1.5)	170	Kumazawa and Mitsui, 1994; Luo and Mitsui, 1994
Green algae					
Chlamydomonas reinhardtii	water			2	Reeves and Greenbaum, 1985
C. reinhardtii cc124	water			16–28**	Melis *et al.*, 2000 Ghirardi *et al.*, 2000b
C. reinhardtii F60	acetate	0.9		40	Gibbs *et al.*, 1986
Chlamydomonas sp. MGA 161					
dark fermentation	starch	0.3*		~6*	Miyamoto *et al.*, 1990
hydrogenase activity	MV red	15*		~300*	Miyamoto *et al.*, 1990
Chlorella vulgaris	water	~0.7		20	Pow and Krasna, 1979
Scenedesmus obliquus	water	~1		30	Pow and Krasna, 1979
hydrogenase activity	MV red	~17*		~500*	Urbig *et al.*, 1993

In some nitrogenase-containing species, the enzyme activity is given as an acetylene photoreduction to ethylene rate (in parentheses). In the references, a single asterisk indicates a hydrogenase-mediated, dark H_2 evolution assay when an exogenous donor is provided. The double asterisks indicate H_2 produced in bulk and collected as a pure gas; this rate lasted for up to 35 h (see the end of Section 8.6.1 for more information).

uptake hydrogenases reincorporate released molecular H_2. In *Hup*$^-$ mutants of purple bacteria (Jahn *et al.*, 1994; Kern *et al.*, 1994) and cyanobacteria (Mikheeva *et al.*, 1995), which lack uptake hydrogenase, the efficiency and rate of H_2 photoproduction are enhanced in comparison to those of wild-type (WT) cultures.

The nitrogenase system operates with a turnover rate of only 5 per second (Thorneley and Lowe, 1983) and has a relatively short half-life (40 h) at saturating irradiances (Kim *et al.*, 1980). To compensate, N_2-fixing purple bacteria synthesise large amounts of this enzyme, which constitutes up to the 25% of the soluble protein in nitrogen-starved cells (Vignais *et al.*, 1985). Under these conditions, enzyme accumulation is probably stimulated by the high rate of photophosphorylation that ensures high capacity for H_2 photoproduction (Klemme, 1993).

8.2.2 *Cyanobacteria*

As early as 3.5 billion years ago, oxygenic prokaryotic cyanobacteria acquired the ability to oxidise water (Blankenship, 1992), using PSII in tandem with PSI to reduce ferredoxin (see Fig. 8.1). A by-product of this key photoreaction, O_2, was toxic but necessary for evolution of higher life forms. Consequently, special adaptive mechanisms were required for N_2 fixation and H_2 metabolism due to O_2-sensitive nitrogenases (Hill, 1988), as described below.

Cyanobacteria have both unicellular and filamentous forms. The latter group includes heterocystous species, containing ~10% distinct cells known as heterocysts that are capable of fixing nitrogen (Lambert and Smith, 1981; Houchins, 1984) as a result of repressed PSII activity in these specialised cells. Energy for N_2 fixation in the thick-walled heterocysts is supplied by PSI-mediated cyclic photophosphorylation, and enhanced respiration, using carbohydrates imported from CO_2-fixing vegetative cells, helps protect nitrogenase from O_2 inactivation. Recently, Janson *et al.* (1994) showed in a nonheterocystous filamentous cyanobacterium (*Trichodesmium* spp.) that ~10% of the cells in central regions of the trichome are also specifically adapted for N_2 fixation. In other nonheterocystous filamentous and unicellular cyanobacteria, O_2 evolution and O_2-sensitive nitrogenase activities are separated temporally (Suda *et al.*, 1992; Rojek *et al.*, 1994) through a regulatory mechanism involving changes of the redox state of the secondary quinone acceptor, Q_b, of PSII (Misra and Desai, 1993).

Hydrogen photoproduction in cyanobacteria, as in photosynthetic bacteria, is mediated primarily by nitrogenase (Benemann and Weare, 1974; Weaver *et al.*, 1980; Lambert and Smith, 1981; Houchins, 1984; Markov *et al.*, 1995b). Under non-optimal

conditions, typical long-term rates of production are rather low, about 1% of the normal O_2 evolution rates (Berchtold and Bachofen, 1979). Nevertheless, some strains of cyanobacteria can photoproduce up to 2 mmol H_2 (g dry mass)$^{-1}$ h^{-1} (Table 8.1) from organic substrates (Luo and Mitsui, 1994; Reddy *et al.*, 1996) or cellular glycogen (Miyamoto *et al.*, 1979a; Kumazawa and Mitsui, 1981, 1994; Suda *et al.*, 1992; Markov *et al.*, 1995a) stored during autotrophic photosynthesis. However, in filamentous cyanobacteria, where O_2 and H_2 evolution are separated spatially, the maximal rate of H_2 photoproduction does not exceed 20% of the 'expected' rate (assuming no thermodynamic losses) based on the electron-transfer rates associated with the photosynthetic CO_2-fixation capacity of the organism. This is about the same as has been observed with green algae. A recalculation of the H_2-production rate in these strains on a heterocyst basis, though, would increase production to ~15 mmol (g dry mass)$^{-1}$ h^{-1} (Reddy *et al.*, 1996). On a chlorophyll basis, reported high rates of 160–330 mmol H_2 (g Chl)$^{-1}$ h^{-1} (Berchtold and Bachofen, 1979; Kumazawa and Mitsui; 1985, Kuwada and Ohta, 1987) are deceptive and may result from the relatively low chlorophyll content of the cells being observed. While cyanobacteria can produce H_2 in the presence of O_2, photosynthetic bacteria and algae have not developed protective systems, and thus cannot produce H_2 under aerobic conditions.

Many cyanobacteria have membrane-bound uptake hydrogenase function and/or soluble bidirectional hydrogenase activity (Adams *et al.*, 1981; Papen *et al.*, 1986; Appel and Schulz, 1998). Uptake hydrogenases have been found in all N_2-fixing filamentous and unicellular strains while bidirectional hydrogenases have been found only in some filamentous strains (Tamagnini *et al.*, 2000). Almost all unicellular strains investigated so far contain a bidirectional hydrogenase (Appel *et al.*, 2000b). Immunological studies have shown that the uptake hydrogenase in unicellular cyanobacteria is a thylakoid-bound enzyme, whereas the reversible (bidirectional) hydrogenase is associated with both the cytoplasmic and thylakoid membranes (Kentemich *et al.*, 1989; Serebryakova *et al.*, 1996). However, only a few findings, shown in Table 8.2, indicate participation of hydrogenase in the initial light-stimulated burst of H_2 production by anaerobically adapted cultures of cyanobacteria grown in nitrate-containing media (Boichenko *et al.*, 1989). The same type of H_2-photoproduction activity has been shown in *Acaryochloris marina* (Boichenko *et al.*, 2000) a unique prokaryotic relative of cyanobacteria that contains chlorophyll *d* (Miyashita *et* al., 1996; Hu *et al.*, 1998). Also, considerable long-term hydrogenase-mediated H_2 photoevolution has been clearly demonstrated in the nonheterocystous cyanobacterium *Oscillatoria limnetica* that was adapted to conditions of anoxygenic, sulphide-dependent CO_2 photoassimilation (Belkin and Padan, 1978). In non-nitrogen-fixing cells of heterocystous *Anabaena variabilis*, anaerobic incubation

Table 8.2 Maximum initial rates of H_2 photoproduction on a time scale of seconds to minutes, aerobic light-saturated rates of O_2 evolution, ratios of these rates and relative maximum quantum yields of these processes in various species of cyanobacteria and green algae

Species	Maximum photoproduction rate mmol (g Chl)$^{-1}$ h^{-1}		Rate ratio H_2/O_2	Quantum yield ratio H_2/O_2	References
	H_2	O_2			
Cyanobacteria					
Anabaena cylindrica	6	130	0.05	1.0	Boichenko *et al.*, 1989
Chamaesiphon confervicola	9	200	0.05	0.13	Boichenko *et al.*, 1989
Chroococcus globosus	8	280	0.03	0.4	Boichenko *et al.*, 1989
Chroococcidiopsis thermalis	12	360	0.04	0.9	Boichenko *et al.*, 1989
Cyanothece minerval	11	200	0.06	0.1	Boichenko *et al.*, 1989
Gloeocapsa alpicola	10	220	0.05	0.06	Boichenko *et al.*, 1989
Plectonema boryanum	12	360	0.04	0.3	Boichenko *et al.*, 1989
Synechocystis aquatilis	25	320	0.08	0.4	Boichenko *et al.*, 1989
Oscillatoria sp. Miami BG7	200				Kumazawa and Mitsui, 1985
Green algae					
Chlamydomonas moewusii	100				Brand *et al.*, 1989
C. reinhardtii 137c(+)	60–120				Greenbaum, 1980
					Lien and San Pietro, 1981
					McBride *et al.*, 1977
C. reinhardtii CALU 449	300	210	1.4	3.8	Boichenko *et al.*, 1989
C. reinhardtii 24g.1	454				Ghirardi *et al.*, 1998
Chlamydobotrys stellata	70				Boichenko *et al.*, 1992

Table 8.2 cont'd.

Species	Maximum photoproduction rate mmol (g Chl)$^{-1}$ h^{-1}		Rate ratio H_2/O_2	Quantum yield ratio H_2/O_2	References
	H_2	O_2			
Chlorella vulgaris	70	200	0.35	3.7	Boichenko *et al.*, 1989
Chlorococcum humicolum	220	320	0.7	3.2	Boichenko *et al.*, 1989
C. littorale	120				Schnackenberg *et al.*, 1995
Coelastrum proboscideum	120	240	0.5	1.9	Boichenko *et al.*, 1989
Coccomyxa lacustris	50	220	0.22	3.2	Boichenko *et al.*, 1989
Dictyosphaerum pulchellum	140	140	1.0	2.1	Boichenko *et al.*, 1989
Kirchneriella obesa	56	340	0.16	1.2	Boichenko *et al.*, 1989
Pandorina morum	68				Brand *et al.*, 1989
Pediastrum boryanum	90	300	0.3	4.3	Boichenko *et al.*, 1989
Scenedesmus obliquus D3	50–180				Francis and Senger, 1985 Urbig *et al.*, 1993
S. obliquus CALU 13	220	240	0.9	5.3	Boichenko *et al.*, 1989
Scotiella nivalis	60	140	0.43	1.4	Boichenko *et al.*, 1989
Tetraedron caudatum	64	270	0.25	2.9	Boichenko *et al.*, 1989

induces reversible hydrogenase activity despite the fact that little difference in the amount of enzyme detected immunologically is observed before and after induction (Troshina *et al.*, 1996). Low but significant H_2-uptake activity was always observed in these cells, but this was attributed to the reversible, not the uptake, hydrogenase. Consistent with this is the fact that the uptake hydrogenase is normally regulated in concert with nitrogenase in the absence of combined nitrogen (Appel and Schulz, 1998) to conserve reductant (H_2 produced as a waste product of nitrogen fixation) as in photosynthetic bacteria. Since nitrogenase activity in cyanobacteria is also inhibited by H_2 (Appel and Schulz, 1998), removal of H_2 by uptake hydrogenase, a light-dependent reaction, also appears to protect nitrogenase activity. The reader is cautioned that differences in structural and functional observations in different cyanobacteria may be explained by differences in strain, methods of measurement, cultivation conditions and growth phase of the culture.

8.2.3 Algae

Although many eukaryotic algae contain hydrogenase activity (Kessler, 1974), the capacity for H_2 photoproduction is found only in species of thirty genera of green algae, two species of yellow-green algae and one species of diatom (Boichenko and Hoffmann, 1994). Hydrogen photoproduction has never been observed in macroalgae (Ben-Amotz *et al.*, 1975; Greenbaum and Ramus, 1983) or in those unicellular algae, such as *Porphyridium*, *Euglena* and *Dunaliella* (Boichenko *et al.*, 1989; Brand *et al.*, 1989), which belong to the list of hydrogenase-containing species (Kessler, 1974). On the other hand, there are scattered reports of H_2 photoproduction by terrestrial plant cells (Kulandaivelu *et al.*, 1988; Schulz, 1996), but these are probably artifacts attributable to microbial contamination. There is a diversity of H_2-metabolising pathways in eukaryotes, perhaps as a result of different properties of individual hydrogenases, as seen in prokaryotes. Furthermore, an extremely wide variation in H_2 photoproduction capacity exists among the various algae, among different strains of the same species and within the same strain under various growth and adaptation conditions (Boichenko and Hoffmann, 1994).

Unlike cyanobacteria, with their advanced protective mechanisms for their essential nitrogenase systems, hydrogenases of green algae are very sensitive to O_2 inactivation as a result of the irrelevance of these enzymes for aerobic metabolism. Effective donation of electrons from PSII-driven water-splitting function to H_2 production therefore critically depends on the removal of evolved O_2 (Pow and Krasna, 1979). Studies with D_2O (Abdel-Basset *et al.*, 1998), mutants and inhibitors

(*e.g.*, Schulz, 1996; Ghirardi *et al.*, 1998) have confirmed the old results that water is the source of H_2 in both cyanobacteria and algae. In the presence of the O_2 scavenger dithionite, the maximum reported longer-term rates of H_2 photoproduction are in the range 0.7–1 mmol (g dry mass)$^{-1}$ h^{-1} or 20–30 mmol (g Chl)$^{-1}$ h^{-1} in *Chlorella* and *Scenedesmus* (Table 8.1). With continuous purging of evolved gases by an inert carrier gas stream, the sustained (up to 160 h) rates of H_2 photoproduction did not exceed 2–3 mmol (g Chl)$^{-1}$ h^{-1} (Greenbaum, 1980, 1984; Reeves and Greenbaum, 1985). Sustained PSII-supported H_2 production rates were, therefore, not more than 1–20% of the maximum CO_2 fixation rates observed during oxygenic photosynthesis. These rates are comparable with PSI-dependent H_2 production rates during dehydrogenation (photodissimilation) of acetate (Gibbs *et al.*, 1986) and with dark H_2 production rates during fermentation of stored starch in some strains of algae (Miura *et al.*, 1986; Brand *et al.*, 1989; Miyamoto *et al.*, 1990).

In contrast to the moderate, sustained rates of H_2 production observed *in vivo*, short-term (minutes) *in vitro* assays for hydrogenase activity in crude cell extracts using reduced methyl viologen as a donor indicate potential maximum rates of 10–17 mmol (g dry mass)$^{-1}$ h^{-1} or about 300–500 mmol (g Chl)$^{-1}$ h^{-1} (Miyamoto *et al.*, 1990; Urbig *et al.*, 1993).

In fact, under optimal conditions the maximum short-term H_2 production rates of the most active producers among green algae (Table 8.2) reach 100–300 mmol (g Chl)$^{-1}$ h^{-1}. These are close to the rates of hydrogenase activity *in vitro* and to the maximal steady-state rates of CO_2-dependent O_2 evolution (Boichenko *et al.*, 1989). The highest transient rate ever reported for a green alga was 454 mmol (g Chl)$^{-1}$ h^{-1} in a *C. reinhardtii* variant selected from a WT population under conditions where the organisms were able to grow on H_2 (Ghirardi *et al.*, 1998). However, these high rates were recorded only during the first few seconds (Boichenko *et al.*, 1989, 1992) or minutes (McBride *et al.*, 1977; Greenbaum, 1980; Brand *et al.*, 1989; Ghirardi *et al.*, 1998) of irradiation. Subsequently, they decay rapidly (the decay is multiphasic on the scale of seconds to hours) to the low sustainable levels reported in Table 8.1. Steady-state levels of H_2 production probably depend on several interrelated factors, including the equilibrium between rates of hydrogenase inactivation and re-synthesis, capacity for alternative electron donation from reduced organic donors of fermentation, competitive reactions involving ferredoxin re-oxidation by other endogenous electron acceptors, cyclic electron transport around PSI and redox control of electron transport. Electron donation from organic substrates controls the redox state of the photosynthetic electron-transfer chain between PSII and PSI, and hence this redox state modulates the activity of water oxidation *via* a feedback mechanism (Diner and Mauzerall, 1973; Boichenko and Hoffmann, 1994). A recent advance in

sustainable algal H_2-production activity was observed in a sulphur-deprived system (Melis *et al.*, 2000; Ghirardi *et al.*, 2000) that is described in Section 8.6.1.

Using data on the properties of purified algal reversible hydrogenases (Happe and Naber, 1993; Schnackenberg *et al.*, 1993), one can calculate that the enzyme content in anaerobically adapted algae constitutes <1% of the soluble proteins. This corresponds to a ratio of one hydrogenase per >10,000 Chl, or one hydrogenase per >10 PSI units. Therefore, increasing the amount of hydrogenase and/or its synthesis rate in the organisms could well lead to substantially improved, longer-term H_2 production rates.

8.3 Structure and mechanism of the enzymes catalysing H_2 production

We have already noted that H_2 photoproduction in phototrophs is catalysed by two different types of enzymes, nitrogenases and hydrogenases. Although these enzymes have some similarities (Kim and Rees, 1992, 1994) and probably originate from a common molecular ancestor of FeS proteins, their basic properties and functions are quite different. Hydrogenases can in turn be classified as uptake or bidirectional (reversible) depending on their function. X-ray crystal structures have been reported for (i) the heterodimeric NiFe uptake hydrogenase from *Desulfovibrio gigas* (Volbeda *et al.*, 1995); (ii) the heterodimeric NiFe bidirectional hydrogenase from *Desulfovibrio vulgaris* Miyazaki F (Higuchi *et al.*, 1997); and (iii) the Fe-only hydrogenases from *Clostridium pasteurianum* (monomeric and bidirectional; Peters *et al.*, 1998) and *Desulfovibrio desulfuricans* (heterodimeric and uptake; Nicolet *et al.*, 1999). Several nitrogenase structures have also been solved, and we shall discuss these next.

8.3.1 Nitrogenases

Biological nitrogen fixation is catalysed by the nitrogenase enzyme system. This can mediate three different reactions under physiological conditions:

$$N_2 + 8Fd_{red} \rightarrow 2NH_3 + 8Fd_{ox} + H_2 \quad (8.2)$$

$$2Fd_{red} \rightarrow 2Fd_{ox} + H_2 \quad (8.3)$$

$$ATP + H_2O \rightarrow ADP + P_i \quad (8.4)$$

These are for one-electron Fd reactions only. According to the thermodynamics of these reactions (Alberty, 1994), the third is necessary for the first two reactions to

occur, but it is not coupled to them stoichiometrically. However, it is generally accepted (Burris, 1991; Smith and Eady, 1992; Kim and Rees, 1994; Howard and Rees, 1994) that, under optimal conditions, two ATP molecules are hydrolysed per electron pair transferred. Normally one molecule of H_2 is produced per molecule of N_2 fixed, but higher ratios have been reported (Eady, 1996). Irreversible H_2 evolution is an inherent property of the nitrogenase mechanism, and the enzyme can be described as an ATP-dependent hydrogenase.

The nitrogenase enzyme system is a dynamic supercomplex of two proteins, a homodimeric Fe protein (*nif*H gene product, dinitrogenase reductase) of 60 kDa and an $\alpha_2\beta_2$ tetrameric MoFe protein (*nif*D/*nif*K gene product, dinitrogenase) of ~240 kDa (see Fig. 8.2). The crystal structure of these proteins (Georgiadis *et al.*, 1992; Kim and Rees, 1992, 1994; Howard and Rees, 1994) explains their functional arrangement. The Fe protein contains a [4Fe–4S] cluster at the interface between two subunits near the surface of the globular dimer, and two nucleotide-binding sites for MgATP/MgADP located at the interface, 20 Å from the cluster. This arrangement suggests an allosteric coupling mechanism between these functional sites. Each of the two heterodimeric units of MoFe protein contains: (i) a substrate-binding FeMo cofactor (buried in the α subunit about 10 Å below the protein surface) formed from [Mo–3Fe–3S] and [4Fe–3S] clusters bridged by three sulphides and (ii) a P-cluster pair of two disulphide-bridged [4Fe–4S] clusters (see Fig. 8.2) located on the interface between the α and β subunits. The tetrameric MoFe protein is mainly packed by interactions of β subunits and further stabilised by a calcium-binding site.

The slow catalytic turnover of nitrogenase involves a sequence of electron pair transfer events each involving the same rate-limiting step prior to the final concerted N_2 fixation reaction. According to the docking model (Kim and Rees, 1992, 1994; Howard and Rees, 1994), the overall kinetics of the nitrogenase-catalysed reactions critically depends on the formation of a complex between the Fe and MoFe proteins, and the rate-limiting step is the dissociation of this complex (Thorneley and Lowe, 1983). As suggested above, only the ferredoxin-reduced Fe-protein $[ATP]_2$ complex has the appropriate configuration to form a supercomplex with the MoFe protein in the functional orientation of an almost straight electron path, where the distances from the Fe protein [4Fe–4S] cluster to the P-cluster pair and from the latter to the FeMo cofactor are 18 Å and 14 Å respectively. Further unidirectional electron transfer to the FeMo cofactor may be triggered by (i) configurational changes of both supercomplex partners arising from ATP hydrolysis within the Fe protein and resulting in more intimate contact between the [4Fe–4S] cluster and the P-cluster pair at <15 Å; and (ii) a rotation-dependent change in the environment of the P-cluster pair.

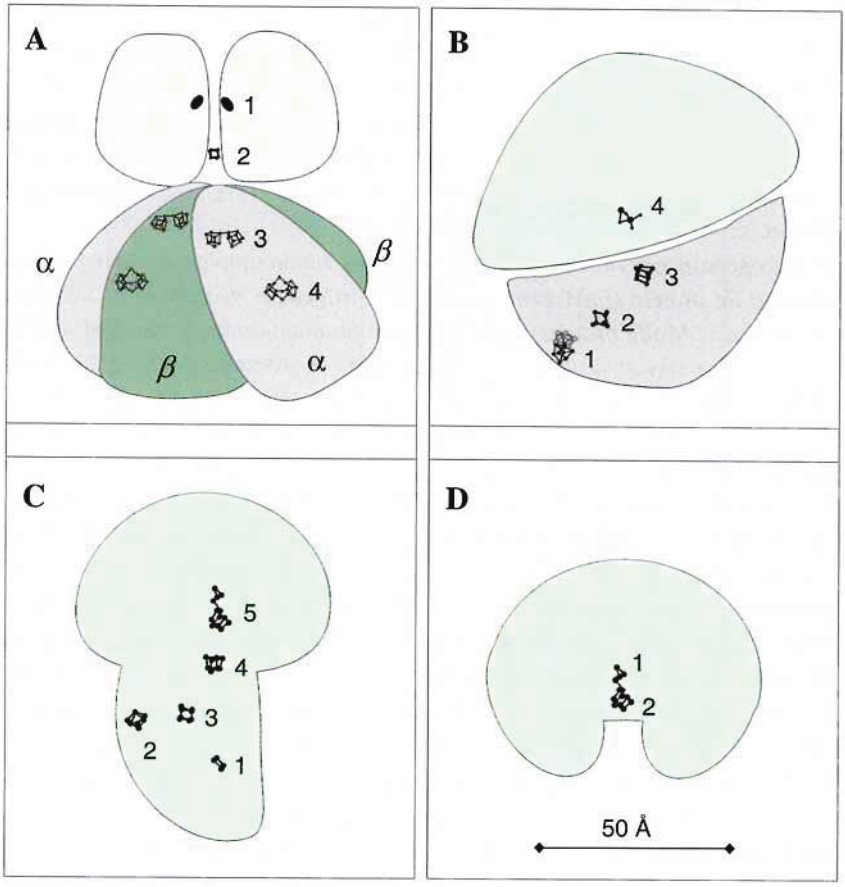


Figure 8.2 Structural models of nitrogenase and hydrogenases based on crystallographic data. **A**, supercomplex of dimeric Fe–protein and tetrameric MoFe-protein of nitrogenase from *Azotobacter vinelandii* (adapted from Kim and Rees, 1992): 1, nucleotide-binding site; 2, [4Fe–4S] cluster; 3, P-cluster pair; 4, FeMo-cofactor. **B**, heterodimeric NiFe-hydrogenase from *Desulfovibrio gigas* (adapted from Volbeda *et al.*, 1995): 1, distal [4Fe–4S] cluster; 2, [3Fe–4S] cluster; 3, proximal [4Fe–4S] cluster; 4, active centre NiFe-cluster. **C**, monomeric Fe-hydrogenase from *Clostridium pasteurianum* (adapted from Peters *et al.*, 1998): 1, [2Fe–2S] cluster; 2, $[4Fe–4S]_C$ cluster; 3, $[4Fe–4S]_B$ cluster; 4, $[4Fe–4S]_A$ cluster; 5, active centre H-cluster. **D**, monomeric Fe-hydrogenase from green algae containing an active-centre with: 1, [2Fe–2S] and 2, [4Fe–4S] clusters; no other metal clusters are found in the algal enzyme (based on reconstructions of Florin *et al.*, 2001 and Happe and Kaminski, 2002). The images are proportional to the protein and cofactor sizes, each panel width being 100 Å.

Alternative vanadium- and iron-only nitrogenases have very similar amino acid sequences to the usual enzyme form. However, unlike the MoFe-protein, both the FeV-protein (*vnf*DKG gene product) and the Fe-only protein (*anf*DKG gene product) are hexamers, containing two small additional subunits that stabilise their quaternary structures. Interestingly, the alternative nitrogenases have diminished N_2-fixation activity and increased H_2-production ability compared with Mo nitrogenase (Davis *et al.*, 1996).

8.3.2 Hydrogenases

Hydrogen-acceptor oxidoreductases catalyse the reversible reaction

$$2H^+ + 2e^- \leftrightarrow H_2$$

and are a widespread group of different classes of iron–sulphur proteins (Adams *et al.*, 1981; Adams, 1990; Przybyla *et al.*, 1992; Wu and Mandrand, 1993; Albracht, 1994; Vignais *et al.*, 2001). On the basis of the metal ions of their active centres, hydrogenases are divided into Fe-hydrogenases (Adams, 1990) and NiFe(Se)-hydrogenases (Albracht, 1994). Fe-hydrogenases exhibit much greater specific activities than NiFe-hydrogenases (Adams, 1990). There are also a couple of reports in the methanogenic archaea literature of a hydrogenase that possesses no metal ions (Zirngibl *et al.*, 1992; Thauer *et al.*, 1996). The enzymes are further classified according to their physiological electron carriers (ferredoxin, cytochrome, NAD, F420, *etc.*) and physiological functions (H_2-uptake, H_2-evolving, bi-directional, or H_2-sensor). With the accumulation of new data on the molecular biology of different species of microorganisms, there are now detailed specialised reviews comparing the protein structures of hydrogenases and their phylogeny (Wu and Mandrand, 1993; Vignais *et al.*, 2001). The Fe-hydrogenase family is especially interesting from the point of view of the origin and evolution of eukaryotes (reviewed in Horner *et al.*, 2002). Furthermore, detailed X-ray analyses of the structure of NiFe-hydrogenases (Volbeda *et al.*, 1995; Higuchi *et al.*, 1997) and Fe-hydrogenases (Peters *et al.*, 1998; Nicolet *et al.*, 1999) now make it possible to describe both the molecular arrangement of the proteins and the active sites of the enzyme (Fig. 8.2). Several excellent reviews that examine NiFe- and Fe-hydrogenases have appeared quite recently (Vignais *et al.*, 2001; Frey, 2002; Tamagnini *et al.*, 2002; Happe *et al.*, 2002).

In photosynthetic bacteria, the membrane-bound H_2-uptake hydrogenase functions physiologically in an energy-conserving mode (alone or in tandem with nitrogenase) (Kovács and Bagyinka, 1990) and is a Class I NiFe-hydrogenase (Wu and Mandrand,

1993). The heterodimeric protein consists of 40-kDa (*hup*S gene product) and 66-kDa (*hup*L gene product) subunits (Vignais and Toussaint, 1994). The bacterial enzymes possess two different types of clusters, the H_2-interacting, active-site H-cluster and at least two electron-transferring F-clusters containing [4Fe–4S] moieties (Adams, 1990). From the crystal structure of the Class II uptake hydrogenase from *D. gigas*, a non-photosynthetic organism (Fig. 8.2; Volbeda *et al.*, 1995), the [NiFe–4S] cluster (the active site) is buried in the large subunit, close to the interface of the globular heterodimer. The other two [4Fe–4S] clusters (distal to the Ni ion) and an intermediary [3Fe–4S] cluster are distributed in the small subunit along nearly a straight line from its surface to the interface near the Ni active site. The observed arrangement of this hydrogenase also indicates a possible proton transport pathway to the active site *via* several histidine residues that are highly conserved among NiFe(Se)-hydrogenases. The Fe–S centres, constituting the electron pathway to, or from, the active site in the two Fe-only hydrogenases with crystal structures exhibit different geometries, but only the *D. desulfuricans* uptake hydrogenase is similar to the analogous *D. gigas* NiFe enzyme geometry. In the case of the evolving *C. pasteurianum* Fe-only hydrogenase, the three Fe–S centres distal to the active site are branched (Fig. 8.2; Peters *et al.*, 1998). One can speculate that different electron carriers may interact by different molecular pathways in the enzyme.

Although the currently identified NiFe- and Fe-hydrogenases from bacteria differ in structure and functional properties, an infrared spectroscopic study by van der Spek *et al.* (1996) indicates that their active sites (required for the activation of H_2) exhibit similar architecture. In particular, groups of infrared bands at 2100–1800 cm^{-1} may correspond to three small non-protein ligands that coordinate Fe in the NiFe active site (Volbeda *et al.*, 1995). The active sites of the two Fe-only hydrogenases for which there are crystal structures (Peters *et al.*, 1998; Nicolet *et al.*, 1999) are similar to each other in that they contain a [2Fe-2S] subcluster linked by a single cysteinyl S to a proximal [4Fe–4S] cubane subcluster. One of the two irons in the [2Fe] subcluster has a vacant coordination site and is probably the primary catalytic site of the protein (Nicolet *et al.*, 1999). The [2Fe-2S] subcluster is unusual in that it incorporates a unique ligand bridging the two Fe atoms (Nicolet *et al.*, 1999). X-ray structure and spectroscopic analysis of the *D. desulfuricans* iron hydrogenase, and DFT analysis of the active site model compounds have identified the ligand as di(thiomethly)amine (Nicolet *et al.*, 2001; Fan *et al.*, 2001). The X-ray crystal studies (Nicolet *et al.*, 1999) also indicate the following common features of active sites in NiFe- and Fe- hydrogenases: (i) coordination of diatomic ligands (probably CO and CN^-; Volbeda *et al.*, 1995; however, an SO may substitute for one of two CN^- ligands in some NiFe species, Higuchi *et al.*, 1997) to an Fe ion, probably helping to stabilise

a low redox state; (ii) a vacant coordination site on one of the metal ions, representing a possible substrate-binding site; (iii) a thiolate-bridged binuclear centre; and (iv) plausible proton-transfer channels and electron-transfer pathways. Both types of hydrogenases have newly identified cavities that connect the surface of the protein to the active site and may be pathways for the diffusion of molecular H_2 (Montet *et al.*, 1997).

Irrespective of the hydrogenase type, it is well established that activation of H_2 by each of the metal (M)-containing enzymes (Adams *et al.*, 1981; Adams, 1990; Albracht, 1994) involves a heterolytic cleavage of a H_2 molecule with the formation of a hydride and a proton: $MH_2M \leftrightarrow MH^-M + H^+$. This mechanism was assumed to require the presence of an appropriate basic site in the *D. gigas* enzyme, probably a histidine (Volbeda *et al.*, 1995), for stabilisation of the released proton. According to the proposed reaction scheme, there are no changes in the oxidation state of the metal(s) coordinating the hydride, and exchange with the solvent is independent at the two sites. The mechanism proposed for H_2 production *via* the *C. pasteurianum* hydrogenase (Peters *et al.*, 1998) involves displacement of a terminal water ligand bound to the [2Fe] subcluster on reduction, and subsequent generation of an Fe-hydride intermediate. A proximal cysteine residue (Cys299 in the clostridial Fe-hydrogenase, CpI probably acts as the proton donor for release of H_2 gas. Carbon monoxide, known to inhibit hydrogenase function, actually binds to the [2Fe] cluster, coordinately saturates the irons, and thereby directly inhibits the active site (Lemon and Peters, 2000).

In cyanobacteria, membrane-bound uptake hydrogenases have not as yet been characterised in detail; however, they probably belong to Class I. Surprisingly, the sequenced bidirectional (or reversible) hydrogenases from cyanobacteria (Schmitz *et al.*, 1995) show homology to Class IV NAD(P)-linked microbial NiFe-hydrogenases. Along with two structural subunits (*hox*H and *hox*Y), they contain two additional diaphorase subunits (*hox*U and *hox*F) that can bind flavin and [2Fe–2S] clusters (Serebryakova *et al.*, 1996). If other reversible hydrogenases of cyanobacteria also belong to this class, it seems reasonable to assume that their atypical participation in H_2 photoevolution (Belkin and Padan, 1978; Boichenko *et al.*, 1989) should involve ferredoxin-$NADP^+$ oxidoreductase. In fact, these hydrogenases have rather low catalytic activity and react poorly with ferredoxin (Houchins, 1984) but evolve H_2 with NADPH as a donor (Schmitz *et al.*, 1995). As in the algal case (Section 8.1), the function of the bidirectional hydrogenase in cyanobacteria may be to act as a redox-regulator 'valve' for low-potential electrons at the onset of light under rapidly changing light conditions. This could be done *via* coupling of the electron-transport chain to H_2 as a temporary storage vehicle (Appel *et al.*, 2000a).

At present, less information exists about the genetics and molecular biology of algal hydrogenases than of bacterial and cyanobacterial enzymes. In all active H_2 photoproducers, hydrogenases are considered to be reversible (but see evidence below for multiple hydrogenase genes in some algae) H_2-ferredoxin oxidoreductases (*e.g.* Schulz, 1996) that catalyse the following reaction:

$$2H^+ + 2Fd_{red} \leftrightarrow H_2 + 2Fd_{ox} \quad (8.5)$$

Some years ago, purified enzymes from *Chlamydomonas* (Roessler and Lien, 1984; Happe and Naber, 1993), *Scenedesmus* (Schnackenberg *et al.*, 1993) and *Chlorococcum littorale* (a marine green alga; Ueno *et al.*, 1999) were isolated and partially characterised. These hydrogenases were not thought to be very similar, except in so far as all are soluble, very sensitive to O_2 (Urbig *et al.*, 1993) and highly active in H_2 production, with a catalytic rate of about 700 turnovers per second using reduced methyl viologen as a donor.

Chlamydomonas hydrogenase is a monomeric protein of 48 kDa that contains twelve Fe atoms (Happe *et al.*, 1994), twelve cysteines and eleven histidines (Happe and Naber, 1993). The nickel content, 0.05 atoms per protein molecule (Happe *et al.*, 1994), is much too low to be functional, and addition of this element during culture growth does not stimulate enzyme activity. Hence this hydrogenase may be a Class III Fe-hydrogenase (Adams, 1990; Wu and Mandrand, 1993), a type that has not as yet been found in photosynthetic prokaryotes. However until recently (see below), there was no evidence for a close relationship between algal Fe-hydrogenases and any of the bacterial hydrogenases, at least on the basis of the 24-residue N-terminal amino acid sequence of the *Chlamydomonas* hydrogenase (Happe and Naber, 1993). In contrast with *Chlamydomonas*, the first biochemical analysis of isolated *Scenedesmus* hydrogenase (Schnackenberg *et al.*, 1993) showed that it contained two subunits (55 and 36 kDa) with a native molecular weight of 150 kDa. This indicated a unique subunit stoichiometry in this protein (Schnackenberg *et al.*, 1993). Furthermore, specific incorporation of labeled Ni into the isolated protein fraction with hydrogenase activity has been observed (Zinn *et al.*, 1994). These findings suggested that *Scenedesmus* has a NiFe-hydrogenase, and Southern hybridisation experiments indicated that it is nuclear-encoded (Schulz, 1996).

Results reported at the Sixth International Conference on the Molecular Biology of Hydrogenases (Berlin, 5–10 August 2000) and Biohydrogen 2002 (Ede, 21-24 April 2002) show a great deal of progress in the area of algal hydrogenases, with several reports of Fe-only hydrogenase sequence information in *C. reinhardtii, S. obliquus*, and *C. fusca* (Florin *et al.*, 2000; Forestier *et al.*, 2000; Kaminski and

Happe, 2000) that were subsequently reported in Genbank and published (Florin *et al.*, 2001; Forestier *et al.*, 2001; Happe and Kaminski, 2002; Winkler *et al.*, 2002).

The previously reported number of Fe atoms in the *Chlamydomonas* hydrogenase (twelve) implied some differences in the arrangement of its FeS clusters compared with the bacterial Fe-hydrogenases (the *C. pasteurianum* CpI enzyme contains 20 Fe atoms). Sequence analyses of the *C. reinhardtii hydA1* previously called the *hydA* or *hyd1*) gene (Happe and Kaminski, 2002; see also Forestier *et al.*, 2001, and L. Mets under Genbank accession no. AF289201) has revealed a highly conserved domain of ~130 amino acids on the C-terminal part of the protein, including four cysteine ligands associated with the coordinating sphere of the active site H-cluster. On the other hand, the conserved cysteines of the accessory [Fe–S] clusters at the N-terminus of the bacterial Fe-hydrogenases were not found in the alga. Thus, the algal Fe-hydrogenase contains only 6 Fe atoms (Fig. 8.2.D), and the preliminary value was an overestimate (T. Happe, personal communication). All the algal Fe-hydrogenases sequenced at this point have large open reading frames and several introns. It appears that Fe-hydrogenases from *C. reinhardtii* (*hydA1*), *S. obliquus* and *C. fusca* are all monomeric enzymes with a molecular weight of 45–55 kDa and are regulated at the transcriptional level. They also exhibit highly conserved H-clusters. Since they lack the FeS centres that act as an electron conduit to the active site, they may engage in direct electron transfer from ferredoxin to the active site of the enzyme (Kaminski *et al.*, 2000; Florin *et al.*, 2001; Happe and Kaminski, 2002; see also Wünschiers *et al.*, 2001a). In striking contrast to observations of a NiFe-hydrogenase in *S. obliquus* (Schnackenberg *et al.*, 1993; Wünschiers *et al.*, 2001a), the recent study of Florin *et al.* (2001) revealed only an Fe-hydrogenase in this alga. The latter authors could not detect the two subunits of a NiFe-hydrogenase nor a Ni-dependency related to the hydrogenase activity, and the discrepancy is unresolved. Wünschiers *et al.* (2001b) have confirmed the presence of an Fe-hydrogenase in *S. obliquus*, but sequence information indicates that it may be a different Fe-hydrogenase from that reported by Happe's laboratory above. A second Fe-hydrogenase gene (*hydA2*) has now also been reported in *C. reinhardtii* (Forestier *et al.*, 2001; Genbank accession no. AY055756), and the putative protein has very high homology with both HydA1 and all other algal hydrogenases. Also characteristic of algal hydrogenases, it contains two short inserts not seen in bacterial Fe-hydrogenases that map to surface regions of the protein (Forestier *et al.*, 2003).

8.4 Metabolic versatility and conditions for hydrogen evolution

The H_2-production activity of nitrogenases and hydrogenases in phototrophic organisms requires strict anaerobiosis at the site of action. Oxygen is therefore an important factor in expression and synthesis of these proteins and regulation of their turnover. Other factors, especially the presence of organic substrates and nitrogen sources, also strongly affect the development of the nitrogenase system in prokaryotes (Vignais *et al.*, 1985; Sasikala *et al.*, 1993), as well as the induction of hydrogenase activity in algae (Lien and San Pietro, 1981; Francis and Senger, 1985; Aparicio *et al.*, 1985; Boichenko *et al.*, 1992). Regulation of reversible hydrogenase activity in at least one green alga (*S. obliquus*) appears to be under redox control at the level of thioredoxin, an alternative ferredoxin acceptor. Wünschiers *et al.* (1999) have demonstrated evidence for a thioredoxin-accessible site on the *Scenedesmus* enzyme (a NiFe-hydrogenase). Astonishingly, more than 50 *nif* genes may be involved in the synthesis and regulation of vital N_2-fixing enzyme systems. These systems include two distinct environmentally regulated Mo nitrogenases in cyanobacteria (Thiel *et al.*, 1995; Schrautemeier *et al.*, 1995), as well as alternative V- and Fe-only nitrogenases (Kentemich *et al.*, 1988, 1991; Masepohl and Klipp, 1996; Davis *et al.*, 1996). Nitrogenase and uptake hydrogenase are co-regulated in cyanobacteria, whereas bidirectional hydrogenases are regulated independently (Lindblad, 2000). Specific ferredoxins (*fdx*H1/2 gene products) associated with the heterocyst (or nitrogen-fixing, non-heterocystous cyanobacteria) differ from the *pet*F-gene-encoded ferredoxin that mediates electron transport from PSI to $NADP^+$ in vegetative cells (Schrautemeier *et al.*, 1994, 1995; Razquin *et al.*, 1995). Although not a photosynthetic organism, the bacterium, *Ralstonia eutropha* (formerly *Alcaligenes eutophus*), has evolved a signal transduction chain that involves a third hydrogenase to regulate hydrogenase synthesis in the presence of H_2. This O_2-insensitive NiFe enzyme senses the presence of H_2 gas. Along with a histidine protein kinase (HoxJ) in combination with a transcription activator (HosA), it constitutes a two-component regulatory system that activates the synthesis of two other O_2-sensitive NiFe-hydrogenases that use H_2 as an energy source (Kleihues *et al.*, 2000).

In general, the pathways of electrons to nitrogenase or hydrogenase with participation of a photoexcited reaction centre (RC*) are as follows (different ferredoxins may be involved):

$$\text{Substrate} \rightarrow \text{Fd/4 ATP (RC*)} \rightarrow N_2\text{ase} \rightarrow H_2 \quad (8.6)$$

$$\text{Substrate} \rightarrow \text{RC*} \rightarrow \text{Fd} + 4\ \text{ATP (RC*)} \rightarrow N_2\text{ase} \rightarrow H_2 \quad (8.7)$$

$$\text{Substrate} \rightarrow \text{RC*} \rightarrow \text{Fd} \rightarrow H_2\text{ase} \rightarrow H_2 \quad (8.8)$$

Figure 8.3 shows more detailed schemes of the electron pathways in anoxygenic and oxygenic photosynthesis. Excitation of the reaction centre occurs after energy migration from light-harvesting antenna, as evidenced by action spectra for H_2 photoproduction in different species (Fig. 8.4). The former figure also shows the requirement for ATP generation (~) from cyclic photophosphorylation when nitrogenase is the mediating enzyme.

In purple bacteria, generation of both reduced ferredoxin and ATP depends on photophosphorylation coupled to cyclic electron flow around the reaction centre (eq. 8.6; dark, reversed electron transfer plus photophosphorylation). Hence, the rate of photophosphorylation may limit H_2 production by nitrogenase in some cultures (Klemme, 1993). Photoreduction of ferredoxin in green bacteria (eq. 8.7), cyanobacteria (eqs. 8.7, 8.8) and green algae (eq. 8.8) appears to be directly mediated by the reaction centre.

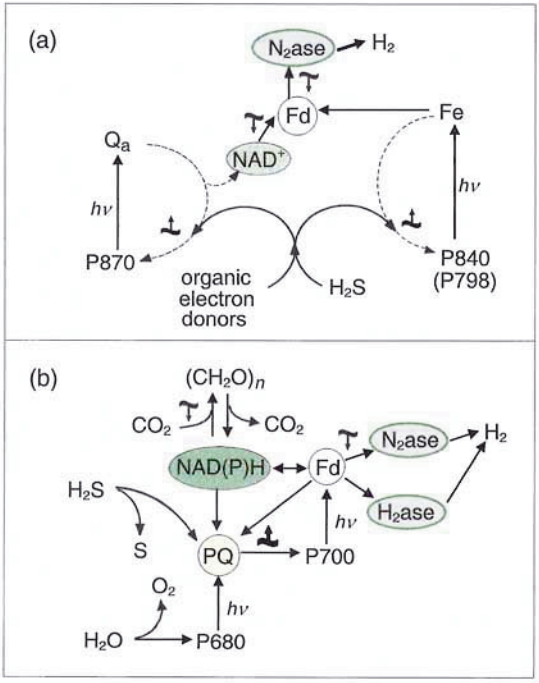


Figure 8.3 Electron transport to nitrogenase and hydrogenase in photosynthetic microorganisms. (a) Pathways in purple bacteria (left route), green bacteria and heliobacteria (right route) and (b) a common pathway in cyanobacteria (to nitrogenase) and green algae (to hydrogenase). Sites of ATP synthesis and hydrolysis are shown by wavy lines with upward and downward arrows, respectively. Light-driven reactions are denoted by $h\nu$.

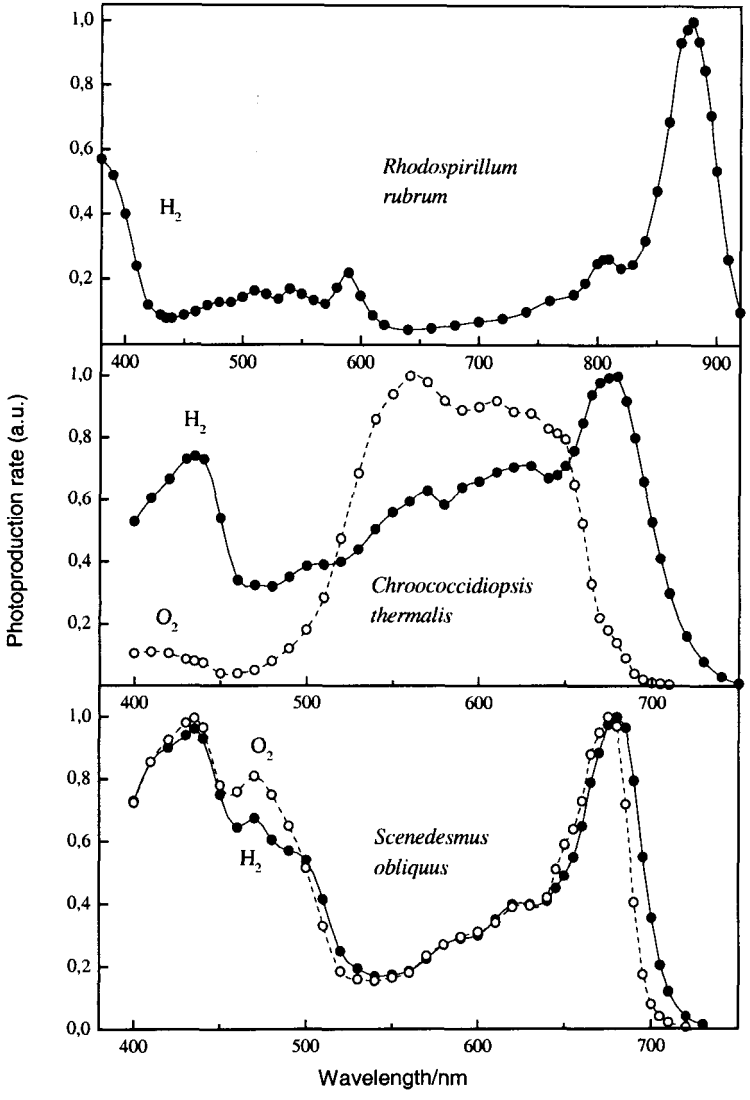


Figure 8.4 Action spectrum of H_2 photoproduction in the purple bacterium *Rhodospirillum rubrum*, and action spectra of H_2 and O_2 photoproduction in the cyanobacterium *Chroococcidiopsis thermalis* and the green alga *Scenedesmus obliquus* (Boichenko, unpublished data). In the latter two cases, the action spectra for O_2 and H_2 production differ, but correspond to PSII- and PSI-driven reactions, respectively.

In purple bacteria and cyanobacteria, the most efficient electron donors for nitrogenase-dependent H_2 production are organic acids (lactate, malate, pyruvate) and sugars, with yields for substrate conversion to H_2 on the order of 50–100% (Hillmer and Gest, 1977a; Sasikala *et al.*, 1993; Luo and Mitsui, 1994; Reddy *et al.*, 1996). The dehydrogenation of the organic substrates proceeds through the operation of specific dehydrogenases (in particular, pyruvate-ferredoxin oxidoreductase) and the anaerobic citric acid cycle, yielding NADH. In cyanobacteria, this metabolic route involves the oxidative pentose phosphate pathway, yielding NADPH. In green bacteria (Warthmann *et al.*, 1992, 1993) and some cyanobacteria (Belkin and Padan, 1978), photoreduction of ferredoxin for nitrogenase- and hydrogenase-dependent H_2 evolution can be coupled to sulphide oxidation, possibly *via* a sulphide–quinone reductase (Arieli *et al.*, 1991).

Algal hydrogenases participate in a number of different metabolic pathways (for a brief review, see Appel and Schulz, 1998) in addition to the hydrogen photoproduction mode using water as a substrate. Photoreduction (H_2-uptake) activity can be induced under anaerobic conditions in the presence of the PSII inhibitors, H_2 and CO_2. The hydrogenase involved seems to donate electrons to the PQ pool and photophosphorylation occurs, whereas H_2 production is independent of ATP metabolism. Fermentative H_2 production and the oxy-hydrogen reaction also occur in algae, suggesting the possibility of multiple hydrogenases in these organisms. The anoxygenic mode of H_2 production depends on glycolytic breakdown of endogenous starch (Klein and Betz, 1975; Gfeller and Gibbs, 1984), which occurs more rapidly in the dark than the light. Hydrogen is also produced by the anaerobic metabolism of acetate in the light *via* the glyoxylate and citric acid cycles (Gibbs *et al.*, 1986), involving electron flow through NAD(P)H–plastoquinone oxidoreductase or chloroplastic succinate dehydrogenase to PSI (Willeford and Gibbs, 1989).

In oxygenic phototrophs, action spectra for H_2 and O_2 photoevolution correspond to PSI and PSII, respectively (see Fig. 8.4 and its legend). Hence it is generally accepted that H_2 photoproduction is necessarily PSI-dependent in green algae and cyanobacteria, while PSII plays a role in specific water–plastoquinone photoreductase activity that ensures continuous electron donation to PSI under aerobic conditions (Fig. 8.3). However, some experimental data on mutants of *Chlamydomonas* (Boichenko *et al.*, 1986; Greenbaum *et al.*, 1995; Lee *et al.*, 1996; Greenbaum *et al.*, 1997a) cannot be quantitatively explained by the Z-scheme. Small amounts of PSI were postulated to be present in these mutants (Boichenko *et al.*, 1986; Boichenko, 1996), and subsequent action-spectral measurements showed that, in all reported mutants, H_2 photoproduction clearly depends on the presence of some PSI units (Greenbaum *et al.*, 1997b, 1997c; Boichenko, 1998; Boichenko and Bader, 1998).

Moreover, Cournac *et al.* (1997) and Redding *et al.* (1999) have demonstrated that photoautotrophic growth, CO_2 fixation and H_2 evolution do not occur in PSI deletion mutants of *C. reinhardtii*. However, since the Z-scheme requires more than the mere presence of PSI, quantitative measurements correlating the amount of PSI with the amount of reduced photoproduct may merit additional investigation.

8.5 Quantum and energetic efficiencies of hydrogen photoproduction

As long as H_2 photoevolution in phototrophs proceeds through the absorption and utilisation of light energy, the electron transport pathway (eqs. 8.6–8.8) and the organisation of the photosynthetic unit together define the maximum efficiency of light conversion in the organism. Thus, the maximum quantum yield of H_2 production (ϕ_{H_2}, the molar ratio of H_2 evolved to the quanta absorbed) depends on the molecular mechanism of the reaction. However, natural photosynthesis saturates with increasing light intensity because the maximum rate of electron transport between PSII and PSI limits the amount of optical excitation energy that the photosynthetic apparatus can convert into chemical energy. Consequently, only measurements of conversion efficiencies under strictly light-limiting conditions reveal the inherent maximum capabilities of the photophysical machinery of photosynthesis independently of the subsequent dark biochemistry. Under non-light-limiting conditions (*e.g.*, incident solar intensities), net conversion efficiencies can be 10–100 times lower, depending on cell concentration, photobioreactor design, antenna content and other parameters.

Theoretically, the expected values of ϕ_{H_2} for the nitrogenase-catalysed reaction are 0.166 in green bacteria (with sulphide as the electron donor), 0.15 in purple bacteria (with malate as the donor) and 0.083 in cyanobacteria (with water as the donor). These values are based on the general assumptions of 1 quantum/$2H^+$ pumped, 1 ATP/$3H^+$ used, and 1 ATP/$2e^-$ reversed from NADH to ferredoxin. Likewise, in green algae (and perhaps in some cyanobacteria that can produce H_2 using a reversible, Fd-linked hydrogenase), the maximum theoretical quantum yield of hydrogenase-mediated H_2 production with water as the sole electron donor is 0.25. The experimental values for bacteria, shown in Table 8.3 (see also Warthmann *et al.*, 1993), are 30–90% of these limiting values, but at least the upper end of the range is somewhat consistent with the predictions. However, in algae the experimental value for the H_2 quantum yield (Boichenko and Litvin, 1988) measured using the flashing light method (Ley and Mauzerall, 1982) is often approximately two-fold higher than the theoretical prediction. This is also the case for the ratios of the maximum relative quantum yields of anaerobic H_2 to aerobic O_2 production (Table 8.2; Boichenko *et al.*,

Table 8.3 Maximum quantum yields, energy conversion efficiencies and net energy yields of H_2 photoproduction by nitrogenase-containing photosynthetic prokaryotes and hydrogenase-containing green algae

Species	Electron donor	Quantum yield (ϕ_{H_2})	Energy conversion efficiency (η_{H_2})	Energy yield	References
Photosynthetic prokaryotes					
Anabaena variabilis	glycogen		0.08	<0.08	Hall *et al.*, 1995
Chlorobium vibrioforme	sulphide	0.116	0.195	0.15	Warthmann *et al.*, 1993
Ectothiorhodspira shaposhnikovii	malate	0.043	0.075	0.00	Warthmann *et al.*, 1993
Rhodospirillum rubrum K100	malate	0.134	0.235	0.01	Warthmann *et al.*, 1993
Rhodobacter sphaeroides 8703	lactate		0.08	0.00	Miyake and Kawamura, 1987
Green algae					
Chlamydobotrys stellata	starch	0.29	0.39	0.34*	Boichenko *et al.*, 1992
Clamydomonas moewusii	water		0.24	0.24	Greenbaum, 1988a
C. reinhardtii UTEX 90	water		0.21	0.21	Greenbaum, 1988a
C. reinhardtii CALU 449	starch	0.41	0.555	0.34*	Boichenko and Litvin, 1988
Chlorella vulgaris CALU 246	starch	0.44	0.595	0.34*	Boichenko and Litvin, 1988
Scenedesmus obliquus D3	water		0.23	0.23	Greenbaum, 1988a
Scenedesmus obliquus D3	starch	0.37	0.5	0.34*	Boichenko and Litvin, 1988

The energy conversion efficiency is calculated as the ratio of the free energy of combustion of evolved H_2 to the absorbed radiant energy at the threshold wavelength that corresponds to the energy level of the relevant reaction centre. The energy yield was calculated as the ratio of the difference between the free energies of combustion of evolved H_2 and oxidised substrate to absorbed radiant energy at the threshold wavelength. Asterisks indicate theoretical maximum values for water splitting to H_2 and O_2 with a ratio of 2.

1989). This apparent inconsistency might be explained as follows: under extremely low irradiances (≤ 1 mW m^{-2}), H_2 photoproduction may proceed mainly *via* a one-photoelectron reaction which involves a semi-reduced state of the hydrogenase, formed during slow ATP-dependent dark electron transport from organic donors.

From a biotechnological point of view, the energy conversion efficiency and the net energy yield characterise the net maximum productivity of photosynthetic systems in terms of the thermodynamics of H_2 formation. The energy conversion efficiency η_{H_2} is the ratio of the free energy of oxidation of evolved H_2 to the absorbed light energy. In general, the relationship between the energy conversion efficiency and the quantum yield is described by the equation

$$\eta_{H_2} = \frac{\Delta G^{\circ}_{H_2} \times \phi_{H_2}}{U_\lambda} \tag{8.9}$$

where $\Delta G^{\circ}_{H_2}$ is the Gibbs free energy content of hydrogen (237 kJ mol^{-1}), and U_λ is the energy of a mole of quanta ($1.2 \times 10^5/\lambda$ kJ nm^{-1}) at wavelength λ.

The net energy yield is the ratio of the difference in free energies of combustion of the evolved H_2 and the oxidisable substrate (glycogen, sulphide *etc.*) divided by the absorbed light energy. Since the energy conversion efficiencies are proportional to wavelength, the maximum values can be measured at the longest wavelength that drives the photochemical reaction. Conventionally, the reaction centre energy level is usually used as a wavelength threshold for these calculations. In the case of oxygenic photosynthesis, the wavelength threshold is about 680 nm, corresponding to the far-red limit of the maximum quantum yield of O_2 evolution (this limit is caused by differences in excitation of the two photosystems above 680 nm). Taking into account the maximum quantum yield and wavelength threshold for a given photosystem, the theoretical maximum monochromatic energy conversion efficiency should be 0.28 for green bacteria (at 840 nm), 0.265 for purple bacteria (at 880 nm), 0.113 for cyanobacteria (at 680 nm) and 0.34 for algae (at 680 nm). Since the substrate energy for water is zero, the maximum net energy yield should be the same as the energy conversion efficiency for oxygenic phototrophs, but 0.215 for green bacteria (with sulphide) and only 0.015 for purple bacteria (with malate). On the other hand as shown in Table 8.3, the energy yield for water splitting to O_2 and H_2 in green algae was at least 0.24 under continuous broadband, steady-state illumination with a projector lamp (this result was actually calculated by converting the light to 680 nm quanta; Greenbaum, 1988a). However, the value could reach 0.34 under flashing light conditions (Boichenko and Litvin, 1988). In this latter case, H_2 derived by the use as oxidisable substrates of both water and the stored organic products of previous

photosynthesis in algae is observed. All the above conversion efficiencies were obtained using very low light intensity (<10 mW m^{-2}). Under outdoor conditions with solar light levels, the net energy yield of H_2 photoproduction in a photobioreactor has a theoretical maximum of ~0.27. Here the calculation was simplified by assuming a monochromatic wavelength of 550 nm, the maximum in the spectral distribution of solar radiant energy, rather than integrating over the whole solar spectrum. However, it is practically impossible to maintain this energy yield because of the kinetic limitations of photosynthesis at high incident light intensities. Another major loss of H_2 in both algae and cyanobacteria is the oxy-hydrogen or 'Knallgas' reaction

$$O_2 + 2H_2 \rightarrow 2H_2O \tag{8.10}$$

Appel and Schulz (1998) have suggested several specific mechanisms to explain this reaction in organisms of different types.

A more thorough analysis of energy conversion efficiencies referenced to light absorption properties of microorganisms in culture and broadband solar energy is beyond the scope of this review, but this was well set out by Bolton and Hall (1990).

8.6 Hydrogen production biotechnology

With the background presented above, what are the prospects for practical application of 'biohydrogen' technology in the future? Fortunately, the news is encouraging because there is an in-place industry that is currently selling high-value (on a per kg basis) products produced photosynthetically using cyanobacteria and algae; these include astaxanthin, β-carotene, chlorophyll, omega–3 fatty acids, phycocyanin, protein and vitamins (Seibert *et al.*, 1996). Other products (*e.g.* pesticides and pharmaceuticals) that can be produced photosynthetically from microorganisms are also under development. Hydrogen at the May 2002 merchant value of $4.70 per GJ (produced by reforming natural gas, the price of which was $2.50/1000 scf in May 2002; W. Amos, personal communication) is, of course, not a high-value product. However, this industrial base is the logical starting point for a future trend toward photosynthetic production of lower-value products, including H_2. Certainly the amount of H_2 in use (6.4×10^9 GJ per year of gas energy equivalent is produced world-wide) makes a serious examination of the biological option worthwhile. Furthermore, current environmental concerns support the search for non-polluting energy alternatives for the future, and this could be based on an electricity/H_2 economy. Fortunately, many governments have recognised the need, and there are active programmes in Japan, Europe and the USA (Table 8.4 at the end of the chapter

lists relevant websites) with the goal of producing H_2 biologically using photosynthetic prokaryotes and algae, as well as thermally from traditional biomass. The potential for large impact of hydrogen on the world's society and economy is real, and in the following sections we shall highlight only a few of the recent activities aimed at developing H_2-production technologies around the world.

8.6.1 Hydrogen-producing systems

Probably the greatest amount of R&D effort over the years has gone into H_2-photoproduction technology using photosynthetic bacteria and waste products such as organic acids, organic alcohols, organic gases, aromatic acids, inorganic donors and natural substrates (*e.g.* fermented food waste, spent beer, cheese whey). The work in the 1970s (for general reviews see Benemann *et al.*, 1977; Miyamoto *et al.*, 1979b; Weaver *et al.*, 1980) and 1980s (Sasikala *et al.*, 1993) was successful in that important advances were made in demonstrating energy conversion efficiency improvements. However, the "energy conversion efficiencies" of the bacterial nitrogenase-based systems being investigated at the time were limited to a maximum of 0.05 (based on low intensity, incident white light and of course ignoring energy in the substrate) in the laboratory. Furthermore, contamination problems (mostly from methanogenic or sulphate-reducing bacteria that consume H_2) and lower efficiencies were observed when the systems were taken outdoors and run under non-sterile conditions for extended periods of time.

The Japanese Research Institute of Innovative Technology for the Earth (RITE) Programme has been particularly active in this area recently (Takasaki *et al.*, 1996; Mitsugi *et al.*, 1998; *www.aist.go.jp/NIBH/ourpages/iea/index.html*) and improving on the older technology. RITE research activities have included biological screening aimed at identifying high H_2-producing organisms, engineering work on large-scale cultivation and gas-refining techniques, co-product investigations to enhance the potential economics of H_2 production, and system studies to develop integrated processes. One of the most interesting processes developed was a three-stage system that used *Chlamydomonas* sp. MGA161 to convert sunlight, water and CO_2 to stored carbohydrate. When the cultures were subsequently exposed to anaerobic conditions in the dark, fermentation products (acetic acid, ethanol, and glycerol) were excreted into the medium. At that point, the substrates could be processed anaerobically in the light by a marine photosynthetic bacterium (*Rhodopseudomonas sulfidophilum* FERM P–15320, available from the Fermentation Research Institute, Ibaraki, Japan) to produce H_2 (Maeda *et al.*, 1998). An earlier version of this system was scaled up in a

greenhouse located at the Nankoh power plant (Kansai Electric Power Co.) in Osaka, Japan (Akano *et al.*, 1996) and produced H_2 at an "energy conversion efficiency" of less than 0.005; this estimate was based on incident broadband light energy and excluded mechanical energy input. This was a very exciting result because the system actually worked, producing H_2 from water (though indirectly), apparently for many days. A new Japanese H_2 Program entitled "Development of High Efficiency Hydrogen–Methane Fermentation Processes using Organic Wastes" supported by NEDO started in March 2002 (personal communication, Dr. Y. Asada, Nihon University). Researchers from national institutes, universities and private companies will develop a highly efficient anaerobic digestion system in tandem with an H_2 fermentation process. R&D for monitoring and controlling microbial populations, which are involved in H_2 metabolism, hydrolysis, and methane formation in the anaerobic digestion process, will also be supported.

Major European efforts in biohydrogen research during the past decade have included the German Biological Hydrogen and the European Union COST 818 Programmes. The emphasis of both was at the fundamental level and much useful knowledge was generated. The German (BMBF) programme (Benemann, 1998) emphasised six areas of R&D: biophotolysis using nitrogenase- and hydrogenase-based systems; photoproduction from organics using nitrogen-fixing photosynthetic bacteria; fermentations of organic substrates using dark, fermentative bacteria; biomimetic H_2 production using inorganic (Pt) catalysts instead of hydrogenase; structure, function and stability of hydrogenases and nitrogenases; and technological evaluation of economic, environmental and social aspects. 45 projects were supported and over 400 papers were published in the literature on these subjects during the period 1990–1995. One of the few applied projects was continued at the University of Aachen for two years after the programme ended in order to demonstrate the long-term project goal of producing 2 litres of $H_2\ m^{-2}\ h^{-1}$. This outdoor system, employing photosynthetic bacteria and acetate as a substrate, did meet that goal (Benemann, personal communication), but the project ended before a planned two-stage system (using algae at the front end to supply substrate) could be built.

The EU's COST Programme (1994–1999) emphasised the areas of 'hydrogenases and their biotechnological applications'. Research activities in hydrogenase structure, enzyme mechanisms, molecular biology, physiology and biotechnological applications were encouraged through workshop sponsorship, scientific exchanges and travel. One of the few applied projects associated with this programme was a cyanobacterial system located at King's College, London. There, the late Professor David Hall, with the help of several Russian collaborators and some Japanese RITE funding, developed both hollow-fibre (Markov *et al.*, 1995a) and helical tubular

(Tsygankov *et al.*, 1999) photobioreactor systems that successfully employed the *Anabaena variabilis* PK84 mutant developed by Mikheeva *et al.* (1995). This mutant lacks H_2-uptake activity and, consequently, produces more H_2 than the WT. (In the case of N_2 fixation, H_2-uptake activity in the WT can save at least 25% of the energy in H_2 that otherwise would be lost to the N_2-fixation process (Hansel and Lindblad, 1998) since re-use of the H_2 evolved decreases the loss of reductant.) Laboratory studies with hollow-fibre photobioreactors demonstrated the mutant's capability of producing H_2 from water more efficiently than the WT, and continuous H_2 production was observed for a period of up to 18 months (Markov *et al.*, 1995a; Ghirardi *et al.*, 1997). More recent outdoor results with the same PK84 mutant using a helical, tubular, airlift photobioreactor with automatic controls and monitoring have demonstrated H_2 production in sunlight for several months (Tsygankov *et al.*, 2002).

Other groups in Europe have reported H_2 production from *Nostoc* sp. (Tredici, 1990), and several groups have demonstrated sustained rates of H_2 production with various strains of WT (Fedorov *et al.*, 1998) and *hup*$^-$ photosynthetic bacteria using organic acids as substrates (Kern *et al.*, 1994; Jahn *et al.*, 1994; Krahn *et al.*, 1996; Zorin *et al.*, 1996). Kern *et al.* (1992) have also reported increases in bacterial H_2 production when EDTA, a metal chelater, was added to the culture. EDTA apparently inhibited the biosynthesis of uptake hydrogenase and activated the biosynthesis of the nitrogenase complex. Finally, the Enricherche (now Enitecnologie) group in Rome, with RITE support, reported production rates from *Rhodobacter sphaeroides* R.V. of 1.15 litres of H_2 per litre of reactor culture per day, using food waste as a substrate (d'Addario *et al.*, 1996). Fortunately, the COST Programme with its emphasis on hydrogenases has been renewed (COST 841; *http://h2-ase.szbk.u-szeged.hu/*) for another 5-year period (1999–2004). Benemann (1998) has written a much more detailed assessment of the European H_2 efforts, and Hansel and Lindblad (1998) have reviewed biotechnology aspects of cyanobacterial H_2 production.

Recent, biohydrogen production research in the USA has been sponsored by the US Department of Energy's Hydrogen Program. Two approaches have received recent emphasis. The first approach, a near-term option, is a hybrid biomass gasification and subsequent biological gas conditioning (a water-gas shift) process that produces H_2 pure enough to be fed directly into a phosphoric acid fuel cell. The idea was to use photosynthetic bacteria as room-temperature CO biocatalysts in a dark, single-step gas-conditioning process to convert thermally generated fuel gases (H_2 + CO) into H_2-rich, CO-free gas that is usable without further purification. Weaver *et al.* (1998) reported H_2 production rates from CO of up to 3 mmol (g cell dry wt)$^{-1}$ min^{-1} using a CO-linked hydrogenase in WT whole cells collected in the Denver area (a recent value of 8.3 mmol [g cell dry wt]$^{-1}$ min^{-1} has been observed;

Maness and Weaver, 2002). The shift reaction *occurs in the dark* under ambient conditions and produces gas with less than 0.1 ppm residual CO from initial levels of up to 20% (P.-C. Maness, personal communication). Mass transfer of gases into liquid cultures is the major problem that has to be solved, and currently various bioreactor designs are being tested and scaled-up for proof of concept. The economics of the process appear very favorable and at a biomass cost of $22/ton along with some improvements in current biological activity, H_2 competitive with that produced from natural gas may become a reality.

The second US activity is a longer-term team effort to develop efficient green algal strains that will produce H_2 from water under ambient conditions. Two major biological problems—the low photosynthetic light conversion efficiencies at solar light levels common to all photosynthetic organisms (Greenbaum and Lee, 1998; Melis *et al.*, 1998, 1999) and the O_2-sensitivity of algal hydrogenases (Ghirardi *et al.*, 1998)—are being addressed. Progress in the former area has demonstrated improvements in photosynthetic efficiency when the algal antenna size is decreased as the result of exposure to high light intensity, although the effect is a transient phenomenon (Melis *et al.*1998, 1999). More recently, Polle *et* al. (2001) reported improvements in photosynthetic efficiencies on a chlorophyll basis in a genetically truncated *Chlamydomonas* mutant. It is expected that this or a similar type of antenna mutant will demonstrate improvements in H_2-production capability of algal cultures. Progress in the latter area has included the development of mutant selection and screening procedures that have improved the O_2 tolerance of H_2 production in *Chlamydomonas* by a factor of 10 (i.e. the O_2 I_{50} for H_2 production on a time scale of minutes was increased from about 0.2% to 2%; Seibert *et al.*, 1998; Ghirardi and Seibert, 1999; Ghirardi *et al.*, 1999 and 2000a; Seibert *et al.*, 2001; Flynn *et al.*, 2002). An alternative approach, transferring prokaryote hydrogenase genes encoding more O_2-resistant enzymes (*e.g.* the stable *Thiocapsa roseoersicina* NiFe-hydrogenase; Rákhely *et al.*, 1998) into microalgae, has been "plagued with problems" (Schulz, 1996), although some progress has been made in expressing bacterial hydrogenase genes in *Synechococcus* (Koike *et al.*, 2000).

For the last three years, the US programme has utilised physiological manipulation of *C. reinhardtii* to sustain H_2 photoproduction in green algae. Cultures grown to late-log phase were deprived of sulphate (patent pending), resulting in substantial, but reversible inactivation of O_2-evolution function in the algae and subsequent production of large amounts of pure H_2 in laboratory-scale experiments (Ghirardi *et al.*, 2000b; Melis *et al.*, 2000). The purpose of this approach is to partially inactivate photosynthetic O_2 evolution, which prevents the accumulation of O_2 and resultant inhibition of hydrogenase function.

This physiological approach substitutes for inert gas purging, gas pumping or exogenous reductant addition to the cultures since any of these may be impractical for use in future commercial systems. This is the first algal system that has actually been able to produce H_2 bubbles and substantially more than the few microlitres of H_2 gas previously collected by others. From a biochemical perspective, sulphur deprivation decreases O_2 evolution (Wykoff *et al.*, 1998) to the point that cellular respiratory processes, which remain fairly stable under these conditions (Melis *et al.*, 2000), can metabolise any residual O_2 released. There seems to be an energy requirement for the inhibition of O_2 evolution since the presence of acetate during the initial stage of sulphur deprivation speeds up the process (Ghirardi *et al.*, 2000a). When the cells remove the O_2, the culture goes anaerobic, the Fe-hydrogenase is induced, and H_2 gas is produced for 3–4 days. At least 80% of the electrons for H_2 production come from residual water-splitting function (Ghirardi *et al.*, 2000a and b; Seibert *et al.*, 2000; Antal *et al.*, 2001). Reduced cellular materials, including protein and starch, stored during the sulphur-replete growth phase, supply the reductant to respire the O_2 and the balance of the electrons required for H_2 not supplied by water (Ghirardi *et al.*, 2000 a and b; Kosourov *et al.*, 2002; Zhang *et al.*, 2002). The regulation of the process seems to involve the redox state of the plastoquinone pool, which controls the amount of electron transport from PSII during all stages of sulphur deprivation, and this in turn depends on the rates of photosynthesis, respiration, chlororespiration and H_2 production (Antal *et al.*, 2001). After H_2 production stops, the process can be repeated if sulphate is re-added to the depleted cells during a recovery period that allows for normal photosynthetic growth (Ghirardi *et al.*, 2000b). Interestingly, this work has demonstrated that algal Fe-hydrogenase activity can be induced by anaerobic adaptation in the light, contrary to previous belief (Appel and Schulz, 1998).

The sulphur-deprivation process for H_2 production is still at an early stage of development, and the production rates and/or yields will have to be increased substantially for practical application. Nevertheless, substantial progress has been made recently by (a) optimising the sulphate concentration at the time of sulphur-deprivation (Kosorouv *et al.*, 2002), (b) growing the cells under defined light/dark cycles (Kosorouv *et al.*, 2002), and (c) eliminating the requirement for sulphate removal from the cultures by centrifugation (Laurinavichene *et al.*, 2002). If the theoretical potential of this process as currently understood can be reached, a one-acre bioreactor could produce enough H_2 on average each day to drive a fuel-cell-powered car 800 miles (W. Amos, pers. comm.). Cost is, of course, as yet unknown. Finally, Melis and Happe (2002) have provided a recent perspective on algal H_2 production, and the website *www.eren.doe.gov/hydrogen/publications.html* gives more detailed information about the US Hydrogen Program.

8.6.2 Photobioreactors

There is a lot of information available in the literature about photobioreactors suitable for biomass growth, but less information specifically aimed at H_2-production systems. Benemann (1999) has reviewed this field in detail, but several efforts are worth noting briefly. Japanese activities in the area are well documented, with a number of pictures of bioreactor and fibre-optic illumination systems specifically designed for bacterial H_2 production. The reader can view many examples at the Japanese website *www.aist.go.jp/NIBH/ourpages/iea/index.html*. A major current effort in Europe, as mentioned previously, uses helical, tubular photobioreactor systems suitable for

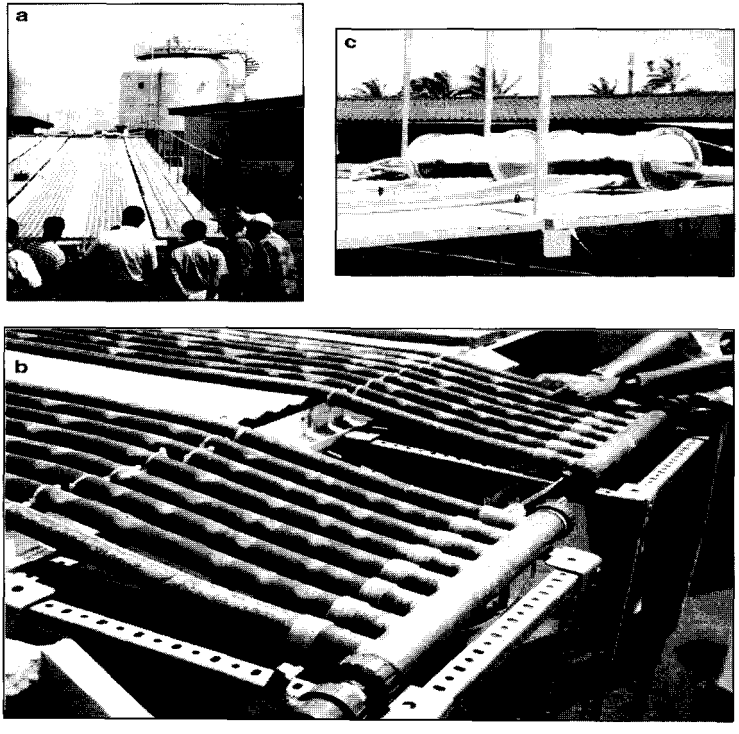


Figure 8.5 A Tredici-type tubular photobioreactor system located at the Kewalo Basin site of the University of Hawaii at Manoa. (a) Two eight-tube, 20-m bioreactors are shown on a slightly inclined platform. The tubes are 4 cm in diameter and are being used in this case to grow *Anthrospira* sp. Future plans for this facility include adapting the system to produce H_2; (b) gas-injection manifold allowing bubbles to form in the six centre tubes. The bubbles rise up the incline, mixing the culture, removing excess gas from the liquid and cleaning the insides of the tubes. The outer two tubes in each case are return tubes; (c) degasser assembly located at the top of the incline seen in (a).

outdoor production of H_2 from water and a heterocystous cyanobacterium. An example of this type of photobioreactor can also be seen at the above-mentioned website. The major US effort in photobioreactor technology has been located on Oahu at the Hawaii Natural Energy Institute, University of Hawaii. Figure 8.5 shows one of their Tredici-type sealed, tubular photobioreactors (Szyper *et al.*, 1998). These use compressed gas (air in this case for demonstration purposes) bubbles to circulate culture medium; keep the proprietary, transparent plastic tubing clean; and prevent excess gas from building up in the liquid phase. The system is currently being used by the Marine Bioproducts Engineering Centre (MARBEC), University of Hawaii, to search for high-value products that could be produced by marine organisms.

8.7 Future prospects

Economics will be the prime future driver for commercial success of any biological H_2-production technology. In the near term, depending on the price of oil (which has recently risen from a historical low in constant dollars) and environmental regulations (including global climate change issues), hybrid systems using biomass gasification combined with biological gas conditioning (photosynthetic bacteria functioning in the dark) may have the greatest promise as the biohydrogen-production technology that will come online first. Mid-term technology will probably utilise wastewater treatment in combination with H_2 production as a byproduct or implement improvements of the algal H_2-production system described in Section 8.6.1 that separates O_2 evolution and H_2 production temporally. In the long term, photobiological water-splitting systems combining antenna-depleted microalgae with O_2-tolerant hydrogenases in principle have the potential to supply large fractions of our future chemical fuel needs. Finally, Table 8.4 lists useful websites on the topics of this review.

Acknowledgments

The authors thank M. L. Ghirardi, P. King, J. W. Lee, P.-C. Maness, A. San Pietro and P. F. Weaver for their critical reading of the manuscript, and W. Amos, J. R. Benemann, J. C. Fontecilla-Camps, C. G. Padró and the late D. O. Hall for many helpful suggestions. This work was supported by the Russian Foundation for Basic Research and the Programme of Physical and Chemical Biology (VAB), the US DOE Hydrogen Program (MS and EG), and the Division of Energy Biosciences, Office of Science, US DOE (MS).

Table 8.4 Websites treating hydrogen and biological hydrogen production

Subject	Website
AAA protein superfamily; ATP protein superfamily; ATPases associated with various cellular activities	*http://yeamob.pci.chemie.uni-tuebingen.de/Default.html*
Atmospheric gas composition	*www.c-f-c.com/charts/atmosph.htm*
The Chlamydomonas Genetics Center	*www.biology.duke.edu/chlamy/*
Chlorophyta—Green algae	*http://seaweed.ucg.ie/Algae/Chlorophyta.html*
Chlorophyta Index	*www.ucmp.berkeley.edu/greenalgae/chlorophyceae.html*
Department of Physiological Botany, Uppsala University	*www.fysbot.uu.se/fysbot/Cyano/index.html*
European COST Action 841 Program, Biological and Biochemical Diversity of Hydrogen Metabolism	*http://h2-ase.szbk.u-szeged.hu/*
German Hydrogen Association	*www.dwv-info.de/indexe.htm*
German Hydrogen Projects	*www.hydrogen.org/germanh2/index.html*
Global Energy Marketplace	*http://gem.crest.org/*
Hydrogen InfoNet, U.S. Department of Energy	*www.eren.doe.gov/hydrogen/*
Hydrogen Energy Center	*www.h2eco.org/links.htm*
International Association for Hydrogen Energy	*www.iahe.org/*
International Energy Agency (IEA) Biological Hydrogen Network	*www.aist.go.jp/NIBH/ourpages/iea/*
International Energy Agency (IEA) Hydrogen Program	*www.eren.doe.gov/hydrogen iea.html*
The Netherlands Biohydrogen Program	*www. biohydrogen.nl*
Introduction to the Cyanobacteria	*www.ucmp.berkeley.edu/bacteria/cyanointro.html*

Table 8.4 cont'd.

Kingdom Monera: The Cyanobacteria	*http://fig.cox.miami.edu/Faculty/Dana/cyano. html*
Metabolic Pathways database	*http://wit.mcs.anl.gov/WIT2/*
National Hydrogen Association	*www.ttcorp.com/nha/*
National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, METI	*www.nibh.go.jp/English/index_e.html*
National Renewable Energy Laboratory	*www.nrel.gov/*
NREL Hydrogen Research	*www.nrel.gov//lab/pao/hydrogen.html*
Russian Academy of Sciences, Institute of Basic Biological Problems, Laboratory of Biochemistry and Biotechnology of Phototrophic Microorganisms	*http://phototrophs. net.ru*
Schatz Energy Research Center (SERC)	*www.humboldt.edu/~serc/index.shtml*
Solstice: Center for Renewable Energy and Sustainable Technology (CREST)	*http:// solstice.crest.org/index.shtml*
University of Hawaii, Hawaii Natural Energy Institute	*www.soest.hawaii.edu/ HNEI/index.html*
WE-NET International Clean Energy Network Using Hydrogen Conversion	*www.enaa.or.jp/WE-NET/index.html*

The above web-based resources relevant to the areas of hydrogen and biological hydrogen production are not intended to be comprehensive, but will give the reader a starting point for accessing available in formation in this emerging area of interest. The authors encourage readers to explore the links that are available at many of these websites. Information researched by E. Kalim and L. Westdal.

Note added in proof

This article was completed during the first part of 2002. Since that time, many new articles have appeared. In particular, the authors would like to call the reader's attention to a special issue of the *International Journal of Hydrogen Energy* (Volume 27 [11,12], 1123–1505), published at the end of 2002. This issue contains papers from presentations made at *Biohydrogen 2002*, an international symposium held in the Netherlands, 21–24 April 2002. This collection of papers represents an updated synopsis of what is currently happening in the area of biological hydrogen research. Furthermore, the following are examples of relevant new reviews that have appeared over the last year: Nicolet *et al.* (2002); Rees (2002); Zehr *et al.* (2003).

References

Abdel-Basset R., Spiegel S. and Bader K. P. (1998), 'Saturation of cyanobacterial photoevolution of molecular hydrogen by photosynthetic redox components', *J. Photochem. Photobiol. B* **47**, 31–38.

Adams M. W. W., Mortenson L. E. and Chen J.-S. (1981), 'Hydrogenase', *Biochim. Biophys. Acta* **594**, 105–176.

Adams M. W. W. (1990), 'The structure and mechanism of iron hydrogenases', *Biochim. Biophys Acta* **1020**, 115–145.

Akano T., Muiro Y., Fukatsu K., Miyasaka H., Ikuta Y., Matsumoto H., Hamasaki A., Shioji N., Mizoguchi T., Yagi K. and Maeda I. (1996), 'Hydrogen production by photosynthetic microorganisms', *Appl. Biochem. Biotechnol.* **57–58**, 677–688.

Alberty R. A. (1994), 'Thermodynamics of the nitrogenase reactions', *J. Biol. Chem.* **269**, 7099–7102.

Albracht S. P. J. (1994), 'Nickel hydrogenases: in search of the active site', *Biochim Biophys. Acta* **1188**, 167–204.

Antal T. K., Krendeleva T. E., Laurinavichene T. V., Makarrova V. V., Tsygankov A. A., Seibert M. and Rubin A. B. (2001), 'The relationship between photosystem 2 activity and hydrogen production in sulfur-deprived *Chalmydomonas reinhardtii* cells', *Dokl. Akad. Nauk.* **381**, 119–122.

Aparicio P. J., Azuara M. P., Ballesteros A. and Fernandez V. M. (1985), 'Effects of light intensity and oxidized nitrogen sources on hydrogen production by *Chlamydomonas reinhardtii*', *Plant Physiol.* **78**, 803–806.

Appel J. and Schulz R. (1998), 'Hydrogen metabolism in organisms with oxygenic photosynthesis: hydrogenases as important regulatory devices for a proper redox poising?', *J. Photochem. Photobiol. B* **47**, 1–11.

Appel J., Phunpruch S., Steinmüller K. and Schulz R. (2000a), 'The bidirectional hydrogenase of *Synechocystis* sp. PCC 6803 worked as an electron valve during photosynthesis', *Arch. Microbial.* **173**, 333–338.

Appel J., Vossen P. and Schulz R. (2000b), 'Distribution of hydrogenases in different classes of cyanobacteria', *Abstr. 6th. Int. Conf. on the Molecular Biology of Hydrogenases*, Potsdam, 5–10 August 2000, p. PB1.

Arieli B., Padan E. and Shahak Y. (1991), 'Sulfide-induced sulfide–quinone reductase activity in thylakoids of *Oscillatoria limnetica*', *J. Biol. Chem.* **266,** 104–111.

Belkin S. and Padan E. (1978), 'Sulfide-dependent hydrogen evolution in the cyanobacterium *Oscillatoria limnetica*', *FEBS Lett.* **94**, 291–294.

Ben-Amotz A., Erbes D. L., Riederer-Henderson M. A., Peavey D. G. and Gibbs M. (1975), 'H_2 metabolism in photosynthetic organisms. I. Dark H_2 evolution and uptake by algae and mosses', *Plant Physiol.* **56**, 72–77.

Benemann J. R and Weare N. M. (1974), 'Hydrogen evolution by nitrogen-fixing *Anabaena cylindrica* cultures', *Science* **184**, 174–175.

Benemann J. R., Weissmann J. C., Koopman B. L. and Oswald W. J. (1977), 'Energy production by microbial photosynthesis', *Nature* **268**, 19–23.

Benemann J. R. (1996), 'Hydrogen biotechnology: progress and prospectives', *Nature Biotechnol.* **14**, 1101–1103.

Benemann J. R. (1998), *European Biohydrogen R&D Activities*, Unpublished report submitted to NREL, 30 December 1998.

Benemann J. R. (1999), 'Photobioreactors for biohydrogen production: technology review and assessment', Unpublished report submitted to NREL, 15 March 1999.

Berchtold M. and Bachofen R. (1979), 'Hydrogen formation by cyanobacteria cultures selected for nitrogen fixation', *Arch. Microbiol.* **123,** 227–232.

Blankenship R. E. (1992), 'Origin and early evolution of photosynthesis', *Photosynth. Res.* **33**, 91–111.

Bockris J. O'M. (1976), *The Solar–Hydrogen Alternative*, Architectural Press, London.

Boichenko V. A., Allakhverdiev S. I., Ladygin V. G. and Klimov V. V. (1986), 'Functional conjunction of hydrogenase with Photosystem II in the whole cells of *Chlamydomonas reinhardtii* mutants', *Dokl. Akad. Nauk SSSR* **290**, 995–998.

Boichenko V. A. and Litvin F. F. (1988), 'Maximum quantum yield of hydrogen photoproduction by cells of green algae', *Dokl. Akad. Nauk SSSR* **301**, 1497–1500.

Boichenko V. A. and Hoffmann P. (1994), 'Photosynthetic hydrogen production in prokaryotes and eukaryotes: occurrence, mechanism, and functions', *Photosynthetica* **30**, 527–552.

Boichenko V. A., Satina L. Y. and Litvin F. F. (1989), 'Efficiency of hydrogen photoproduction in algae and cyanobacteria', *Fiziol. Rast.* **36**, 239–247.

Boichenko V. A., Wiessner W., Klimov V. V., Mende D. and Demeter S. (1992), 'Hydrogen photoevolution indicates an increase in the antenna size of photosystem I in *Chlamydobotrys stellata* during transition from autotrophic to photoheterotrophic nutrition', *Plant Physiol.* **100**, 518–524.

Boichenko V. A. (1996), 'Can photosystem II be a photogenerator of low potential reductant for CO_2 fixation and H_2 photoevolution?', *Photosynth. Res.* **47**, 291–292.

Boichenko V. A. (1998), 'Photosynthetic production of O_2 and H_2 in Photosystem I-deficient mutants of *Chlamydomonas*', *Biochemistry (Moscow)* **63**, 56–63.

Boichenko V. A. and Bader K. P. (1998), 'Verification of the Z-scheme in *Chlamydomonas* mutants with Photosystem I deficiency', *Photosynth. Res.* **56**, 113–115.

Boichenko V. A., Klimov V. V., Miyashita H. and Miyachi S. (2001), 'Functional characteristics of chlorophyll-*d*-predominating photosynthetic apparatus in intact cells of *Acaryochloris marina*', *Photosynth. Res.* **65**, 269–277.

Bolton J. R. and Hall D. O. (1990), 'The maximimum efficiency of photosynthesis', *Photochem. Photobiol.* **53**, 545–548.

Brand J. J., Wright J. N. and Lien S. (1989), 'Hydrogen production by eukaryotic algae', *Biotech. Bioengin.* **33**, 1482–1488.

Burris R. H. (1991), 'Nitrogenases', *J. Biol. Chem.* **266**, 9339–9342.

Cournac L., Redding K., Bennoun P. and Peltier G. (1997), 'Limited photosynthetic electron flow but no CO_2 fixation in *Chlamydomonas* mutants lacking Photosystem I', *FEBS Lett.* **416**, 65–68.

d'Addario E., Fascetti E. and Valdiserri M. (1996), 'Hydrogen production from organic wastes by continuous culture of *Rhodobacter sphaeroides* RV', *Proc. 11th. World Hydrogen Conf.*, Stuttgart, pp. 2577–2582.

Davis R., Lehman L., Petrovich R., Shah V. K., Roberts G. P. and Ludden P. W. (1996), 'Purification and characterization of the alternative nitrogenase from the photosynthetic bacterium *Rhodospirillum rubrum*', *J. Bacteriol.* **178**, 1445–1450.

Diner B. and Mauzerall D. (1973), 'Feedback controlling oxygen production in a cross-reaction between two photosystems of photosynthesis', *Biochim. Biophys. Acta* **305**, 329–352.

Eady R. R. (1996), 'Structure–function relationships of alternative nitrogenases', *Chem. Rev.* **96**, 3013–3030.

Fan H. J. and Hall M. B. (2001) 'A capable bridging ligand for Fe-only hydrogenase: density functional calculations of a low-energy route for heterolytic cleavage and formation of dihydrogen', *J. Am. Chem. Soc.* **123**, 3828–3829.

Fedorov A. S., Tsygankov A. A., Rao K. K. and Hall D. O. (1998), 'Hydrogen photoproduction by *Rhodobacter sphaeroides* immobilized on polyurethane foam', *Biotechnol. Lett.* **20**, 1007–1009.

Florin L., Tsokoglou A. and Happe T. (2000), 'The first characterization of an [Fe] hydrogenase in the green alga *Scenedesmus obliquus*', *Abstr. 6th. Int. Conf. on the Molecular Biology of Hydrogenases*, Potsdam, 5–10 August 2000, p. PB7.

Florin L., Tsokoglou A. and Happe T. (2001), 'A novel type of Fe-hydrogenase in the green alga *Scenedesmus obliquus* is linked to the photosynthetic electron transport chain', *J. Biol. Chem.*, **276**, 6125–6132.

Flynn T., Ghirardi M. L. and Seibert M. (2002) 'Accumulation of O_2-tolerant phenotypes in H_2-producing strains of *Chlamydomonas reinhardtii* by sequential applications of chemical mutagenesis and selection', *Int. J. Hydrogen Energy* **27**, 1421–1430.

Forestier M., Ghirardi M. L. and Seibert M. (2000), 'Are there multiple Fe-hydrogenases in the green alga *Chlamydomonas reinhardtii?*', *Abstr. 6th. Int. Conf. on the Molecular Biology of Hydrogenases*, Potsdam, 5–10 August 2000, p. PT1.

Forestier M., Zhang L., King P., Plummer S., Ahmann D., Seibert M. and Ghirardi M. (2001), 'The cloning of two hydrogenase genes from the green alga *Chlamydomonas reinhardtii*', *Proc. 12th. Int. Congress on Photosynthesis*, 18–23 August 2001, Brisbane, Australia, CSIRO Publishing, Melbourne, Australia (*www.publish.csiro.au/ps2001*).

Forestier M., King P., Schwarzer S., Happe T., Zhang L., Ghirardi M. L. and Seibert M. (2003), 'A second putative [Fe]-hydrogenase gene (hydB) in *Chlamydomonas reinhardtii* is also induced under anaerobic conditions,' *Eur. J. Biochem.* **270**, 2750–2758.

Francis K. and Senger H. (1985), 'Correlation between respiration and hydrogenase adaptation in *Scenedesmus obliquus*', *Physiol. Plantarum* **65**, 167–170.

Frey M. (2002), 'Hydrogenases: hydrogen-activating enzymes', *ChemBioChem.* **3**, 153–160.

Gaffron H. and Rubin J. (1942), 'Fermentative and photochemical production of hydrogen in algae', *J. Gen. Physiol.* **26**, 219–240.

Georgiadis M. M., Komiya H., Chakrabarti P., Woo D., Kornuc J. J. and Rees D. C. (1992), 'Crystallographic structure of the nitrogenase iron protein from *Azotobacter vinelandii*', *Science* **257**, 1653–1659.

Gest H. and Kamen M. D. (1949), 'Photoproduction of molecular hydrogen by *Rhodospirillum rubrum*', *Science* **109**, 558–559.

Gfeller R. P. and Gibbs M. (1984), 'Fermentative metabolism of *Chlamydomonas reinhardtii*. I. Analysis of fermentative products from starch in dark and light', *Plant Physiol.* **75**, 212–218.

Ghirardi M. L., Flynn T., Markov S. and Seibert M. (1997), 'Development of an efficient algal H_2 production system', *Proc. 1997 US Hydrogen Program Review,* Vol. I, pp. 11–24, NREL/CP–430–23722.

Ghirardi M. L., Flynn T., Forestier M. and Seibert M. (1998), 'Development of an efficient algal H_2 production system', *Proc. 1998 US Hydrogen Program Review,* Vol. I, pp. 43–60, NREL/CP–570–25315.

Ghirardi M. L. and Seibert M. (1999), 'Process for selection of oxygen-tolerant algal mutants that produce H_2 under aerobic conditions', US Patent No. 5,871,952.

Ghirardi M. L., Flynn T., Forestier M. and Seibert M. (1999), 'Development of an efficient algal H_2 production system', *Proc. 1999 US Hydrogen Program Review,* Vol. I (*www.eren.doe.gov/hydrogen/publications.html*).

Ghirardi M. L., Flynn T., Forestier M. and Seibert M. (2000a), 'Development of an efficient algal H_2 production-system', *Proc. 2000 US Hydrogen Program Review,* Vol. I (*www.eren.doe.gov/hydrogen/publications.html*).

Ghirardi M. L., Zhang L., Lee J. W., Flynn T., Seibert M., Greenbaum E. and Melis A. (2000b), 'Microalgae: a green source of renewable H_2', *Tibtech.* **18**, 506–511.

Gibbs M., Hollaender B., Kok B., Krampitz L. O. and San Pietro A. (1973), *Proc. Workshop of Biosolar Conversion*, Indiana University, Bloomington, pp. 1–6.

Gibbs M., Gfeller R. P. and Chen C. (1986), 'Fermentative metabolism of *Chlamydomonas reinhardii*: III. Photoassimilation of acetate', *Plant Physiol.* **82**, 160–166.

Greenbaum E. (1980), 'Simultaneous photoproduction of hydrogen and oxygen by photosynthesis', *Biotechnol. Bioengin. Symp.* No. **10**, pp. 1–13.

Greenbaum E. and Ramus J. (1983), 'Survey of selected seaweeds for simultaneous photoproduction of hydrogen and oxygen', *J. Phycol.* **19**, 53–57.

Greenbaum E. (1984), 'Biophotolysis of water: the light saturation curves', *Photobiochem. Photobiophys.* **8**, 323–332.

Greenbaum E. (1988a), 'Energetic efficiency of hydrogen photoevolution by algal water splitting', *Biophys. J.* **54**, 365–368.

Greenbaum E. (1988b), 'Hydrogen production by algal water splitting', in Lembi C. A. and Waaland J. R., eds., *Algae and Human Affairs*, Cambridge University Press, Cambridge.

Greenbaum E., Lee J. W., Tevault C. V., Blankinship S. L. and Mets L. J. (1995), 'CO_2 fixation and photoevolution of H_2 and O_2 in a mutant of *Chlamydomonas* lacking photosystem I', *Nature* **376**, 438–441.

Greenbaum E., Lee J. W., Blankinship S. L. and Tevault C. V (1997a), 'Hydrogen and oxygen production in mutant Fud26 of *Chlamydomonas reinhardtii*', *Proc. 1997 US DOE Hydrogen Program Review*, pp. 1–10, 21–23 May 1997, Herndon, VA, NREL/CP–340–23722.

Greenbaum E., Lee J. W., Tevault C. V., Blankinship S. L., Owens T. G. and Mets L. J. (1997b), 'CO_2 fixation and photoevolution of H_2 and O_2 in a mutant of *Chlamydomonas* lacking Photosystem I', *Nature* **376**, 438–441.

Greenbaum E., Lee J. W., Tevault C. V., Blankinship S. L., Owens T. G. and Mets L. J. (1997c), 'Photosystem I measurements in mutants B4 and F8 of *Chlamydomonas*', *Science* **277**, 167–168.

Greenbaum E. and Lee J. W. (1998), 'Renewable hydrogen production by photosynthetic water splitting', *Proc. 1998 US Hydrogen Program Review,* Vol. I, pp. 61–64, NREL/CP–570–25315.

Hall D. O., Markov S. A., Watanabe Y. and Rao K. K. (1995), 'The potential applications of cyanobacterial photosynthesis for clean technologies', *Photosynth. Res.* **46**, 159–167.

Hansel A. and Lindbald P. (1998), 'Towards optimization of cyanobacteria as biotechnologically relevant producers of molecular hydrogen, a clean and renewable energy source', *Appl. Microbiol. Biotechnol.* **50**, 153–160.

Happe R. P., Roseboom, W., Plerik A. J., Albracht S. P. J. and Bagley K. A. (1997), 'Biological activation of hydrogen', *Nature* **385**, 126.

Happe T. and Naber J. D. (1993), 'Isolation, characterization and N-terminal amino acid sequence of hydrogenase from green alga *Chlamydomonas reinhardtii*', *Eur. J. Biochem.* **214**, 475–481.

Happe T., Mosler B. and Naber J. D. (1994), 'Induction, localization and metal content of hydrogenase in the green alga *Chlamydomonas reinhhardtii*', *Eur. J. Biochem.* **222**, 769–774.

Happe T. and Kaminski A. (2002), 'Differential regulation of the Fe-hydrogenase during anaerobic adaptation in the green alga *Chlamydomonas reinhardtii*', *Eur. J. Biochem.* **269**, 1022–1032.

Happe T., Hemschemeier A., Winkler M. and Kaminski A. (2002), 'Hydrogenases in green algae: do they save the algae's life and solve our energy problems?', *Trends Plant Sci.* **7**, 246–250.

Higuchi Y., Yagi T. and Yasuoka N. (1997), 'Unusual ligand structure in Ni–Fe active center and an additional Mg site in hydrogenase revealed by high resolution X-ray structure analysis', *Structure* **5**, 1671–1680.

Hillmer P. and Gest H. (1977a), 'H_2 metabolism in the photosynthetic bacterium *Rhodopseudomonas capsulata*: H_2 production by growing cultures', *J. Bacteriol.* **129**, 724–731.

Hillmer P. and Gest H. (1977b), 'H_2 metabolism in the photosynthetic bacterium *Rhodopseudomonas capsulata*: production and utilization of H_2 by resting cells', *J. Bacteriol.* **129**, 732–739.

Hill S. (1988), 'How is nitrogenase regulated by oxygen?', *FEMS Microbiol. Rev.* **54**, 111–130.

Hollaender A., Monty K. J., Pearlstein R. M., Shmidt-Bleek F., Snyder W. T. and Volkin E. (1972, eds.), 'An inquiry into biological energy conversion', NSF–RANN, Gatlinburg.

Horner D.S., Heil B., Happe T. and Embley T. M. (2002), 'Iron hydrogenases—ancient enzymes in modern eukaryotes', *Trends Biochem. Sci.* **27**, 148–153.

Houchins J. P. (1984), 'The physiology and biochemistry of hydrogen metabolism in cyanobacteria', *Biochim. Biophys. Acta.* **768**, 227–255.

Howard J. B. and Rees D. C. (1994), 'Nitrogenase: a nucleotide-dependent molecular switch', *Ann. Rev. Biochem.* **63**, 235–264.

Hu Q., Miyashita H., Iwasaki I., Kurano N., Miyachi S., Iwaki M. and Hoh S. (1998), 'A Photosystem I reaction center driven by chlorophyll *d* in oxygenic photosynthesis', *Proc. Nat. Acad. Sci. USA* **95**, 13319–13323.

Jahn A., Keuntje B., Doerffler M., Klipp W. and Oelze J. (1994), 'Optimizing photoheterotrophic H_2 production by *Rhodobacter capsulatus* upon interposon mutagenesis in the *hup*L gene', *Appl. Microbiol. Biotechnol.* **40**, 687–690.

Janson S., Carpenter E. J. and Bergman B. (1994), 'Compartmentalisation of nitrogenase in a non-heterocystous cyanobacterium *Trichodesmium contortum*', *FEMS Microbiol. Lett.* **118**, 9–14.

Kaminski A. and Happe T. (2000), '*Hyd*A, encoding the [Fe]-hydrogenase of *Chlamydomonas reinhardtii*, is rapidly induced during anaerobic adaption', *Abstr. 6th. Int. Conf. on the Molecular Biology of Hydrogenases*, Potsdam, 5–10 August 2000, p. PR16.

Kaminski A., Florin L., Tsokoglou A. and Happe T. (2000), 'Molecular biological characterization of [Fe] hydrogenases in green algae', *Abstr. 6th. Int. Conf. on the Molecular Biology of Hydrogenases*, Potsdam, 5–10 August 2000, p. LB6.

Kentemich T., Danneberg G., Hundeshagen B. and Bothe H. (1988), 'Evidence for the occurrence of the alternative, vanadium-containing nitrogenase in the cyanobacterium *Anabaena variabilis*', *FEMS Microbiol. Lett.* **51**, 19–24.

Kentemich T., Bahnweg M., Mayer F. and Bothe H. (1989), 'Localization of the reversible hydrogenase in cyanobacteria', *Z. Naturforsch.* **44c**, 384–391.

Kentemich T., Haverkamp G. and Bothe H. (1991), 'The expression of a third nitrogenase in the cyanobacterium *Anabaena variabilis*', *Z. Naturforsch.* **46c**, 217–222.

Kern M., Koch H.-G. and Klemme J.-H. (1992), 'EDTA activation of H_2 photoproduction by *Rhodospirillum rubrum*', *Appl. Microbiol. Biotechnol.* **37**, 496–500.

Kern M., Klipp W. and Klemme J.-H. (1994), 'Increased nitrogenase-dependent H_2 photoproduction by *hup* mutants of *Rhodospirillum rubrum*', *Appl. Environm. Microbiol.* **60**, 1768–1774.

Kerr R. A. (1998), 'The next oil crisis looms large—and perhaps close', *Science* **281**, 1128–1131.

Kessler E. (1974), 'Hydrogenase, photoreduction and anaerobic growth', in Stewart W. D. P. (1974, ed.), *Algal Physiology and Biochemistry*, Blackwell, Oxford, pp. 456–473.

Kim J. and Rees D. C. (1992), 'Crystallographic structure and functional implications of the nitrogenase molybdenum–iron protein from *Azotobacter vinelandii*', *Nature* **360**, 553–560.

Kim J. and Rees D. C. (1994), 'Nitrogenase and biological nitrogen fixation', *Biochem.* **33**, 389–397.

Kim J. S., Ito K. and Takahashi H. (1980), 'The relationship between nitrogenase activity and hydrogen evolution in *Rhodopseudomonas palustris*', *Agric. Biol. Chem.* **44**, 827–833.

Kimble L. K. and Madigan M. T. (1992), 'Nitrogen fixation and nitrogen metabolism in heliobacteria', *Arch. Microbiol.* **158**, 155–161.

Kleihues L., Lenz O., Bernhard M., Buhrke T. and Friedrich B. (2000), 'The H_2 sensor of *Ralstonia eutropha* is a member of the subclass of regulatory [NiFe] hydrogenases', *J. Bacteriol.* **182**, 2716–2724.

Klein U. and Betz A. (1975), 'Fermentative metabolism of hydrogen-evolving *Chlamydomonas reinhardtii*', *Plant Physiol.* **61**, 953–956.

Klemme J.-H. (1993), 'Photoproduction of hydrogen by purple bacteria: a critical evaluation of the rate-limiting enzymatic steps', *Z. Naturforsch.* **48c**, 482–487.

Koike Y., Schnackenberg J., Miyake M., Miyake J. and Asada Y. (2000), 'Functional expression of clostridial [Fe-only] hydrogenases in the cyanobacterium *Synechococcus* sp. PCC7942', *Abstr. 6th. Int. Conf. on the Molecular Biology of Hydrogenases*, Potsdam, 5–10 August 2000, p. LR13.

Kosourov S., Tsygankov A., Seibert M. and Ghirardi M. (2002), 'Sustained hydrogen photoproduction by *Chlamydomonas reinhardtii*: effects of culture parameters', *Biotechnol. Bioengin.* **78**, 731–740.

Kovács K. L. and Bagyinka C. (1990), 'Structural properties, functional states and physiological roles of hydrogenase in photosynthetic bacteria', *FEMS Microbiol. Rev.* **87**, 407–412.

Krahn E., Schenider K. and Mueller A. (1996), 'Comparative characterization of H_2 production by the conventional Mo nitrogenase and the alternative "iron-only" nitrogenases of *Rhodobacter capsulatus hup*$^-$ mutants', *Appl. Microbiol. Biotechnol.* **46**, 285–290.

Kulandaivelu G., Ravi V. and Thangaraj A. (1988), 'Hydrogen photoproduction by algae and higher plants: optimal conditions for polarographic determination', *Bull. Mat. Sci.* **10**, 393–400.

Kumazawa S. and Mitsui A. (1981), 'Characterization and optimization of hydrogen photoproduction by a salt-water blue-green alga, *Oscillatoria* sp. Miami BG7. I. Enhancement through limiting the supply of nitrogen nutrients', *Int. J. Hydrogen Energy* **6**, 339–348.

Kumazawa S. and Mitsui A. (1985), 'Comparative amperometric study of uptake hydrogenase and hydrogen photoproduction activities between heterocystous cyanobacterium *Anabaena cylindrica* B629 and nonheterocystous cyanobacterium *Oscillatoria* sp. strain Miami BG7', *Appl. Environm. Microbiol.* **50**, 287–291.

Kumazawa S. and Mitsui A. (1994), 'Efficient hydrogen photoproduction by synchronously grown cells of a marine cyanobacterium, *Synechococcus* sp. Miami BG 043511, under high cell density conditions', *Biotechnol. Bioengin.* **44**, 854–858.

Kuwada Y. and Ohta Y. (1987), 'Hydrogen production by an immobilized cyanobacterium *Lyngbya sp.*', *J. Ferment. Technol.* **65**, 597–602.

Lambert G. R. and Smith G. D. (1981), 'The hydrogen metabolism of cyanobacteria (blue-green algae)', *Biol. Rev.* **56**, 589–660.

Laurinavichene T. V., Tolstygina I. V., Galiulina R. R., Ghirardi M. L., Seibert M. and Tsygankov A. A. (2002), 'Dilution methods to deprive *Chlamydomonas reinhardtii* cultures of sulfur for subsequent hydrogen photoproduction', *Int. J. Hydrogen Energy* **27**, 1245–1249.

Lee J. W. and Greenbaum E. (1995), 'Bioelectronics and biometallocatalysis for production of fuels and chemicals by photosynthetic water splitting', *Appl. Biochem. Biotechnol.* **51/52**, 295–305.

Lee J. W., Tevault C. V., Owens T. G. and Greenbaum E. (1996), 'Oxygenic photoautotrophic growth without Photosystem I', *Science* **273**, 364–367.

Lee J. W. and Greenbaum E. (1997), 'A new perspective on hydrogen production by photosynthetic water splitting', in Saha B. D. and Woodward J. (eds.), *Fuels and Chemicals from Biomass*, ACS Symposium Series **666**, Am. Chem. Soc., pp 209–222.

Lemon B. J. and Peters J. W. (2000), 'Photochemistry at the active site of the carbon monoxide inhibited form of the iron-only hydrogenase (CpI)', *J. Am. Chem. Soc.* **122**, 3793–3794.

Ley A. C. and Mauzerall D. C. (1982), 'Absolute absorption cross-sections for Photosystem II and the minimum quantum requirement for photosynthesis in *Chlorella vulgaris*', *Biochim. Biophys. Acta.* **680**, 95–106.

Lien S. and San Pietro A. (1981), 'Effect of uncouplers on anaerobic adaptation of hydrogenase activity in *Chlamydomonas reinhardtii*', *Biochem. Biophys. Res. Commun.* **103**, 139–147.

Lindblad P. (2000), 'Cyanobacterial hydrogenases', *Abstr. 6th. Int. Conf. on the Molecular Biology of Hydrogenases,* Potsdam, 5–10 August 2000, p. LB3.

Luo Y. H. and Mitsui A. (1994), 'Hydrogen production from organic substrates in anaerobic nitrogen-fixing marine unicellular cyanobacterium *Synechococcus* sp. strain Miami BG 043511', *Biotechnol. Bioengin.* **44**, 1255–1260.

Maeda I., Chowdhury W. Q., Idehara K., Yagi K., Mizoguchi T., Akano T., Miyasaka H., Furutani T., Ikuta Y., Shioji N. and Miura Y. (1998), 'Improvement of substrate conversion to molecular hydrogen by three-stage cultivation of a photosynthetic bacterium *Rhodovulum sulfidophilum*', *Appl. Biochem. Biotechnol.* **70–72**, 301–310.

Madigan M., Cox S. S. and Stegeman R. A. (1984), 'Nitrogen fixation and nitrogenase activities in members of the family *Rhodospirillaceae*', *J. Bacteriol.* **157**, 73–78.

Maness P.-C. and Weaver P. (2002), 'Hydrogen production from a carbon monoxide oxidation pathway', *Int. J. Hydrogen Energy* **27**, 1407–1411.

Markov S. A., Bazin M. J. and Hall D. O. (1995a), 'Hydrogen photoproduction and carbon dioxide uptake by immobilized *Anabaena variabilis* in a hollow-fiber photobioreactor', *Enzyme Microbiol. Technol.* **17**, 306–310.

Markov S. A., Bazin M. J. and Hall D. O. (1995b), 'The potential of using cyanobacteria in photobioreactors for hydrogen production', *Adv. Biochem. Eng. Biotech.* **52**, 61–86.

Markov S. A., Weaver P. F. and Seibert M. (1997), 'Spiral tubular bioreactors for hydrogen production by photosynthetic microorganisms: design and operation', *Appl. Biochem. Biotechnol.* **63–65**, 577–584.

Masepohl B. and Klipp W. (1996), 'Organization and regulation of genes encoding the molybdenum nitrogenase and the alternative nitrogenase in *Rhodobacter capsulatus*', *Arch. Microbiol.* **165**, 80–90.

McBride A. C., Lien S., Togasaki R. K. and San Pietro A. (1977), 'Mutation analysis of *Chlamydomonas reinhardtii*: application to biological solar energy conversion', in Mitsui A., Miyachi S., San Pietro A. and Tamura S., eds., *Biological Solar Energy Conversion*, Academic Press, New York, 77–86.

Melis A., Neidhardt J., Broli I. and Benemann J. R. (1998), 'Maximizing photosynthetic productivity and light utilization in microalgae by minimizing the light-harvesting chlorophyll antenna size of the photosystems', in Zaborski O. R., ed., *Biohydrogen*, Plenum Press, New York, pp. 41–52.

Melis A., Neidhardt J. and Benemann J. R. (1999), '*Dunaliella salina* (Chlorophyta) with small chlorophyll antenna sizes exhibit higher photosynthetic productivities and photon use efficiencies than normally pigmented cells'. *J. Appl. Phycol.* **10**, 515–525.

Melis A., Zhang L., Forestier M., Ghirardi M. L. and Seibert M. (2000), 'Sustained photobiological hydrogen gas production upon reversible inactivation of oxygen evolution in the green alga *Chlamydomonas reinhardtii*', *Plant Physiol.* **122**, 127–136.

Melis A. and Happe T. (2002) 'Hydrogen production. Green algae as a source of energy', *Plant Physiol.* **127**, 740–748.

Mikheeva L. E., Schmitz O., Shestakov S. V. and Bothe H. (1995), 'Mutants of the cyanobacterium *Anabaena variabilis* altered in hydrogenase activities', *Z. Naturforsch.* **50**, 505–510.

Misra H. S. and Desai T. S. (1993), 'Involvement of acceptor side components of PSII in the regulatory mechanism of *Plectonema boryanum* grown photoautotrophically under diazotrophic condition', *Biochem. Biophys. Res. Commun.* **194**, 1001–1007.

Mitsugi C., Haram A. and Keno F. (1998), 'WE–NET: Japanese hydrogen program', *Int. J. Hydrogen Energy* **23**, 159–165.

Miura Y., Ohta S., Mano M. and Miyamoto K. (1986), 'Isolation and characterization of a unicellular marine green alga exhibiting higher activity in dark hydrogen production', *Agric. Biol. Chem.* **50**, 2837–2844.

Miyake J. and Kawamura S. (1987), 'Efficiency of light energy conversion to hydrogen by the photosynthetic bacterium *Rhodobacter sphaeroides*', *Int. J. Hydrogen Energy* **12**, 147–149.

Miyamoto K., Hallenbeck P. C. and Benemann J. R. (1979a), 'Hydrogen production by the thermophilic alga *Mastigocladus laminosus*: effects of nitrogen, temperature, and inhibition of photosynthesis', *Appl. Environm. Microbiol.* **38**, 440–446.

Miyamoto K., Hallenbeck P. C. and Benemann J. R. (1979b), 'Solar energy conversion by nitrogen-limited cultures of *Anabaena cylindrica*', *J. Ferment. Technol.* **57**, 287–293.

Miyamoto K., Nawa Y., Matsuoka S., Ohta S. and Miura Y. (1990), 'Mechanism of adaptation and H_2 photoproduction in a marine green alga, *Chlamydomonas* sp. MGA 161', *J. Ferment. Bioengin.* **70**, 66–69.

Miyashita H., Adachi K., Kurano N., Ikemoto H., Ehihara M. and Miyachi S. (1996), 'Chlorophyll *d* as a major pigment', *Nature* **383**, 402.

Montet Y., Amara P., Volbeda A., Vernede X., Hatchikian E. C., Field M. J., Frey M. and Fontecilla-Camps J. C. (1997), 'Gas access to the active site of Ni–Fe hydrogenases probed by X-ray crystallography and molecular dynamics', *Nat. Struct. Biol.* **4**, 523–526.

Nicholls D. G. and Ferguson S. J. (1992), *Bioenergetics 2*, Academic Press, London, p. 160.

Nicolet Y., Piras C., Legrand P., Hatchikian C. E. and Fontecilla-Camps J. C. (1999), '*Desulfovibrio desulfuricans* iron hydrogenase: the structure shows unusual coordination to an active site Fe binuclear center', *Structure* **7**, 13–23.

Nicolet Y., de Lacey A. L., Vernede X., Fernandez V. M., Hatchikian C. E. and Fontecilla-Camps J. C. (2001), 'Crystallographic and FTIR spectroscopic evidence of changes in Fe coordination upon reduction of the active site of the Fe-only hydrogenase from *Desulfovibrio desulfuricans*', *J. Am. Chem. Soc.* **123**, 1596–1601.

Nicolet Y., Cavazza C. and Fontecilla-Camps J. C. (2002), 'Fe-only hydrogenases: structure, function and evolution', *J. Inorg. Biochem.* **91**, 1–8.

Papen H., Kentemich T., Schmulling H. and Bothe H. (1986), 'Hydrogenase activities in cyanobacteria', *Biochemie* **68**, 121–132.

Peters J. W., Lanzilotta W. N., Lemon B. J. and Seefeldt L. C. (1998), 'X-ray crystal structure of the Fe-only hydrogenase (CpI) from *Clostridium pasteurianum* to 1.8 Angstrom resolution', *Science* **282**, 1853–1858.

Polle J., Kanakagiri S., Benemann J. R. and Melis A. (2001) 'Maximizing photosynthetic efficiencies and hydrogen production in microalga cultures', in Miyake J., Matsunaga T. and San Pietro A., eds, *Biohydrogen II*, Pergamon, Amsterdam, pp. 111–130.

Pow T. and Krasna A. I. (1979), 'Photoproduction of hydrogen from water in hydrogenase-containing algae', *Arch. Biochem. Biophys.* **194**, 413–421.

Przybyla A. E., Robbins J., Menon N. and Peck H. D. Jr. (1992), 'Structure–function relationships among the nickel-containing hydrogenases', *FEMS Microbiol. Rev.* **88**, 109–136.

Rákhely G., Colbeau A., Garin J., Vignais P. M. and Kovács K. L. (1998), 'Unusual organization of the genes coding for HydSL, the stable [NiFe] hydrogenase in the photosynthetic bacterium *Thiocapsa roseopersicina* BBS', *J. Bacteriol.* **180**, 1460–1465.

Razquin P., Schmitz S., Peleato M. L., Fillat M. F., Gómez-Moreno C. and Böhme H. (1995), 'Differential activities of heterocyst ferredoxin, vegetative cell ferredoxin and flavodoxin as electron carriers in nitrogen fixation and photosynthesis in *Anabaena* sp.', *Photosynth. Res.* **43**, 35–40.

Redding K., Cournac L., Vassiliev I., Golbeck J. H., Peltier G. and Rochaix J. D. (1999), 'Photosystem I is indispensable for photoautrophic growth, CO_2 fixation and H_2 photoproduction in *Chlamydomonas reinhardtii*', *J. Biol. Chem.* **274**, 10466–10473.

Reddy P. M., Spiller H., Albrecht S. L. and Shanmugam K. T. (1996), 'Photodissimilation of fructose to H_2 and CO_2 by a dinitrogen-fixing cyanobacterium, *Anabaena variabilis*', *Appl. Environm. Microbiol.* **62**, 1220–1226.

Rees D. C. (2002), 'Great metalloclusters in enzymology', *Ann. Rev. Biochem.* **71**, 221–246.

Reeves M. and Greenbaum E. (1985), 'Long-term endurance and selection studies in hydrogen and oxygen photoproduction by *Chlamydomonas reinhardtii*', *Enzyme Microbial Technol.* **7**, 169–174.

Roessler P. G. and Lien S. (1984), 'Purification of the hydrogenase from *Chlamydomonas reinhardtii*', *Plant Physiol.* **75**, 705–709.

Rojek R., Harms C., Hebeler M. and Grimme L. H. (1994), 'Cyclic variations of photosynthetic activity under nitrogen fixing conditions in *Synechococcus* RF–1', *Arch. Microbiol.* **162**, 80–84.

Sasikala K., Ramana C. V., Rao P. R. and Kovács K. L. (1993), 'Anoxygenic phototrophic bacteria: physiology and advances in hydrogen production technology', *Adv. Appl. Microbiol.* **38**, 211–295.

Schmitz O., Boison G., Hilscher R., Hundeshagen B., Zimmer W., Lottspeich F. and Bothe H. (1995), 'Molecular biological analysis of a bidirectional hydrogenase from cyanobacteria', *Eur. J. Biochem.* **233**, 266–276.

Schnackenberg J., Schulz R. and Senger H. (1993), 'Characterization and purification of a hydrogenase from the eukaryotic green alga *Scenedesmus*', *FEBS Lett.* **327**, 21–24.

Schnackenberg J., Ikemoto H. and Miyachi S. (1995), 'Relationship between oxygen evolution and hydrogen evolution in a *Chlorococcum* strain with high CO_2 tolerance', *J. Photochem. Photobiol.* **28**, 171–174.

Schrautemeier B., Cassing A. and Boehme H. (1994), 'Characterization of the genome region encoding an *Fdx*-type ferredoxin and new 2(4Fe–4S) ferredoxin from the nonheterocystous, nitrogen-fixing cyanobacterium *Plectonema boryanum* PCC 73110', *J. Bacteriol.* **176**, 1037–1046.

Schrautemeier B., Neveling U. and Schmitz S. (1995), 'Distinct and differently regulated Mo-dependent nitrogen-fixing systems evolved for heterocysts and vegetative cells of *Anabaena variabilis* ATCC 29413: characterization of the *fdx*H1/2 gene regions as part of the *nif*1/2 gene clusters', *Molec. Microbiol.* **18**, 357–369.

Schulz R. (1996) 'Hydrogenases and hydrogen production in eukaryotic organisms and cyanobacteria', *J. Mar. Biotechnol.* **4**, 16–22.

Seibert M., Ghirardi M., Maness P.-C. and Weaver P. (1996), 'Photobiological production of renewable fuels, chemicals and materials', in *Proc. AAA BIOTEC Conf.*, Ferrara, Italy, Oct. 1996, **IV**, pp. 122–136.

Seibert M., Flynn T., Bensen D., Tracy E. and Ghirardi M. (1998), 'Development of selection and screening procedures for rapid identification of H_2-producing algal mutants with increased O_2 tolerance', in Zaborski O. R. (1998, ed.), *Biohydrogen*, Plenum Press, New York, pp. 227–234.

Seibert M., Forestier M., Zhang L., Melis A. and Ghirardi M. (2000), 'A new direction for algal biohydrogen production', *Abstr. 6th. Int. Conf. on the Molecular Biology of Hydrogenases*, Potsdam, 5–10 August 2000, p. LT4.

Seibert M., Flynn T. Y. and Ghirardi M. L. (2001), 'Strategies for improving oxygen tolerance of algal hydrogen production', in Miyake J., Matsunaga T. and San Pietro A., eds, *Biohydrogen II*, Pergamon, Amsterdam, pp. 67–77.

Serebryakova L. T., Medina M., Zorin N. A., Gogotov I. N. and Cammack R. (1996), 'Reversible hydrogenase of *Anabaena variabilis* ATCC 29413: catalytic properties and characterization of redox centres', *FEBS Lett.* **383**, 79–82.

Smith B. E. and Eady R. R. (1992), 'Metalloclusters of the nitrogenases', *Eur. J. Biochem.* **205**, 1–15.

Steinborn B. and Oelze J. (1989), 'Nitrogenase and photosynthetic activities of chemostat cultures of *Rhodobacter capsulatus* 37b4 grown under different illuminations', *Arch. Microbiol.* **152**, 100–104.

Suda S., Kumazawa S. and Mitsui A. (1992), 'Changes in the H_2 photoproduction capability in a synchronously grown aerobic nitrogen-fixing cyanobacterium *Synechococcus* sp. Miami BG 043511', *Arch. Microbiol.* **158**, 1–4.

Szyper J. P., Yoza B. A., Benemann J. R., Tredici M. R. and Zaborski O. R. (1998), 'Internal gas exchange photobioreactor', in Zaborski O. R. (ed.), *Biohydrogen*, Plenum Press, New York, pp. 441–446.

Takasaki K., Kohno M. and Hattori T. (1996), 'Development of environmentally friendly technology for the production of hydrogen', in *Hydrogen Energy Progress* XI, Vol. 1, *Proc. 11th. World Hydrogen Energy Conf.*, Int. Assoc. for Hydrogen Energy, Schön & Wetzel, Frankfurt, pp. 61–70.

Tamagnini P., Costa J.-L., Almeida L., Oliveira M.-J., Salema R. and Lindblad P. (2000), 'Diversity of cyanobacterial hydrogenases, a molecular approach', *Curr. Microbiol.* **40**, 356–361.

Tamagnini P., Axelsson R., Lindberg P., Oxelfelt, F., Wünschiers R. and Lindblad P. (2002), 'Hydrogenases and hydrogen metabolism of cyanobacteria', *Microbiol. Mol. Biol. Rev.* **66**, 1–20.

Thauer R. K., Klein A. R. and Hartmann G. C. (1996), 'Reactions with molecular hydrogen in microorganisms: evidence for a purely organic hydrogenation catalyst', *Chem. Rev.* **96**, 3031–3042.

Thiel T., Lyons E. M., Erker J. C. and Ernst A. (1995), 'A second nitrogenase in vegetative cells of a heterocyst-forming cyanobacterium', *Proc. Nat. Acad. Sci. USA* **92**, 9358–9362.

Thorneley R. N. F. and Lowe D. J. (1983), 'Nitrogenase of *Klebsiella pneumoniae*. Kinetics of the dissociation of oxidized iron protein from molybdenum–iron protein: identification of the rate-limiting step for substrate reduction', *Biochem. J.* **215**, 393–403.

Tredici M. R., Margheri M. C., de Phillippis R. and Materassi R. (1990), 'The role of hydrogen metabolism in the photoheterotrophic cultures of the cyanobacterium *Nostoc* sp. strain Cc isolated from *Cycas circinalis* L', *J. Gen. Microbiol.* **136,** 1009–1015.

Troshina O. Y., Serebryakova L. T. and Lindblad P. (1996), 'Induction of H_2-uptake and nitrogenase activities in the cyanobacterium *Anabaena variabilis* ATCC 29413; effects of hydrogen and organic substrate', *Curr. Microbiol.* **33**, 11–15.

Tsygankov A. A., Borodin V. B., Rao K. K. and Hall D. O. (1999), 'H_2 photoproduction by batch culture of *Anabaena variabilis* ATCC 29413 and its mutant PK84 in a photobioreactor', *Biotechnol. Bioengin.* **64**, 709–715.

Tsygankov A. A., Fedorov A. S., Kosourov S. N. and Rao K. K. (2002), 'Hydrogen production by cyanobacteria in an automated outdoor photobioreactor under aerobic conditions', *Biotechnol. Bioengin.* **80**, 777–782.

Ueno Y., Kurano N. and Miyachi S. (1999), 'Purification and characterisation of hydrogenase from the marine green alga *Chlorococcum littorale*', *FEBS Lett.* **443**, 144–148.

Urbig T., Schulz R. and Senger H. (1993), 'Inactivation and reactivation of the hydrogenases of the green algae *Scenedesmus obliquus* and *Chlamydomonas reinhardtii*', *Z. Naturforsch.* **48c**, 41–45.

van der Spek T. M., Arendsen A. F., Happe R. P., Yun S., Bagley K. A., Stufkens D. J., Hagen W. R. and Albracht S. P. J. (1996), 'Similarities in the architecture of the active sites of Ni-hydrogenases and Fe-hydrogenases detected by means of infrared spectroscopy', *Eur. J. Biochem.* **237**, 629–634.

Vignais P. M., Colbeau A., Willison J. C. and Jouanneau Y. (1985), 'Hydrogenase, nitrogenase, and hydrogen metabolism in the photosynthetic bacteria', *Adv. Microbial Physiol.* **26**, 155–234.

Vignais P. M. and Toussaint B. (1994), 'Molecular biology of membrane-bound H_2 uptake hydrogenases', *Arch. Microbiol.* **161**, 1–10.

Vignais P. N., Billoud B. and Meyer J. (2001), 'Classification and phylogeny of hydrogenases', *FEMS Microbiol.Rev.* **25**, 455–501.

Volbeda A., Charon M.-H., Piras C., Hatchikian E. C., Frey M. and Fontecilla-Camps J. C. (1995), 'Crystal structure of the nickel–iron hydrogenase from *Desulfovibrio gigas*', *Nature* **373**, 580–587.

Warthmann R., Cypionka H. and Pfennig N. (1992), 'Photoproduction of hydrogen from acetate by synthrophic co-cultures of green sulfur bacteria and sulfur-reducing bacteria', *Arch. Microbiol.* **157**, 343–348.

Warthmann R., Pfennig N. and Cypionka H. (1993), 'The quantum requirement for H_2 production by anoxygenic phototrophic bacteria', *Appl. Microbiol. Biotechnol.* **39**, 358–362.

Weaver P. F., Lien S. and Seibert M. (1980), 'Photobiological production of hydrogen', *Solar Energy* **24**, 3–45.

Weaver P. F., Maness P.-C., Rhodes C., Scahill J., Dundorf S. and Martin S. (1998), 'Biological H_2 from fuel gases and from H_2O', in *Proc. 1998 US Hydrogen Program Review,* Vol. I, NREL/CP–570–25315, pp. 1–8.

Willeford K.O. and Gibbs M. (1989), 'Localization of the enzymes involved in the photoevolution of H_2 from acetate in *Chlamydomonas reinhardtii*', *Plant Physiol.* **90**, 788–791.

Winkler M., Heil Bu., Heil Be. and Happe T. (2002), 'Isolation and molecular characterization of the [Fe]-hydrogenase from the unicellular green alga *Chlorella fusca*', *Biochim. Biophys. Acta* **1576**, 330–334.

Wu L.-F. and Mandrand M. A. (1993), 'Microbial hydrogenases: primary structure, classification, signatures and phylogeny', *FEMS Microbiol. Rev.* **104**, 243–270.

Wünschiers R., Heide H., Follmann H., Senger H. and Schulz R. (1999), 'Redox control of hydrogenase activity in the green alga *Scenedesmus obliquus* by thioredoxin and other thiols', *FEBS Lett.* **445**, 162–164.

Wünschiers R., Senger H. and Schulz R. (2001a), 'Electron pathways involved in H_2-metabolism in the green alga *Scenedesmus obliquus*', *Biochim. Biophys. Acta* **1503**, 271–278.

Wünschiers R., Stangier K., Senger H. and Schulz R. (2001b), 'Molecular evidence for a Fe-hydrogenase in the green alga *Scenedesmus obliquus*', *Curr. Microbiol.* **42**, 353–360.

Wykoff D. D., Davies J. P., Melis A. and Grossman A. R. (1998), 'The regulation of photosynthetic electron transport during nutrient deprivation in *Chlamydomonas reinhardtii*', *Plant Physiol.* **117**, 129–139.

Zehr J. P, Jenkins B. D., Short S. M. and Seward G. F. (2003), 'Nitrogenase diversity and microbial community structure: a cross-system comparison', *Environ. Microbiol.* **5**, 539–554.

Zhang L., Happe T. and Melis A. (2002) 'Biochemical and morphological characterization of sulfur-deprived and H_2-producing *Chlamydomonas reinhardtii* (green algae)', *Planta* **214**, 552–561.

Zinn T., Schnackenberg J., Haak D., Romer S., Schulz R. and Senger H. (1994), 'Evidence for nickel in the soluble hydrogenase from the unicellular green alga *Scenedesmus obliquus*', *Z. Naturforsch.* **49c**, 33–38.

Zirngibl C., van Dongen W., Schwörer B., von Bünau R., Richter M., Klein A. and Thauer R. K. (1992), 'H_2-forming methylenetetrahydromethanopterin dehydrogenase, a novel type of hydrogenase without iron–sulfur clusters in methanogenic archaea', *Eur. J. Biochem.* **208**, 511–520.

Zorin N. A., Lissolo T., Colbeau A. and Vignais P. M. (1996), 'Increased hydrogen photoproduction by *Rhodobacter capsulatus* strains deficient in uptake hydrogenase', *J. Mar. Biotechnol.* **4**, 28–33.

This page intentionally left blank

CHAPTER 9

PHOTOCONVERSION AND ENERGY CROPS

M. J. BULLARD
Bio-Renewables Ltd, ADAS Arthur Rickwood, Mepal, Ely, Cambridgeshire CB6 2BA, UK
mike.bullard@adas.co.uk

He had been eight years upon a project for extracting sun-beams out of cucumbers, which were to be put into vials hermetically sealed, and let out to warm the air in raw inclement summers.

Jonathan Swift, *Gulliver's Travels : A Voyage to Laputa*, 1726.

9.1 Introduction

Any plant species has the potential to be an energy crop, whether it be an undomesticated species, an agricultural waste or a crop grown specifically for energy use. The principle underlying energy generation from crops is that, during photosynthesis, the plant harvests a proportion of the radiant energy incident on it and stores this energy as fixed carbon (mainly in sugars, oils or ligno-cellulose). Thermo-chemical conversion (with or without an intermediate preparatory stage) releases this stored energy into a form that can be used as heat, power or electricity.

An energy crop can be either one that yields a specific product with a high energy content, for example ethanol from sugarcane (*Saccharum*) or rape methyl ester from oilseed rape (*Brassica napus*)—these are the so-called liquid biofuels—or one that yields a non-specific product, ligno-cellulosic material—these are the so-called solid biofuels. The latter can be either above-ground ligno-cellulosic material such as that harvested from *Salix*, *Eucalyptus* or *Miscanthus*, or below-ground material from 'root fuels' such as *Cucurbita foetidissima*. The waste product from a liquid biofuel can in itself form a solid biofuel (*e.g.* sugarcane bagasse). There are three primary requirements of viable energy crop planting: high product yields, removal of excessive moisture from the product prior to harvest, and reduction in the volume of the product.

The burning of ligno-cellulosic biomass or liquid biofuels to produce heat or electricity is appropriate at a local, regional or national scale. It is equally applicable to developing and industrialised nations, although the two may have differing

requirements and potentials. While the three scales of production have different requirements in terms of combustion technology and socio-economic application, all have similar requirements as regards the crop, particularly in terms of photo-conversion: the highest possible quantity of fixed (harvestable) radiant energy per unit area of land. In this chapter, we shall look at the primary sources of biomass and energy crops, which energy crops are most important in different regions, and the requirements of production at different scales and in different regions, concentrating on the processes that can be employed by the farmer to maximise the harvestable commodity. We shall also look at the role of biomass and energy crops under current energy production scenarios and survey current scientific and political developments in cropping and biomass utilisation.

9.1.1 Definitions

The term 'Biomass and Energy Crop' (BEC) is not as tautologous as one might first think. Scurlock and Hall's (1989) definition of 'biomass' refers to the totality of biological material that is used globally to generate energy. As such, biomass includes woodfuel, liquid and solid biofuel crops, charcoal, forestry residues, agricultural residues and dung. Thus biomass contains both materials that are waste or by-products of other biological processes (forestry waste, agricultural residues, dung) and those that are grown specifically for the purpose of harvest and combustion to produce energy (woodfuel, liquid and solid biofuel crops). In this chapter, I refer to natural vegetation, waste and by-products as *biomass*, and crops and their derivatives grown specifically for energy exploitation as *energy crops*. Table 9.1 lists the major biomass and energy crops, and Appendix 9.1 (at the end of the chapter) gives a full list.

One can further distinguish energy crops on the basis of the end product. Those providing solid ligno-cellulosic material and requiring no intermediate processing between harvest and thermochemical conversion can be classified as *direct energy crops*. Those that yield energy following oil extraction or fermentation are classified as *indirect energy crops*. This chapter covers only direct and indirect energy crops, except in the introductory sections, where we shall consider the world's total biomass resource in environmental and socio-economic terms.

Table 9.1 Examples of biomass and direct and indirect energy crops[#]

Biomass	**Direct energy crops**	**Indirect energy crops**
Wastes	Trees	Sugar
Manures	Eucalyptus	Sugarcane
Industrial, commercial and	Willow	Beet
domestic waste	Poplar	Millet
Wood wastes	Pines	Sorghum
Sewage	Acacias	Starch
	Albizia	Potatoes
		Maize
Residues		
Forestry residues	Grasses	
Bagasse	Sugarcane	Oils
Straw	Napier grass	Oilseed rape
Molasses	*Miscanthus*	Linseed
Husks	*Spartina*	Sunflower
Stalks	*Panicum*	
Orchard thinnings	Cereals	
Nutshells		

[#]Appendix 9.1 gives a full list.

9.2 Why grow energy crops?

Burning wood is the oldest form of anthropogenic energy production. It is only extremely recently that other forms of primary energy generation have supplanted wood burning as the world's principal energy source. Indeed, in many regions the burning of wood has remained the sole source of energy generation and there has been little or no improvement in the technology.

9.2.1 The importance of renewables

It is now almost universally agreed that anthropogenic emission of carbon dioxide (CO_2) during the last 250 years has contributed significantly to global warming and climate change (Watson, 2000). The International Panel on Climate Change (IPCC) has recently amended its predictions on the effect of CO_2 emissions, to suggest that

ambient CO_2 levels may treble from 350 to 1000 ppm by 2050, causing a possible 6 C rise in mean global temperatures (Watson, 2000). This rate of temperature change is unprecedented in the last 10,000 years and, if unabated, will have adverse consequences, undermining the very foundation of sustainable development.

The main mechanisms for reversing this trend are improvement in the efficiency of electricity generation and use, and the development of alternative, renewable technologies. The IPCC now state that 'the weight of scientific evidence suggests that the observed changes in the earth's climate are, at least in part, due to human activities' (Watson, 2000). Furthermore, human activities are changing atmospheric concentrations of greenhouse gases, with atmospheric CO_2 concentrations increasing by 30% since 1750, methane more than doubling and NO_x increasing by 15%. Greenhouse gases will continue to increase, with IPCC predictions of CO_2 emissions from fossil fuel combustion increasing from 6.3 GtC yr^{-1} to as much as 35 GtC yr^{-1}. These changes, unless reversed, will have unprecedented impacts on human land use. The details of global warming scenarios are beyond the scope of this chapter but are well described in IPCC (2000). However, central to CO_2 mitigation strategies is the switch from fossil-fuel-derived energy sources to renewable sources. BECs offer almost complete carbon neutrality, and must be a central component of any renewables strategy. Indeed they may even be considered carbon-positive if below-ground carbon sequestration is also considered (Grubb, 2001). BECs can be expected to contribute significantly for a number of reasons (FAO, 1999):

1. BECs can substitute directly for fossil fuels within existing energy supply infrastructures.
2. The potential resource is extremely large.
3. In countries where biomass use is traditional and remains the principal feedstock, population and thus demand is increasing.
4. In countries where fossil fuels are currently predominant, there is already evidence of a shift in attitude towards renewables.
5. In developed countries, agro-business is seeking to diversify from traditional food production to non-food revenue streams.
6. BEC generation systems offer continuity of supply since they are not limited by the sun shining, the wind blowing and so forth, and consequently there is no electricity storage requirement.
7. BECs may offer the additional benefit of short-term sequestration of carbon in unharvested top growth and medium-term sequestration in below-ground root biomass.

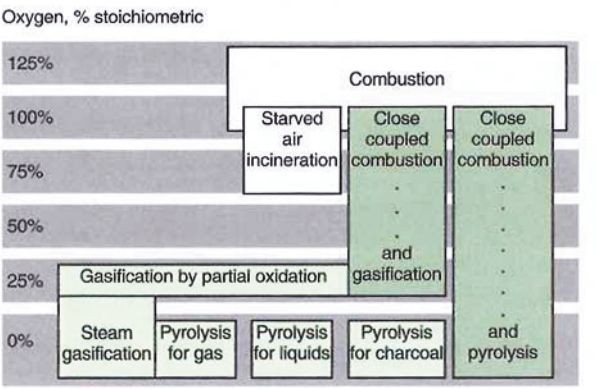


Figure 9.1 Worldwide biomass resources (Bauen, 2001). Current biomass use: 33–55 EJ (world primary energy consumption: 400 EJ) (FAO 1999). Future scenario estimates: 2025: 60–145 EJ; 2050: 100–280 EJ (Hall, 1997). All figures are EJ yr^{-1}.

Estimates suggest that biomass and energy crops currently contribute 5–20% of global energy demand, or 20–55 EJ yr^{-1} depending on the authority (Scurlock and Hall, 1989; IPCC, 1995; FAO, 1999). However, the geographic usage of biomass is uneven, as Fig. 9.1 shows. BECs are the most important source of energy for most of the world's population. Developing countries (in which three-quarters of the world's population resides) derive 38% of their primary energy requirements from biomass (Hall *et al.*, 1996). Precise levels vary from 90% in Nepal, Rwanda, Tanzania and Uganda to 45% in India, and 28% in China and Brazil (FAO, 1999). Levels of electricity generation from renewables are lower in developed countries. For example, they are 14% in Austria, 20% in Finland, 18% in Sweden and 3% in the UK. However, the total energy consumption in less developed countries represents only 34% of the world total. Clearly this average figure disguises many situations where biomass and energy crops are the *only* source of fuel. For as many as 2 billion people it is the only source of primary energy (Pasztor and Kristoferson, 1990).

Figure 9.2 shows the growth in global energy consumption since 1860. Although fossil fuels have supplanted biomass in many regions during the last 250 years, it still remains the fourth largest source of global primary energy. Developed countries generate and consume two-thirds of the world's energy, and 85% of this is generated from fossil fuels (Table 9.2). Woods and Hall (1994) and ENEP (1996) have published an exhaustive list of individual country's total energy production and the

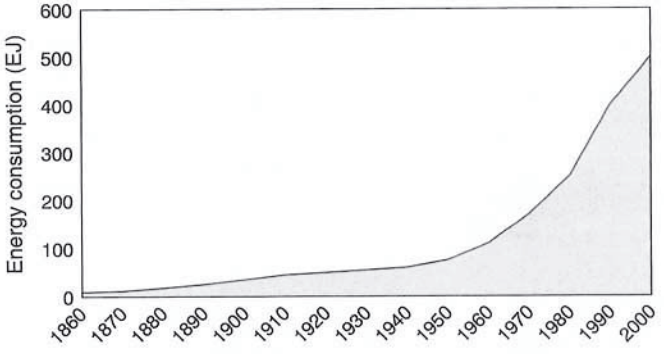


Figure 9.2 Global energy consumption 1860–2000 (Shell Oil plc, 1994).

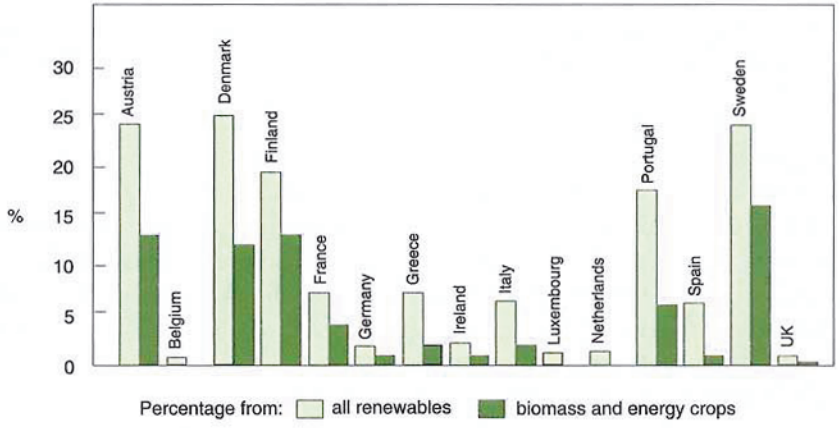


Figure 9.3 Energy generation in Western European countries from all renewable sources, and from biomass and energy crops (most recent available data), both expressed as a percentage of total energy production in that country. Total renewables data from EUROSTAT (1994) and CEC (1996); biomass and energy crops data from Scurlock and Hall (1994), except for Austria, which are from Hall (1997). Data therefore represent a spread of years (1988–1996) and should be treated with some caution.

proportion of that which is derived from biomass. Figure 9.3 shows the proportion of energy derived from biomass in Western European countries, and illustrates the extent to which exploitation of biomass varies within this region.

Table 9.2 Global and regional energy use by fuel source

Source	World primary energy consumption in 2000 (%)[a]	EU average in 2001 (%)[b]	UK in 2000 (%)[c]	OECD average in 2000 (%)[a]	OECD Europe in 2000 (%)[a]	Non-OECD average in 2000 (%)[a]
Oil	39.04	40	32	62.26	20.33	37.74
Gas	22.82	23	43	54.70	18.55	45.30
Coal	23.85	15	15	47.17	13.61	52.83
Nuclear	6.49	16	9	86.40	36.17	13.60
Hydro	7.04	–	–	50.43	19.96	49.57
Other[e]	0.76	6 (renewables and electricity imports)	1 (including all renewables)	80.60	28.76	19.40

IEA (2000).

[a] EIA (2002).

[b] IEA Bioenergy (2001).

[c] DTI (2000).

[d] Adapted from Hall and House (1995).

[e] Includes biomass and other renewables except hydro.

Energy production from biomass growing as native vegetation or from waste from separate processes (*e.g.* agricultural residues or forestry waste) far exceeds energy production from crops grown specifically for that purpose. For example, currently all of the UK's 200 MW_e of biomass capacity comes from forestry thinning, cereal residues or poultry litter, although a government-sponsored energy crop scheme is in place to stimulate cropping. In most areas where biomass is widely used, land is at a premium for food production. Within specific regions, notably the EU, however, the gap is closing, due principally to policy measures which have environmental stimuli.

The higher energy density of fossil fuels and their proven exploitation technology, relatively low cost and plentiful supply have all led to the rapid increase in their exploitation since 1860 shown in Fig. 9.2. UK domestic electricity and gas costs are 20% lower in 2002 than they were in 1990, twelve years during which the real impacts of climate change have become more widely understood. UK government is clearly supportive of the principle of renewable energy yet the interests of the environment are clearly still ranked behind consumer's short-term interests and political ambition.

The production of 1 GJ of energy from oil results in the net release of 73 kg CO_2. The figure for coal is 91 kg and that for natural gas is 52 kg (IPCC, 1995). The burning of fossil fuels puts 7.6 ± 1.5 Gt yr^{-1} of carbon into the atmosphere annually (28.58 ± 5.6 $GtCO_2$ yr^{-1}). Natural processes cannot adapt to remove this on the same time scale, so a large proportion adds to the net CO_2 in the atmosphere; unexploited fossil fuels represent sequestered carbon that would not normally significantly contribute to rising atmospheric levels. Atmospheric CO_2 levels are at their highest for 160,000 years and this net addition, which started at the beginning of the industrialised era *c.* 1750, has been identified as the main cause of climate change. Whilst there is still tremendous uncertainty over the manner in which raised CO_2 levels will affect the biosphere, it is generally acknowledged that there is a need to first halt and then reverse the trend of increasing atmospheric CO_2 concentrations. Renewable energy sources offer a way forward here. Renewables generally result in less CO_2 emitted per GJ of energy generated than do fossil fuels. Most potential for CO_2 mitigation is shown by biomass and energy crops grown over the long term in a sustainable manner, even though CO_2 release (per GJ energy generated) is highest, as shown in Table 9.3. Their benefit lies in the fact that regrowing vegetation will re-absorb the CO_2 released during combustion. Sustainable energy cropping on short rotations offers a more efficient strategy for CO_2 mitigation in the long term than does 'carbon storage' in vegetation, because the latter would saturate in time (maximum tree growth rates decline after 10–60 years).

Table 9.3 Carbon emissions per unit of energy from some primary energy sources

Fuel	kg C GJ^{-1}
Wood	26.1–29.9
Peat	30.0
Coal	23.9—25.8
Crude oil	19.0–21.4
Natural gas	13.6–15.4

Adapted from IPCC (1995).

Replacement of fossil fuels with biomass and energy crops is close to 'carbon neutrality' because any CO_2 emitted is reabsorbed during the photosynthesis of successor crops that have replaced those which were harvested or have regrown. Increased awareness of the potential of energy crops has come in response to the requirements for CO_2 abatement and other environmental considerations, increased global demand and regional land use issues. Whilst renewables and biomass systems for large-scale energy generation are often relatively expensive, most renewable systems in industrialised countries are seeing a 10–15% reduction in costs of production year on year.

It is worth noting that 100 years ago oil represented only 2% of the total energy market, and only entered wide-spread use as a by-product from niche-market developments such as kerosene (Dupont-Roc *et al.*, 1994). After 1890, the development of the automotive industry and the switch from coal to oil in industries where the higher energy density of oil was at a premium acted as 'drivers' for the oil industry. Coincidental was a decline in the costs of production as extraction technology improved. Thus in a short space of time a marginal and expensive product became widely used and relatively inexpensive. It is quite possible that, although dependent on a different set of drivers, an analogous situation could exist with biomass. This is already happening in developed countries, where scale-up of steam turbine systems and newer gasification and pyrolysis generation systems are improving efficiencies of electrical conversion.

Global warming is not the sole reason for increasing the utilisation of BECs. At a rural scale, cooking-fire fuel is a scarce commodity in many areas. Chege (1993) estimated that as many as 2.7 billion people would subsist with inadequate access to fuel wood by 2000. This has in many cases been the result of increasing populations coinciding with a history of unsustainable management of existing wood fuel resources. International efforts are not simply looking to re-forest such denuded areas—above ground biomass often produces great quantities of smoke and is

implicated in chronic health problems. Some studies are looking to increase the use of 'root fuels': taproots from plants, for example members of the Cucurbitaceae such as *Cucurbita foetidissima*, are being examined as fuel wood sources for arid areas of Mexico, Brazil, Zimbabwe and India (Shultz and Bragg, 1995).

9.2.2 *Biomass and energy crop classification by resource sector*

Bioresources are potentially the world's largest source of fuel and chemicals, a renewable resource of 220 billion dry tonnes of annual primary production, equal to about 4×10^{18} MJ of energy (FAO, 1999). The energy content of standing biomass on the planet has been calculated to be 36×10^{18} MJ (Hall and Rao, 1999). Within geographic regions the proportion of total renewables generation from biomass and energy crops varies significantly. Within the EU, for example, the proportion varies from <1% to 18%, as Fig. 9.3 shows. Much more could be used in the future: Table 9.4 shows Swisher and Wilson's assessment of the potential for energy crop production in different regions of the world by 2030, and Table 9.5 a complementary assessment to 2050.

Estimates of land availability for long-term energy plantations and agro-forestry (*e.g.* timber production) vary greatly. Grübler *et al.* (1993) estimated that 265 million hectares are available for forest plantations and an additional 85 million hectares for agroforestry, but Winjum *et al.* (1992) suggested a figure of 600–1200 million hectares. The energy cropping area of developing countries has been estimated to be 500 million hectares (Hall, 1991), and the available land for tropical plantations to be 420–620 million hectares (Grainger, 1990; Houghton *et al.*, 1991). The scale of this variation indicates the difficulties in formulating precise figures for bio-productivity and will clearly have an impact on the likely contribution BECs can make to global energy requirements and CO_2 abatement strategies.

9.2.3 *Future trends*

For a significant proportion of the world's population, fossil fuels remain the least expensive, mass energy source. For geopolitical reasons it is unlikely that this will change in the foreseeable future. Therefore we must look for alternative stimuli for increased BEC use. We have already noted that the major stimulus for industrialised nations to increase BEC energy generation in the next century will be the need to reverse the trend in increasing global CO_2 concentrations (IPCC, 1992, 1995; Watson,

2000), largely caused by dramatic increases in fossil fuel consumption over the last 100 years. Currently, industrialised nations contribute a disproportionate amount (75%) of the net CO_2 emitted. As developing nations strive to 'industrialise' and attain higher standards of living, a secondary global objective must be that this is achieved using the cleanest possible technology so that reductions made by currently industrialised nations are not offset (or overtaken) by new nations.

Table 9.4 Estimates of potential of renewable energy sources in 2030 (TWh yr^{-1})

Region	Total renewables[a]	Biomass[b]	Energy crops[c]	Forests[d]
Canada	2,218	211	233	0
USA	3,353	889	734	0
Central America	984	303	84	178
Andean Countries	2,391	220	100	1,187
Brazil	3,417	678	536	1,603
Argentina and Chile	840	165	105	0
Nordic Countries	489	119	103	0
Western Europe	1,577	458	211	0
Eastern Europe	424	248	59	0
FSU	3,471	733	354	0
Japan	379	0	55	0
Australia/New Zealand	788	156	81	0
China	2,784	843	60	0
India	1,743	926	62	196
Four Tigers	38	18	0	0
Asia	2,887	376	103	1,679
Pacific Islands	2,194	669	17	924
Middle East	118	0	0	0
North Africa	167	55	40	0
Sub-Saharan Africa	3,359	880	720	1,101
TOTAL	33,621	8,003	3,603	6,868

Source: Swisher and Wilson (1993). [a] Excludes direct building and heating technologies and non-commercial biomass use; [b] Original data from Hall (1991), assuming a conversion efficiency of 33%; [c] Assumes 150 GJ ha^{-1} yr^{-1} on 10% of the total land (crop, forest, woodland) area available (higher percentage used for US and Nordic countries). A lower energy yield per hectare (75 GJ ha^{-1} yr^{-1}) is assumed for developing countries because of infrastructure deficiencies; [d] Assumes sustainable development of tropical forestland and the annualised energy content of 35-year rotations.

Table 9.5 Projection of regional technical biomass energy potential by 2050

Region	Population in 2050 (billion)	Total land with crop production potential (Gha)	Cultivated land in 1990 (Gha)	Additional cultivated land required in 2050 (Gha)	Available area for biomass production in 2050 (Gha)	Maximum additional energy from biomass[a] ($EJ\ yr^{-1}$)
Developed world[b]		0.820	0.670	0.050	0.100	30
Latin America						
Central and Caribbean	0.286	0.087	0.037	0.015	0.035	11
South America	0.524	0.865	0.153	0.082	0.630	189
Africa						
Eastern Africa	0.698	0.251	0.063	0.068	0.120	36
Middle Africa	0.284	0.383	0.043	0.052	0.288	86
Northern Africa	0.317	0.104	0.04	0.014	0.050	15
Southern Africa	0.106	0.044	0.016	0.012	0.016	5
Western Africa	0.639	0.196	0.090	0.096	0.010	3
Asia						
China[c]	–	–	–	–	–	2
Western Asia	0.387	0.042	0.037	0.010	–0.005	0
South-Central Asia	2.521	0.200	0.205	0.021	–0.026	0
Eastern Asia	1.722	0.175	0.131	0.008	0.036	11
Southeast Asia	0.812	0.148	0.082	0.038	0.028	8
Total for above	8.296	2.495	0.897	0.416	1.28	396
Total biomass energy potential ($EJ\ yr^{-1}$)						441[d]

Sources: Fischer and Heilig (1998); D'Apote (1998); IIASA/WEC (1998). [a] Assumed 15 odt (oven-dry tonnes) $ha^{-1}\ yr^{-1}$ and 20 $GJ\ odt^{-1}$; [b] Here, OECD and Economies in Transition; [c] For China, the numbers are projected values from d'Apote (1998) and not maximum estimates; [d] Includes 45 $EJ\ yr^{-1}$ of current traditional biomass.

9.2.3 Future trends

Fossil fuels remain the least expensive energy source for a significant proportion of the world's population; for geopolitical reasons it is unlikely that this will change in the foreseeable future. Therefore we must look for alternative stimuli for increased BEC use. The major stimulus for industrialised nations to increase BEC energy generation in the next century will almost certainly be the need to reverse the trend in increasing global CO_2 concentrations (IPCC, 1992, 1995; Watson, 2000), largely caused by dramatic increases in fossil fuel consumption over the last 100 years. Industrialised nations currently account for a disproportionate amount (75%) of the net CO_2 emitted to the atmosphere. As developing nations strive to 'industrialise' and attain higher standards of living, a secondary global objective must be that this is achieved using the cleanest possible technology, so that the reductions made by currently industrialised nations are not offset (or overtaken) by new nations.

By no means secondary to this imperative is the need to develop sustainable biomass feedstock systems in many parts of the world where centralised fossil fuel energy generation is not feasible, but where inappropriate harvesting of biomass is leading to environmental degradation and loss of agricultural suitability.

Land availability issues may act either as stimulants or deterrents to increased BEC production. Many industrialised countries produce surplus food and this may act as a stimulus to grow BECs on land that would otherwise be economically surplus and therefore likely to degenerate to wasteland. However, land availability limits food production in most countries, and there will consequently be a direct conflict between energy and food crop needs. Indeed, in many areas, biomass availability is linked to land clearance for agriculture, although clearly this is not sustainable since the biomass is replaced by food production. It is one of our greatest challenges to incorporate biomass and energy cropping *per se* in these situations.

9.2.4 Discounting carbon sinks

Uncropped vegetation, both aquatic and terrestrial, acts as a sink for carbon. Much consideration has been given to the large-scale establishment of forests to soak up CO_2. FAO (1999) estimates that such forests could increase the amount of carbon sequestered from the atmosphere by a factor of five (up to 10 GtC yr^{-1}). This has been much advocated by the USA, Japan and Australia as an alternative to the 'more economically damaging switches of power technology' that would otherwise be needed. This would be a short-term palliative but it offers no real long-term solution.

The rate of carbon sequestration in a perennial plant declines with age, until at some point the CO_2 losses from respiration become equal to the CO_2 uptake in photosynthesis. Thus, this approach could only postpone the inevitable increase in atmospheric CO_2. Although carbon sequestration may have a short-term role in displacement while alternative technologies are developed, a long-term abatement strategy must use energy generation from biomass.

Having said that, sequestration in energy cropping systems offers an additional short-term buffer to atmospheric CO_2 increase. Figure 9.4 shows not only that a considerable additional carbon offset would be achieved through the accumulation of carbon in the soil and litter of energy tree systems, but also that trees are sequestering carbon even before the above-ground feedstock is harvested. In other words, energy crops are abating climate change as soon as they begin growth.

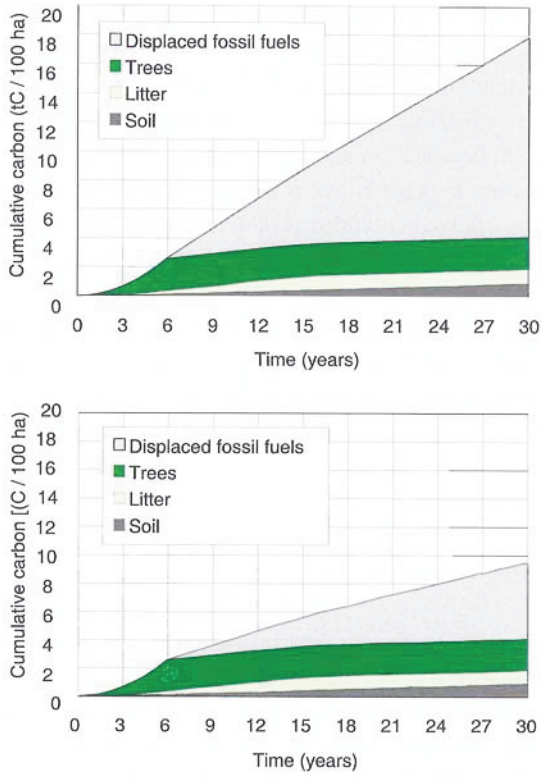


Figure 9.4 Cumulative carbon offset from trees grown for energy when replacing (a) coal for heat; (b) gas for electricity generation (Grubb, 2001).

9.2.4 The contribution of BECs to CO_2 abatement

The evidence for global climate change caused by elevated atmospheric CO_2 and other greenhouse gases is compelling. Reversal of these trends is a great challenge, but the contribution that BECs can make is probably larger than any other renewable energy sector, when considered globally.

The role for renewables generally, and energy crops specifically, is indicated by three major 'replacement scenario' reports (IPCC, 1992 and 2001; Shell Oil, 1994) and various policy documents from the Commission of the European Communities.

Under all scenarios, energy crops will have a key role in the abatement of atmospheric CO_2 levels. According to Shell Oil, additional global energy generation from crops may range from 5% to 15% by 2050 depending on the scenario that is realised. Within Europe, energy crops are projected to contribute 100—200 GJ annually and take up to 10 M ha of agricultural land by 2050.

In the UK, Smith (1998) calculated that 3.5–4 Gt of residues are available annually (with an energy content of 65 EJ) and Hall *et al.* (1993) estimated that on a global basis residues from major agricultural crops alone could yield sufficient biomass to generate 38 EJ yr^{-1}. In the European Community, the market share of renewable energy sources in Europe in 2020 is projected to be between 7.5% and 13.7% of the total inland consumption, compared with present consumption of 5.4% (CEC, 1996). Of the four long-term energy forecasts established by the EC, the one that leads to the greatest energy share from renewables, the 'forum' scenario, assumes the highest level of international stability, economic growth and therefore harmonised political intervention, and a stable oil price. Under this 'ecological' scenario the contribution of all renewable energy sources could amount to 220 Mt annually, a 2.5-fold increase on the present level.

In the USA, biomass currently provides 3–4% of total energy needs. 10.3 GW of installed electrical capacity produces 65 GWh_e annually, which amounts to 1.3% of the USA's electrical demand. NREL (2002) suggest that this figure could increase by a factor of 4.5 by 2020. Worldwide, BECs have the potential to substitute 0.5–1.6 Gt of fossil-fuel carbon yr^{-1}, or 8–27% of current global consumption (Paustian *et al.*, 1997). These figures do not include additional long-term sequestration of carbon in soils or afforested vegetation.

BEC resources may be boosted by improved varieties and management techniques. FAO (1999) predicts that the use of dedicated BECs can contribute significantly to energy production. The sugarcane-derived ethanol programme of Brazil, the maize-derived ethanol programme of the USA, Sweden's willow programme and the UK's *Miscanthus* and SRC (Short Rotation Coppice) programme are all examples of this

potential. According to FAO (1999), some studies indicate the potential replacement of fossil fuel by energy crops in the Tropics alone to be as high as 150–510 MtC yr^{-1}. In temperate zones, the figure may be 80–490 MtC yr^{-1}. Many rural farmers could increase yields by 50%. Africa only uses 20% of its available cropland. If new technologies were adopted Africa could still have 75% remaining by 2025, yet generate ten times its present energy consumption.

9.2.5 *Available resources for biomass and energy cropping*

A significant amount of energy is generated from the residues of forestry and agriculture on all scales today (*e.g.* 8,000 MW_e in the USA are already generated from crop residues). McKeever (1995) estimated that 170 Mt of solid wood waste is generated annually in the USA, of which nearly 40 Mt may be recoverable for energy generation systems.

In addition, significant quantities of agricultural waste such as cereal straw and bagasse could also be used to generate energy without any need for cropping. For example, annual EU cereal residues[1] total 64 Mt yr^{-1} and it is estimated that in the pacific northwest of the USA alone, 8.1 Mt yr^{-1} of agricultural residues could become available for energy generation (Kroll and Forester, 1995). 282.5 kg (dry basis) of bagasse are available from every tonne of sugarcane harvested for sugar extraction (Beeharry, 1996). Thus, a small sugarcane producer such as Mauritius could produce approximately 1.8 Mt of biomass for energy generation annually. However, likely as it is that waste commodities will be fully utilised before significant areas of new crop are planted, these resources are finite, and totally dependent on the existence of the mother industry. If a sustained level of biomass generation of 5–10% is to be attained in industrialised countries, as required by the IPCC and EC renewables scenarios, then there simply is not sufficient waste feedstock. Much greater amounts of energy could be produced from energy crops; Hall *et al.* (1993) estimated a global potential of 267 EJ yr^{-1}. Furthermore, many wastes will remain unutilised because they are spread too thinly geographically to warrant the siting of a conversion facility and competition from other more lucrative markets may reduce the quantity of available material. Thus while there is considerable interest in sitting straw-burning power stations in the south-east of the UK, and the first such station was opened near Ely in Cambridgeshire in late 2000, it is unlikely that one would be built in the southwest, a region

[1]Assuming a 'harvest index' of 0.4, and 1991 figures of ~160 Mt of grain produced annually (EUROSTAT, 1994).

which is a net importer of straw at consequently higher prices. Also, some crop waste incorporation into soil is required to maintain soil carbon cycles and fertility, reduce soil erosion and enhance moisture retention. Indeed, even if crop wastes were available in quantity and concentration the sustainability of using, say, all forestry residues in energy generation, is being seriously questioned in some quarters.

It is quite possible that the first phase of large-scale energy cropping in industrialised countries will derive not from an energy source stimulus, but from the need to augment existing supplies for the forest products and agricultural residue industries.

9.2.6 The policy framework for energy cropping

In subsistence economies, policies for energy cropping are aimed not at stimulating the use of environmentally benign renewables but rather at encouraging the efficient production of the *only* available source of primary energy. In the developed world, policy measures where the primary aim is to reduce reliance on fossil fuels have their origins in the 'oil shock' of 1973. More recently, national and international policy agendas for the advancement of renewable energy systems have been driven mainly (but not exclusively) by environmental considerations. For example, within the EU the preponderance of surplus agricultural land has led to renewed interest in the exploitation of non-food crops. Additional policies aimed at reducing SO_2 and NO_x emissions have favoured renewable energy sources. In Brazil, the 90-year development of their fuel-alcohol programme has been motivated by a desire to reduce reliance on foreign oil (Goldemberg *et al.*, 1992). This approach is replicated in other countries (Rosillo-Calle, 1990). Renewables technologies may generally find increasing favour since they offer indigenous fuel supply security (PIU, 2002).

On a global scale, a powerful political stimulus for the development of energy crops came from the Intergovernmental Panel on Climate Change (IPCC, 1992). At the so-called Earth Summit UN conference in Rio de Janeiro 1992, the IPCC called for a global reduction of CO_2 emissions levels in 2010 to 1990 levels (the 'Rio' Declaration). Signatories to the Rio agreement implemented energy efficiency and new generation policy measures (with varying degrees of vigour and success). The Rio Declaration was followed by the Kyoto Protocol of 1997, which proposed measures aimed at reducing global greenhouse gas emissions by 6% relative to 1990 levels by the period 2008–2012 and attempted to assign individual targets for reductions for nation states (Kyoto Protocol, 1997). The proposed targets varied between countries, with a few (*e.g.* Australia) permitted an increase, but with most required to reduce emissions by 6–8%.

Ratification of the Kyoto Protocol was finally achieved in 2001 at the Seventh Conference of Parties (COP–7) in Marrakech. Although the proposals themselves have been watered down and international consensus impaired by the USA's refusal to ratify the Protocol, this nevertheless represents an important start in the creation of a legally binding framework for the global reduction of greenhouse gas emissions. The Protocol allows for a number of mechanisms for nation states to reduce CO_2 emissions: increased energy efficiency, reduced fossil fuel consumption, increased use of renewable resources, carbon and emissions trading, and limited carbon sequestration development.

9.2.7 *Examples of existing biomass and energy crop production programmes*

United Kingdom

The UK has a Kyoto Protocol target of an 8% reduction of greenhouse gas emissions relative to 1990 levels and a self-imposed target reduction of 12.5%, both of which it has already exceeded. The UK has in place one of the best developed strategies for combating climate change (DETR, 2000a), and now aims to cut CO_2 emissions by 23% relative to 1990 levels. There are proposals that suggest that the UK should cut emissions by 60% relative to 1990 levels (RCEP, 2000) and that the contribution of renewable energy to UK requirements should increase to 20% by 2020 (PIU, 2002), but these targets were not incorporated in legislation at the time of writing this chapter. These laudable targets are set in stark contrast to the consumer energy markets, which currently enjoy the lowest gas and electricity prices in history. There is therefore little economic incentive to become more efficient in energy use or to switch to other technologies, other than where stimulated with grant aid by government.

Before 2000, the main stimulus for the development of renewable energy systems in the UK was the Non-Fossil Fuel Obligation, under which contracts of up to 15 years duration and paying a premium price for electricity generated from non-fossil fuel sources were awarded. NFFO aimed to provide a level playing field in which developing (and thus expensive technologies) could compete with coal, oil and gas. Successive rounds of NFFO aimed to reduce the scale of the premium, ultimately to the point at which fossil and non-fossil fuel sources achieved price parity. This approach was successfully demonstrated with on-shore wind energy systems. From 1995, NFFO tranches offered contracts for energy-crop power stations. Unfortunately, significant local planning objections and/or difficulties in raising finance have seen the demise of all but one coppice/forestry thinning gasifiers (the 8 MW_e output Arbre

gasifier), whose future also hangs in the balance at the time of writing, and one cereal straw/*Miscanthus* power station (the 36 MW_e output Elean Power Station, opened in early 2002). Many projects are stalled at the planning stage. In 2001, the Renewables Obligation (RO) replaced the NFFO. Under the RO, electricity suppliers will be required to supply an increasing proportion (5% IN 2003 rising to 10% by 2010) of their electricity from renewable sources. Every unit of RE generated is awarded a Renewable Obligation Certificate (ROC) by the electricity regulator OFGEM. Failure to do so will result in penalties of £0.03 kWh^{-1}, levied by OFGEM. Those levies will be passed on to those electricity suppliers who are compliant, thus enabling the latter to invest in further RE. The current deficit in ROCs, combined with the reapportioning of money to compliant suppliers has been sufficient to increase the value of the ROC way past the apparent cost of the penalty. Thus the penalty when combined with the baseline electricity generating price of £0.02 kWh^{-1} for fossil-derived fuels suggests that a maximum price of approximately £0.05 kWh^{-1} could be paid for renewable-sourced electricity, whereas spot prices in 2002 of £0.067 kWh^{-1}. The RO is not banded and it is likely that wind energy projects will proliferate, since these are among the lowest-cost renewable technologies available. This will be at the expense of energy crop systems. However, opportunities and funds are now available for smaller-scale combined heat and power, industrial heat and electricity generation from biomass, and these should make a significant contribution to the UK's renewables mix in the next twenty years.

In the UK, an area of 125,000 ha of energy crops is proposed by 2006 (DTI, 1999) and both SRC and *Miscanthus* are eligible for planting grants in addition to 'set-aside' annual payments. It is proposed that the resultant biomass (about 1.5 Gt yr^{-1}) will be used in conventional combustion systems for the generation of electricity, in combined heat and power (CHP) units, or in gasification/pyrolysis systems. The scale of conversion will range from 50kW_e to 50 MW_e. Opportunities for co-firing biomass with fossil fuel powered plants are also being considered.

A significant review (PIU, 2002) of UK energy policy was completed in 2002, covering the options for energy generation until the middle of the century. Renewable energy is considered central to the UK's future energy security, environmental aims under a low-carbon economy, and rural diversification requirements (the travails of the farming community following BSE and the Foot and Mouth outbreak have focussed governmental attention). This review proposes that a broad blend of renewable technologies should contribute 20% of the UK's energy demands by 2020. Table 9.6 shows the predicted tranches of RE and their costs in 2020 and 2050.

Table 9.6 Adapted from the PIU (2002)

Technology	2020 cost (p/kWh)	Basis for assessment	2050 cost (p/kWh)
End use	Low	Engineering assessment	Decrease, but variable
Fuel cells	Unclear	Engineering assessment	Sustained decrease
Large CHP	Under 2	Engineering assessment	Limited decrease
Micro-CHP	2.5–3.5	Engineering assessment	Sustained decrease
Transport efficiency	Low	Engineering assessment	Unclear—fuel switching
Photovoltaics	10–16	Learning rate and market growth rate	Sustained decrease
Onshore wind	1.5–2.5	Learning rate and market growth rate	Limited decrease
Offshore wind	2.0–3.0	Engineering assessment and onshore learning rate	Decrease
Energy crops	2.5–4.0	Engineering assessment and learning rate	Decrease
Wave	3.0–6.0	Engineering assessment	Uncertain
Fossil generation with CO_2, C and S	3.0–4.5	Engineering assessment	Uncertain
Nuclear	3.0–4.0	Engineering assessment	Decrease
CCGT	2.0–3.0	Engineering assessment and learning rates	Limited decrease
Coal (IGCC)	3.0–3.5	Engineering assessment	Decrease

United States of America

Energy generation from biomass in the USA, while of a significant scale (8,000 MW_e in 1993 rising to 10,000 MW_e by 1998), is derived predominantly from municipal and agricultural wastes and forestry industry by-products (Robertson and Shapouri, 1993; Anon., 2000). In addition, according to DTI (1999), 50,000 hectares of woody plantations have also been planted. Wood and wood wastes constitute 80% of the utilised resource, municipal solid waste 16% and alcohol 3% (Brettler Bereneyi, 1995). Current energy generation from biomass accounts for 3.5% of USA's total energy production. Cook and Beyea (2000) estimate that agricultural and forestry

residues alone could offset 4% (3EJ yr^{-1}) of the nation's fossil energy use. In common with most developed countries, the further development of the US renewables industry to the extent of adopting specific energy crops is dependent on economic and environmental stimulants. There is currently no commercial planting of energy crops, owing to a lack of demand: economic stimuli are not in place and the bulkiness of the raw materials, leading to high transportation costs, limits the size of conversion facilities and thus generation capacity. Forestry waste-powered systems exist as either dedicated boilers for biomass or co-generation facilities. There are currently 149 stand-alone biomass units and 367 co-generation power plants (Robertson and Shapouri, 1993). It is estimated that there is the potential to co-fire 2,000 generation facilities. Biomass supply to these would demand 24,000 tonnes per day.

A number of studies (Robertson and Shapouri, 1993; Keener and Roller, 1975; Hall *et al.*, 1993) have identified the tremendous potential of energy crops in the USA. Williams (1994) suggests 15 EJ could be produced annually without confronting significant land use constraints. It is estimated that 370 Mh of land could support energy cropping, although only 14–30 Mt of this could be considered land with high potential (Williams, 1994; Robertson and Shapouri, 1993). The crop most likely to be adopted in the medium term, which has the additional support of environmental pressure groups because it is an indigenous species, is *Panicum virgatum* (switch grass). Additional crops which are being evaluated include Bahia grass, Bermuda grass, eastern Gama grass, reed canary grass, napier grass, rye, Sudan grass, tall fescue, timothy, weeping love grass, alfalfa, bird's-foot trefoil, crown vetch, flatpea, clover and *Sericea lespedeza* (Wright *et al.*, 1993).

It is quite possible that the first stimulus for large scale-cropping of woody biomass and herbaceous species will come, not from energy demands, but as a response to dwindling forest product reserves and increased protection of existing forests (Kroll and Forester, 1995).

Brazil

The ProAlcool programme of Brazil, aimed at substituting bio-ethanol in petrol engines, was launched in 1975. The programme was stimulated on the one hand by escalating world oil prices and on the other by the rapid decline in world sugar prices of the mid-1970s. Thus the ProAlcool programme was aimed at maintaining traditional markets by fuel substitution. By 1998, Brazil was producing 13 billion litres of ethanol, the equivalent of 220,000 barrels of petrol imports per day (Rosillo-Calle and Cortez, 1998). Macedo (1992), calculating annual production to be 11.8M m^3, suggested that this offset 9.75M m^3 of gasoline, avoiding 7.41M m^3 carbon

emissions annually. At its peak, the scale of bio-ethanol production in Brazil was such that all transport vehicles run either on ethanol or gasohol (an ethanol/gasoline mixture). The development of the Brazilian programme at the expense of fossil fuels had, at its peak, reduced net annual CO_2–C emissions by 88 Mt (figure derived from Goldemberg *et al.*, 1992). That bio-energy competed to the exclusion of fossil fuels is due entirely to the use of appropriate economic stimulation measures:

1. The state-owned gasoline company was obliged to purchase some ethanol;
2. Economic incentives (low interest rates) were provided to potential developers;
3. Discounts at the pump made bio-ethanol attractive to consumers.

However, fuel alcohol did not succeed in out-competing gasoline costs and its production is vulnerable to competition from other markets. Escalating world sugar markets in the early 1990s demonstrated the vulnerability of an energy source based on agricultural commodities with alternative markets. Sugar processors switched their supply from alcohol production to the more lucrative sugar markets and the production of alcohol-fuelled cars collapsed. This decline was exacerbated by a review of the economic support of fuel production (Rosa and Ribeiro, 1998). By 2002, production was increasing again as government intervention in price setting has ceased, and market rates and competition enable a more robust market to exist.

Brazil is now also looking to develop a direct energy-crop market through exploitation of *Pennisetum purpureum*. Several varieties of this forage grass adapted to Brazil and capable of yielding ~60 odt ha^{-1} yr^{-1} exist (Mazzarella *et al.*, 1999).

Sweden

The Swedish biomass programme relies extensively upon forestry thinnings (with 23 Mha of forestry available) but willow plantations on Sweden's 3 Mha of arable land make a small but significant contribution. Between 16,000 and 18,000 ha of willow provide feedstock for district CHP schemes (DTI, 1999). Establishment is currently at the rate of 1500–2000 ha annually. In winter 2001, 1700 ha were harvested, yielding 30,000 odt (0.5 PJ). Currently most of this biomass is utilised in district heating systems involving small-scale generation of heat from modified oil and coal boilers. Adoption of energy cropping and renewables in Sweden has been stimulated by fossil-fuel taxes and the imposition of set-aside. In addition, the Swedes are considering alcohol production from grain and briquetting and combustion of the energy grass *Phalaris arundinacea*. Sweden's first bio-ethanol demonstration plant is planned to produce 50,000 m^3 of ethanol annually for blending with gasoline, with a similar amount of animal protein feed as a by-product.

9.3 The nature of biomass

9.3.1 Chemical composition, energy and moisture content

The energy content of bone-dry plant biomass material ranges from 17 to 19 MJ kg^{-1}. Differences in this rather narrow range are generally caused by different ratios of the major chemical constituents of plant material: cellulose, hemi-cellulose, lignin, starch and oils. Table 9.7 shows the energy content (*i.e.* the energy released on combustion) of a selection of compounds; this is inversely related to the proportion of oxygen atoms in the molecular formula. Thus methane and other hydrocarbons contain no or little oxygen and have high energetic values, while monomer carbohydrates have low energy values and high oxygen contents. Importantly, ligno-cellulosic compounds contain progressively greater amounts of energy as their structure becomes more complex. Consequently a glucose polymer (cellulose) contains more energy than free glucose (Alexander, 1985).

Table 9.7 Energy content of selected organic compounds

Compound	Basic formula	MJ kg^{-1}
Acetic acid	CH_3COOH	9.5
Glucose	$C_6H_{12}O_6$	13.3
Cellulose	$C_6H_{10}O_5$	15.2
Lignin	$C_6H_8O_4$	28.5
Turpentine	$C_6H_6O_3$	38.0
Methane	CH_4	47.5

Adapted from Alexander (1985).

Table 9.8 shows the chemical composition of a range of potential energy crops. Some plant species store part of their photosynthate as oils rather than carbohydrate and consequently have very high energetic values per unit biomass. Examples of these that could be used to advantage as energy crops are *Euphorbia* spp., *Asclepias* spp., *Hevea* spp., *Copaifera langsdorfi*, *Parthenium argentatum*, *Brassica napus*, *Linum* spp., *Crambe abysinicca*, *Creosote* spp. and milkweed. However, the comparison with crude oil, coal and gas (Table 9.9) shows the relatively narrow variation in energy content of different plants.

Table 9.8 The chemical composition (% by weight, as received) of BECs

	Cellulose	Hemi-cellulose	Lignin	Ash	Other
Saccharum bagasse[1]	48	22	20	6	4
Saccharum bagasse[3]	36	28	20	2	13
Wheat[1]	51–54	26–30	16–18	7–8	
Bamboo[1]	34–36	15–16	26–28	3	
Pine[1]	42	24	27	0.2	6.8
Poplar[1]	48	23	19	0.4	9.6
Poplar[4]	70	23	4	4	
Eucalyptus[1]	49	15	28	0.4	7.6
Miscanthus[2]	44	24	17	1.5	13.5
Phalaris[2]	28	22	14	8	28
Lucerne[2]	25	23	12	6	34
Betula[2]	41	33	21	<1	4

[1]McDougall *et al.* (1993); [2]Berggren (1993); [3]Alexander (1985); [4]Kheshgi *et al.* (2000).

The energy content of a direct energy crop and its ease of thermochemical conversion are inversely related to the moisture content of the material, as Table 9.9 also shows. Quite small variations in moisture content can have significant effects on energy yield. Thus moisture content at harvest and yield form the two most important criteria for a direct energy crop. Typical moisture contents for actively growing plants lie in the region 70–95%. The harvested biomass may have a moisture content as variable as 90–15%. Considerable effort is made to reduce moisture content during post-harvest processing.

9.3.2 *Conversion routes, current species used and expected yields*

There are five principal conversion routes for energy crops in large-scale energy generation: combustion, gasification, pyrolysis, fermentation, oil extraction and anaerobic digestion. These are discussed in detail in the following chapter and are merely summarised here. The first three are used more for direct energy crops, while fermentation and oil extraction are the most likely conversion routes for indirect energy crops.

Table 9.9 Energy values of selected crops and the influence of moisture content

Energy crop	**Moisture content (%)**	**Dry yields (t ha^{-1} yr^{-1})**	**Predicted yield for 2015**	**Energy content (GJ/t)**
Crude oil[a]	0			45.37
Washed coal[a]	0			28.84
Power station coal[a]	0			24.00
Gas				
Biomass[a]	0			17.00
Biomass[a]	15			14.45
Biomass[a]	20			13.60
Biomass[a]	25			12.75
Biomass[a]	30			11.90
Logging residues—after final felling[b]	25	0.26	0.38	20.8
Logging residues—after first thinning[b]	50	0.49	0.60	20.8
Miscanthus x giganteus[c]	48[c]	20.3[c]		
Wheat (grain)[b]	17	5.1	7.5	18.5
Rape (seed)[b]	18	2.5	3.1	27.8
Potatoes[b]	78	7.7	10.0	17.0
Sugar beet[b]	76	11.0	14.0	17.4
Clover-grass ley[b]	77	7.5	11.0	18.4
Lucerne[b]	77	8.0	14.0	19.0
Reed canary grass[b]	15	6.5	12.0	18.4
Salix[b]	50	9.3	17.0	19.5
Straw[b]	15	2.0	2.0	18.1

[a]Rutherford and Bell (1992); [b]Börjesson (1996); [c]DTI Sustainable Energy Programmes, *Evaluating Grasses as a Long-Term Energy Resource.*

Combustion

Simple combustion systems are by far the most common methods of obtaining energy from biomass, for the generation of heat or electricity via steam production for turbines. Conversion to electricity with this technology has particularly low conversion efficiencies (~20–35%). On the other hand, the technology is reliable and proven and therefore appropriate for immediate uptake and exploitation. In Sweden, 12 TWh of energy was generated for home heating from combustion of biomass in 1994 alone. About 8000 MW_e of electricity is produced in the USA through combustion of forestry thinnings in boilers. At the other end of the spectrum, simple combustion systems form the basis for log stoves and other domestic heating systems throughout the world.

Gasification

This involves the conversion of the biomass into a combustible gas through initial combustion under a reduced atmosphere (air/O_2) and temperatures of 800–1300 C. The main combustible components of the resultant so-called *producer gas* are CO, H_2 and CH_4. Producer gas can be used as a substitute fuel in oil-fired furnaces or boilers or in (diesel or gas turbine) engines (Nordin and Kjellstrom, 1996). Gasification technologies for biomass are based on existing systems for coal and, whilst relatively new and unproven, offer significant gains in efficiency for electricity generation at the 1–30 MW_e scale. A technical option that is receiving widespread interest in the UK is the co-combustion and co-gasification of coal/wood mixtures (with 20% w/w of wood) in existing coal-fired stations. However, the major limitations to biomass co-firing are that: a) biomass is difficult to pulverise in the manner used for coal in advanced systems, and b) biomass tends to produced fouling gases and slogging, which limits the enthusiasm of industry to take up this technology.

Pyrolysis

Pyrolysis is the thermal degradation of biomass in the absence of oxygen. The resulting liquid biofuel (bio-crude) has a high bulk density and can be used for firing boilers with relatively high efficiency (35–50%). Pyrolysis systems are still at the developmental stage but offer significant promise for the future. While the conversion technologies for energy crops and biomass are dealt with in detail in the following chapter, it is relevant to our task of defining the available range of energy-cropping systems to consider the basic mechanisms of conversion for the two main liquid

biofuels: bio-ethanol and biodiesel. Korbitz and Walker (1996) and Mortimer (2002) have discussed the current status of bio-ethanol and biodiesel, respectively.

Bio-ethanol

Ethanol can be produced from sugars, starch or cellulose. Ethanol production from BECs involves four main steps (Rosillo Calle, 1990):

1. Pre-treatment of the raw material into a suitable substrate;
2. Enzymatic hydrolysis or acid degradation of starch and cellulose into sugar;
3. Yeast fermentation of the sugars to ethanol and CO_2;
4. Purification of the ethanol from its by-products.

The potential of ethanol as a BEC-derived fuel for transport and centralised energy can be seen in the Brazilian bio-ethanol programme (Goldemberg *et al.*, 1992), where 2.3 million hectares of sugar cane cultivated for the bio-ethanol programme produce 16.3 billion litres of ethanol annually. In the USA, approximately 3 billion litres of ethanol are produced annually from corn (*Zea mays*) (Swisher and Wilson, 1993).

Any plant material (forestry and agricultural residues, municipal solid waste, annual and perennial crops) can be fermented, but the most successful bio-ethanol crops are those with a high yield of hydrolysable starch and cellulose. Sugarcane yields 70 litres of ethanol per tonne of cane processed. The ethanol yield from 1 tonne of wood is 330 litres, although the energy input to convert the wood to ethanol is higher. Table 9.10 shows the yields of ethanol from some of the principal starch/sugar crops that might be grown commercially as fuel crops.

Biodiesel

Extractable vegetable oils can be used in conventional diesel engines after refining. The use of vegetable oils in diesel engines dates to the beginning of the 20th. century, when groundnut oil was used. Korbitz and Walker (1996) describe the production and exploitation of biodiesel in detail. Crops suited to biodiesel extraction include rape, sunflower, soybean, palm, tallow and groundnut. Vegetable oils have similar energetic values to liquid hydrocarbons and share similar physical properties. The oil is generally extracted from the seed by crushing and then refined to the correct specification. This is followed by *trans*-esterification (through the addition of methanol) forming fatty acid mono-esters and glycerine. The ester is decanted as the fuel element. Suitable vegetable oil for biodiesel must be flowable at low temperatures and have a low propensity to oxidation or polymerisation (Korbitz and Walker, 1996).

Table 9.10 Yields of current and potential indirect energy crops

Crop	Fresh yield (t ha^{-1} yr^{-1})	Ethanol yield (litres t^{-1})	Ethanol yield (litres ha^{-1} yr^{-1})
Saccharum spp.	50–90	70–90	3,500–8,000
Echinocloa frumentacea	45–80	60–80	1,750–5,300
Sugar beet	15–50	90	1,350–5,500
Sugar beet (fodder)	100–200	90	4,400–9,350
Triticum aestivum	1.5–2.1	340	510–714
Hordeum vulgare	1.2–2.5	250	300–625
Rice	2.5–5.0	430	1,075–2,150
Zea mays	1.7–5.4	360	600–1,944
Grain sorghum	1.0–3.7	350	350–1,295
Potato	10–25	110	1,110–2,750
Cassava	10–65	170	1,700–11,050
Sweet potatoes	8–50	167	1,336–8,350
Grapes	10–25	130	1,300–3,250
Nipa palm			2,300–8,000
Sago palm			1,350
Sugar cane[a]	56.2		

Adapted from Rosillo Calle (1990). [a]Average between 1984 to 1995 (Hartemink and Kunita, 1996).

The attraction of both bio-ethanol and biodiesel is that they are high energy density liquid products that can be used in internal combustion engines, *i.e.* as transport fuels. The lack of widespread adoption comes from: (a) poor comparative economics of production; (b) poor energy ratios;[2] and (c) concerns about wider environmental impact of cropping (in the case of biodiesel). Critical to indirect energy crops is the need to increase the harvest index of the valuable commodity (the seed) and to maximise the oil content of the seeds. This demands the attention of both the plant breeder and the agronomist.

[2] The energy ratio of a crop is the ratio of the energy yielded by the final commodity to the energy used in growing the crop. Bullard and Metcalfe (2000) give a detailed description of the derivation of energy ratios and the importance of system boundary in its application.

9.3.3 Crop species and yields

A tremendous diversity of crop species is grown for direct energy generation throughout the world. A non-exhaustive list of both direct and indirect energy crops (Tables 9.10 and 9.11) serves to show this diversity. The current annualised yields indicate the variation in productivity currently seen in different geographic regions from the crops currently best suited to those individual conditions.

Cannell (1989) has drawn our attention to the inherent risks of comparing yield data across localities, sites, years and researchers. Major errors may be introduced by poor sampling techniques, extrapolation from small sample areas to large areas where sample variances are high, multiplication of average tree weights by the number of trees per hectare, edge effects in small plots and the inclusion of total above-ground estimates of bio-productivity regardless of the amount of material that will probably be lost through commercial harvesting operations. The figures in Table 9.11 do, however, serve to provide a broad context for current achievable yields. Potential yields based on physiological principles are generally significantly higher than the figures quoted in Table 9.11 and are discussed in later sections.

The waste plant material from ethanol production can itself form a valuable primary energy source. For example, for every tonne of cane processed in Brazil, 283 kg (wet basis) of bagasse residue remains (Goldemberg *et al.*, 1992). The overall viability of the ethanol production industry can be improved by increasing the efficiency with which this bagasse is used to produce heat and electricity. To the bagasse can be added 150 kg (dry basis) of the tops and leaves of sugar cane plants ('barbojo'). Equally, lignin by-products from wood fermentation may be used to produce electricity and steam to fuel the ethanol production process.

9.3.4 Questions of scale

In the UK, a coal-burning power station of medium size is likely to generate >1000 MW_e. The largest feasible scale for a biomass-burning plant is likely to be about 50 MW_e, an order of magnitude less. This is entirely due to supply economics. A 50_e MW power station will require approximately 300,000 t of biomass annually. Assuming that this comes from crops with an annual yield of 20 t ha^{-1} (currently an optimistic prediction for temperate Europe), 15,000 ha or 150 km^2 of land is needed. The actual cropping area, at least in lowland Britain, is most unlikely to exceed 25% of available land. Therefore 600 km^2 is the minimum supply area for the biomass to the power station.

Table 9.11 A selection of crop species and yields for direct energy production in different geographical areas

Crop	Clone	Yield (t ha^{-1})	Area	Reference
Salix	–	8–11	Canada	Robertson (1984)
Salix viminalis	–	9–10	Scotland	Cannell *et al.* (1987)
Salix	–	15–20	Sweden	Nilsson (1985)
Miscanthus	–	12–24	UK	Bullard *et al.* (1995)
Miscanthus x giganteus	–	7.7	Denmark	Jørgensen (1997)
Miscanthus sinensis	–	8.9	Denmark	
Eucalyptus globules	–	≥30	Spain	San Miguel (1986)
Populus spp.	–	14	Italy	FAO (1958)
Saccharum (energy cane)	–	105	Puerto Rico	Alexander (1985)
Pennisetum purpurescens	– Columbia River Fritzi Pauley Trichobel	67 0.34 0.21 0.54	Puerto Rico Belgium Belgium Belgium	Alexander (1985) Deraedt and Ceulemans (1998) Deraedt and Ceulemans (1998) Deraedt and Ceulemans (1998)
Poplar	–	10–15	Germany	Dimitri (1984)

Table 9.11 cont'd.

Crop	Clone	Yield (t ha^{-1})	Area	Reference
P. trichocarpa Poplar clones	Beaupre Boelare	0.224 0.0890	Belgium	Deraedt and Ceulemans (1998)
P. trichocarpa x P. Deltoides Poplar clones	Hazendans Hoogvorst	0.241 0.375	Belgium	Deraedt and Ceulemans (1998)
	Unal Raspalje IBW1 D*T	0.247 0.106 0.256		Deraedt and Ceulemans (1998)
P. deltoids x P. trichocarpa	IBW2 D*T IBW3 D*T	0.048 0.096	Belgium	
P. trichocarpa x P. balsamifera	Balsam Spire	0.282	Belgium	Deraedt and Ceulemans (1998)
P. deltoids x P. nigra	Gaver Gibecq Primo	0.021 0.021 0.211	Belgium	Deraedt and Ceulemans (1998)
P. nigra	Wolterson	0.147	Belgium	Deraedt and Ceulemans (1998)

Transportation of feedstocks is a significant cost in energy cropping; the low energy density of biomass combined with transportation costs mean that a larger supply area renders energy generation uneconomic. This is in spite of efficiency gains in electricity generation that could be achieved by moving from 30 to 100 MW_e in generation scale. As generator efficiencies improve, with the movement towards gasification and pyrolysis for biomass conversion (Chapter 12), plant size can probably increase; 100 MW_e biomass boilers for use with forestry residues are already in use in the USA.

9.4 Physiological and agronomic basis of energy capture and the selection of appropriate energy crop species

The selection of an appropriate energy crop for a given region, or the improvement of existing crops, can be undertaken with a basic understanding of the physiological principles that govern crop growth. These are discussed briefly below. Cannell (1989) and Hall and House (1992) consider these topics in detail.

9.4.1 Photosynthesis—an inefficient process

Put simply, any plant species has the potential to be an energy crop. The principle underlying energy generation from these crops is that the plant, during photosynthesis, harvests the highest possible proportion of the radiant energy incident on a unit area of ground and stores this energy as fixed carbon in the appropriate part of the plant; either above-ground harvestable dry matter (ligno-cellulose) in the case of direct energy crops, or specific oils or lipids for indirect energy crops. The process of combustion releases this stored energy in a useful form (heat or electricity).

The objective of energy cropping is thus to maximise the amount of solar radiation intercepted, and to convert intercepted solar energy into ligno-cellulosic biomass as efficiently as possible. All other considerations are secondary to this, or are inextricably linked to this central requirement.

Although current estimates suggest that annual global phyto-productivity is an order of magnitude greater than fossil fuel use (IPCC, 1992; Pasztor and Kristoferson, 1990; Hall, 1984), and thus on the face of it more than sufficient to meet energy needs, the efficiency with which solar radiation is converted into fixed carbon is remarkably poor. Supply economics dictate that improved efficiencies of energy capture will be needed if energy crops and biomass are to be economically viable.

Depending on latitude, altitude and geographic location, 1 m^2 of surface land area receives between 2500 and 8000 MJ of solar radiation annually (Davidson, 1985). If all of this radiant energy were converted into fixed carbon, global productivities would range between 1100 and 4400 t d.m. ha^{-1} yr^{-1}. Regardless of the annual energy receipt, global phyto-productivities are much lower than this—in other words, our ability to harvest solar energy as dry matter plant material is extremely poor. There are several reasons for this. Of the 100% of light energy falling on a unit area of land, only 50% is in the PAR wavelength range (300–700 nm) used in the photosynthetic process. Of the remainder, up to 90% is reflected, dissipated or lost through photosynthetic inefficiencies. These inefficiencies occur because either other climatic variables are not conducive to efficient photosynthesis, or there is no crop leaf canopy in place (typically the case for deciduous temperate species), or the crop's photosynthetic system is unable to deal with all the energy, or because of respiratory and photo-respiratory losses. Only the 3–6% of energy remaining is theoretically available and only 0.3–1.0% is typically harvested in cropping systems. The remainder is lost through the activity of weeds, pests and diseases, through moisture stress, temperature stress or because the biomass is non-harvestable. The proportion harvested from ecosystems is generally even lower.

Global productivity patterns

Global productivity and land suitability for energy cropping do not necessarily coincide. Whilst the tropics and sub-tropics offer the environments with the highest potential bio-productivity (per unit area of land) and certainly the largest untapped biomass resource, much of this area should not be considered a primary energy source for environmental reasons. Where land is cleared and is potentially available for energy cropping, significant competition for the resource will come from food and high-value cash crops.

9.4.2 *Striving for the ideal energy crop*

The ability of a plant to utilise the radiant energy incident on it is dependent first on the presence of a green canopy; the photosynthetic capacity of a crop is usually directly proportional to the photosynthetically active radiation intercepted by that canopy (Monteith, 1977). The presence and efficiency with which this canopy operates is dependent on temperature, nutrient and water supplies and a host of interactions with the biotic and abiotic environment. Losses of solar energy at each

stage identified above may in future be reduced improved understanding of how these processes operate for each potential energy crop. Equally, this knowledge allows us to define the ideal energy crop. Specifically, the ideal energy crop will be one which intercepts all photosynthetically active radiation, whose conversion efficiency of that energy into fixed carbon is not limited by temperature, light intensity, nutrients or water, and which suffers minimal respiratory losses. An ideal energy crop growing in the UK would be capable of producing as much as 55 t d.m. ha^{-1} yr^{-1}. Yields from 'ideal' energy crops growing in tropical latitudes may be three times as high.

The principal activities of the agronomist and scientist in selecting a suitable energy crop and husbandry strategy for a given area are to improve the amount of 'fixed' energy that is actually harvested. With our knowledge of the ideal energy crop, species selection and husbandry can be defined in terms of strategy to reduce energy losses. The following sections define the biological principles that underlie the identification of the ideal energy crop and introduce husbandry principles that will ensure that the gap between theoretical maximal yields and realised yields is as small as possible.

9.4.3 Photosynthetic pathways

The plant kingdom has evolved three major forms of photosynthesis able to exploit different sets of climatic conditions optimally. Consequently, crops of any given pathway will be more likely to achieve a high percentage energy efficiency in some, but not all, agri-climatic zones. Matching the correct photosynthetic pathway to the correct climate will go a long way to ensuring high yields. These pathways were considered in detail in Chapter 4, but we shall summarise them again here.

The *C_3 pathway* is the most basic photosynthetic mechanism. On uptake, CO_2 is fixed by the 5-carbon atom ribulose bisphosphate (RuBP). The first metabolic product from this (mediated by the ubiquitous enzyme ribulose bisphosphate carboxylase/oxidase—rubisco) is the three-carbon molecule phosphoglyceric acid (PGA), which then enters the Calvin Cycle. Most plant species (95% of global biomass) are C_3's, and they predominate in cooler climatic zones. They include all trees and almost all temperate crops. Three key elements of this photosynthetic process influence cropping suitability:

1. Rubisco is equally capable of oxidation and carboxylation of ribulose bisphosphate; thus photorespiration by reaction with atmospheric O_2 competes directly with CO_2 fixation. This photorespiration consumes as much as half the

carbon fixed during photosynthesis. Consequently rates of net photosynthesis in C_3 plants tend to exhibit a positive response to increases in ambient CO_2 levels (as the CO_2/O_2 ratio increases).

2. At high temperatures and light intensities, the photosynthetic efficiency of C_3's is limited by the plant's ability to re-synthesise rubisco. At low temperatures C_3 plants are the most photosynthetically efficient.
3. The maximum theoretical solar conversion efficiency attainable by a C_3 plant is 3.3% (Hall *et al.*, 1996)

C_4 plants have an additional CO_2-concentrating mechanism within the bundle sheaths (Kranz anatomy) of the leaves by means of which they can increase the local CO_2 concentration to several times above ambient levels. CO_2 is initially fixed in a 4-carbon atom molecule (*e.g.* oxaloacetate, OAA) in the mesophyll cells by phosphoenol–pyruvate (PEP), and this molecule is then transported to the bundle sheaths, where it is reduced. Decarboxylation of the product produces PGA, some of which returns to the mesophyll cells while the remainder enters the Calvin Cycle. Consequently C_4 plants have a CO_2-concentrating mechanism and are not generally limited by ambient CO_2 concentrations. Collins and Jones (1985) have provided a comprehensive description of C_4 plants found in temperate Europe. Key aspects of C_4 photosynthesis are:

1. As decarboxylation occurs in the bundle sheaths, photorespiration does not cause photosynthetic inefficiency.
2. CO_2-concentrating mechanisms allow C_4 photosynthesis to proceed without becoming saturated up to much higher radiation levels (>1,500 μmol PAR m^{-2} s^{-1}).
3. C_4 photosynthesis and leaf expansion generally cease at relatively high temperatures (6 C and 10 C, respectively). Thus C_4 plants tend to be restricted to warmer climatic zones (tropics, sub-tropics and semi-arid zones); their lack of low-temperature tolerance does not allow competitive growth rates in temperate zones.
4. Where radiation and temperature levels are not limiting, the radiation use efficiency (RUE) of C_4 plants can be much higher that of C_3 plants.
5. C_4 plants generally have a higher water use efficiency (WUE) because photosynthesis occurs at lower internal CO_2 concentrations.
6. C_4 plants generally have a higher nutrient use efficiency (NUE) than C_3's (Sage *et al.*, 1987).
7. The maximum theoretical solar conversion efficiency attainable by a C_4 plant is 6.7% (Hall *et al.*, 1996)

The final photosynthetic type, Crassulacean acid metabolism (CAM), is a variant of the C_4 mechanism, where CO_2 is concentrated but also stored. CO_2 uptake occurs at night when leaf temperatures and the leaf-to-air water vapour concentration difference are lowest. Therefore less water is lost through transpirational demand and WUE is higher than for either C_3 or C_4 plants. CO_2 taken up at night is used the following day in normal photosynthetic reactions. CAM is typical of arid zone and desert species.

While C_4 and CAM species may offer more potential in tropical and arid zones respectively, they may not be suited to the low temperatures and relatively low annual radiation receipts in temperate regions. However, considerable research has been undertaken to select C_4 species and varieties that have low-temperature tolerance in order to exploit their higher theoretical productivities in temperate zones.

The opportunities here can be exemplified by considering *Miscanthus* spp., a genera of sub-tropical grasses endemic to Asia. Unlike other C_4 species introduced to temperate zones, *Miscanthus* does not demonstrate springtime photoinhibition, and the threshold temperature at which low-temperature impairment of leaf development occurs is lower (Beale *et al.*, 1996). Combined with high radiation use efficiencies (Bullard *et al.*, 1995; Beale and Long, 1995), high nitrogen efficiency and efficient nutrient cycling (Christian *et al.*, 1997; Christian, 1997), this suggests that *Miscanthus* spp. has more potential for successful growth and biomass yield under temperate conditions than any other C_4 species, and therefore more potential than any other crop to be a successful energy species. Indeed, peak yields of 24 odt yr^{-1} have been recorded in unirrigated field situations and Fig. 9.5 shows that the species *Miscanthus x giganteus* has significant adaptation to most of mainland England and Wales (Nixon, 2001). However, improved low-temperature tolerance does not prevent canopy loss following sub-zero temperature episodes that are often experienced during late spring. As a consequence of canopy loss, as much as two months of crop growth is lost. Furthermore, radiation use efficiency in this species is particularly sensitive to moisture deficit (Bullard *et al.*, 1997), even though the gross water use efficiency of the crop is very high (S. Long, personal communication). Yields in excess of 35 odt ha^{-1} can be achieved where moisture supply is non-limiting (S. Constantino, personal communication).

9.4.4 Radiation interception

We noted in the last section that radiation interception by a green canopy is one of the keys to high-productivity energy cropping. Different groups of energy crops show significant variation in interception profile. Measurements of radiation interception

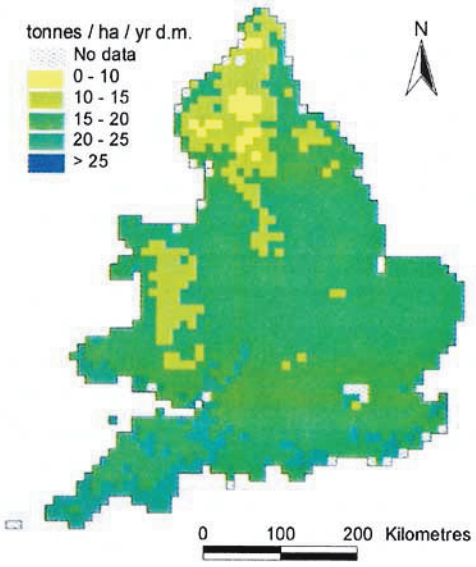


Figure 9.5 Yield map of *Miscanthus* (10 × 10 km grid) based on historic climatic data sets (1961–1990) and assuming: a) unlimited soil water availability; b) limited soil water availability (Price *et al.*, 2002).

form the basis of crude assessments of radiation interception as they merely express the difference between radiation incident on the surface of the crop canopy and the proportion of radiation that reaches the base of the canopy. Thus canopy reflectance, light interception by senescent (*i.e.* non-photosynthetic) material and differences in the photosynthetic potential of different plant tissues are not taken into account. Canopy reflectance may be as low as 4% in cane grasses and as high as 15% in broad-leaved tree canopies (Cannell, 1989).

There is tremendous variation in the speed with which crops develop a full canopy: at equivalent spacings, conifers take longer than broad-leaved species, and these in turn take longer than cane grasses grown at similar latitudes. There are large inherent differences within species and clones of energy crop in the speed with which they can develop a canopy, and this often forms an important area of potential genetic exploitation in the development of new lines. This was the basis on which the 'Green Revolution' increased rice yields three-fold during the 1960s and 70s. Broad-leaved crops develop a closed canopy more quickly than conifers because, although conifers may produce leaf primordia at a rate 10–20 times faster than broad-leaves, an individual needle has only 0.2–2% of the projected green area (Cannell, 1989).

9.4.5 *Canopy structure and duration*

The efficiency of radiation use depends on canopy architecture as well as duration. The growth area index (GAI) is an expression of the total area of green leaf material present over a unit area of ground. The proportion of light intercepted is an asymptotic function of GAI, described by Beer's Law. The proportion of radiation *usefully* intercepted within a crop canopy also depends on the internal orientation of the constituent photosynthetic parts. This is commonly measured by the attenuation coefficient k, which is the proportion of radiation intercepted per unit green area. This decreases towards zero as leaves become more erect (as in grasses) but increases towards 1 as leaf structure becomes more planar (as with broad-leaved trees). The significance of this is twofold: first, the amount of green canopy necessary for the interception of, say 95%, of incident radiation will vary depending on k; second, according to Beer's law, there will be an optimum GAI above which additional light interception does not occur and indeed mutual shading of leaves begins to reduce biomass accumulation efficiencies. Consequently it should be possible to manipulate crop husbandry through fertiliser application and density choice in order to gain the optimal GAI as quickly as possible but not produce a super-optimal crop canopy. The speed with which a canopy develops within a new crop stand may be offset by the duration of the crop canopy. For example, deciduous trees in the UK may hold their leaves for 8 months, while spruce canopies hold their needles for 5–8 years. In Sweden, this latter figure may be 18 or more years. Thus short-term differences in canopy development may be offset by long-term accumulated radiation interception.

Nonetheless, selection of varieties within a species that demonstrates earlier bud break or leaf emergence can significantly increase yield, particularly in regions with cool springs. Cannell *et al.* (1987) estimated that for a species with a radiation use efficiency of 1 g d.m. MJ^{-1} radiation intercepted and mean daily radiation receipts during May/June of 12.5 MJ at latitude 56°N, a one-week delay in canopy emergence will cause a yield suppression of 0.9 t ha^{-1} d.m. Delaying the onset of leaf fall in the same crop may be less important because radiation receipts will have fallen to 2.5 MJ day^{-1} by November. However, the maintenance of a fully functioning canopy remains important in Mediterranean and tropical zones.

It is clear from this that an early canopy should be in place at the start of the season to capture as much radiant energy as possible. Equally, crop spacing should be such that all light is captured at the earliest possible time. The delivery of radiation in temperate and tropical zones is significantly different. Temperate zones with a distinct seasonality receive 80% of their total energy receipts in a 6-month period. Tropical zones receive the radiant energy in a more uniform pattern. As a consequence,

perennial energy crops in temperate systems tend to be deciduous: the canopy is lost by leaf fall during the winter when, were it to remain in place, respiratory losses of energy would far exceed any net gain from intermittent photosynthesis. Leaf fall also acts as a mechanism for reducing long-term pest and disease damage. The key for these systems is to ensure that a new crop canopy develops early in the following spring. For annuals, autumn-sown crops are likely to have at least some functioning photosynthetic material present in the following spring. For example, the photosystems of *Triticum aestivum* photosynthesis are active at 5 C. Those temperate energy species, which are evergreen, have thicker leaf cuticles and modified leaf shapes to minimise summertime water loss, and the metabolic costs of these compensate for the reduced energy expended on replacement of leaves on a roughly biennial cycle (Woods and Hall, 1994).

Strategies for spring-sown annuals (for example *Brassica napus*) are that the crop should be sown as early as is feasible and that weed control should be optimised to ensure unfettered canopy development.

In tropical climates the challenge comes not so much from seasonality but from leaf shading, senescence and pests and diseases. All leaves have a finite photosynthetically productive lifetime, and those that are no longer productive may act to reduce the amount of intercepted radiation that is converted. Consequently, the harvest interval becomes critical in maintaining optimal efficiency.

9.4.6 Pests and pathogens

Pest and disease control are paramount in energy crops because pests and diseases reduce the leaf area available for photosynthesis, and they impose a metabolic cost on the plant either through avoidance or disease symptoms. Several control strategies are available which utilise petroleum-based chemicals to varying degrees. Growing non-indigenous crops offers a degree of protection, at least until pests and diseases co-evolve with the new crop, followed by the opportunity to introduce natural predators when the need arises. With the case of yellow rust *(Melampsora* spp.) on SRC willow species, a devastating pathogen that can entirely defoliate crops in very short time, research has identified seven distinct 'pathotypes', each of which has a different varietal activity profile (Pei *et al.*, 1997). By judiciously selecting a range of varieties, the farmer can ensure that not only will any given pathotype outbreak defoliate only the susceptible proportion of his crop, but also that the mixture of varieties actually presents a physical barrier to the rapid spread of the pathogen (McCracken and Dawson, 1997).

Just as it is true that a plant population has an optimum density per unit area of land, for the maximisation of resource exploitation and minimisation of competition between individuals, so it is the case that a crop has an optimal leaf area index. Where this leaf area index is known, targeting fertilisers, planting density and husbandry can optimise yield whilst at the same time minimising the cost and environmental impact caused by excessive application of agrochemicals.

9.4.7 *Radiation use efficiency*

Radiation use efficiency (RUE) is the field measurement of the amount of intercepted solar radiation that is needed to produce a unit increment in dry biological material (units: g d.m. MJ^{-1} PAR m^{-2}; that is, tonnes of dry mass per megajoule of incident photosynthetically active radiation per square metre). The RUE of a crop species is a key determinant of its suitability as an energy crop. RUE provides a measurement of the inherent productive potential of the crop species *under non-limiting conditions*. C_3 species are capable of a maximum RUE of approximately 1.4 g d.m. MJ^{-1}. C_4 crops can achieve significantly higher figures when growing under suitable conditions of temperature, radiation levels and moisture supply but, as Fig. 9.6 shows, they are at a disadvantage at high temperatures and light intensities.

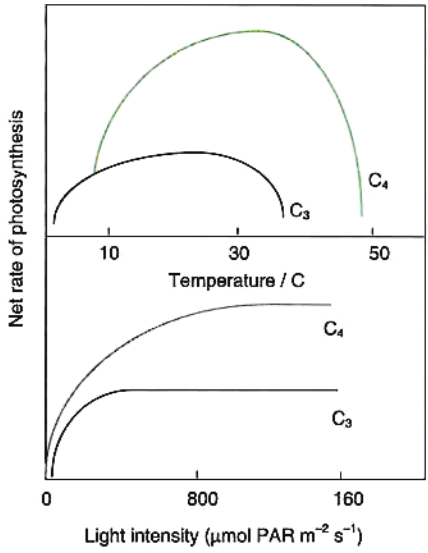


Figure 9.6 Idealised photosynthetic response of C_3 and C_4 plants to temperature and light intensity (adapted from Murphy and Helel, 1996).

Table 9.13 Radiation use efficiencies (RUE) and solar energy conversion efficiencies (ECE) for a range of temperate and tropical plants

Species	Clone	Common name	Country	RUE ($g\ MJ^{-1}\ m^{-2}$)	ECE (%)	Yield ($t\ d.m.\ ha^{-1}\ yr^{-1}$)	Authority
Eucalyptus cameldulensis	–		–		5.8	51	Davidson (1987)
Eucalyptus teritcornis	–		–		4.8	42	Davidson (1987)
Sesbania grandiflora	–		–		6.8*	>50	Davidson (1987)
Saccharum (plant)	–	Sugarcane—18 month harvest interval	–	2.3		117	Muchow *et al.* (1991)
Saccharum (ratoon)	–	Sugarcane—12 month harvest interval	–	2.65		76.5	Muchow *et al.* (1991)
Saccharum	–	Sugarcane	–			58[a]	Ogden *et al.* (1990)
	–	Sorghum	–	3.2			Muchow and Davis (1988)

Table 9.13 cont'd.

Species	Clone	Common name	Country	RUE ($g\ MJ^{-1}\ m^{-2}$)	ECE (%)	Yield ($t\ d.m.\ ha^{-1}\ yr^{-1}$)	Authority
	–	Maize	–	2.5			Muchow and Davis (1988)
Miscanthus x giganteus	–		–	1.4–2.3			Bullard *et al.* (1997)
Miscanthus sacchariflorus	–		–	3.6			Beale and Long (1995)
Salix viminalis	–	Willow	–	1.4			Cannell *et al.* (1987)
Populus trichocarpa	–	Poplar	–	1.0			Cannell *et al.* (1988)
Eucalyptus globules	–	Eucalyptus	–				
Brassica napus	–	RME oilseed rape	–				

Table 9.13 cont'd.

Species	Clone	Common name	Country	RUE (g MJ ^{1} m ^{2})	ECE (%)	Yield (t d.m. ha ^{1} yr ^{1})	Authority
Populus	Beaupre Fritzi Pauley Robusta	Poplar	Scotland	0.56 0.47 0.59		8 t ha ^{1} 7 t ha ^{1} 5 t ha ^{1}	Milne *et al.* (1992)
Pinus taeda L.		Loblolly pine	Summervill, USA	1.33 1.48 g MJ ^{1}		Above ground net primary production 32.9 38.2 Mg ha ^{1}	McCrady and Jokela (1998)
Cannabis sativa L.		Fibre hemp	Italy Netherlands	2.26 before flowering; 0.95 after flowering 2.16 before flowering; 1.09 after flowering		22.5 dry yield above ground	Struik *et al.* (2000)

[a] Global annual average sugar cane yield.

Table 9.13 gives a range of RUEs for energy crops growing in different regions and in non-limiting conditions of water and nutrients. Stress induced by a lack of either of these will significantly reduce RUE. As already noted, crops vary in the amount of radiation intercepted during a season. In the UK, 500–2500 MJ of radiation may be intercepted and the biomass increment (for trees) can vary between 2.5 and 25 t yr^{-1} (Cannell, 1989). Variations in RUE can occur between genotypes, environments and within and between seasons, as Table 9.14 illustrates.

Table 9.14 Radiation use efficiency (RUE) of *Miscanthus x giganteus* growing at two densities in the Cambridgeshire fens (Bullard *et al.*, 1997)

	1993	**1994**	**1995**	**1996**
	RUE (r^2)[1]	RUE (r^2)	RUE (r^2)	RUE (r^2)
High density	1.17 (0.84)	1.12 (0.95)	0.85 (0.98)	1.05 (0.81)
Low density	0.71 (0.75)	0.98 (0.93)	1.16 (0.90)	0.78 (0.54)

[1] r^2 is the regression coefficient.

Calculation of the leaf specific weight (LSW), that is, the weight per unit area of leaf, can be a useful surrogate for measurements of maximum photosynthetic capacity (F_{max}) in leaves. It has been demonstrated in a number of agricultural crops that F_{max} differs considerably between varieties and clones, and that this can be directly correlated against LSW (Cannell, 1989). Thus selection of clones with a high SLW may imply higher RUE at a given GAI, presumably because of increased photosynthetic capacity of individual leaves. However, Charles-Edwards (1982) has noted that if increased resource allocation leads to an increase in SLW while simultaneously reducing the rate of leaf canopy expansion, then there may not be a net increase in RUE. It follows that, on a unit area basis, tropical and sub-tropical zones will be the most productive areas for the production of energy crops. Indeed, this is the approach that Shell Oil took during the 1990s when they invested heavily in BECs by locating their eucalyptus biomass programme in Brazil rather than Europe.

9.4.8 Plant–water relations

There are both direct and indirect implications of water on energy crops. In a direct manner water is crucial to energy crop production, because it drives all physiological processes, as with any other crop. While the physiological requirements for water are well understood, there are also environmental effects on water supply which can be altered by appropriate crop selection. As global climate patterns alter, there may be even more emphasis on water-efficient mechanisms of agricultural production. However, in many areas there will be a direct conflict between water required for domestic and food crop growth and that required for energy crop production.

Selection of suitable energy crops and energy-cropping strategies will therefore take into account both the most efficient internal use of water in any given crop and also the wider implications of the whole cycle of cropping on the surrounding ecosystem and climatic environment.

The need for an economically viable yet environmentally sustainable cropping system in industrialised countries points to the necessity of crops with a high water use efficiency (WUE) wherever possible. However, high WUE must be combined with high potential productivity; drought-tolerant plants with CAM photosynthetic pathways may be extremely efficient water users but they will not produce sufficient dry matter to be viable energy species. Under many circumstances, the C_4 photosynthetic pathway confers a higher WUE on plants than does the C_3 pathway. Generally C_4 plants will require only 200–400 litres H_2O kg^{-1} d.m. whereas C_3 plants require 500–1000 litres H_2O kg^{-1} d.m. Clearly, when taking into account other climatic factors, there may be scope to select crops with relatively high WUEs from either group. Even so, uncontrolled dense plantings of either crop type may have significant impacts on catchment hydrology owing to high absolute water demands (Stephens *et al.*, 2001). Plantation design can have a profound impact on the quantity of water used by the crop. Long, thin plantations maximise convective evaporative losses and are thus extremely inefficient. An efficient energy crop will ensure complete ground cover as quickly as possible to reduce evaporative water loss from soils. The perennial nature of many energy crops aids long-term soil structure and the build-up of organic matter, and may be self-mulching, thereby enhancing moisture-retaining ability. Harvesting systems, particularly of non-renewable biomass sources, must take into account the effects of large-scale cropping from inclined areas.

9.4.9 *Moisture content at harvest*

Dry harvested crops are preferred to wet ones because they reduce transport costs and, in the case of direct energy crops, contain more energy per unit of supplied biomass. The agronomist can attempt to reduce moisture content at harvest by modifying harvest time, applying crop desiccants or field-drying baled or chipped materials. Modifying harvest time can also affect the dry-matter yield of the crop; for example, delaying the Northern Hemisphere harvest from August to December in *Phalaris arundinacea* decreases moisture content from 60% to 30%, but with a concomitant reduction in yield of 20% (Nixon, 1997). Nellist (1997) considers field and covered drying systems for energy crops in detail.

9.4.10 *Crop density*

Competition for light, nutrients and water occurs within assemblages of the same species as well as between crop and weed. A population is an aggregation of individuals, and the optimum number of individuals per unit area of land must be achieved if radiation receipts are to be used efficiently. The optimum population for an energy crop will be the minimum number of plants which allow full exploitation of the physical environment by *all* individuals with no plant death, resource depletion or metabolic losses due to competition.

The importance of optimal planting density can be seen from the data of Kopp *et al.* (1997) for willow, shown in Table 9.15. The initial planting density had a profound impact on annual yield, cumulative yield, yield profile and plant survival. Here, planting at high density (0.3 m × 0.3 m) gave rise to an initial yield advantage. However, this yield benefit was not sustained as the crop aged within an annual harvest cycle. It is unlikely that the additional costs of cuttings would be recouped by higher yield at any density. Indeed, even at the annual harvest cycle, only 65% of the original plants survived after four seasons. With the triennial harvest system, only half survived. In a similar study in the UK, Bullard *et al.* (2001a and b) noted a significant increase in yield at the first harvest cycle of SRC willow harvested after 2 or 3 years as plant density increased from 10,000 to 11,000 plants ha^{-1} (Fig. 9.7). However, by the second cycle of biennial harvests, all density-related yield benefits had been lost, due to the increased impact of intraspecific competition.

Table 9.15 The effect of plant density and harvest cycle on the productivity of willow in New York

Cutting cycle	Density (plants ha^{-1})	1991	1992	1993	1994	Summer 91–92	Summer 92–93	Summer 93–94	Mean
Annual	111,111	19.3	11.2	10.1	11.4	30.5	40.6	52.0	13.0
	37,037	19.1	11.2	11.4	12.6	30.3	41.7	54.3	13.6
	15,151	15.0	10.1	10.1	8.7	25.1	35.2	43.9	11.0
Mean		17.8	10.8	10.5	10.9	28.6	39.2	50.1	
Biennial	111,111		28.0		31.4	28.0		59.4	14.9
	37,037		34.8		35.0	34.8		69.8	17.5
	15,151		30.3		33.4	30.3		63.7	15.9
Mean			31.0		33.3	31.0		64.3	
Triennial	111,111			54.9			54.9		18.3
	37,037			71.3			71.3		23.8
	15,151			67.3			67.3		22.4
Mean				64.5			64.5		

Source: Kopp *et al.* (1997). The figures in the year columns are in t/ha.

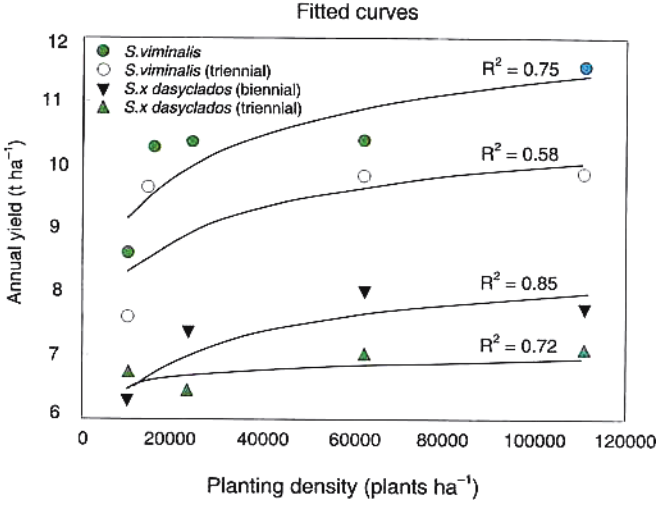


Figure 9.7 The effect of planting density on annualised yield (t ha^{-1} yr^{-1}) with a biennial or triennial harvest, for *Salix viminalis* and *S. x dasyclados* (Bullard *et al.*, 2001a).

Refined food production systems will allow high plant densities to be maintained through the judicious application of nutrients and careful control of weeds, pests and diseases. The same approach should be taken with energy crops, wherever financially feasible and environmentally acceptable.

9.4.11 Nutrient supply, nutrient status and soils

An ideal energy crop, whether an annual oil crop or a perennial, will have an efficient nutrient metabolism and, in the case of perennials, will cycle nutrients internally. The photosynthetic capacity of a plant will depend on the amount and distribution of nitrogen in the canopy (Hirose and Werger, 1987; Evans, 1989; del Pozo and Dennett, 1991), because nitrogen directly affects green area development and consequently the amount of light energy captured (Monteith, 1977; Gallagher and Biscoe, 1978). Inadequate nitrogen supply to the crop may limit the maximum rate of photosynthesis under light-saturated levels. This has been demonstrated for *Eucalyptus* spp. (Mooney *et al.*, 1978), *Pinus sylvestris* (data from Linder in Jarvis and Leverenz, 1983), *Betula* (Linder *et al.*, 1981) and willow (McDonald *et al.*, 1981).

Crops which have a high inorganic fertiliser demand will be unsustainable because: a) large areas of the planet are nutrient-deficient, and areas of high nutrient

status are more likely to be used for food crop production; b) inorganic fertilisers are expensive, consequently increasing costs of production; c) it is increasingly accepted that there is a direct environmental cost of agricultural fertilisers entering aquatic and terrestrial non-crop systems; and d) a large quantity of energy is expended during the manufacture of inorganic fertiliser and this will reduce the energy ratio of the crop.

Reductions in the reliance of the energy-cropping system on inorganic fertiliser inputs for soil fertility maintenance can be attained by a number of means. Perennial crop species which internally cycle at least a proportion of the nitrogen needed for annual growth can be grown (*e.g.* willow, *Miscanthus*, *Eucalyptus* spp.). Intercropping with ground-covering legumes will not cause competition for light, but decomposition of the legumes during the season will release the nitrogen fixed by associated *Rhizobia* bacteria (diazotrophs). Equally, selecting crops which form diazotrophic relationships with N-fixing bacteria or vesiculararbuscular mycorrhizae will reduce reliance on inorganic fertilisers. Application of agricultural and municipal sewage can in many situations provide valuable soil conditioning, nutrients and water to the crop while at the same time disposing of large volumes of waste material that are not appropriate for disposal into water courses or food crops.

Many crops, such as willow and *Miscanthus*, will lose all their leaves before harvest, and these leaves will contribute recycled nutrients to the cropping system. In addition, ash material and distillation by-products can be applied to the field, thus returning a proportion of those lost. Finally, atmospheric deposition of small quantities of nutrients (sometimes as much as 50 kg ha^{-1} yr^{-1} in the case of nitrogen) may complement the soil-available nutrient supply.

The photosynthetic system of the crop will also influence fertiliser use. The nitrogen use efficiency of C_4 crops may be as much as 50% higher than that of C_3's, because of the more efficient cycling of rubisco in the former group. Reliance on inorganic fertilisers may reduce the extent to which energy crops mitigate global warming. Additional ammonia-based fertiliser N may give rise to volatilisation of N_2O, a greenhouse gas 320 times more potent than CO_2 (Callender *et al.*, 1995).

9.4.12 Potential sites for energy cropping

Most countries have a system of land-use classification to differentiate high-quality agricultural land from inferior sites. For example, the Department for Food and Rural Affairs in the UK recognises five classes of land. Energy crops, by nature 'high-volume, low-value' commodities, will tend to be grown on the poorest quality lands. In temperate and Mediterranean regions, these will include marginal and derelict

farmland, derelict land, neglected woodland, exploited peatlands, infertile land and areas of inherent low productivity. The one possible exception to this is where policy stimulation measures encourage cropping on agricultural land in order to manipulate food commodity production. The principal areas for energy cropping in tropical and sub-tropical areas are shrublands, grasslands or sites that have been deforested, where productivity potential is low due to erosion, acidity and nutrient deficiency.

Annual crops, by definition, are more likely to be grown in an existing agricultural infrastructure, and the relatively short term within which they must develop requires that the best available land is used for them. Generally, the higher the quality of land, the higher the expected yield. Growing a crop on more marginal land will increase the interval between harvests.

The positioning of an energy crop plantation should be selected carefully and it should not displace land uses of high agricultural and ecological value. This can be achieved by considering; biodiversity, landscape and visibility, soil type, water use, vehicle access, nature conservation, pests and diseases, access and perhaps most importantly, consultation with local people at an early stage.

Weed control during the immediate post-planting period is critical to the successful cropping of many crops. Weed competition affects not only percentage survival and individual plant growth in the initial season, but produces a season-to-season carry-over of effects with perennials. Minimal acceptable conditions can be characterised for each crop species with respect to soil depth, soil type, pH, trafficability, workability and fertiliser level. For coppiced trees, where repeated access is required, soil structure must be resistant to compaction. Selection of appropriate species for any given combination from this list, for example planting calcifuge plants such as alder on nutrient-depleted, low pH soils, will increase yields.

9.4.13 Soil preparation, crop planting, harvest and storage

Annual crops in both tropical and temperate zones require a high-quality seedbed for successful establishment. This means a tilth that contains a high proportion of aggregates with a size similar to that of the seed in order to maximise soil/seed moisture contact. It is essential also for perennial cropping that a suitable seedbed is produced and that adequate account is taken at the outset of long-term needs such as sub-soiling and pH control, in order to reduce the effects of compaction and soil acidity, respectively. There is substantial evidence to show the effects of poor seedbed preparation in a range of energy crops, such as depressed yield, increased period to crop maturity and a reduced ability to tolerate winter disturbance.

Harvest cycles, times and techniques vary tremendously depending on the crop and location in question: the reader is referred to standard agricultural texts and specialist papers, *e.g.* Wall and Deboys (1997) for comprehensive reviews. Mechanical harvesting systems must combine efficient recovery of as much potentially harvestable biomass with appropriate comminution in order to present the feedstock in an appropriate form either for field drying or direct removal to the energy utilisation site. This could mean that material is cut whole, baled, chipped or bundled. Efficiency of recovery dictates that, where possible, the cutting and comminution/baling should occur concurrently using one piece of equipment. Some estimates of harvesting loss and post-harvest losses of biomass are extremely high. For example, Hall (1984) calculates that 25% of tree biomass may be lost at harvest and during transport.

Crop storage is required for two reasons. First, field drying may reduce the moisture content of the crop sufficiently to allow more cost-effective transportation and energy conversion and, second, a twelve-month supply cycle is required to the power plant whereas harvest is a seasonal activity. Power stations would not have the capacity to store large quantities of feedstock. For example, a 10 MW coppice-fired power station would require an annual supply of 135,000 t (wet) biomass (Allen *et al.*, 1997). Field storage is the most likely method for overcoming these problems, principally because it is prohibitively expensive to store under cover. However, the storage process is likely to lead to a decline in the energy value of the biomass, if stored incorrectly, and also in the gross amount of biomass present, because of the presence of microflora which degrade cellulose slowly over time. Thus over a six-month storage period, improvements in dry matter content of the biomass may be offset by a reduction in the actual weight.

In many cases it is more efficient to transport direct to the power station and use waste heat to dry the feedstock rather than to attempt field drying (Nellist, 1997).

9.4.14 Energy balance

The importance of energy balances should not be overlooked; they form one of the principal tools by which policy makers evaluate a given technology. It must, therefore, be a high priority to formulate a universally accepted system for calculating energy ratios. Direct comparison of energy ratios for different crops and different conversion processes is problematic because there is no universal convention for calculating energy ratios. However, the ratios in Tables 9.16 and 9.17 show that, for many crops and processes, the energy output derived from the technology is often little more than the energy input; in some cases the 'energy crop' is in fact a net energy consumer.

Table 9.16 Energy ratios for various indirect and direct energy crops

Crop and conversion process	Energy ratio	Reference
Sugarcane/bio-ethanol	6.45	Goldemberg *et al.* (1992)
Wheat/bio-ethanol	0.467–1.785	Mortimer (2002)
Hybrid poplar	16	Williams (1994)
Sorghum	12	Williams (1994)
Switchgrass	11	Williams (1994)
Miscanthus	25	Bullard and Metcalfe (2000)
SRC	11	Mortimer (2002)
Biodiesel	2	DEFRA (2002)

9.5 Conclusions

We have discussed the technical and socio-economic factors that must be addressed in determining if production of energy crops is viable at a given location, and the subsequent selection of a suitable crop. The decision processes can be aided by the summary of the positive and negative aspects of energy cropping shown in Table 9.18. In industrialised countries, which generate 75% of global warming gases, there are signs that the use of BECs will increase in the next decade, on account of: a) heightened environmental awareness; b) the deliberate choice of consumers who can pay higher prices for energy; c) the availability of the resource; and d) government policies and support. However, it is difficult to see sufficient activity stimulated to produce the 60% in CO_2 emissions said to be required (Watson, 2000). There is insufficient political urgency about the need to act, presumably because the remedy would be unpalatable to many voters. As public reaction to the severe flooding in the UK and the USA in 1999–2000 demonstrated, this may not continue to be the case.

Energy cropping is a technology whose time has come. Rapid improvement in the efficiency of thermoconversion systems is bettering the feasibility of large-scale generation of electricity from biomass. Woods and Hall (1994) summarise biomass and energy crops perfectly: 'Biomass is a flexible feedstock capable of conversion into solid, liquid and gaseous fuels which can substitute for fossil-based fuels with relatively small-scale investment. Global expansion of bio-energy systems could be influential in bettering both the socio-economic conditions and the environment of countries, and sustainable bio-energy production and commercialisation could open new opportunities for improving the quality of life of rural people.'

Table 9.17 Energy ratios for biofuels and transport fuels

Biofuels	**Energy ratio**	**Location**
SRC	29	UK
	19–30	Ireland
	14	Germany (poplar)
	17	Sweden (willow chips)
	8	Austria
	14	Denmark (willow)
Miscanthus	15–20	Germany
	18	Denmark
Reed canary	14	Sweden (bales)
Cereals	8.5	Germany

Liquid transport fuels	**Energy ratio**	**Location**
Rape (biodiesel)	2.5	Ireland; RME excluding straw
	5.5	Ireland; RME including straw
	1.9	Germany; RME excluding straw
	1.1–4.5	Belgium; RME, three scenarios
	2.8	Austria; RME excluding straw
	6	Denmark, including straw
	2.1	Finland; RME
Sunflower (biodiesel)	3.3	Austria; SME without straw
Winter wheat (bioethanol)	1.1–1.7	Germany; without residue and straw
	1.4–1.9	Germany; with residue, without straw
	4.0–5.0	Germany; with residue and straw
	1.1–5.9	Belgium; three scenarios
	1.3	Finland

All data from Venendaal *et al.* (1997). RME = rape methyl ester; SME = sunflower methyl ester.

Table 9.18 Strengths and weaknesses of biomass and energy crops: a summary

Factors acting against energy cropping	**Response: factors acting in support of energy cropping**
Potential direct conflict between food and fuel use for land	Offers sustainable use for surplus agricultural land in developed countries
Energy ratios may not be sufficiently high to warrant systems	Utilisation of biomass and energy crops offers one of the most direct routes to atmospheric CO_2 abatement
Energy density of energy crops is low	High efficiencies of conversion are possible with modern technologies
Technical barriers to use of existing thermo-conversion systems	Enhances rural land scale and rural economy
Lack of suitable political motivation to achieve goals	Recycled ash can be used as fertiliser
Internalisation of externalities biased against energy crops in favour of fossil fuels	Could improve energy price stability
Sufficiently high, reliable yields are difficult to achieve in some areas	Suitable use for tropical degraded areas

A bright outlook exists for biomass in tropical and sub-tropical regions on account of the climate and the greater diversity of biomass, greater yields and lower unit costs of production than can be achieved in temperate regions. Indeed, the only large-scale systems where crops are specifically grown for energy are in tropical regions (bio-ethanol and eucalyptus programmes in Brazil; energy cane in Puerto Rico). Existing tropical biomass should not be seen as a directly exploitable resource; it is more likely that sophisticated inter-cropping programmes that minimise disruption to local ecosystems can be developed. Production economics for energy crops are far more attractive where it can be demonstrated that secondary products with added value can also be derived from the feedstock. Finally, many governments, the UK's included, insist on price convergence during the development of renewable energy programmes. These countries need to invest in the research and infrastructure needed to exploit BECs while at the same time ensuring that domestic energy markets (*i.e.* costs to the consumer) reflect the true environmental costs of fossil fuel. This dual approach will quickly make biomass and energy crops economically viable.

APPENDIX 9.1

Crop and plant species with potential as energy species

Tropical and sub-tropical agricultural systems		Boreal, temperate and Mediterranean agricultural systems	
Trees and shrubs	**Common name**	**Trees and shrubs**	**Common name**
Acacia auriculiformis	Australian Acacia		
A. catechu	Khayer	*Alnus incana*[b]	Grey alder
A. dealbata[a]			
A. mangium	Mangium	*Ceratonia siliqua*[c]	Carob
A. nilotica	Babul		
Albizia lebbek	Kala Koroi		
A. procera	Jat Koroi	*Euphorbia lathyrus*[d]	
Alnus acuminata	Breadfruit, Jackfruit	*Liquidambar styraciflua*[e]	Sweetgum
A. nepalensis	Kathal		
Artocarpus heterophyllus	Neem	*Pinus radiata*	
Azadirachta indica	Pigeon pea, Arhar	*Populus*	
Cajanus cajan	Calliandra		
Calliandra clothyrsus	Minjiri		
Cassia siamea	Casuarina, Yar	*Salix*	
Casuarina equisetifolia	Australian pine, Jau		
Cucurbita foetidissima	Buffalo gourd[9]		
Dalbergia sissoo	Shisham		
Derris indica	Saw, Kerong		

Appendix 9.1 cont'd.

Tropical and sub-tropical agricultural systems		**Boreal, temperate and Mediterranean agricultural systems**	
Emblica officinalis	Amloki	**Root crops**	
Eucalyptus brassiana	Cape York red gum	*Cucurbita foetidissima*	Buffalo gourd[f]
Eucalyptus camaldulensis	River-red gum		
E. citriodora	Lemon-scented gum		
E. deglupta	Kamarere		
E. globulus	Blue gum		
E. grandis	Flooded gum		
E. regans[a]			
E. tereticornis	Forest red gum		
E. urophylla	Timor mountain gum		
Gliricidia sepium	Gliricidia, Mexican Lilac		
Gmelina arborea	Gamar		
Leucaena leucocephala	Ipil-Ipil		
Melia azedarach	Paradise tree, Bakain		
Moringa oleifera	Ben, Sajina		
Paraserianthes falcataria	Moluccana		
Pinus radiata[2]	Monterey pine		
Samanea saman	Saman, Randi Koroi		
Sesbania bispinosa	Dhaincha		
S. grandiflora	Bakphul		
S. sesban	Sesban		
S. cumini	Jam		

Appendix 9.1 cont'd.

Tropical and sub-tropical agricultural systems		**Boreal, temperate and Mediterranean agricultural systems**	
Grasses			
	Bahia grass[g]		
	Bermuda grass[g]		
	Eastern gamma grass[g]		Eastern gamma grass[g]
	Reed canary grass[g]		Reed canary grass[g]
	Napier grass[g]		
	Rye[g]		Rye[g]
	Sudan grass[g]		
	Switch grass[g]		Switch grass[g]
	Tall fesue[g]		Tall fescue[g]
	Timothy[g]		Timothy[g]
	Weeping love grass[g]		Weeping love grass[g]

[a]Stjernquist (1992); [b]Saarsalmi and Palmgren (1984); [c]Mervwin (1990); [d]Tenorio *et al.* (1985); [e]Francis and Baker (1982); [f]Shultz and Bragg (1995); [g]Wright *et al.* (1993).

Acknowledgement

I would like to thank Miss Alexandra Anghel for assistance with the production of tables and referencing and comments on the text.

References

Ahmed K. (1994), *Renewable Energy Technologies: A Review of the Status and Costs of Selected Technologies*, World Bank Technical Paper Number 40, World Bank, Washington DC.

Alexander A. G. (1985), *The Energy Cane Alternative*, Elsevier, Oxford.

Allen J., Browne M., Hunter A., Boyd J. and Palmer H. (1997), 'Supply systems for biomass fuels and their delivered costs', *Aspects Appl. Biol.* **49**, *Biomass and Energy Crops*, 369–378.

Anon. (1996), *A Working Countryside for Wales*, Cm 3180, HMSO, London.

Anon. (2000), *Renewable Energy Annual 1999*, US Department of Energy Report DOE/EIA0603(99).

Bauen A. (2001), 'Biomass energy, greenhouse gas abatement and policy integration', *Biomass and Energy Crops II Conference*, York, UK.

Beeharry R. P. (1996), 'Extended sugarcane biomass utilisation for exportable electricity production in Mauritius', *Biomass Bioenergy* **11**, 441–449.

Berggren H. (1993), 'Project Agro-Fibre: an investigation into the possibility for using agricultural crops for pulp production in Sweden', in *New Crops for Temperate Europe*, Anthony K. R. M., Meadley J. and Robbelen G., eds., Chapman & Hall, London.

Börjesson P. I. I. (1996), 'Energy analysis of biomass production and transportation', *Biomass Bioenergy* **11**, 305–318.

Brettler Bereneyi E. (1995), 'An overview of waste-to-energy systems in the USA: waste as an energy source', *Proc. 2nd. Conf. of the Americas: Energy, Environment, Agriculture, and Industry*, National Renewable Energy Laboratory, Colorado, pp. 88–99.

Bullard M.J., Mustill S.J., Nixon P.M.I., McMillan S D and Britt C.P. (2001) 'The effect on yield of planting density of short rotation coppice', *Aspects Appl. Biol.* **65**, *Biomass and Energy Crops*, 167–172.

Bullard M. J. and Metcalfe P. (2000), 'Energy ratios and carbon balances for perennial herbaceous energy grasses', ETSU Report **291**, Energy Technology Support Unit, Didcot, UK.

Bullard M. J., Nixon P. M. I. and Heath M. C. (1997), 'Quantifying the yield of *Miscanthus giganteus* in the UK', *Aspects Appl. Biol.* **49**, *Biomass and Energy Crops*, 199–206.

Bullard M. J., Mustill S. J., Carver P. and Nixon P. M. I. (2002), 'Yield improvements through modification of planting density and harvest frequency in short rotation coppice *Salix spp.* —2. Resource capture and use in two morphologically diverse varieties', *Biomass Bioenergy* **22**, 27–39.

Callender B., Harris N., Maskell K., Mills F. and Kettenberg A. (1995), 'Summary for policy makers: radiative forcing of climate change', in *Climate Change 1994, Radiative Forcing of Climate Change and an Evaluation of the IPCC IS92 Emissions Scenarios*, Houghton L. G., Filho M., Bruce J., Lee H., Callender B. A., Haites E., Harris N. and Maskell K., eds., Cambridge University Press, Cambridge.

Cannell M. G. R., Milne R., Sheppard L. J. and Unsworth M. H. (1987), 'Radiation interception and productivity of willow', *J. Appl. Ecol.* **24**, 261–278.

Cannell M. G. R., Sheppard L. J. and Milne R. (1988), 'Light use efficiency and woody biomass production of poplar and willow', *Forestry* **61**, 125–136.

Cannell M. G. R. (1989), 'Physiological basis of wood production: a review', *Scand. J. For. Res.* **4**, 459–490.

CEC (1996), *Energy Green Paper*, Commission of the European Community.

Charles-Edwards D. A. (1982), *Physiological Determinants of Crop Growth*, Academic Press, London and New York.

Chege N. (1993), 'Not-yet-fossil fuel', *World Watch* **6**, 8–10.

Christian D. (1997), 'Wintertime use of hybrid poplar plantations by deer and medium-sized mammals in the Midwestern US', *Biomass Bioenergy* **12**, 35–40.

Christian D. G., Poulton P. R., Riche A. B. and Yates N. E. (1997), 'The recovery of ^{15}N-labelled fertilizer applied to *Miscanthus x Giganteus*', *Biomass Bioenergy* **12**, 21–24.

Clark W. C. (1982, ed.), *Carbon Dioxide Review: 1982*, Oxford University Press, New York.

Collins R. P. and Jones M. B. (1985), 'The influence of climatic factors on the distribution of C_4 species in Europe', *Vegetation* **64**, 121–129.

Cook J. and Beyea J. (2000), 'Bioenergy in the United States: progress and possibilities', *Biomass Bioenergy* **18**, 441–455.

Deraedt W. and Ceulemans R. (1998), 'Clonal variability in biomass production and conversion efficiency of poplar during the establishment year of a short rotation coppice plantation', *Biomass Bioenergy* **15**, 391–398.

Davidson J. (1985), 'Species and sites: what to plant and where to plant', *Food and Agriculture Organization of the United Nations UNDP/FAO Project BGD/79/017*, Assistance to the Forestry Sector of Bangladesh.

Davidson J. (1987), 'Bioenergy tree plantations in the tropics. Ecological implications and impacts', *IUCN Commission on Ecology Papers Number 12*, Dorchester, Dorset Press.

d'Apote S. L. (1998), 'IEA biomass energy analysis and projections', in *Biomass Energy: Data, Analysis and Trends, Proc. OECD/IEA Conf.*, March 23–24, OECD/IEA, Paris.

DEFRA (2002), Annual Report of the Government–Industry Forum on Non-Food Uses of Crops, *www.defra.gov.uk/farm/acu/non-food/forum-ann-rep-02.pdf.*

del Pozo A. and Dennett M. D. (1991), 'Modelling the effect of leaf nitrogen content on crop photosynthesis and radiation use efficiency', *Aspects Appl. Biol.* **26**, 285–289.

DETR (2000), Climate Change Levy—2000, *www.ceramfed.co.uk/Climate Levy2000.html.*

Dimitri L. (1984), 'Production of forest biomass through growth of fast-growing tree species in short rotation', *Bioenergy* **84**, 52–62.

DTI (1999), *New and Renewable Energy—Prospectus for the 21st. Century*, URN 99/744.

DTI (2000), *Digest of UK Energy Statistics*, Stationery Office, Norwich, UK.

Dupont-Roc G., Khor A. and Chris A. (1994), *The Evolution of the World's Energy Systems*, Shell International, London.

EIA (2002) Energy Information Administration, *International Energy Annual 2000, http://www.eia.doe.gov/pub/international/ieapdf/t01_08.pdf.*

ENEP (1996), Energy Efficiency Promotion Program, *www.gtzth.org/service/enep php?il=02&in=1.*

EUROSTAT (1994), *http://europa.eu.int/comm/eurostat/Public/datashop/print-catalogue/EN?catalogue=Eurostat&theme=2-Economy%20and%20Finance*, Statistical Office of the European Communities, Luxemburg.

Evans J. R. (1989), 'Photosynthesis and nitrogen relationship in leaves of C_3 plants', *Oecologia* **78**, 9–19.

FAO (1999), *Statistical Databases on the Internet*, Food and Agriculture Organization of the United Nations, Rome, Italy.

Fisher G. and Heilig G. K. (1998), *Population Momentum and the Demand on Land and Water Resources, Report IIASA-RR–98–1*, International Institute of Applied Systems Analysis (IIASA), Laxenburg, Austria.

Gallagher J. N. and Biscoe P. V. (1978), 'Radiation absorption, growth and yield of cereals', *J. Agric. Sci.* **91**, 47–60.

Goldemberg J., Monaco L. C. and Macedo I. C. (1992), 'The Brazilian fuel–alcohol program', Chapter 20 in Johansson T. B., Kelly H., Reddy A. K. N. and Williams R. H., eds., *Renewable Energy: Sources for Fuels and Electricity*, Island Press, Washington DC.

Grainger A. (1990), 'Modelling the impact of alternative afforestation strategies to reduce carbon dioxide emissions', *Proc. Conf. on Tropical Forestry Response Options to Global Climate Change*, São Paulo, Brazil.

Grübler A., Nilsson S. and Nakicenovic N. (1993), 'Enhancing carbon sinks', *Energy* **18**, 499–522.

Hall D. O. (1991), 'Biomass energy', *Energy Policy* **19**, 711–737.

Hall D. O. (1997), 'Biomass energy in industrialised countries—a view of the future', *Forest Ecol. Management* **91**, 17–45.

Hall D. O. and House J. I. (1995), 'Biomass energy in Western Europe to 2050', *Land Use Policy* **12**, 37–48.

Hall D. O. and Rao K. K. (1999), *Photosynthesis*, 6th. ed., *Studies in Biology*, Cambridge University Press, Cambridge.

Hall D. O., Rosillo-Calle F., Williams R. H. and Woods J. (1992), 'Biomass for energy: supply prospects', in *Renewable Energy: Sources for Fuels and Electricity*, Johansson T. B., Kelly H., Reddy A. K. N. and Williams R., eds., Island Press, Washington DC.

Hall D. O., Rosillo-Calle F. and Scrase J. I. (1996), 'Biomass: an environmentally acceptable and sustainable energy source for the future', Report to the UN Department for Policy Coordination and Sustainable Development (DPCSD), New York.

Hartemink A. E. and Kuniata L. S. (1996), *Outlook on Agriculture* **25**, 227–234.

Harvey J. J. and Sylvester-Bradley R. (1992), 'The potential of *Miscanthus* for the UK', ETSU Report, Energy Technology Support Unit, Didcot, UK.

Hirose T. and Werger M. J. A. (1987), 'Maximising daily canopy photosynthesis with respect to the leaf nitrogen allocation pattern in the canopy', *Oecologia* **72**, 520–526.

Houghton R. A., Unruh J. and Lefèbre P. A. (1991), 'Current land use in the tropics and its potential for sequestering carbon', *Proc. Technical Workshop to Explore Options for Global Forestry Management*, 24–29 April 1991, Bangkok, Thailand. International Institute for Environment and Development, UK.

IEA Bioenergy (2000), Key World Energy Statistics, *http://www.iea.org/statist/keyworld2002/key2002/keystats.htm.*

IEA Bioenergy (2001), *Annual Report 2001, http://www.ieabioenergy.com/library/139_ieaarweb.pdf.*

IIASA/WEC (1998), *Global Energy Perspectives*, Nakicenovic N., Grubler A. and McDonald A., eds., International Institute for Applied Systems Analysis (IIASA) and World Energy Council (WEC), Cambridge University Press, Cambridge, UK.

IPCC (1992), *1992 IPCC Supplement*, Intergovernmental Panel on Climate Change, Geneva, Switzerland.

IPCC (1995), *Climate Change 1995—The Science of Climate Change*, Cambridge University Press, Cambridge.

IPCC (2001), *Special Report on Emissions Scenarios*, Intergovernmental Panel on Climate Change, UNEP/WMO, New York.

Jarvis P. G. and Leverenz J. W. (1983), 'Productivity of evergreen and deciduous forests', in *Encyclopedia of Plant Physiology, New Series, Vol 12D, IV*, Lange O. L., Nobel P. S., Osmond C. B. and Ziegler H., eds., Springer-Verlag, Berlin.

Jørgensen U. (1997), 'Genotypic variation in dry matter accumulation and content of N, K and Cl in *Miscanthus* in Denmark', *Biomass Bioenergy* **12**, 155–169.

Keener H. M. and Roller W. L. (1975), *Energy Production by Field Crops*, Agricultural Engineering Department, Ohio Agricultural Research and Development Centre, Wooster, Ohio.

Kheshgi H.S., Prince R. C. and Marland G (2000). 'The potential of biomass fuels in the context of global climate change: focus on transport fuels', *Ann. Rev. Energy Environment* **25**, 199–244.

Kopp R. F., Abrahamson L. P., White E. H., Burns K. F. and Nowak C. A. (1997), 'Cutting cycle and spacing effects on biomass production by a willow clone in New York', *Biomass Bionergy* **12**, 313–319.

Korbitz W. and Walker K. (1996), 'Biodiesel—production and exploitation', in *Energy from Crops*, Murphy D. P. L., Bramm A. and Walker K. C., eds., Semundo Ltd., Cambridge, UK.

Kroll T. and Forester B. S. (1995), 'Large-scale biomass plantings in Minnesota: scale-up and demonstration projects in perspective', *Proc. 2nd. Conf. of the Americas: Energy, Environment, Agriculture, and Industry*, National Renewable Energy Laboratory, Colorado, pp. 21–29.

Kyoto Protocol (1997), *http://unfccc.int/resource/docs/convkp/kpeng.html.*

Linder S., MacDonald J. and Lohammer T. (1981), 'Effects of nitrogen status and irradiance during cultivation on photosynthesis and respiration in birch seedlings', *Energy For. Proj., Tech. Rep.* **12**, 19, Swedish University of Agricultural Science, Uppsala (in Swedish with English summary).

Long S. P., private communication.

Macedo I C. (1992), 'The sugar-cane industry—its contribution to reducing CO_2 emissions in Brazil', *Biomass Bioenergy* **3**, 77–80.

Mazzarella V. N. G. and Katayama M. T. (1999), 'BIP–Biomass Integrated Project—present status of an integrated development for some high-productivity biomass species', IUAPPA Conference, Brighton, England.

McCracken A. R. and Dawson W. M. (1997), 'Using mixtures of willow clones as a means of controlling rust disease', *Eur. J. Forest Pathol.* **27**, 319–329.

McCrady R. L. and Jokela E. J. (1998), 'Canopy dynamics, light interception, and radiation use efficiency of selected loblolly pine families', *Forest Sci.* **44**, 64–72.

McDonald J., Lohammar T. and Linder S. (1981), 'Effect of leaf nitrogen content on CO_2 exchange in a number of *Salix* clones', *Energy Forests Project Tech. Rep.* **16**, Swedish University of Agricultural Science, Uppsala.

McDougall G. J., Morrison I. M., Stewart D., Weyers J. D. B. and Hillman J. R. (1993), 'Plant fibres: botany, chemistry and processing for industrial use', *J. Sci. Food Agric.* **62**, 1–20.

McKeever D. B. (1995), 'Resource potential of wood-based wastes in the United States', in *Proc. 2nd. Conf. of the Americas: Energy, Environment, Agriculture, and Industry,* National Renewable Energy Laboratory, Colorado, pp. 77–87.

Milne R., Sattin M., Deans J. D., Jarvis P. G. and Cannell M. G. R. (1992), 'The biomass production of three poplar clones in relation to intercepted solar radiation', *Forest Ecol. Management* **55**, 1–14.

Monteith J. L. (1977), 'Climate and efficiency of crop production in Britain', *Phil. Trans. R. Soc. London, Ser. B* **281**, 277–294.

Mooney K. A., Ferrar P. J. and Slayter R. O. (1978), 'Photosynthetic capacity and carbon allocation patterns in diverse growth forms of *Eucalyptus*', *Oecologia* **36**, 103–111.

Mortimer N. D., Cormack P., Elsayad M. A. and Home R. E. (2002), 'Evaluation of the comparative energy environmental and social costs and benefits of biodiesel', Sheffield Hallam University, Report for DEFRA, pp. 82.

Muchow R. C. and Davis R. (1988), 'Effect of nitrogen supply on the comparative productivity of maize and sorghum in a semi-arid tropical environment. II. Radiation interception and biomass accumulation', *Field Crops Res.* **18**, 17–30.

Muchow R. C., Hammer G. L. and Kingston G. (1991), 'Assessing the potential yield of sugar cane', in *Proc. Austral. Soc. Sugar Cane Technologists*, pp. 146–151.

Murphy D. P. L. and Helel H. M. (1996), 'Farming for energy—agronomic principles and environmental impacts', in *Energy from Crops*, Murphy D. P. L., Bramm A. and Walker K. C., eds., Semundo Ltd., Cambridge, UK

Nellist M. E. (1997), 'Storage and drying of arable coppice', *Aspects Appl. Biol.* **49**, *Biomass and Energy Crops*, pp. 349–360.

Nilsson L. O. (1985), 'Growth and yields of willow stands in relation to climate and nutrition', Dept. of Ecology and Environment Research, Swedish University of Agricultural Science, Uppsala, Report No. 21.

Nixon P. M. I. (1997), 'Does the common rustic moth *Mesapamea secalis (L.)* represent an economic threat to *Miscanthus*?', *Aspects Appl. Biol.* **49**, *Biomass and Energy Crops*, pp. 137–142.

Nixon P. M. I., Bullard M. J. and Price L. (2001), 'Is *Miscanthus* suited to the whole of England and Wales? Preliminary studies', *Aspects Appl. Biol.* **65**, *Biomass and Energy Crops II Conference*, York, UK.

Nordin A. and Kjellström B. (1996), 'Biomass for electricity and heat production—engineering aspects', in *Energy from Crops*, Murphy D. P. L., Bramm A. and Walker K. C., eds., Semundo Ltd., Cambridge, UK.

NREL (2002). Bioenergy: An Overview. *www.eren.doe.gov/consumerinfo/refbriefs/nb2.html*

Ogden J. M., Williams R. H. and Fulmer M. E. (1990), 'Cogeneration applications of biomass gasifier/gas turbine technologies in the cane sugar and alcohol industries: getting started with bioenergy strategies for reducing greenhouse gas emissions', in *Global Warming and Climate Change: Perspectives from Africa,* Nairobi, Kenya.

Pasztor J. and Kristoferson L. A. (1990, eds.), *Bioenergy and the Environment.* Westview Press, Oxford.

Paustian K., Andren O., Janzen H. H., Lal R., Smith P., Tian G., Tiessen H. and van Noordwijk M. (1997), 'Agricultural soils as a sink to mitigate CO_2 emissions', *Soil Use and Management* **13**, 230–244.

Pei M. H., Parker S. R., Hunter T. and Royle D. J. (1997), 'Variation in populations of *Melampsora* willow rust and the implications for design and short rotation coppice plantations', *Aspects Appl. Biol.* **49**, *Biomass and Energy Crops*, pp. 91–96.

PIU (2002), *The Energy Report*, UK Performance and Innovation Unit, Feb. 2002 (*www.cabinet-office.gov.uk/innovation/2002/energy/report/*).

Price L., Bullard M. J., Lyons H., Anthony S. and Nixon P. M. I. (2002), 'Identifying the yield potential of *Miscanthus x giganteus*: an assessment of the spatial and temporal variability of biomass productivity across England and Wales', submitted to *Biomass and Bioenergy.*

Robertson A. (1984), 'Willow plantations in agroforestry', *Span* **27**, 32–34.

Robertson T. and Shapouri H. (1993), 'Biomass: an overview in the United States of America', *Proc. 1st. Biomass Conf. of the Americas*, Burlington, Vermont. NREL/CP–200–5768, National Renewable Energy Laboratory, Golden, Colorado.

Rosa L. P. and Ribeiro S. K. (1998), 'Avoiding emissions of carbon dioxide through the use of fuels derived from sugar cane', *Ambio* **27**, 465–470.

Rosillo-Calle F. (1990), 'Liquid biofuels', in *Bioenergy and the Environment*, Westview Press, Oxford.

Rosillo-Calle F. and Cortez L. A. B. (1998). 'Towards ProAlcool II—a review of the Brazilian bioethanol programme', *Biomass Bioenergy* **14**, 115–124.

RCEP (2000), *Energy—The Changing Climate*, Royal Commission on Environmental Pollution, 22nd. Report, Cm 5459, ISBN 0–10–154592 4, *www.rcep.org.uk/ reports2.html#22.*

Rutherford I. and Bell A. (1992), 'Economic appraisal', in Rutherford I. and Heath M. C., eds., *The Potential of Miscanthus as a Fuel Crop*, ETSU B 1354, Energy Technology Support Unit, Didcot, UK.

Saarsalmi A. and Palmgren K. (1984), 'Biomass production and nutrient requirements of a young grey alder plantation', *Bioenergy* **84**, 52–62.

Sage R. F., Pearcy R. W. and Seemann J. R. (1987), 'The nitrogen use efficiency of C_3 and C_4 plants', *Plant Physiol.* **85**, 355–359.

San Miguel A. (1986), *Forest Biomass Plantations in Spain*, Country Report to EEC, Hummel F., ed., EEC, Brussels.

Scurlock J. M. O. and Hall D. O. (1989), 'Biological fuels', *New Scientist* **121**, 73–73.

Shell Oil plc (1994), *www.shell.com/home/Framework?siteId=home.*

Shultz E. B. Jr. and Bragg W. G. (1995), 'Carbohydrate biofuels I: rootfuel studies in Mexico, Brazil, Zimbabwe and India', in *Proc. 2nd. Conf. of the Americas: Energy, Environment, Agriculture, and Industry,* National Renewable Energy Laboratory, Colorado, pp. 313–322.

Smith P., Powlson D. S., Glendining M. J. and Smith J. U. (1998), 'Preliminary estimates of the potential for carbon mitigation in European soils through no-till farming', *Global Change Biol.* **4**, 679–685.

Stephens W., Hess T. M. and Knox J. W. (2001) 'The effect of energy crops on hydrology', *Aspects Appl. Biol.* **65**, *Biomass and Energy Crops*, 101–108.

Struik P. C., Amaducci S., Bullard M. J., Stutterheim N. C., Venturi G. and Cromack H. T. H. (2000), 'Agronomy of fibre hemp (*Cannabis sativa L.*) in Europe', *Industrial Crops and Products* **11**, 107–118.

Swisher J. and Wilson D. (1993), 'Renewable energy potentials', *Energy* **18**, 437–459.

Venendaal R., Jørgensen U. and Foster C. A. (1997), 'European energy crops: a synthesis', *Biomass Bioenergy* **13**, 147–185.

Wall M. and Deboys R. S. (1997), 'Forestry Commission ETSU field trials of SRC harvesting and comminution machinery', *Aspects Appl. Biol.* **49**, *Biomass and Energy Crops*, 361–368.

Watson R. T. (2000), Presentation from Chair of the IPCC at the 6th. Conference of the Parties at the UNFCCC, 13 November 2000 (*http://www.ipcc.ch/press/spech.htm*).

Williams R. H. (1994), 'Roles for biomass energy in sustainable development', in *Industrial Ecology and Global Change*, Socolow R., Andrews C., Berkhout F. and Thomas V., eds., Cambridge University Press, Cambridge.

Winjum J. K., Dixon R. K. and Schroeder P. E. (1992), 'Estimating the global potential of forest and agroforest management practices to sequester carbon', *Water, Air and Soil Pollution* **62**, 2131–2227.

Woods J. and Hall D. O. (1994), 'Bioenergy for development: technical and environmental dimensions', FAO Environment and Energy Paper **13**, Rome, 1–71.

Wright L. L., Cushman J. H., Ehrenshaft A. R., McLaughlin S. B., McNabb W. A., Ranney J. W., Tuskan G. A. and Turhollow A. F. (1993), *Biofuels Feedstock Development Program*, Annual Progress Report for 1992, Environmental Science Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee.

This page intentionally left blank

CHAPTER 10

THE PRODUCTION OF BIOFUELS BY THE THERMOCHEMICAL PROCESSING OF BIOMASS

TONY BRIDGWATER
Bio-Energy Research Group, Aston University, Birmingham B4 7ET, UK
a.v.bridgwater@aston.ac.uk
and
KYRIAKOS MANIATIS
European Commission, DG TREN, Brussels B1049, Belgium
kyriakos.maniatis@ cec.eu.int

The plants are unsurpassed masters of—or marvellous workshops for—photochemical synthesis of the fundamental substances, building up from carbon dioxide with the help of solar energy.

Giacomo Ciamician, *Eighth International Congress of Applied Chemistry*, Washington and New York, September 1912.

Bioenergy is now accepted as having the potential to provide the major part of the projected renewable energy provisions of the future as biofuels in the form of gas, liquid or solid fuels or electricity and heat. There are three main routes to providing these biofuels—thermal conversion, biological conversion and physical conversion — all of which employ a range of chemical reactor configurations and designs. In this chapter we focus on thermochemical conversion processes for their higher efficiencies, lower costs and greater versatility in providing a wide range of energy, fuel and chemical options. We describe the technologies of gasification and fast pyrolysis, particularly the reactors that have been developed to provide the necessary conditions to optimise performance. The primary products that can be derived as gas, liquid and solid fuels are characterised, as well as the secondary products of electricity and/or heat, liquid fuels and a considerable number of chemicals. We also identify the main technical and non-technical barriers to the market deployment of the various technologies and briefly discuss them.

10.1 Introduction

Renewable energy is of growing importance in responding to concerns over the environment and security of energy supply. Biomass is unique in providing the only renewable source of fixed carbon, which is an essential ingredient in meeting many of our fuel and consumer goods requirements. Wood and annual crops and agricultural and forestry residues are some of the main renewable energy resources available. The biodegradable components of MSW (Municipal Solid Waste) and commercial and industrial wastes are also significant bio-energy resources, although particularly in the case of MSW, they may require extensive processing before conversion. Biomass is considered the renewable energy source with the highest potential to contribute to the energy needs of modern society for both the developed and developing economies worldwide (European Commission, 1997; IEA, 2000). In addition, energy from biomass based on short rotation forestry and other energy crops can contribute significantly towards the objectives of the Kyoto Protocol in reducing greenhouse gas emissions and alleviating problems related to climate change (IEA Bioenergy, 1998).

Biomass fuels and residues can be converted to energy via the thermal, biological and mechanical or physical processes summarised in Fig. 10.1. Thermal processing currently attracts the most interest, and gasification receives the most RD&D support as it offers higher efficiencies compared with combustion, while fast pyrolysis is still at a relatively early stage of development but offers the benefits of a liquid fuel with concomitant advantages of easy storage and transport as well as comparable power generation efficiencies at the smaller scales of operation that are likely to be realised

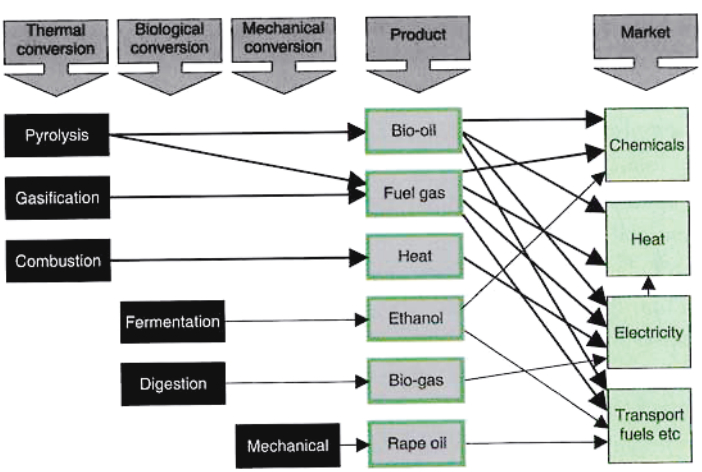


Figure 10.1 Biomass conversion processes, products and applications.

from bio-energy systems as compared with fossil fuelled systems. The higher efficiency of gasification systems arises from high efficiency in converting to a gas (up to 98% hot gas efficiency is realisable), and higher efficiencies in utilising heat from combustion of gas. This includes larger scale power generation of up to 100 MW_e with Integrated Gasification Combined Cycle (IGCC) processes, with predicted electricity production efficiencies of 45–50% compared with only 25–35% via combustion; and small-scale power generations systems of up to 5 MW_e using engines that offer up to 30% efficiency compared to 10–15% using combustion and a steam cycle. Both of these thermochemical conversion processes offer high conversion efficiencies, as explained above, potentially competitive costs and considerable flexibility in scale of operation and range of products and we shall therefore concentrate on these technologies in this review. Combustion, biological conversion processes (fermentation and digestion) and mechanical processing (*e.g.* vegetable oils) are well established and are commercially offered with performance guarantees and therefore we have described these only in summary.

The key difference between thermal and biological conversion is that biological conversion gives single or specific products such as ethanol or biogas (which contains up to 60% methane) and is a slow process, typically taking hours, days, weeks (anaerobic fermentation and farm digestion) or years (landfill gas by digestion) for reactions to be completed. Thermal conversion gives multiple and often complex products, with catalysts often used to improve the product quality or spectrum, and takes place in very short reaction times of typically seconds or minutes. Table 10.1 summarises some of the main products that can be obtained by processing biomass.

10.1.1 Biological conversion summary

To produce ethanol by fermentation, the cellulose and hemicellulose in lignocelluloses need to be hydrolysed to sugars before fermentation, and enzyme and acid hydrolysis are both employed for this purpose. Carbohydrates such as starch also require hydrolysis. Only the cellulose in biomass is converted to ethanol, although it is now possible to convert some hemicellulose in integrated processes such the SSF (Simultaneous Saccharification and Fermentation) process, which reduces costs and improves efficiency. The lignin residue can be burned for process heat, particularly for ethanol concentration. Another recent development is fermentation of synthesis gas from thermal gasification. The carbon monoxide, carbon dioxide and hydrogen are biologically converted to ethanol in high yield and at very high reaction rates (Klasson *et al.*, 1990). This process is still at an early stage of development. Overall

efficiencies of ethanol production are rather low, owing to the loss of about half the carbon in holocellulose as carbon dioxide; the loss of the carbon in lignin, which is not converted; and the need to concentrate the dilute ethanol solution. Fermentation is particularly suitable for materials with high moisture content, as drying is not required. Ethanol can be readily converted to ETBE (ethyl tertiary butyl ether), which can be directly used as a gasoline additive. Fermentation technology is commercial and tends to be high cost unless subsidies are applied and low efficiency unless credits are applied to the by-products. After a lull in RD&D, there is now renewed interest in bio-ethanol, with enzyme hydrolysis in particular attracting more attention. A substantial demonstration plant can be expected soon, but there is little RD&D because the technology is established and viability relies on financial support.

Table 10.1 Major products from biomass conversion

Product		**Biological processing**	**Physical processing**	**Thermal processing**
Fuels	Additives	✓	✓	✓
	Alcohols			✓
	Charcoal			✓
	Diesel-type fuels		✓	✓
	Fischer–Tropsch liquids			
	Fuel oil			✓
	Gas	✓		✓
	Gasoline			✓
	Hydrogen	✓		✓
Electricity	Electricity			✓
Chemicals	Acetone	✓		
	Activated carbon			✓
	Butanol	✓		
	Ethanol	✓		✓
	Fertilisers	✓		✓
	Fine chemicals	✓	✓	✓
	Food additives	✓		✓
	Hydrogen	✓		✓
	Methane	✓		✓
	Methanol			✓
	Resins			✓

There are several books on biological conversion, such as Waymand and Parekh (1990), and reports on current activities can be found the "grey" literature, particularly from the USA. A useful source of ongoing activities is IEA Bioenergy (IEA Bioenergy, 2002), which publishes occasional reviews (*e.g.* $(S\&T)^2$ Consultants Inc., 2001; IEA 1994).

Biodiesel is the ester formed by reacting vegetable oils or animal fats with methanol or ethanol. Vegetable oil can be recovered from oil seeds such as rape (canola or colza), or other crops such as linseed, sunflower *etc.* The product in its raw form is unsuitable for many applications because it is highly viscous and has other deleterious properties, so the methyl or ethyl ester is formed by esterification. However, there have been limited attempts to use the cold pressed oil as transport fuel. The most common product is Rape Methyl Ester or RME. The raw oil is recovered by pressing, usually accompanied by solvent extraction to improve yields. This raw oil is then subjected to esterification with methanol or ethanol over a catalyst giving a lower viscosity and more stable product and glycerine as a by-product. The ester is entirely compatible with diesel in use and applications and biodiesel is thus an attractive renewable transport fuel. The yield of vegetable oil per unit land area is, however, very low at around 1 t/ha/y and is accompanied by around 10 t/ha/y of solid residues—rape straw for example. The product cost is therefore high. The technology is simple and proven. Animal fats and waste cooking oils can also be processed in an analogous way and, while the scope is limited, this route offers significant economic opportunities while the waste materials continue to be available at low cost. Again, IEA Bioenergy is a useful source of updated information, particularly through the liquid fuels Task (IEA Bioenergy, 2002; Eibensteiner and Danner, 2001).

Anaerobic digestion is microbial conversion of organic materials to methane and carbon dioxide in the absence of oxygen. The product gas from farm digesters is typically around 60% methane, although higher levels have been reported. The typical methane content from landfill sites is lower at 50–55%. Digestion is particularly suitable for residues with high moisture content, as drying is not required. The biogas can be used for heat with minimal processing, or for power generation in engines or turbines and (after upgrading to methane quality by removal of carbon dioxide and other components) in fuel cells or as gaseous fuel for transport applications. This requires increasingly stringent quality specifications. Landfill sites tend to be very large and typically produce gas over a 20–25 year life. Gas is collected through a system of wells, which collect and pipe the gas to the user.

The gas is wet and contains acid components that require careful management to avoid or minimise problems in using the gas for heat and/or power production. As landfill gas collection is required in many countries to comply with legislation to

minimise hazards, the additional costs of distributing gas to users is very small, so landfill gas is an economically attractive renewable energy resource. However as pressure grows to reduce land filling by legislation in the European Union, this resource is expected to reduce in the long term. On-farm or industrial digesters using farm wastes, industrial food wastes and household wastes are becoming more widely used for smaller scale applications. The wastewater industry in particular has successfully and effectively used digestion for in-plant power generation for many years. Dedicated digesters tend to be costly and inefficient as well as making a significant amount of solid residues that must be disposed of. Thus small-scale digestion is not often cost-effective. Although there is widespread interest in anaerobic digestion for both waste disposal and energy generation, economic opportunities are limited to niche applications.

New developments in solid-state fermentation have allowed the dry-solids content of the substrate to be as high as 30–35 wt%, compared with only 3–10 wt% for traditional liquid phase fermentation. This increases biogas productivity by a factor of about three, and reduces digester volumes. This technology has found applications in the processing of the biodegradable fraction of MSW or source-separated waste (De Baere, 2000). In all types of digestion, the carbon dioxide component can be reduced or removed to give higher quality gas, up to natural gas qualities. There is a range of available technologies, including membrane processes and conventional scrubbing processes, according to the scale of operation. All are costly, and careful assessment of the added value of the resultant higher quality gas is needed. The purified methane can either be used as transport fuel or added to a natural gas pipeline. Useful texts include Wheatley (1990) and Lusk *et al.* (1996). IEA Bioenergy (2002) is another useful source of updated information.

10.1.2 Biomass resources

Biomass is a diffuse resource, arising over very large areas, and thus requiring large land areas with substantial logistical problems in collection and transport as well as high costs. Typically a sustainable crop of 10 dry t/ha/y of woody biomass can be produced in Northern Europe, rising to perhaps 15 or maybe 20 dry t/ha/y for energy crops in Southern Europe. Thus an area of 1 km^2 or 100 ha will produce 1000 dry t/y, enough for a power output of 150 kW_e at low conversion efficiencies or 300 kW_e at high conversion efficiencies. It is therefore difficult to visualise power generation plants much bigger than around 30–40 MW_e anywhere in Europe and even these will require a planted area of around 100 km^2. A further complication with almost all

forms of biomass is their seasonality; forestry and coppiced crops can only be harvested during the winter months, and energy crops and agricultural residues are even more seasonal, typically growing for a only few months a year. Extensive provision for storage thus has to be made. One solution to this problem is a multi-fuel system and increasing efforts are underway to develop processes that can handle a number of different fuels, either mixed or separately. The current view is that even these plants are limited in size, with a typical plant size of 5–15 MW_e likely to dominate the market in the short term. However, in locations where extensive industrial operations are located close to managed forests, it is technically feasible and economically attractive to install large-scale bioenergy Combined Heat and Power (CHP) plants and burn the process residues to provide heat for the local industry. For example, the Alhomens boiler in Finland, commissioned in 2001, has a design capacity of 500 MW_{th} and is designed to operate with up to 100% biomass or on mixtures of biomass and peat. Biomass and agricultural wastes are very similar in their arisings, with most European industries individually producing comparable quantities of material, although overall regional and national totals may be substantial.

10.2 Thermal conversion processes

There are three main thermal processes—pyrolysis, gasification and combustion—available for converting biomass to a more useful energy form. Figure 10.2 summarises their oxygen requirements and relationships to each other, and Fig. 10.3 their products and applications. Combustion is already a well-established commercial technology with applications in most industrialised and developing countries, and

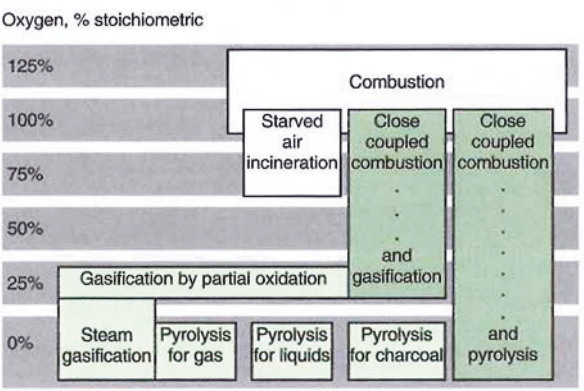


Figure 10.2 Thermal conversion processes

development is concentrated on resolving environmental problems. In this chapter, we therefore focus on the advanced technologies of gasification and pyrolysis, as these are expected to offer the greatest contribution in the short term in terms of versatility, improved efficiency and environmental acceptability.

In all cases, a commercial process comprises four main stages from feed reception to delivery of one or more useful products:

1. Feed reception, storage, handling and pre-treatment;
2. Conversion of solid biomass to a more usable form of energy by means such as gasification or pyrolysis;
3. Primary product refining or clean-up;
4. Conversion of the primary product to a marketable end-product such as electricity, heat, liquid biofuels or chemicals.

While this chapter focuses on the thermal conversion of prepared biomass into a biofuel and its subsequent transformation into a usable product, the overall process requirements must not be forgotten, and these are described at the end of each main section on gasification and pyrolysis.

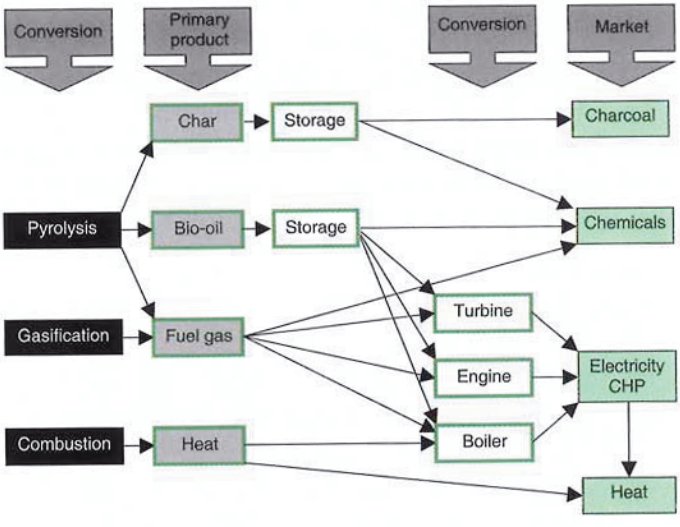


Figure 10.3 Products from thermal biomass conversion

10.3 Gasification

Fuel gas can be produced from biomass and related materials either by partial oxidation to give a mixture of carbon monoxide, carbon dioxide, hydrogen and methane with nitrogen if air is used as the oxidant, or by steam or pyrolytic gasification. Table 10.2 summarises the main products in each case. The process of gasification occurs in a number of sequential steps:

- drying to evaporate moisture;
- pyrolysis to give gas, vaporised tars or oils and a solid char residue;
- gasification or partial oxidation of the solid char, pyrolysis tars and pyrolysis gases.

The first step, drying, is a relatively fast process. The second step, pyrolysis, is also relatively fast but it is a complex process that gives rise to the tars that cause so many problems in gasification processes. When a solid fuel is heated to 300–500 C in the absence of an oxidising agent, it pyrolyses to solid char, condensable hydrocarbons or tar and gases. The relative yields of gas, liquid and char mainly depend on the rate of

Table 10.2 Modes of thermal gasification

Gasification mode	**Characteristics**
Partial oxidation with air	The main products are CO, CO_2, H_2, CH_4, N_2 and tar, giving a low heating value gas of ~5MJ/m^3. Utilisation problems can arise in combustion, particularly in gas turbines.
Partial oxidation with oxygen	The main products are CO, CO_2, H_2, CH_4 and tar (no N_2), giving a medium heating value gas of ~10–12 MJ/m^3. The cost of providing and using oxygen is compensated by a better quality fuel gas. The trade-off is finely balanced.
Steam (pyrolytic) gasification	The main products are CO, CO_2, H_2, CH_4 and tar giving a medium heating value gas of ~15–20 MJ/m^3. The process has two stages: the primary reactor produces gas and char, and the sand and char is passed to a second reactor where the char is burned with air to reheat the sand, which is then re-circulated to the first reactor to provide the heat for reaction. The gas heating value is maximised due to a higher methane and higher hydrocarbon gas content, but at the expense of lower overall efficiency due to loss of carbon in the second reactor.

heating and the final temperature, and this is discussed later in the section on fast pyrolysis. In gasification by partial oxidation, the gas, liquid and solid products of pyrolysis then react with the oxidising agent—usually air—to give the permanent gases CO, CO_2, H_2, and lesser quantities of hydrocarbon gases.

Generally in gasification processes, pyrolysis proceeds much faster than char gasification, which involves relatively slow gas–solid reactions between oxygen and char and is the rate-controlling step. Char gasification is the interactive combination of several gas–solid and gas–gas reactions in which solid carbon is oxidised to carbon monoxide and carbon dioxide, and hydrogen is generated through the water-gas shift reaction. The gas–solid reactions of char oxidation are the slowest and limit the overall rate of the gasification process. Scheme 10.1 shows the main chemical reactions. Many of these are catalysed by the alkali metals present in wood ash, but still do not reach equilibrium. The composition of the gas from char gasification and partial oxidation of the other pyrolysis products is influenced by many factors, including feed composition, water content, reaction temperature and the extent of oxidation of the pyrolysis products. However, the overall composition is essentially the equilibrium composition of the C–H–O system at the temperature of gasification.

Overall conceptual reaction

$C_6H_{10}O_5$ (average biomass) $\rightarrow$ $CO_2 + CO + H_2O + CH_4 + C_6H_{10}O_4$ (tar) + C

Heterogeneous reactions			Heat of reaction ΔH (kJ/mole at 20 C)
$C + \frac{1}{2} O_2$	$\rightarrow$	CO	− 110.6
$C + O_2$	$\rightarrow$	CO_2	− 393.8
$C + CO_2$	$\rightarrow$	2 CO	+ 172.6
$C + 2H_2$	$\rightarrow$	CH_4	− 74.9
$C + H_2O$	$\rightarrow$	$CO + H_2$	+ 131.4
Homogeneous reactions			
$CO + H_2O$	$\rightarrow$	$CO_2 + H_2$	− 41.2
$CH_4 + H_2O$	$\rightarrow$	$CO + 3H_2$	− 201.9
$2H_2 + 2CO$	$\rightarrow$	$CO_2 + CH_4$	− 243.1

Scheme 10.1 The main chemical reactions in biomass char gasification

Not all the liquid products resulting from the pyrolysis step are completely converted owing to the physical or geometrical limitations of the reactor and the chemical limitations of the reactions involved, and these give rise to contaminant tars in the final product gas. These tars tend to be refractory and are difficult to remove by thermal, catalytic or physical processes. This aspect of tar cracking or removal in gas clean-up is the most important technical uncertainty in implementation of gasification technologies and we discuss it below in Section 10.3.16.

A number of reactor configurations have been developed and tested. A recent survey (Knoef, 2000) of gasifier manufacturers found that 75% of gasifiers offered commercially were downdraft, 20% were fluid beds (including circulating fluid beds), 2.5% were updraft and 2.5% were other types. In the rest of this section, we shall review the range of gasifier technologies and their advantages and disadvantages.

10.3.1 Downdraft—fixed bed reactors

In this fixed bed configuration, solid biomass moves slowly down a vertical shaft and air is introduced and reacts at a throat that supports the gasifying biomass, as shown in Fig. 10.4. The reaction products are intimately mixed in the turbulent high-temperature region around the throat, which aids tar cracking. This is also referred to as co-current gasification. The technology is simple, reliable and proven for fuels that are relatively uniform in size and have a low content of fines below 5mm. A relatively clean gas is produced with low tar and usually with high carbon conversion. The low tar is achieved by cracking on a hot bed of char below the grate. Scale-up is limited to about 500 kg h^{-1} feed rate (dry biomass basis), owing to the need to retain a bed of

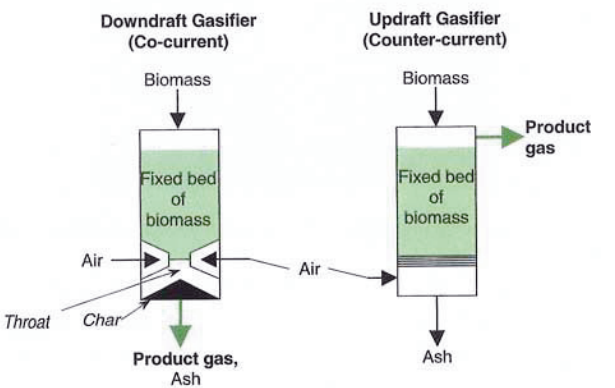


Figure 10.4 Fixed bed gasifiers

char across the throat where the air is introduced. There is a maximum feed moisture content of around 35% wet basis to avoid the heat for water evaporation consuming so much energy that the gasification reactions are quenched. Examples include the gasifiers made by Biomass Engineering (Walker *et al.*, 2001) shown in Fig. 10.5, Rural Energy (UK DTI, 2001), BTG and KARA (Knoef *et al.*, 2000), Fluidyne (Fluidyne, 2001) and Johanssen (System Johanssen, 2001).

This configuration is widely favoured for small-scale electricity generation with an internal combustion engine because of the low content of tars in the gas. A variation on the throated gasifier is the stratified or open-core downdraft gasifier in which there is no throat, the bed is supported on a grate, and char and ash are removed intermittently. Several companies in China and Indonesia use this gasifier type for rice husk gasification and power generation (Beenackers and Maniatis, 1998; Bridgwater *et al.*, 1999), and it has been further developed by Syngas Inc. (Grabowski and Brogan, 1988).

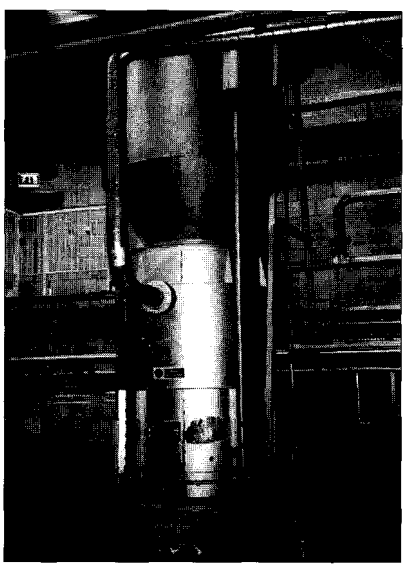


Figure 10.5 Biomass Engineering Ltd 30 kW_e downdraft gasifier at Ballymena, N. Ireland, UK.

Atmospheric downdraft gasifiers are attractive for small-scale applications up to about 1.5 MW_{th} or 300 kW_e as there is a very big market in both developed and developing economies (van Swaaij, 1994). However, the problem of efficient tar removal is still a major technical barrier and a higher level of automation is needed, especially for small-scale industrial applications.

10.3.2 Updraft—fixed bed reactors

In this fixed-bed configuration, solid moves down a vertical shaft and contacts a counter-current upward moving product gas stream (see the right-hand diagram in Fig. 10.4). The technology is simple, reliable and proven for fuels that are relatively uniform in size and have a low content of fines of below 5mm, so that channelling or and blinding the moving packed bed is not a problem. The product gas is very dirty, with high levels of tars from what is effectively distillation of volatiles from the upper part of the bed by heat from the rising hot gas. Tar crackers have been developed that effectively reduce tars to very low and acceptable levels but at the expense of reduction in efficiency and higher cost (ETSU, 1997). Scale-up is limited to around 3 dry t/h feed rate (up to about 5 MW_e) from physical size limitations and the need to ensure uniform solids flow down the reactor. The system has very high thermal efficiency and high carbon conversion and delivers a gas with a low gas exit temperature. There is good turndown capability (ability to operate below design capacity). Examples of direct heat applications include Volund (Volund website, 2001) and Bioneer (Kurkela, 1999). Wellman offers a 2.5 MW_e system for power applications (McClellan, 2000a) with their proprietary tar-cracking unit (ETSU, 1998).

Atmospheric updraft gasifiers seem to have little market attractiveness for power applications owing to the high tar levels in the product gas and the difficulty in reducing these to acceptable levels for engines or turbines. While this may be due to the high tar levels in the fuel gas, recent developments in tar cracking have shown that very low levels can be achieved from dedicated thermal/catalytic cracking reactors downstream of the gasifier (McLellan, 2000). Another possible reason is that the upper size of a single unit is around 5 MW_e so larger plants require multiple units.

10.3.3 Bubbling fluid beds

Bubbling fluid beds (see Figs. 10.6 and 10.7) offer good temperature control and high reaction rates and are much more tolerant of a wide range of feed sizes than are fixed-bed reactors. The product gas contains more particulates than fixed-bed gasifiers but tar levels are moderate. There is good scale-up potential to 10–15 dry t h^{-1} with high specific capacity and the system is easily started and stopped. A typical operating temperature for biomass gasification is about 800–850 C. Most of the conversion of the feedstock to product gas takes place within the bed, but some conversion continues in the freeboard section because entrained small particles react and thermal tar cracking occurs. In most cases, carbon conversion approaches 100%, unless

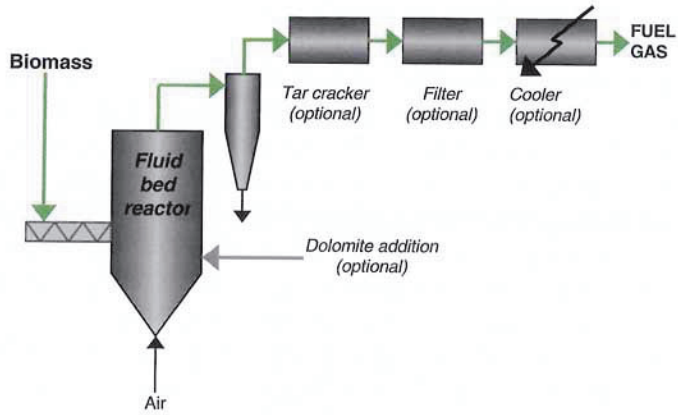


Figure 10.6 Fluid bed gasifier.

excessive carry over of fines takes place, which will occur with top feeding. Generally, there can be significant carbon loss with entrained ash. Turndown is generally limited to around 50% owing to the need to maintain fluidisation.

While tar cracking catalysts such as dolomite and nickel based catalysts can be added to the fluid bed to reduce tar levels, this is not nearly as effective as adding a second reactor in which parameters such as temperature, oxygen levels and residence

Figure 10.7 SEI Fluid bed gasifier in Florida supplying raw gas for drying.

times can be optimised for tar cracking rather than gasification. Tar cracking and conversion are discussed more fully in Section 10.3.15.

Fluidised beds have many features lacking in fixed-bed reactors, including high rates of heat and mass transfer and good mixing of the solid phase, which means that reactions rates are high and the temperature is more or less constant in the bed. Compared with dense-phase gasifiers, a relatively small particle size is desirable and this may require additional size reduction. The ash is elutriated and is removed as fine particulates entrained in the off-gas. Loss of fluidisation due to bed sintering is also a commonly encountered problem depending on the thermal characteristics and composition of the ash, but the inherently lower operating temperature of a fluid bed and better temperature control provide an acceptable control measure. The problem is that alkali metals from the biomass ash form low-melting eutectics with the silica in the sand, resulting in agglomeration and bed sintering with eventual loss of fluidisation. With biomass of high ash/inerts content it is better to use alumina. Fluidised beds are not considered economical for small-scale applications. Examples include Carbona (Salo and Horwath, 1999) and Dinamec (De Ryuck *et al.*, 1996).

Atmospheric bubbling fluidised bed (BFB) gasifiers have proved to be reliable with a variety of feedstocks at pilot scale and commercial applications in the small to medium scale, up to about 25 MW_{th}. They are limited in their capacity size range as they have not been scaled up significantly and the gasifier diameter is significantly larger than that of circulating fluid beds for the same feedstock capacity. Their market attractiveness is thus relatively high, as is their technology strength.

10.3.4 Circulating fluid beds

The fluidising velocity in a circulating fluid bed (CFB) reactor, illustrated in Fig. 10.8, is high enough to entrain large amounts of solids with the product gas. These systems were developed so that the entrained material is recycled back to the fluid bed to improve the carbon conversion efficiency compared with the single fluid bed design. A hot raw gas is produced which, in most commercial applications to date, is used for close-coupled process heat or retrofitting to boilers to recover the sensible heat in the gas. Power generation requires tar cracking and/or removal.

CFBs have all the features of bubbling beds and in addition require a large minimum size for viability of typically above 15 t/h dry feed rates. In-bed catalytic processing is not easy because of high attrition rates and a secondary catalytic cracking unit is preferred. Examples of integrated gasification and catalytic cracking include TPS (Waldheim *et al.*, 2001) and Sydkraft at Värnamo, which is a pressurised

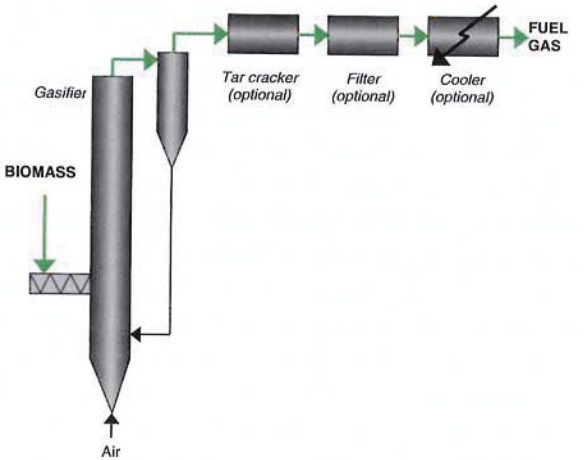


Figure 10.8 Circulating fluid bed gasifier with gas cleaning and cooling.

system (Ståhl *et al.*, 2001). Other examples include gasifiers developed by Lurgi (Vierrath and Greil, 2001) and Foster Wheeler (Nieminen, 1999).

Atmospheric circulating fluidised bed gasifiers have proved very reliable with a variety of feedstocks and are relatively easy to scale up from a few MW_{th} to ~100 MW_{th}. Even for capacities above 100 MW_{th}, there is confidence that the industry can provide reliable gasifiers. Circulating fluidised beds are the preferred system for large-scale applications and the type used by most industrial companies. These systems are technically well proven and therefore have high market attractiveness.

10.3.5 Twin fluid beds

A twin fluid bed consists of a gasifier and a char combustor. The gasifier is effectively a high temperature pyrolyser to which steam is added to improve carbon conversion and increase the hydrogen content of the gas. The by-product char is separated and burnt in a second reactor to heat the fluidising sand, which is then recirculated back to the gasifier as a heat carrier (see Fig. 10.9). The gasifier and the combustor can be either a bubbling fluid bed or a circulating fluid bed. The Austrian Energy plant at Güssing, Austria, for example, has a BFB gasifier and a CFB combustor (Hofbauer and Rauch, 2001: see Fig. 10.12 below), while the Burlington plant in Vermont, USA has a CFB gasifier and a BFB char combustor (Paisley *et al.*, 2001: see Fig. 10.11 below).

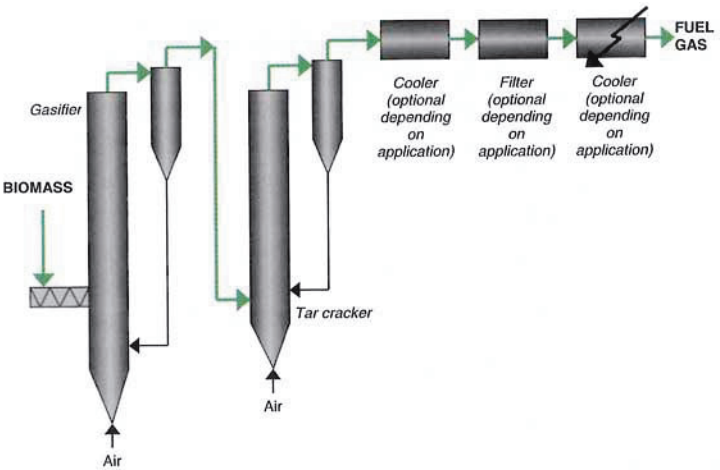


Figure 10.9 Circulating fluid bed (CFB) gasifier with CFB tar cracker as used by TPS at the ARBRE plant (see Section 10.3.10).

Twin fluid beds offer a better quality gas in terms of heating value giving a medium heating-value gas of around 12–18 MJ Nm^{-3} without requiring oxygen, as there is no dilution from the nitrogen in air. However, the gas is of poorer quality in terms of incompletely cracked tars and particulates from incompletely separated char, although catalysts can be added to the bed to improve tar cracking. The carbon conversion to gas is relatively low due to loss of carbon as charcoal, which is used to reheat the sand for the primary gasifier. The process is more complex with two close-coupled reactors with difficult scale-up and high cost and is therefore usually limited to larger scales of operation above ~10 t/h feed rate. The gasification reactor may be either a fluid bed or circulating fluid bed, as indicated in Fig. 10.10. Examples include the reactors made by Ferco, Vermont USA (Paisley *et al.*, 2001), shown in Fig. 10.11, and the Technical University of Vienna with Austrian Energy (Hofbauer and Rauch, 2001), shown in Fig. 10.12.

10.3.6 Entrained beds

In entrained flow gasifiers, no inert material is present but a finely reduced feedstock is required. Entrained bed gasifiers operate at high temperatures, about 1200–1500 C depending on whether air or oxygen is employed, and hence the product gas has low concentrations of tars and condensable gases. However, the high temperature of operation creates problems of materials selection and ash melting. Conversion in

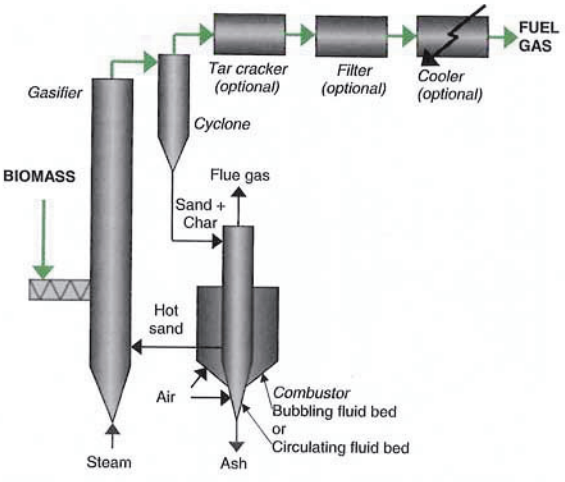


Figure 10.10 Twin fluid bed gasifier with CFB gasifier and char combustor as a CFB or bubbling bed.

entrained beds effectively approaches 100%. There is little experience with biomass in such systems, although it is the preferred technology for coal gasification. An example is the Texaco gasifier, which has been tested on biomass at pilot scale.

Entrained flow reactors have inherently simple reactor design, but are only potentially viable above around 20 dry t/h feed rate although there is good scale-up potential. There is, however, costly feed preparation needed for woody biomass to

Figure 10.11 Ferco 15 MW_e gasifier in Vermont, USA.

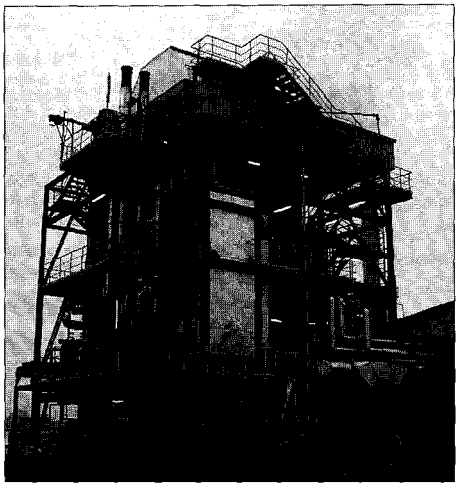


Figure 10.12 Austrian Energy 2 MW_e gasifier in Güssing, Austria.

reduce it to the small size needed for high reaction rates, there is high carbon loss with ash, and little experience with biomass. These reactors are still at an early stage of development and the requirement of a small feedstock size limits their potential.

10.3.7 Other reactors

Many other types of gasifiers have been explored. Moving bed gasifiers can operate under partial oxidation or high temperature pyrolysis conditions with mechanical transport of solids through the reactor. Technologies include multiple hearth, horizontal moving bed, sloping hearth and screw or auger kilns. Rotary kilns have good gas–solid contact, but careful design is needed to avoid solid carry over. They are popular for waste processing because solids control and mixing are both good. Examples of developers include Waste Gas and Siemens (Whiting, 1997).

Multi-stage reactors with separate pyrolysis and gasification stages give improved process control for more difficult feed materials such as sawdust and cotton residue and have been developed particularly for Municipal Solid Waste. Examples include Thermoselect (Whiting, 1997) and Compact Power (Whiting, 1997).

Cyclonic and vortex reactors have high particle velocities and high reaction rates. They have only recently been tested for biomass feedstocks and although their simplicity has some attractions, they are still unproven.

10.3.8 Pressurised gasification

Pressurised gasification is of considerable interest as the extensive gas cleaning and cooling in atmospheric pressure systems can be minimised and efficiency improved. These gasifiers operate under pressures of typically 15–30 bars, with the hot gas being cooled to around 500 C to aid precipitation of alkali metals on the particulates prior to hot gas filtration to remove the particulates but retain tars in the vapour phase. Some heat can be recovered from the hot gas. The tars pass through the filter and are burned with the hot gas in the gas turbine, thus retaining most of the sensible heat and the chemical energy of the tars in the gas. Both tar cracking and gas cleaning and cooling are avoided, reducing complexity and costs, as well as avoiding the gas compressor prior to the gas turbine required in an atmospheric-pressure system.

Pressurised feeders need significant quantities of inert gas for flushing the lock hoppers during the feeding cycle. The pressurised air supply for the gasifier is usually derived from a compressor on the gas turbine shaft. Greater flexibility and potentially better control is derived from a separate air compressor, but at the expense of higher cost and lower efficiency. Figure 10.13 shows a pressurised gasifier with power generation in open cycle. Both capital and operating costs are significantly higher for pressurised operation, although these are to some extent balanced by savings from reduced vessel and piping sizes, the avoidance of a gas compressor for the gas turbine and higher efficiencies. Examples include the circulating fluid bed demonstration plant at Värnamo, built and operated by Foster Wheeler and Sydkraft (Ståhl *et al.*, 2001), which finished operation in 2000, and Carbona (Salo and Horwath, 1999).

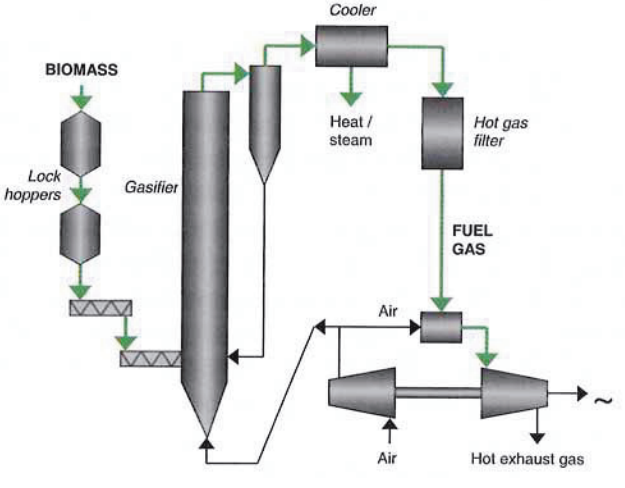


Figure 10.13 Pressurised gasifier with power generation in open cycle.

Pressurised fluidised bed systems, whether circulating or bubbling, are generally considered of limited short-term market attractiveness owing to the more complex operation of the installation and the additional costs of construction of pressurised vessels. Pressurisation offers significant potential efficiency and cost advantages in IGCC (Integrated Gasification Combined Cycle) applications, but large sizes are needed to justify the additional costs, currently viewed as typically above 50 MW_e. At this scale, circulating fluid beds are preferred.

No company is known to be developing pressurised systems for downdraft, updraft, cyclonic or entrained bed gasifiers for biomass feedstocks and it is difficult to imagine that such a technology could ever be developed into a commercial product because of the inherent problems of scale and cost. While the relative advantages and disadvantages of pressurised gasification systems and atmospheric systems have not been fully resolved, Table 10.3 gives a summary.

Table 10.3 Features of pressure and atmospheric gasifiers

Pressurised gasifiers	**Atmospheric gasifiers**
• Feeding is more complex and very costly, and has a high inert gas requirement for purging. • Capital costs of pressure equipment are much higher than atmospheric equipment, although sizes are much smaller (Bridgwater and Evans, 1993). • Gas is supplied to the turbine at pressure, removing the need for gas compression and also permitting relatively high tar contents in the gas. Hot gas clean-up also reduces energy losses and in principle is simpler and has lower overall costs than scrubbing systems, • Overall system efficiency is higher owing to retention of sensible heat and chemical energy of tars in the product gas.	• For gas turbine applications, the product gas is required to be sufficiently clean for compression prior to the turbine. For engine applications the gas quality requirements are less onerous and pressure is not required. • Atmospheric systems have a potentially much lower capital cost at smaller capacities of below around 30 MW_e (Bridgwater and Evans, 1993). • Gas compositions and heating values are not significantly different for either system.

10.3.9 Oxygen gasification

Oxygen can be used to replace air either partially or completely as the oxidant. The advantages of using oxygen include:

1. Higher reaction temperatures, which can lead to lower tar levels and smaller gasifiers;
2. Lower gas volumes from the reduction or absence of nitrogen, leading to smaller vessel and piping sizes and hence lower costs;
3. The reduction or absence of nitrogen leads to more efficient use of gas for synthesis of, for example, liquid fuels, and requires a lower level of modifications in boilers, engines and turbines;
4. Improved heating value of gas, requiring fewer modifications to burner.

There is, however, a significant energy and financial cost associated with the use and supply of oxygen, from both its procurement and the additional measures needed to mitigate hazards in handling and use. The overall impact on process performance is currently considered neutral. In electricity generation, there is no evidence that the benefits of producing higher heating value gas with oxygen gasification justify the cost of providing and using oxygen. However, in applications aiming to produce a clean synthesis gas for chemical synthesis (of, for example, biomethanol or biohydrogen), oxygen gasification is the preferred route in order to avoid having to handle high levels of nitrogen in the process.

10.3.10 Integrated gasification combined cycles

Integrated Gasification Combined Cycles (IGCC) are systems where the gas turbine cycle is followed by a steam cycle after recuperation of the waste heat in a waste-heat boiler (HRSG). They offer high efficiencies of potentially up to 50%. IGCC systems can be operated with an atmospheric pressure gasifier or a pressurised gasifier (see Section 10.3.8). Figure 10.14 shows an atmospheric gasification IGCC system, while Fig. 10.15 shows a pressurised gasification IGCC system for comparison.

For an atmospheric pressure gasifier, the product gas requires compression to be fired in the gas turbine combustor and compression requires a clean, cool gas. The air supply to the gasifier would probably be provided independently, although a bleed from the air compression loop could be used. This latter choice would require extensive compressor modifications and impose control problems on the system. Thus a separate additional compressor is usually specified. A major requirement is for a

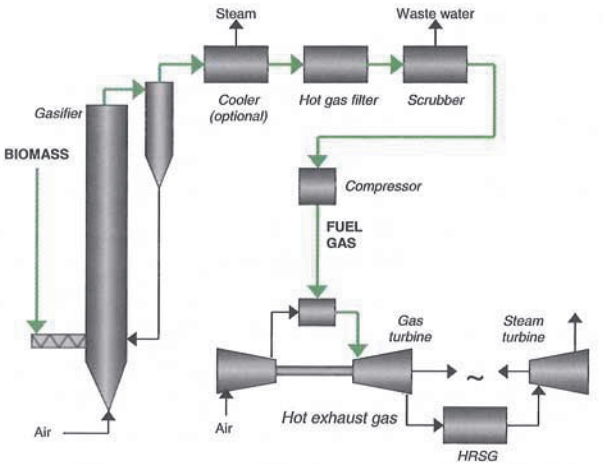


Figure 10.14 Atmospheric gasification with co-generation (IGCC).

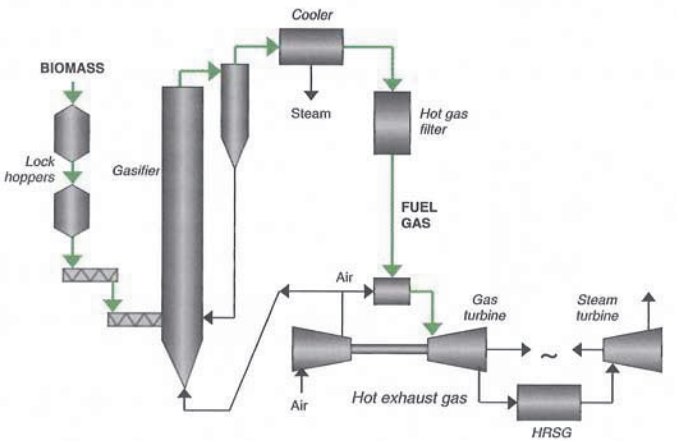


Figure 10.15 Pressurised gasification with co-generation (IGCC).

clean cool gas to be fed to the fuel gas compressor, which requires a high degree of gas cleaning and cooling prior to the compressor. Considerable efforts are being made to minimise the cost of the gas-cleaning step.

Several projects have been initiated for IGCC applications over the last decade but only two have been implemented, the Sydkraft plant at Värnamo, which is based on Foster Wheeler technology (Ståhl *et al.*, 2000), and the ARBRE plant based on TPS technology (Pitcher and Weekes, 2001). Because of their potential significance in the medium to long term, we will describe these in more detail.

The Värnamo Plant in Sweden

The plant in Värnamo (see Fig. 10.16) produces about 6 MW_e electricity for the grid as well as providing 9 MW_{th} to the district heating system of the city of Värnamo, from a total fuel input equivalent to 18 MW_{th}. The accumulated operating experience amounts to about 8500 hours of gasification and more than 3600 hours of gas turbine operation on biomass-derived gas. A successful test programme addressing fuel flexibility and NO_x emission problems was completed in 2000. A range of fuels including wood, bark, forest residues, willow grown as an energy crop, straw and RDF (Refuse-Derived Fuel) have been used without any major operating problems.

However, some problems occurred in the hot gas filtration system, where some ceramic filter candles broke due to micro-cracking in the filter elements. Sintered metal filters were installed in 1999, and have given no trouble. The gas quality has remained at the calculated levels over a wide range of operating conditions with a lower heating value in the range 5.3–6.3 MJ Nm^{-3}. Other gas components, such as tars, were not continuously monitored. Extensive measurements were, however, made during most of the tests, and tars have never caused any problems in the plant. Table 10.4 gives an example of the influence of fuel composition on the tars.

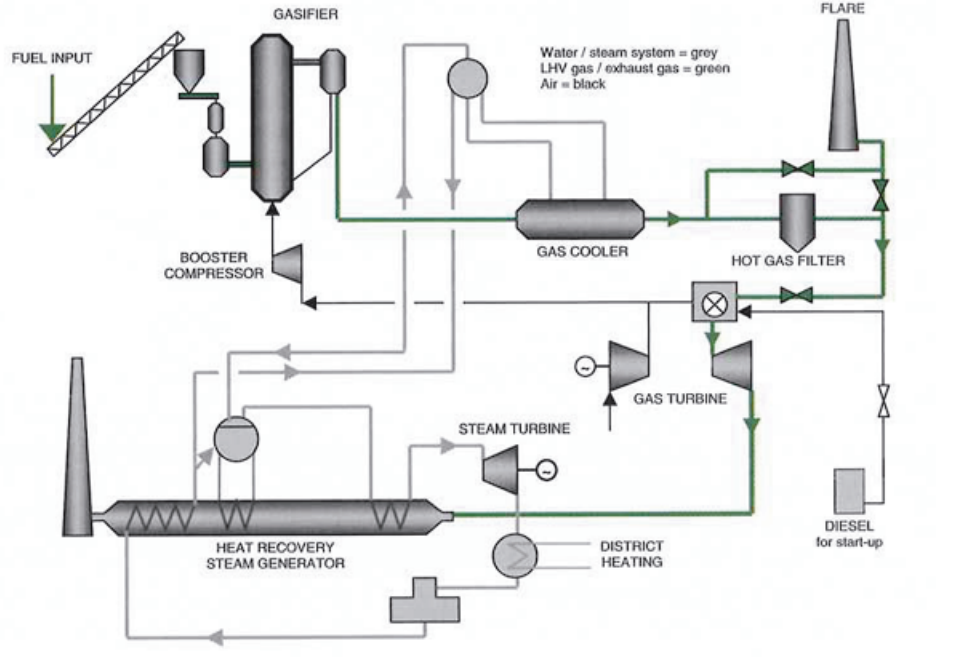


Figure 10.16 Process flow diagram of the Värnamo plant.

Table 10.4 Effect of feedstock on tar content of product gas from Värnamo

Fuel	**Benzene (mg/nm^3)**	**Light tars (mg/nm^3)**
Bark 60% + forest residue 40%	5000 – 6300	1500 – 2200
Cellulose chips	7000 – 9000	2500 – 3700

The ARBRE Plant in Yorkshire, UK

Construction of this plant in Eggborough, Yorkshire, UK was completed in late 2001. All equipment has been commissioned and the IGCC cycle has been demonstrated over limited period of time. This plant will provide a net electrical output of 8 MW_e at an efficiency of 30.6%. The sale of electricity to the grid is supported by the UK's REO (Renewable Energy Obligation) programme. The process flowsheet follows the scheme depicted in Fig. 10.9, and Figs. 10.17 and 10.18 show pictures of the plant. The plant will mainly be fuelled by short rotation coppice grown in the Yorkshire area. A slurry of treated domestic sewage sludge which is low in heavy metals, pathogens and odour is applied to the coppice plantations as organic fertiliser to increase crop yields. Waste ash from both the gasifier and the catalytic cracker will be recycled to the coppice plantations as a soil conditioner and a source of base cations and micronutrients to improve soil fertility.

The TPS circulating fluidised bed gasifier operates at temperatures between 850 and 900 C. In the TPS process, the tars are catalytically cracked in a second circulating fluidised bed reactor, which is built in the same way as the gasifier, and has similar dimensions. The gas is introduced at the bottom of the catalytic reactor, together with a controlled amount of air to raise the temperature, and contacts the bed

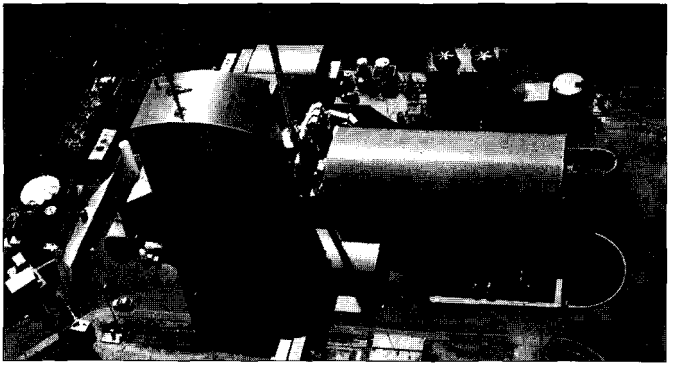


Figure 10.17 Aerial view of the ARBRE plant showing the gasifier building on the left

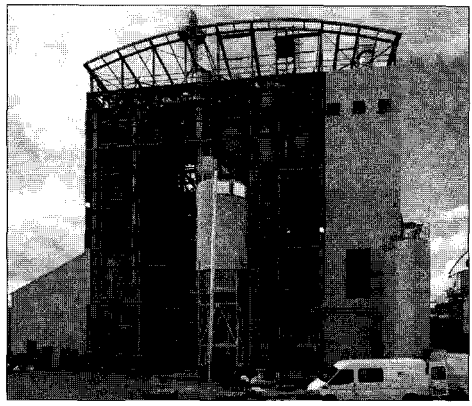


Figure 10.18 ARBRE circulating fluid bed gasifier.

material, dolomite, at about 900 C. The gas is then cooled to 180 C in a cooler, which generates high-pressure saturated steam. The gas leaving the cooler enters a conventional high efficiency bag-house utilising needle fibre bags. The gas then passes to a combination cooler and scrubber where its temperature is reduced to 25 C and any remaining alkalis, tars and ammonia are removed. Liquid effluent from the gas scrubber is treated in a wastewater treatment plant. Facilities will be provided for pelletisation of dolomite-containing ash for use as slow-release fertiliser to be applied in the energy plantations.

After compression to about 20 bars, the gas is fired in an ABB Alstom Typhoon gas turbine (the same model as in the Värnamo plant), shown in Fig. 10.19. The exhaust gas from the gas turbine exits at approximately 475 C and is used to generate

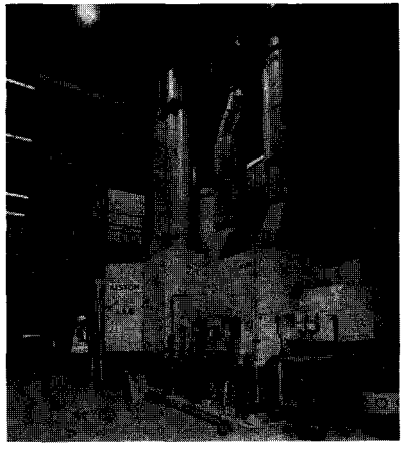


Figure 10.19 Alstom 3 MW_e gas turbine at ARBRE.

exhaust gas from the gas turbine exits at approximately 475 C and is used to generate steam. Further steam is raised by burning addition gas from the gasifier to provide a total of 5.5 MW_e in a steam turbine. Waste heat is used to dry the biomass fuel.

10.3.11 Status of biomass gasification technology

There is still very little information on costs, emissions, efficiencies, turn-down ratios and actual operational experience for gasification technologies. In particular, no manufacturer is willing to give full guarantees for technical performance of their gasification technology. This confirms the limited operating experience and the limited confidence in the technology. Figure 10.20 suggests a relationship between gasification technologies in terms of their strength and their market attractiveness for power generation.

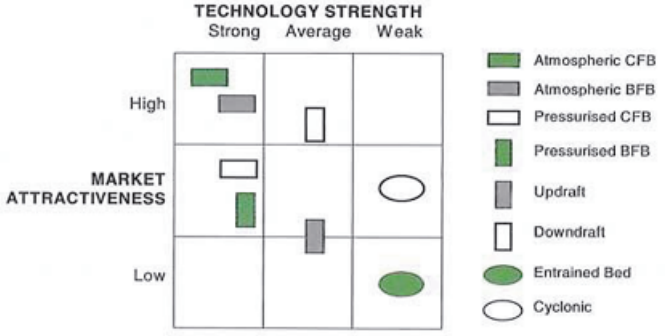


Figure 10.20 Technology status of biomass gasification (derived from Maniatis, 2001).

Atmospheric downdraft gasifiers are attractive for small-scale applications up to about 1.5 MW_{th} as there is a very big market in both developed and developing economies (Limbrick, 2000). However, the difficulty of efficient tar removal is still a major problem and a higher level of automation is needed, especially for small-scale industrial applications. Nevertheless, recent progress in catalytic conversion of tar gives more credible options and these systems can therefore be considered of average technical strength.

Atmospheric updraft gasifiers seem to have little market attraction for power applications. While this may be due to the high tar levels in the fuel gas, recent developments in tar cracking have shown that very low levels can be achieved from dedicated thermal/catalytic cracking reactors downstream of the gasifier (BTG, 2000).

Another possible reason is that the upper size of a single unit is around 2.5 MW_e so larger plant capacities require multiple units.

Atmospheric bubbling fluidised bed gasifiers have proven to be reliable with a variety of feedstocks at pilot scale and commercial applications in the small to medium scale, up to about 25 MW_{th}. They are limited in their capacity size range as they have not been significantly scaled up and the gasifier diameter is significantly larger than that of circulating fluid beds for the same feedstock capacity. On the other hand, they are more economic for small to medium range capacities. Their market attractiveness and technology strength are thus relatively high.

Atmospheric circulating fluidised bed gasifiers have proved very reliable with a variety of feedstocks and are relatively easy to scale up from a few MW_{th} to 100 MW_{th}. Even for capacities above 100 MW_{th}, there is confidence that the industry would be able to provide reliable gasifiers. These gasifiers appear to be the preferred system for large-scale applications and most industrial companies use them; these systems therefore have high market attractiveness and are technically well proven.

Atmospheric cyclonic gasifiers have only recently been tested for biomass feedstocks and although they have medium market attractiveness because of their simplicity, they are still unproven. Finally, atmospheric entrained-bed gasifiers are still at a very early stage of development and since they require feedstock of a very small particle size, their market attractiveness is very low.

Pressurised fluidised bed systems, either circulating or bubbling, are considered of more limited market attractiveness because of their more complex installation and the additional costs of construction of pressurised vessels. However, pressurised fluidised bed systems have the advantage in integrated combined cycle applications as the need to compress the fuel gas prior to utilisation in the combustion chamber of the gas turbine is avoided. No company is known to be developing pressurised systems for downdraft, updraft, cyclonic or entrained-bed gasifiers for biomass feedstocks, and it is difficult to imagine that such a technology could ever be developed into a commercial product due to the inherent problems of scale, tar removal and cost.

In conclusion, for large-scale applications (above 25–50 MW_e) the preferred and most reliable system is the circulating fluidised bed gasifier, while for small-scale applications (up to 0.5 MW_e) downdraft gasifiers are the most extensively studied. Bubbling fluidised bed gasifiers can be competitive for medium-scale applications. Large-scale fluidised bed systems have become commercial by reason of successful co-firing projects (see below), while moving-bed gasifiers are still trying to achieve this. Figure 10.21 shows an overall summary of the range of applications for each technology and representative efficiencies for power generation.

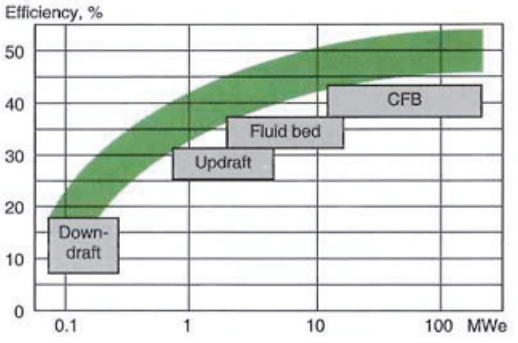


Figure 10.21 Relationship between technology, scale and efficiency for electricity production.

10.3.12 Fuel gas quality

The fuel gas quality requirements, for turbines and liquid fuel synthesis in particular, are very high; Table 10.5 gives some suggested figures. Tar is a particular problem and remains the most significant technical barrier. There are two basic ways of destroying tars (Bridgwater, 1994), both of which have been and continue to be

Table 10.5 Some notional gas turbine fuel specifications

Contaminants		**Tolerance examples**
Minimum gas heating value (LHV)		4–6 MJ Nm^{-3}
Minimum gas hydrogen content		10–20%
Maximum alkali concentration		20–1000 ppb
Maximum delivery temperature		450–600 C
Tars at delivery temperature		All in vapour form or none
Maximum particulates (ash, char *etc.*)	Particle size, μm	Concentration, ppm wt
	>20	0.1
	10–20	1.0
	4–10	10.0
NH_3		No limit
HCl		0.5 ppm
S (H_2S + SO_2 *etc.*)		1 ppm
N_2		No limit
Combinations of contaminants	Total metals	< 1 ppm
	Alkali metals + sulphur	< 0.1 ppm

extensively studied: by catalytic cracking using, for example, dolomite or nickel; or by thermal cracking, for example by partial oxidation or direct contact. The gas is very costly to store or transport so it has to be used immediately. Hot gas efficiencies for the gasifier (total energy in the raw product gas as a fraction of the energy in the feed) can be as high as 95–97% for close-coupled turbine and boiler applications, and up to 85% for cold gas efficiencies. In power generation using combined cycle operation, efficiencies of up to 50% for the largest installations have been proposed, reducing to 35% for smaller applications (Solantausta, 1999; Bridgwater *et al.*, 2002). Maniatis (2001) and Bridgwater (1995) have published comprehensive reviews. Table 10.6 illustrates typical gasifier and product gas characteristics, and Table 10.7 gives typical product gas compositions for the common gasifiers.

Table 10.6 Typical gas and gasifier characteristics

Gasifier type	Gas HHV, MJ Nm^{-3}	Tars	Solids	Turn-down	Scaling up	Size range, dry t h^{-1} [a]
Fixed bed						
Downdraft [A]	5	V. low	Moderate	Good	Poor	0.05 – 0.5
Downdraft [O]	10	V. low	Moderate	Good	Fair	0.5 – 2
Updraft [A]	6	V. high	Moderate	Good	Good	1 – 5
Updraft [O]	10	High	Moderate	Good	Good	3 – 10
Fluid bed						
Bubbling bed [A]	5	Fair	High	Good	Good	1 – 10
Circulating bed [A]	5	Low	V. high	Good	V. good	10 – 50
Circulating bed [O]	10	Low	V. high	Good	V. good	20 – 50
Entrained bed [A]	5	Low	V. high	poor	Good	5 – 30
Twin reactor [P]	15	High	High	Fair	Good	10 – 50
Other						
Rotary kiln [P]	10	High	High	Poor	Fair	2 – 20
Cyclone reactor [A]	5	Low	V. high	Poor	Fair	1 – 10
Horizontal bed [A]	5	High	Low	Fair	Fair	1 – 5
Auger kiln [P]	10	High	Low	Fair	Fair	0.5 – 5

[a] Typical size range based on dry biomass feed rate, dry tonnes h^{-1}; [A] Air-blown gasifier; [O] Oxygen blown; [P] Indirectly heated gasifier (steam gasifier or pyrolyser). Green cells indicate desirable characteristics, black cells indicate undesirable ones, and grey cells moderate ones.

Table 10.7 Typical product gas characteristics from different gasifiers

	Gas composition, dry, vol%					HHV[a]	Gas quality	
	H_2	CO	CO_2	CH_4	N_2	MJ Nm^{-3}	Tars	Dust
Fluid bed air-blown	9	14	20	7	50	5.4	Fair	Poor
Updraft air-blown	11	24	9	3	53	5.5	Poor	Good
Downdraft air-blown	17	21	13	1	48	5.7	Good	Fair
Downdraft oxygen	32	48	15	2	3	10.4	Good	Good
Twin fluid bed	31	48	0	21	0	17.4	Fair	Poor
Pyrolysis for comparison	40	20	18	21	1	13.3	Poor	Good

[a] HHV = Higher Heating Value.

10.3.13 Gas clean-up

Gases formed by gasification will be contaminated by some or all of the constituents listed in Table 10.8. The level of contamination will vary depending on the gasification process and the feedstock. Gas cleaning must be applied to prevent erosion, corrosion and environmental problems in downstream equipment.

Table 10.8 Fuel gas contaminants and their problems

Contaminant	Examples	Problems
Particulates	Ash, char, fluidised bed material	Erosion
Alkali metals	Sodium, potassium compounds	Hot corrosion
Fuel-bound nitrogen	Mainly ammonia and HCN	NO_x formation
Tars	Refractive aromatics	Clogs filters Difficult to burn Deposits internally
Sulphur, chlorine	HCl, H_2S	Corrosion emissions

10.3.14 Hot gas clean-up for particulates

Gas streams from biomass gasification carry very small carbon-containing particles which are difficult to remove by cyclones. Tests using high efficiency cyclones

showed that particulates levels were not reduced to less than 5–30 g Nm^{-3} (Kurkela *et al.*, 1993). For this reason barrier filtration methods such as sintered metal or ceramic filters are preferred. This is particularly important for pressurised systems where the sensible heat of the gas needs to be retained as well as avoiding scrubbing systems for tar removal.

High-temperature ceramic or metal candle filters have been tested with gasification products from peat and coal. Many designs do not give a constant pressure drop across the filter, but rather this increases as the deposits build up. One solution is to layer the filters, and where this is done removal efficiencies in excess of 99.8% have been reported. Tests on wood-derived gases have presented a further problem with filter clogging by soot derived from thermal cracking of tars both in the gas phase and on the filter surface. Cooling the gas to below 500 C and reducing gas face velocities across the filter surface can reduce this problem. However, if temperatures fall below 400 C, there is still a potential problem with tar deposition. Recent developments employ ceramic candle filters with automatic pulsing to strip off the accumulated filter cake. However, there have been problems with the mechanical strength of ceramic filters and their susceptibility to thermal shock. Sintered metal filters are more robust and a guard filter is advisable in all cases to protect sensitive downstream equipment such as the gas turbine.

10.3.15 Tar destruction

Tar concentration is mainly a function of gasification temperature, with tar levels reducing as the temperature increases. The relationship between temperature and tar level is a function of the reactor type and processing conditions. The tars formed in the pyrolysis stage of gasification are subsequently thermally cracked in most environments to refractory tars, soot and gases.

While tar cracking catalysts such as dolomite can be added to the bed, more effective tar conversion comes from a secondary reactor which provides better temperature control and mixing (Corella *et al.*, 1988; Simell and Kurkela, 1997).

Tar levels and characteristics are also dependent on the feedstock. Tests have shown that tar production in wood gasification is much greater than in coal or peat gasification and that the tars tend to be heavier, more stable aromatics (Kurkela *et al.*, 1993). These may partially react to give soot from gas-phase reactions, which can block filters: this appears to be a problem peculiar to biomass gasification.

There are three basic ways of destroying tars (Bridgwater, 1994):

- by catalytic cracking using, for example, dolomite;
- by catalytic reforming using, for example, nickel based catalysts;
- by thermal cracking, for example by partial oxidation or direct contact with a heated surface.

Catalytic cracking and reforming

Tar conversion in excess of 99% has been achieved using dolomite, nickel-based and other catalysts at elevated temperatures of typically 800–900 C. These tests have been performed using both fossil and renewable feeds. Most reported work uses a second reactor. Some work has been carried out on incorporation of the catalyst in the primary reactor, which has often been less successful than use of a second reactor (*e.g.* Corella *et al.*, 1988) although this approach has been selected for the Biopower plant at Värnamo in Sweden (Ståhl *et al.*, 2001; Bridgwater and Evans, 1993). Elevated freeboard temperatures thermally crack tars and can reduce the load on the catalytic cracker.

Catalyst deactivation is generally not a problem with dolomite. An initial loss of activity is sometimes experienced as carbon compounds settle on the catalyst, but these compounds gasify as the bed temperature rises and the catalyst is reactivated. Metal catalysts tend to be more susceptible to contamination. Low hydrogen concentrations in the product gas will reduce the catalytic activity of metal-based systems. The low sulphur content of biomass gases can reduce the activity of metal sulphide catalysts by stripping out the sulphur.

Dolomite and limestone have limited mechanical strength and suffer from severe attrition resulting in losses by elutriation. This requires constant addition of new material, particularly in circulating fluid bed systems, where the highest rates of attrition occur, and thus only relatively inexpensive and readily available catalyst material can be used.

Thermal cracking

Tar levels can be reduced to levels found in downdraft systems by thermal cracking at 800–1000 C. However, biomass-derived tars are more refractory and harder to crack by thermal treatment alone, as indicated in Fig. 10.22. As discussed above, elevated freeboard temperatures in fluid bed gasifiers provide some thermal tar cracking.

There are several ways of achieving thermal cracking:

- Increasing residence time after initial gasification such as in a fluid bed reactor freeboard, but this is only partially effective;
- Direct contact with an independently heated hot surface, which requires a significant energy supply and thus reduces the overall efficiency. This is also only partially effective due to reliance on good mixing;
- Partial oxidation by addition of air or oxygen. This increases CO_2 levels, reduces efficiency and increases cost for oxygen use. It can be very effective particularly at the high temperatures achieved with oxygen gasification of 1300 C or more.

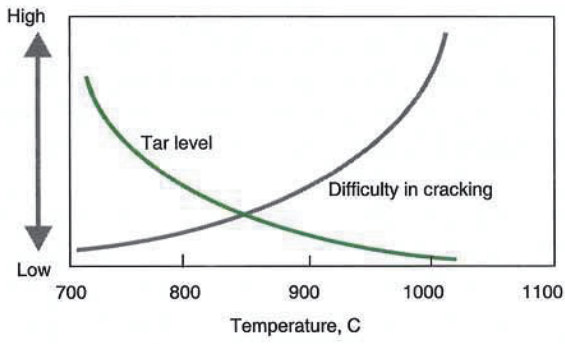


Figure 10.22 Relationship between tar levels, cracking difficulty and temperature.

10.3.16 Tar removal

Water scrubbing is widely assumed to be a proven technique for physical removal of particulates, tars and other contaminants. Unfortunately, most experience is not so reassuring and there are many reported problems, particularly in the poor removal efficiencies of tars although surprisingly little hard data is available (Mackie, 1993). These tars require more physical capture and agglomeration or coalescence than simple cooling. Biomass-derived tars are very difficult to coalesce and a complex treatment system is likely to be required even to attain tar removal levels of 90%.

A typical system will include a saturator to cool and saturate the gas for coalescence of particulates and tars in the next stage. A high-efficiency scrubber then follows to contact the contaminants intimately and reduce the pressure so that the water will condense onto the particulates and tar droplets, thus increasing their size

and improving their susceptibility to agglomeration and coalescence. The final stage is to provide a high-residence time tower to allow the system to equilibrate. Tar levels down to 20–40 mg Nm^{-3} and particulate levels down to 10–20 mg Nm^{-3} have been claimed with such a system. Soluble gases such as ammonia, and soluble solids such as sodium carbonate are effectively removed.

These systems are fairly expensive and create a waste disposal problem by generating large quantities of contaminated water. The wastewater can usually be treated by conventional biological processes unless there is a high recycle ratio, when more concentrated solutions will be produced requiring special disposal methods such as incineration.

Cooling the product will also reduce electrical efficiency, but is essential for applications in engines to provide the highest energy density gas. A few attempts have been made to scrub with oil, which was thought more likely to capture the tars, but the consequential problems outweighed any benefits. Electrostatic precipitation is an effective but costly way of removing tars, but there is little experience on biomass-derived gasification products although it has proved very successful in capturing fast pyrolysis-derived aerosols (Section 10.4.11).

10.3.17 Alkali metals

Alkali metals exist in the vapour phase at high temperatures and will therefore pass through particulate removal devices unless the gas is cooled. The maximum temperature that is considered to be effective in condensing metals is around 600 C. Tests on alkali species have shown that their gaseous concentrations fall with temperature to the extent that concentrations are close to turbine specifications at temperatures below 500–600 C (Kurkela *et al.*, 1993). Thus it is possible that gas cooling to this level will cause alkali metals to condense onto entrained solids and be removed at the particulate removal stage. Alkali metals may also damage ceramic filters at high temperatures, and a hot gas clean-up system will thus first have a cooler before the hot gas filter.

Alkali metals cause high temperature corrosion of turbine blades, stripping off their protective oxide layer. For this reason it is widely believed that their concentration must not exceed 0.1 ppm at entry to the turbine. There is no experience with modern coated blades in such an environment.

10.3.18 Fuel-bound nitrogen

50–80% of fuel-bound nitrogen is converted to ammonia and lesser quantities of other gaseous nitrogen compounds during gasification. These compounds will cause potential emissions problems by forming NO_x during combustion. There are three ways of approaching the problem of NO_x emissions, any of which may be used singly or in combination:

- reduce the formation of NO_x by limiting fuel-bound nitrogen in the feedstock through careful selection of biomass types and/or blending;
- use low-NO_x combustion techniques;
- use selective catalytic reduction (SCR) at the exhaust of the engine or turbine.

Nitrogen-containing contaminants all exist in the vapour phase and will therefore pass through all particulate removal devices. Catalytic conversion methods can sometimes remove ammonia but this is dependent on the catalyst. Some catalysts are reported to increase ammonia content by releasing nitrogen bound in tars. Water scrubbing is effective in removing these soluble impurities, but results in loss of sensible heat and thus poorer efficiencies. SCR involves a reaction between ammonia and NO_x to form nitrogen and water. This is well-established technology and is often specified in exhaust gases from engines and turbines. There is, however, a cost and efficiency penalty.

10.3.19 Sulphur and chlorine

Sulphur is not generally considered to be a problem because biomass feeds have very low sulphur content. However, the specification on turbines is typically 1 ppm or less, and even lower if co-contaminants such as alkali metals are present. Some gas compositions are as high as 0.01% S, which represents 100 ppm. Sulphur removal may therefore be necessary where turbines are used, and this can often be achieved with a conventional sulphur guard. Dolomite (CaO.MgO), often used for tar cracking, will also absorb significant proportions of sulphur. Sulphur concentrations will be lower than those produced by fossil fuel combustion, and hence expensive sulphur removal trains are not necessary. If a dolomite tar cracker is included in the process, this will reduce the sulphur levels considerably, but possibly not to the low levels required. A sulphur guard, consisting of a hot fixed bed of zinc sulphide, is likely to be adequate for the concentrations expected. This would be relatively inexpensive to install but would create a waste disposal problem from the zinc sulphide produced.

Chlorine is another potential contaminant that can arise from pesticides and herbicides and in biomass crops grown near the sea and waste materials. As international trade in biomass grows, sea transport may also increase chlorine levels in the biomass being transported. Levels of 1 ppm are often quoted, but this is a function of the temperature, chlorine species, co-contaminants and materials of construction. The behaviour of chlorine and metals at elevated temperatures is well understood. Chlorine and compounds can be removed by absorption in active material either in the gasifier or in a secondary reactor, or by dissolution in a wet scrubbing system. Dolomite and related materials are less effective at removing chlorine than sulphur. Table 10.9 summarises all the likely contaminants and the methods of clean-up.

Table 10.9 Summary of gas cleaning options

Contaminants	**Clean-up method**
Ash	Filtration, scrubbing
Char	Filtration, scrubbing
Inerts	Filtration, scrubbing
NH_3	Scrubbing, SCR (Selective Catalytic Removal)
HCl	Lime or dolomite, scrubbing, absorption
SO_2	Lime or dolomite, scrubbing, absorption
Tar	Tar cracking, or tar removal
Na	Cooling, condensation, filtration, adsorption
K	Cooling, condensation, filtration, adsorption
Other metals	Cooling, condensation, filtration, adsorption

10.3.20 Applications of product gas

Figure 10.23 summarises the range of fuel, electricity and chemical products that can be derived from the product gas. Medium heating value gas from steam or pyrolytic gasification, or from oxygen gasification, is better suited to synthesis of transport fuels and commodity chemicals because of the absence of diluent nitrogen, which would pass through unchanged but reduce process efficiency and increase costs. The exception is ammonia synthesis, where the nitrogen content derived from air gasification can be utilised in the ammonia synthesis process.

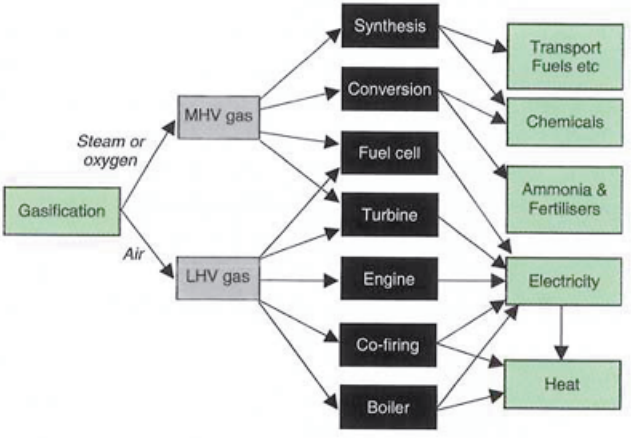


Figure 10.23 Applications for gas from biomass gasification

10.3.21 Electricity

There are two basic machines for generating electrical power from product gas: turbines and engines. There is no clear allocation of choice of machine and system size, but the orthodox view is that engines are more suitable up to 5–10 MW_e and turbines are preferable above 10–20 MW_e for an atmospheric pressure gasifier and above 20–30 MW_e for a pressure gasifier. Turbines become more attractive at larger sizes, particularly for IGCC and similarly advanced cycles when higher efficiencies can be achieved and economies of scale become more noticeable. Engines have the advantages of robustness, high efficiency at low sizes, higher tolerance to contaminants than turbines (up to 30 ppm tars can be tolerated), easier maintenance, and they also have wide acceptability. However, operation in combined cycle mode is rarely justified as only a small increment in efficiency can be gained. There is poor economy of scale as capacity is more a function of the number of cylinders than cylinder size, and specific capital costs are typically independent of size. Engine gen-sets are, however, available up to 50 MW_e, and gas turbines have been successfully used at 3 MW_e. The two main types of turbine are industrial and aero-derived. Industrial turbines are more robust, less demanding and have lower efficiency than aero-derived turbines. There is considerable interest in the potential of fuel cells for power generation, but as yet very little activity in fuel cells based on biomass.

Where gas turbines are used in conjunction with a gasifier operating at atmospheric pressure, the product gas will require compression before combustion. This imposes severe gas quality requirements if damage to the compressor is to be avoided. The air supply to the gasifier would probably be provided independently, although a bleed from the air compression loop could be used. This latter choice would, however, require extensive compressor modifications and impose severe control problems on the system, similar to those for a pressure gasifier. A pressurised gasifier would either use compressed air from the compressed air loop on the turbine set or would have an independent air compressor. The latter solves some of the potential control problems that arise from integration of the gasifier operation with the turbine, but at the expense of higher cost and lower system efficiency.

Engines have more tolerant control requirement since conventional fuel mixing devices and orthodox engine management systems can be used. They tend to react positively and quickly to variations in gasifier output without adversely affecting the gasifier operation. There is extensive practical experience of such systems from small-scale gasifier operations as well as landfill gas operations.

Fuel cells place high demands on gas quality, exceeding the requirements for gas turbines. The absence of any experience of fuel cells using biomass-derived gas limits discussion to speculation that the demands will be difficult to achieve.

The simple gas turbine cycle is not very efficient since there is considerable energy wasted in the hot exhaust gases. Adding a heat recovery system to the gas turbine exhaust, as shown earlier in Fig. 10.15, can increase efficiency. This would usually generate steam to power a steam turbine in a combined cycle mode, or the steam can be mixed with the combustion gases and fed through the gas turbine in a steam injected gas turbine (STIG) cycle. Combined cycle operation will utilise not only the waste heat from the exhaust but also the heat recovered from the primary gas cooler before filtration.

One way of avoiding the problems of gas cleaning is to use a Brayton cycle to burn the fuel in a separate combustor and heat the turbine gases indirectly via a high-temperature gas/gas heat exchanger. This approach is applied at the Free University of Brussels (VUB) CHP plant. (De Ryuck *et al.*, 1994). The main problem is the loss of efficiency caused by indirect heating of the turbine working gases, and the temperature limits imposed by the heat exchanger materials, which limits the attainable efficiency. While indirect heating improves turbine reliability, there are costs incurred in flue gas treatment and the gas/gas heat exchanger.

In summary, system optimisation is a major requirement and requires careful consideration and evaluation.

10.3.22 Transport fuels and other chemicals

The basic raw material for the production of transport fuels and other commodity chemicals is synthesis gas or syngas, as it is usually known. Syngas is a mixture of carbon monoxide (CO) and hydrogen (H_2). There are usually other components arising from gasification such as carbon dioxide (CO_2), methane (CH_4), higher hydrocarbons such as ethylene and ethane, propane and propylene, and nitrogen from air gasification. Generally these act as diluents, but different generic and specific processes have different levels of tolerance for each component. There will also be trace contaminants containing sulphur (*e.g.* H_2S), chlorine (*e.g.* HCl, COCl) and nitrogen (*e.g.* ammonia NH_3) in a range of compounds. The concentrations of these trace components will usually require reduction to a few ppm for most catalyst systems used in synthesising alcohols and hydrocarbons, and each catalyst has its own limitations and tolerances.

Methanol synthesis is commercially available and the major processes are available from ICI or Lurgi. The basic reaction is

$$CO + 2H_2 \rightarrow CH_3OH \tag{10.1}$$

The optimum ratio of hydrogen to carbon monoxide is around 2.2 and there is also an advantage in maintaining a low concentration of carbon dioxide in the syngas. Shift conversion is used to adjust the H_2 : CO ratio

$$CO + H_2O \leftrightarrow H_2 + CO_2 \tag{10.2}$$

The shift reaction is reversible, the equilibrium being determined by temperature, pressure and concentration. Almost any feed gas composition can be readily adjusted to the desired ratio. Finally CO_2 is removed by scrubbing by one of the wide range of proprietary processes. Conversion per pass is ~20% and the unconverted gas is usually recycled to achieve close to 100% conversion, raising conversion efficiency but also increasing capital cost from handling large volumes of gas under pressure. In once-through systems, the residual gas is burned in a gas turbine or combined cycle plant after a single pass through the methanol synthesis reactor. The methanol is either stored for peak power production or exported. This offers higher efficiency at lower cost and increased flexibility. Methanol can be converted to gasoline at high efficiency by the Mobil MTG process using zeolite ZSM–5 catalyst. This process has been commercially proven in New Zealand, where natural gas is converted to methanol and then to gasoline. An analogous process known as MOGD (Methanol to Olefins, Gasoline and Diesel) has been developed for diesel fuel.

Syngas can also be processed by the Fischer–Tropsch synthesis, which produces a broad spectrum of hydrocarbons from methane through to heavy fuel oil. This process is commercially operated by Sasol in South Africa, where over a million tonnes per year of coal are processed into a full range of marketable hydrocarbon products. The major problem currently with using this technology for biomass is the lack of catalyst specificity and the range of products formed. At very high capacities, as at Sasol, all the products can be economically refined into marketable products (as they are in an oil refinery), with some products like transport fuels commanding premium prices and others having a relatively low value. At very small scales of operation, however, no product is produced in quantity sufficient to make refining worthwhile. Nevertheless, progress is being made with catalyst and process development, and there is increasing optimism that this technology could be applied to biomass-derived gas. As with methanol, the once-through concept is also being promoted.

A further option for product gas derived from air gasification routes is synthesis of ammonia, since the nitrogen content of the product gas can be utilised. In all cases there is substantial demand for efficient and economical scaled-down processes, and this will require further process and catalyst development.

10.3.23 Summary

A complete gasification process for power generation consists of three major stages: feedstock reception, storage and pre-treatment; gasification; and power generation. Table 10.10 summarises the typical components of each stage. Although large-scale biomass gasification technologies have been successfully demonstrated and several demonstration projects are in operation or under construction (Maniatis, 1999; Costello, 1999), these approaches are still relatively expensive compared with fossil-based energy and face significant economic and other non-technical barriers (EC, 2000; Beenackers, 2001; Harrisson *et al.*, 1998). Biomass gasification will only penetrate energy markets if it is completely integrated into a biomass system. Thus the innovation in practically all demonstration projects under implementation lies not only in the technical aspects of the various processes but also in the integration of the gasification technologies in existing or newly developed systems, where the overall system demonstrably offers better prospects for economic development (Maniatis and Millich, 1998). The overall challenges that have to be faced include small-scale efficient and cost effective power generation and transport fuel synthesis, while in the short term, the main technical challenge is effective tar reduction in the product gas.

Table 10.10 Typical components of an atmospheric IGCC system [a]

Operation	Equipment	Specification and comment
Pretreatment		
Weigh	Scales × 2	Daylight hours only, 12 h/d
Tip	Tipper × 2	
Temporary storage	Daybin × 2	Lorry load
Transport to storage	Front end loader	
Long-term storage	Radial stacker	2 weeks storage
Reclaim from store	Front end loader	
Temporary store	Bunker	4 hours
Transport to dryer	Conveyor	
Magnet	Overband magnet	Ferrous metal removal
Dry	Rotary dryer	To 30% water. Other possibilities
Fines recovery	Cyclone + bag filter	
Fines storage	Bin	
Fines transport	Conveyor	Can be used in dryer
Transport to surge bin	Conveyor	
Storage	Surge bin	4 hours. Only needed if drying used
Screen	Disc or vibratory	Optional to separate overs
Overs transport	Conveyor or screw	
Overs comminution	Rechip	Optional
Transport of overs	Conveyor	Optional
Storage of overs	Conveyor	Only if no re-chipping
Temporary storage	Surge bin	4 hours
Transport to gasifier	Screw to feeder	Only needed if drying used

Table 10.10 cont'd.

Gasification		
Air blower		Optional
Air preheater		
Feeder		Range of possible configurations
Gasifier		
Cyclone		
Ash transport	Conveyor	
Ash store	Pit	
Ash transport	Conveyor	
Ash disposal	Conveyor	
Steam recovery	Heat exchanger	Optional for combined cycle
Gas clean-up		
Tar cracking	Thermal or catalytic	May be separate or integral
Heat recovery	Heat exchanger	Optional; depends on specification
Hot gas filter	Ceramic or metal	Optional; depends on specification
Scrubbing	Venturi or column	Temperature reduction and impurity removal
Water treatment		Depends on process specification
Power generation		
Air compressor	Coupled to turbine	
Gas turbine		
Heat exchanger	HRSG[b] for steam	
Steam turbine		
Heat exchanger	For CHP	Optional
Heat exchanger	Drying heat recovery	As steam, hot flue gas, hot air *etc.*

[a] Assuming 10 MW_e size and wood chip feed; [b] HRSG = Heat Recovery Steam Generator. See Fig. 10.16 for example.

10.4 Pyrolysis

Pyrolysis is thermal decomposition occurring in the absence of oxygen. It is always also the first step in combustion and gasification, but in these processes it is followed by total or partial oxidation of the primary products. Lower process temperatures and longer vapour residence times favour the production of charcoal. High temperatures and longer residence times increase biomass conversion to gas, and moderate temperatures and short vapour residence time are optimum for producing liquids. Table 10.11 indicates the product distribution obtained from different modes of pyrolysis. Fast pyrolysis for liquids production is currently of particular interest.

Table 10.11 Typical product yields from different modes of pyrolysis of wood

Mode	Conditions	Liquid	Char	Gas
Fast pyrolysis	moderate temperature, around 500 C, short residence time particularly vapour	75%	12%	13%
Carbonisation	low temperature, around 400 C, very long residence time	30%	35%	35%
Gasification	high temperature, around 800 C, long residence times	5%	10%	85%

All percentages on a dry wood basis.

Fast pyrolysis occurs in a time of few seconds or less. Therefore heat and mass transfer processes and phase transition phenomena, as well as chemical reaction kinetics, play important roles. The critical issue is to bring the reacting biomass particles to the optimum process temperature and minimise their exposure to the intermediate (lower) temperatures that favour formation of charcoal. One way this objective can be achieved is by using small particles, for example in the fluidised bed processes that are described later. Another possibility is to transfer heat very fast only to the particle surface that contacts the heat source (this second method is applied in ablative processes that are described later).

10.4.1 Principles

In fast pyrolysis, biomass decomposes to generate mostly vapours and aerosols and some charcoal. After cooling and condensation, a dark brown mobile liquid is formed

which has a heating value about half that of conventional fuel oil. While it is related to the traditional pyrolysis processes for making charcoal, fast pyrolysis is an advanced process, with carefully controlled parameters to give high yields of liquid. The essential features of a fast pyrolysis process for producing liquids are

- very high heating and heat transfer rates at the reaction interface, which usually requires a finely ground biomass feed;
- carefully controlled pyrolysis reaction temperature of around 500 C and vapour phase temperature of 400–450 C;
- short vapour residence times of typically less than 2 seconds;
- rapid cooling of the pyrolysis vapours to give the bio-oil product.

The main product, bio-oil, is obtained in yields of up to 75% wt on a dry-feed basis, together with by-product char and gas, which are used within the process to provide the process heat requirements so there are no waste streams other than flue gas and ash.

A fast pyrolysis process includes drying the feed to typically less than 10% water in order to minimise the water in the product liquid oil (although up to 15% can be acceptable), grinding the feed (to around 2 mm particle size in the case of fluid bed reactors) to give sufficiently small particles to ensure rapid reaction, pyrolysis reaction, separation of solids (char), and quenching and collection of the liquid product (bio-oil).

Virtually any form of biomass can be considered for fast pyrolysis. While most work has been carried out on wood because of its consistency and comparability between tests, nearly 100 different biomass types have been tested by many laboratories, ranging from agricultural wastes such as straw, olive pits and nut shells to energy crops such as miscanthus and sorghum, forestry wastes such as bark and solid wastes such as sewage sludge and leather wastes.

At the heart of a fast pyrolysis process is the reactor. Although it probably represents at most only about 10–15% of the total capital cost of an integrated system, most research and development has focused on the reactor, although increasing attention is now being paid to control and improvement of liquid quality and improvement of collection systems. The rest of the process consists of biomass reception, storage and handling, biomass drying and grinding, product collection, storage and, when relevant, upgrading. The key aspects of these peripheral steps are described later. Bridgwater and Peacocke (1999) have published a comprehensive survey of fast pyrolysis processes for liquids production that have been built and tested in the last 10–15 years.

10.4.2 Bubbling fluid beds

Bubbling fluid beds—usually referred to as just fluid beds as opposed to circulating fluid beds—have the advantages of a well understood technology that is simple in construction and operation, good temperature control and very efficient heat transfer to biomass particles arising from the high solids density. Figure 10.24 shows a typical configuration; electrostatic precipitators are more widely used in laboratory systems. Commercial plants may try to rely on a series of collection stages (as shown in Fig. 10.25), although aerosol capture is notoriously difficult. Heating can be achieved in a variety of ways, as shown in Figs. 10.34 and 10.35 below, and scaling is well understood. However, heat transfer to bed at large scales of operation has to be considered carefully because of the scale-up limitations of different methods of heat transfer. Fluid-bed pyrolysers give good and consistent performance with high liquid yields of typically 70–75% wt. from wood on a dry-feed basis. Small biomass particle sizes of less than 2–3 mm are needed to achieve high biomass heating rates, and the rate of particle heating is usually the rate-limiting step.

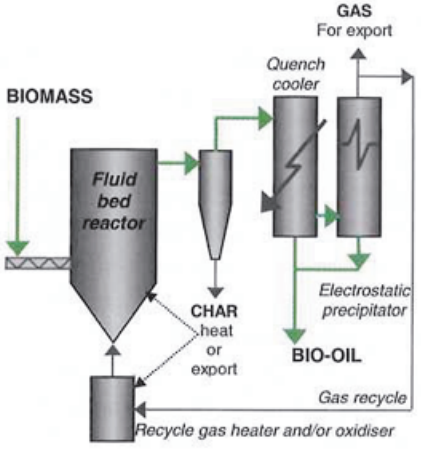


Figure 10.24 Bubbling fluid bed reactor with electrostatic precipitator.

Residence time of solids and vapours is controlled by the fluidising gas flow rate and is higher for char than for vapours. As char acts as an effective vapour cracking catalyst at fast pyrolysis reaction temperatures, rapid and effective char separation/elutriation is important. This is usually achieved by ejection and entrainment followed by separation in one or more cyclones so careful design of sand and biomass/char hydrodynamics is important.

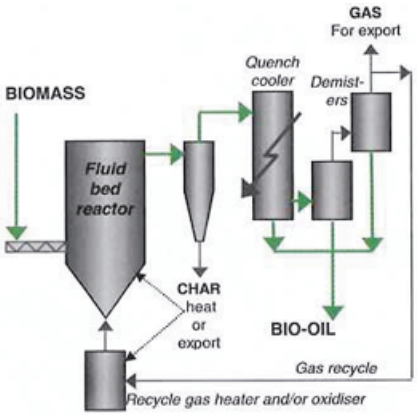


Figure 10.25 Bubbling fluid bed reactor with secondary liquid collection.

All the early work on fluid beds was carried out at the University of Waterloo, Canada, which pioneering the science of fast pyrolysis and established a clear lead in this area for many years (*e.g.* Scott *et al.*, 1995, 1996, 1997). Bubbling fluid beds have been selected for further development by several companies, including Union Fenosa (Cuevas *et al.*, 1995), who built and operated a 200 kg h^{-1} pilot unit in Spain based on the University of Waterloo process; Dynamotive, who have a 75 kg h^{-1} unit and a 400 kg h^{-1} unit (Robson, 2000 and 2001) in Canada based on an RTI design

Figure 10.26 Dynamotive 15 t/d fluid bed pilot plant.

(Fig. 10.26) with a 50 t d^{-1} plant under construction; and Wellman, who have built the 250 kg h^{-1} unit in the UK shown in Fig. 10.27 (McLellan, 2000b). Many research units have been built as they are relatively easy to construct and operate and give good results.

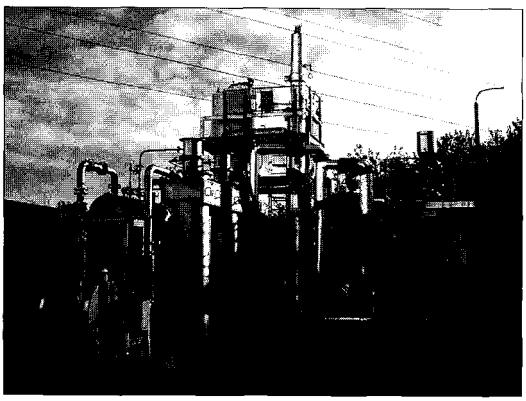


Figure 10.27 Wellman 250 kg/h fluid bed pilot plant.

10.4.3 Circulating fluid bed and transported bed reactors

Circulating fluid bed (CFB) reactors (Fig. 10.28) and transported bed reactor systems have many of the features of bubbling beds described above, except that the residence time of the char is almost the same as for vapours and gas, and the char is more attrited due to the higher gas velocities, which can lead to higher char contents in the collected bio-oil. An added advantage is that CFBs are suitable for very large throughputs even though the hydrodynamics are more complex—this technology is widely used at very high throughputs in the petroleum and petrochemical industry. Heat supply is usually from recirculation of heated sand from a secondary char combustor, which can be either a bubbling or circulating fluid bed. In this respect the process is similar to a twin fluid-bed gasifier except that the reactor (pyrolyser) temperature is much lower and the closely integrated char combustion in a second reactor requires careful control to ensure that the temperature and heat flux match the process and feed requirements. One of the unproven areas is heat transfer at large scales of throughput. Examples include the 650 kg/h ENEL plant built by Ensyn (Trebbi, 1997; Rossi and Graham, 1997) shown in Fig. 10.29, the 1700 kg/h Ensyn unit in the USA (Fig. 10.30) and the CRES pilot plant (Boukis *et al.*, 2001).

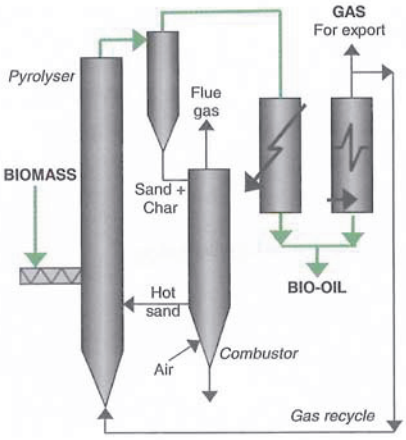


Figure 10.28 Circulating fluid bed reactor

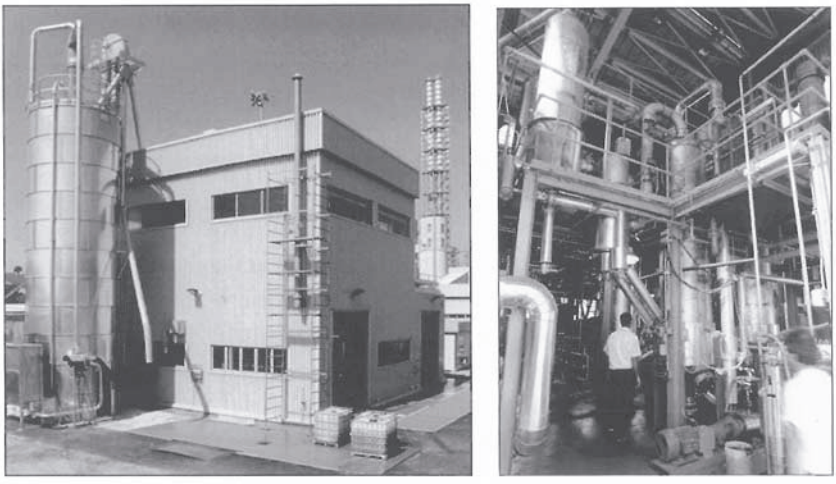


Figure 10.29 650 kg/h pilot plant at ENEL supplied by Ensyn.

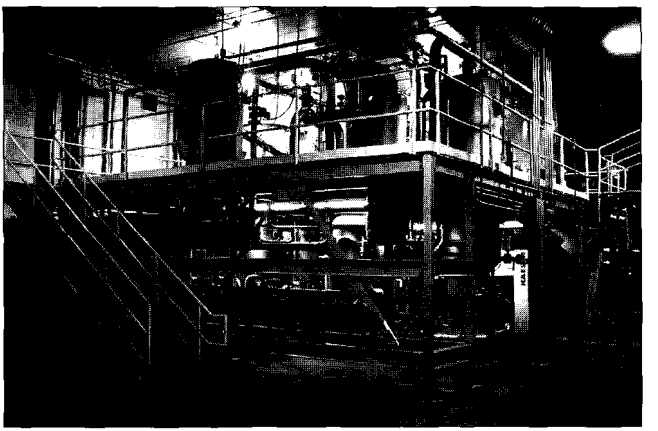


Figure 10.30 1700 dry kg/h commercial plant using the Ensyn RTP™ recirculating transported bed technology.

10.4.4 Ablative pyrolysis

Ablative pyrolysis is substantially different in concept compared with other methods of fast pyrolysis. In all the other methods, the rate of reaction is limited by the rate of heat transfer through the biomass particles, which is why small particles are required. The mode of reaction in ablative pyrolysis is like melting butter in a frying pan—the rate of melting can be significantly enhanced by pressing the butter down and moving it over the heated pan surface. In ablative pyrolysis, heat is transferred from the hot reactor wall to "melt" wood that is in contact with it under pressure. The pyrolysis front thus moves unidirectionally through the biomass particle. As the wood is mechanically moved away, the residual oil film both provides lubrication for successive biomass particles and also rapidly evaporates to give pyrolysis vapours for collection in the same way as other processes. The rate of reaction is strongly influenced by pressure, the relative velocity of the wood and the heat exchange surface and the reactor surface temperature. The key features of ablative pyrolysis are therefore as follows:

- High pressure of particle on hot reactor wall, achieved due to centrifugal force (NREL (Fig. 10.31) or mechanically (Aston (Fig. 10.32));
- High relative motion between particle and reactor wall;
- Reactor wall temperature less than 600 C.

As reaction rates are not limited by heat transfer through the biomass particles, large particles can be used and in principle there is no upper limit to the size that can be processed. The process in fact is limited by the rate of heat supply to the reactor rather than the rate of heat absorption by the pyrolysing biomass, as in other reactors. There is no requirement for inert gas, so the processing equipment is smaller and the reaction system is more intensive. However, the process is surface-area-controlled so scaling is more costly and the reactor is mechanically driven and more complex.

Much of the pioneering work on ablative pyrolysis reactors was performed by the CNRS laboratories in Nancy, France where extensive basic research has been carried out onto the relationships between pressure, motion and temperature (Lédé *et al.*, 1985). The National Renewable Energy Laboratory (NREL) in Boulder, Colorado developed the ablative vortex reactor shown in Fig. 10.31, in which the biomass is accelerated to supersonic velocities to derive high tangential pressures inside a heated cylinder (Diebold and Scahill, 1988). Unreacted particles are recycled and the vapours and char fines leave the reactor axially for collection. Liquid yields of 60–65% wt. on dry-feed basis are typically obtained.

Aston University has developed the ablative plate reactor shown in Fig. 10.32. This has a prototype rotating blade reactor in which pressure and motion is derived mechanically, obviating the need for a carrier gas (Bridgwater and Peacocke, 1999; Peacocke and Bridgwater, 1995). Liquid yields of 70–75% wt. on dry-feed basis are typically obtained. A second-generation reactor has recently been built and commissioned. Other configurations include the coiled tube at Castle Capital (Black and Brown, 1990), now owned by Enervision, and cyclonic reactors (Lédé, 2000).

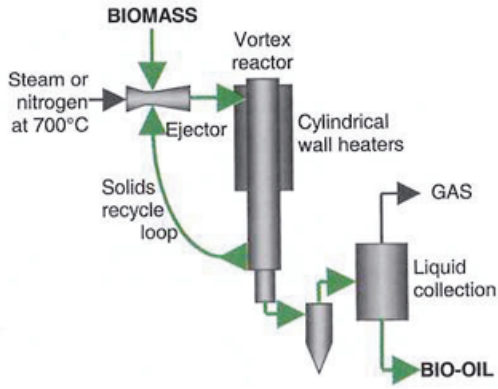


Figure 10.31 NREL Vortex ablative reactor.

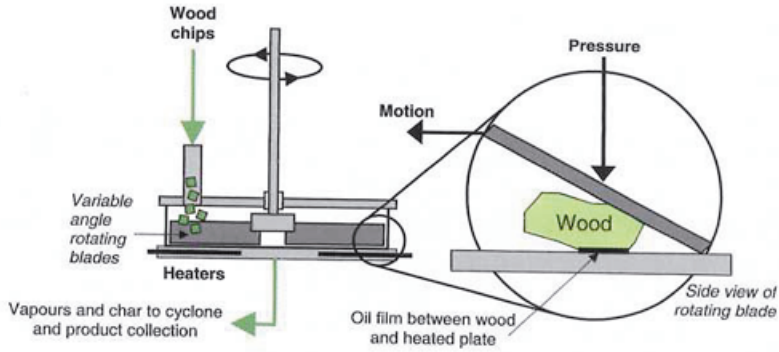


Figure 10.32 Aston University ablative plate fast pyrolysis reactor.

10.4.5 Entrained flow

Entrained flow fast pyrolysis is in principle a simple technology, but most developments have not been as successful as had been hoped, mostly because of the poor heat transfer between a hot gas and a solid particle. High gas flows are required to effect sufficient heat transfer, which requires large plant sizes and entails difficult liquid collection from the low vapour partial pressure. Liquid yields have usually been lower than fluid bed and CFB systems.

This configuration has been developed at Georgia Tech Research Institute (Kovac and O'Neil, 1989) and scaled up by Egemin (Maniatis *et al.*, 1993). Bridgwater and Peacocke (1999) give further details. Neither the GTRI nor Egemin process is now operational and there are no known plans for further development, probably because of the difficulties encountered in achieving good heat transfer from a gaseous heat carrier to solid biomass. Liquid yields of 50–60% wt. on dry feed have been obtained.

10.4.6 Rotating cone

The rotating cone reactor, invented at the University of Twente (Prins and Wagenaar, 1997) and under development by BTG (Wagenaar *et al.*, 2001), is a recent development and effectively operates as a transported bed reactor, but with transport effected by centrifugal forces rather than gas. A 250 kg/h unit is now operational, and plans for scale-up to 25 t d^{-1} have recently been announced. Figure 10.33 shows the basic principle and Fig. 10.34 an early prototype.

The key features are:

- centrifugation (at ~10Hz) drives hot sand and biomass up a rotating heated cone;
- vapours are collected and processed conventionally;
- char and sand drop into a fluid bed surrounding the cone, whence they are lifted to a separate fluid bed combustor where char is burned to heat the sand, which is then dropped back into the rotating cone;
- char is burned in a secondary bubbling fluid bed combustor. The hot sand is recirculated to the pyrolyser;
- carrier gas requirements in the pyrolysis reactor are much less than for fluid bed and transported bed systems; however, gas is needed for char burn-off and sand transport;
- complex integrated operation of three subsystems is required: rotating cone pyrolyser, riser for sand recycling, and bubbling bed char combustor;
- liquid yields of 60–70% on dry feed are typically obtained.

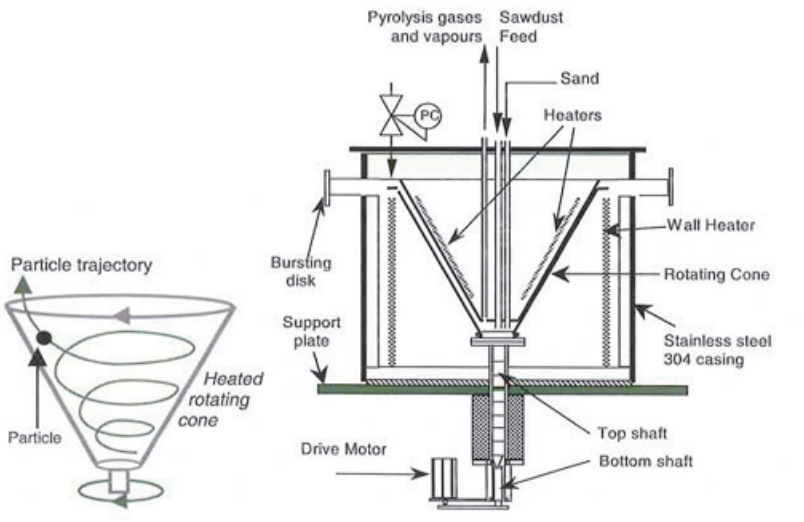


Figure 10.33 Rotating cone pyrolysis unit: principle on left, practice on right.

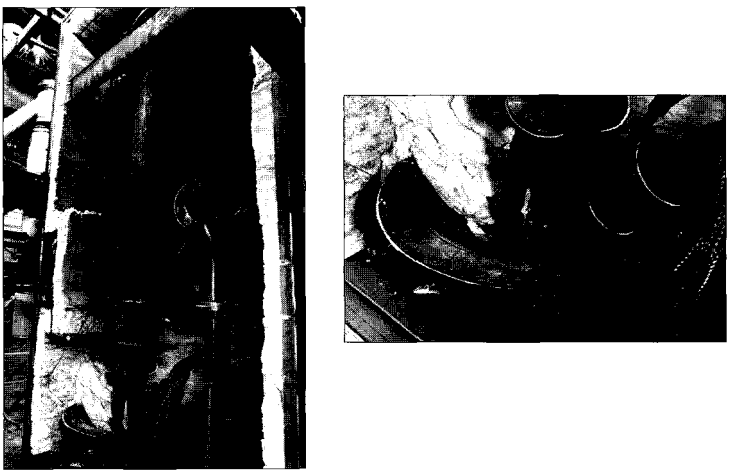


Figure 10.34 Rotating cone fast pyrolysis reactor system. The left-hand picture shows the char combustor coupled to the rotating cone, while the right-hand picture shows the reactor top with sand recycle pipes, biomass feeder and vapour offtake.

10.4.7 Vacuum pyrolysis

Vacuum pyrolysis is arguably not a true fast pyrolysis as the heat transfer rate to and through the solid biomass is much slower than in the previously described reactors. However, the vapour residence time is comparable and the liquid product has similar characteristics.

The basic technology was developed at the University of Laval using a multiple hearth furnace but is now based on a purpose-designed horizontal moving bed. A 50 kg/h unit is available for research and the technology has been scaled up by Pyrovac to a 3.5 t/h unit, which was operating at Jonquiére in Canada up to 2001 (Yang *et al.*, 2001). The process operates at 450 C and 100 kPa. Liquid yields of 35–50% on dry feed are typically obtained with higher char yields than fast pyrolysis systems. Conversely, the liquid yields are higher than in slow pyrolysis technologies because the vapours are removed quickly from the reaction zone. The process is complex and costly because the high vacuum necessitates the use of very large vessels and piping, as illustrated in Fig. 10.35. The advantages of the process are that it can process larger particles than most fast pyrolysis reactors, there is less char in the liquid product because of the lower gas velocities, and no carrier gas is needed.

Figure 10.35 Pyrovac 3 t/h vacuum pyrolysis unit showing the receivers in the foreground and the reactor at the back.

10.4.8 Heat transfer

Pyrolysis is an endothermic process, requiring a substantial heat input to raise the biomass to reaction temperature, although the heat of reaction is insignificant. Heat transfer in commercial reactors is a significant design feature and the energy in the by-product charcoal would typically be used in a commercial process by combustion of the char in air. The char typically contains about 25% of the energy of the feedstock, and about 75% of this energy is required to drive the process. The by-product gas only contains about 5% of the energy in the feed and this is not sufficient for pyrolysis. Figure 10.36 summarises the main methods of providing the necessary heat, which can be added in several ways:

- through heat transfer surfaces located in suitable positions in the reactor (1 and 2 in the figure);
- by heating the fluidisation gas (in the case of a fluid bed or circulating fluid bed reactor) (3 in the figure);
- by removing and re-heating the bed material in a separate reactor (4 in the figure);

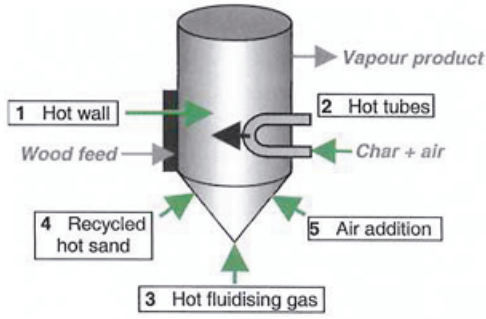


Figure 10.36 Methods of heat transfer to a pyrolysis reactor.

- by the addition of some air, although this can create hot spots and cause cracking of the liquids to tars (5 in the figure).

Figure 10.37 shows how the process heat can be provided, mostly from char combustion in various ways, but also through char gasification and combustion of the resultant fuel gas or biomass combustion, which can be considered if the char by-product has a significant value.

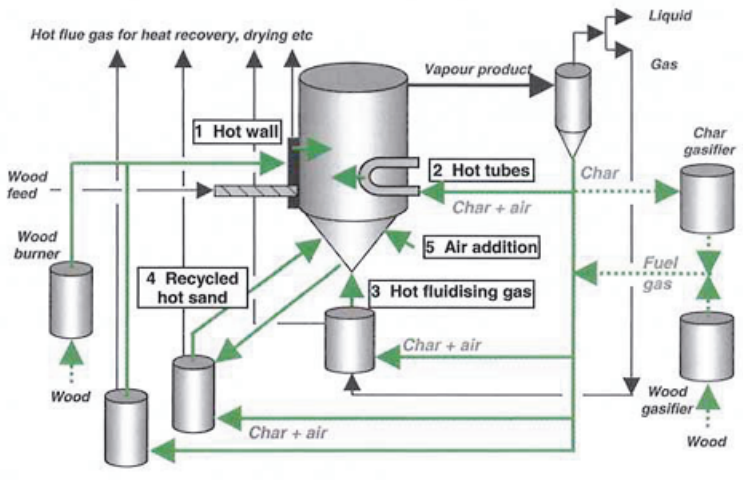


Figure 10.37 Some mechanisms for providing process heat for fast pyrolysis.

There are other ways of adding heat both directly and indirectly, as mentioned above. This facet of pyrolysis reactor design is most important for commercial units and will attract increasing attention as plants become bigger. Examples of options

include combustion of biomass instead of char, particularly where there is a lucrative market for the char; gasification of char and combustion of the LHV gas to provide greater control and avoid alkali metal problems in the char combustor; and use of fossil fuels where these are available at low cost, do not affect any interventions allowable on the process or product, and the by-products have a sufficiently high value.

10.4.9 Summary and status

Table 10.12 summarises some key features of fast pyrolysis reactor systems, and identifies the more promising and less promising features of each type of reactor.

Table 10.12 Overview of fast pyrolysis reactor characteristics for bio-oil production

Property	Status[a]	Bio-oil wt%	Comp-lexity	Feed size	Inert gas need	Specific size	Scale up
Fluid bed	Demo	75	Medium	Small	High	Medium	Easy
CFB	Pilot	75	High	Medium	High	Large	Easy
Entrained	None	65	High	Small	High	Large	Easy
Rotating cone	Pilot	65	High	V small	Low	Small	Hard
Ablative	Lab	75	High	Large	Low	Small	Hard
Vacuum	Demo	60	High	Large	Low	Large	Hard

[a] Demo = demonstration (200 – 2000 kg h^{-1})
Pilot = pilot plant operation (200 – 200 kg h^{-1})
Lab = laboratory operation (1 – 20 kg h^{-1})

Green cells indicate desirable characteristics
Black cells indicate undesirable characteristics
Grey cells indicate moderate characteristics

10.4.10 Char removal

Char acts as a vapour cracking catalyst so rapid and effective separation from the pyrolysis product vapours is essential. Cyclones are the usual method of char removal however, some fines always pass through the cyclones and collect in the liquid product where they accelerate aging and exacerbate the instability problem, which is described below. Hot vapour filtration, analogous to hot gas filtration in gasification processes, gives a high quality char free product (Diebold *et al.*, 1994), however the

liquid yield is reduced by about 10–20% due to the char accumulating on the filter surface that cracks the vapours.

Pressure filtration of the liquid is very difficult because of the complex interaction of the char and pyrolytic lignin, which appears to form a gel-like phase that rapidly blocks the filter. Modification of the liquid microstructure by addition of solvents such as methanol or ethanol that solubilise the less soluble constituents will improve this problem and contribute to improvements in liquid stability, as described below.

10.4.11 Liquids collection

The gaseous products from fast pyrolysis consist of aerosols, true vapours and non-condensable gases. These require rapid cooling to minimise secondary reactions and condense the true vapours, while the aerosols require coalescence or agglomeration. Simple heat exchange can cause preferential deposition of lignin-derived components leading to liquid fractionation and eventually blockage. Quenching in product oil or in an immiscible hydrocarbon solvent is widely practised. Orthodox aerosol capture devices such as demisters and other commonly used impingement devices are not very effective and electrostatic precipitation is currently the preferred method at smaller scales up to pilot plant. The vapour product from fluid bed and transported bed reactors has a low partial pressure of collectible products due to the large volumes of fluidising gas, and this is an important design consideration in liquid collection.

10.4.12 By-products

Char and gas are by-products, typically containing about 25 and 5% of the energy in the feed material respectively. The pyrolysis process itself requires about 15% of the energy in the feed, and of the by-products, only the char has sufficient energy to provide this heat. The heat can be derived from the char by combustion in orthodox reaction system design, as shown in Fig. 10.36. More advanced configurations could gasify the char to a LCV gas and then burn the resultant gas more effectively to provide process heat with the advantage that the alkali metals in the char can be much better controlled and avoid potential slagging problems from direct char combustion.

The waste heat from char combustion and any heat from surplus gas or by-product gas can be used for feed drying and in large installations could be used for export or power generation. An important principle of fast pyrolysis is that there are no waste products from a well-designed and well-run process other than clean flue gas.

10.4.13 Pyrolysis liquid—bio-oil

Crude pyrolysis liquid or bio-oil is dark brown and approximates to biomass in elemental composition. It is composed of a very complex mixture of oxygenated hydrocarbons with an appreciable proportion of water from both the original moisture and reaction product. Solid char may also be present. The product spectrum from aspen wood and the high dependence on temperature is shown in Fig. 10.38.

The liquid is formed by rapidly quenching and thus 'freezing' the intermediate products of flash degradation of hemicellulose, cellulose and lignin. The liquid thus contains many reactive species, which contribute to its unusual attributes. Bio-oil can be considered a micro-emulsion in which the continuous phase is an aqueous solution of holocellulose decomposition products, that stabilises the discontinuous phase of pyrolytic lignin macro-molecules through mechanisms such as hydrogen bonding. Aging or instability is believed to result from a breakdown in this emulsion. In some ways it is analogous to asphaltenes found in petroleum.

Fast pyrolysis liquid has a higher heating value of about 17 MJ kg^{-1} as produced with about 25% wt. water that cannot readily be separated. While the liquid is referred to as 'bio-oil', it will not mix with any hydrocarbon liquids. It is composed of a complex mixture of oxygenated compounds that provide both the potential and challenge for utilisation. Table 10.13 summarises some important characteristics of this liquid. Pyrolysis oil typically is a dark brown, free-flowing liquid. Depending on the initial feedstock and the mode of fast pyrolysis, the colour can be almost black through dark red-brown to dark green, being influenced by the presence of micro-

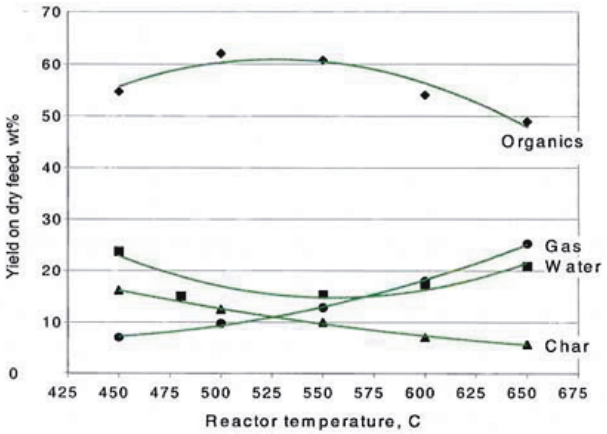


Figure 10.38 Variation of products from Aspen Poplar with temperature (Scott and Piskorz, 1982).

Table 10.13 Typical properties of wood-derived crude bio-oil

Physical property		Typical value	Characteristics
Moisture content		20–30%	• Liquid fuel
pH		2.5	• Ready substitution for conventional fuels in many stationary applications such as boilers, engines, turbines
Specific gravity		1.20	
Elemental analysis	C	55–58%	
	H	5.5–7.0%	
	O	35–40%	• Heating value of 17 MJ/kg at 25% wt. water, is about 40% that of fuel oil / diesel
	N	0–0.2%	
	Ash	0–0.2%	
HHV as produced		16–19 MJ/kg	• Does not mix with hydrocarbon fuels
Viscosity (40C and 25% water)		40–100 cp	
Solids (char)		0.1–0.5%	• Not as stable as fossil fuels
Vacuum distillation residue		up to 50%	• Quality needs definition for each application

carbon in the liquid and chemical composition. Hot vapour filtration gives a more translucent red-brown appearance owing to the absence of char. High nitrogen content can impart a dark green tinge to the liquid.

The liquid has a distinctive odour—an acrid smoky smell due to the low molecular weight aldehydes and acids—which can irritate the eyes on prolonged exposure. The liquid contains several hundred different chemicals in widely varying proportions, ranging from formaldehyde and acetic acid to complex high molecular weight phenols, anhydrosugars and other oligosaccharides. The liquid also contains varying quantities of water, which forms a stable single-phase mixture, ranging from about 15 wt% water to an upper limit of about 30–50 wt%, depending on the feed material and how it was produced and collected. A typical feed material specification is a maximum of 10% moisture in the dried feed material, as this feed moisture and the water of reaction from pyrolysis, typically about 12% based on dry feed, both report to the liquid product. Pyrolysis liquids can tolerate the addition of some water, but there is a limit to the amount that can be added to the liquid before phase separation occurs; in other words, the liquid cannot be dissolved in water. Addition of water reduces viscosity, which is useful: it reduces heating value, which means that more liquid is required to meet a given duty; and can improve stability. The effect of water is therefore complex and important. It is miscible with polar solvents such as methanol, acetone, *etc.* but totally immiscible with petroleum-derived fuels.

The density of the liquid is very high at around 1.2 kg litre^{-1}, compared with light fuel oil at around 0.85 kg/litre. This means that the liquid has about 42% of the energy content of fuel oil on a weight basis, but 61% on a volumetric basis. This has implications for the design and specification of equipment such as pumps and atomisers in boilers and engines.

Viscosity is important in many fuel applications (Diebold *et al.* 1997). The viscosity of the bio-oil as produced can vary from as low as 25 cSt to as high as 1000 cSt (measured at 40 C) or more depending on the feedstock, the water content of the oil, the amount of light ends collected and the extent to which the oil has aged.

Pyrolysis liquids cannot be completely vaporised once they have been recovered from the vapour phase. If the liquid is heated to 100 C or more to try to remove water or distil off lighter fractions, it rapidly reacts and eventually produces a solid residue of around 50 wt% of the original liquid and some distillate containing volatile organic compounds and water. While bio-oil has been successfully stored for several years in normal storage conditions in steel and plastic drums without any deterioration that would prevent its use in any of the applications tested to date, it does change slowly with time, most noticeably there is a gradual increase in viscosity. Recent samples that have been distributed for testing have shown substantial improvements in consistency and stability.

10.4.14 Physical upgrading of bio-oil

The most important properties that adversely affect bio-oil fuel quality are incompatibility with conventional fuels from the high oxygen content of the bio-oil, high solids content, high viscosity, and chemical instability. The field of chemical and physical upgrading of bio-oil has been thoroughly reviewed (Diebold, 2002).

Hot-gas filtration can reduce the ash content of the oil to less than 0.01% and the alkali content to less than 10 ppm, much lower than reported for biomass oils produced in systems using only cyclones. There is limited information available on the performance or operation of hot vapour filters, but they can be specified and perform similar to hot gas filters in gasification processes. Diesel engine tests performed on crude and on hot-filtered oil showed a substantial increase in burning rate and a lower ignition delay for the latter, due to the lower average molecular weight for the filtered oil (Shihadeh, 1998). Hot gas filtration has not yet been demonstrated over a long-term process operation. A consequence of hot vapour filtration to remove char is the catalytic effect of the accumulated char on the filter surface, which cracks the vapours and reduces yield by up to 20%. There is limited

information available on the performance or operation of hot vapour filters, but they can be specified and perform similar to hot gas filters in gasification processes.

Pyrolysis oils are not miscible with hydrocarbon fuels but they can be emulsified with diesel oil with the aid of surfactants. A process for producing stable micro-emulsions with 5–30% of bio-oil in diesel has been developed at CANMET (Ikura *et al.*, 1998) and the University of Florence, Italy, has been working emulsions of 5 to 95% bio-oil in diesel (Baglioni *et al.*, 2001) to make either a transport fuel or a fuel for power generation in engines that does not require engine modification to dual fuel operation. There is limited experience of using such fuels in engines or burners.

Polar solvents have been used for many years to homogenise and reduce the viscosity of biomass oils. The addition of solvents, especially methanol, also showed a significant effect on the oil stability. Diebold and Czernik (1997) observed that the rate of viscosity increase ("ageing") for the oil with 10 wt.% of methanol was almost twenty times less than for the oil without additives.

10.4.15 Chemical upgrading of bio-oil

Bio-oil can be chemically or catalytically upgraded to produce hydrocarbon fuels that can be conventionally processed, for example, in refineries. However, these are more complex and costly than physical methods, but offer significant improvements ranging from simple stabilisation to high-quality fuel products (Maggi and Elliott, 1997). Full deoxygenation to high-grade products such as transportation fuels can be accomplished by two main routes: hydrotreating and catalytic vapour cracking over zeolites, both of which have been reviewed (Bridgwater, 1994; Bridgwater, 1996).

Hydrotreating gives a naphtha-like product that requires orthodox refining to derive conventional transport fuels. The process is separate from fast pyrolysis and processes a liquid feed in a high-pressure, two-stage process over conventional hydro-desulphurisation catalysts in the presence of hydrogen. The catalyst supports are unstable in the high water content environment of bio-oil, though some progress has been made with carbon catalyst supports. There is a substantial hydrogen requirement to both hydrogenate the organic constituents of bio-oil and remove the oxygen content as water. The hydrogen requirement can be represented by a requirement to process an additional amount of biomass equal to about 80% of that required to produce the bio-oil. The process is thus very inefficient and also very high cost from the high-pressure requirements and cost of hydrogen (Cottam and Bridgwater, 1994). Recent cost estimates suggest a diesel product costs between four and eight times the current cost of diesel, which is too high to offer incentives for further development.

Zeolite cracking can be carried out on the primary vapours from fast pyrolysis and can thus be integrated into the fast pyrolysis process, thereby obviating the need for a separate upgrading process and high pressure. Close-coupled operation in a separate reactor is preferred to give better process control and more effective contact with the catalyst. In this process, the oxygen is rejected as CO_2 rather than water, reducing the carbon efficiency but avoiding the need for hydrogen. A further complication of zeolite cracking catalysts is the propensity for rapid coking, so a more complex rapid catalyst regeneration system is required to burn off the coke, as in a fluid catalytic cracking process in a refinery. The hydrocarbon products have a high octane number but contain high levels of hazardous PAH (Poly Aromatic Hydrocarbons) (Williams *et al.*, 1995). While catalyst and process development may reduce this problem, the economics do not currently offer sufficient inducement for further development.

10.4.16 Applications of bio-oil

Bio-oil can substitute for fuel oil or diesel in many static applications including boilers, furnaces, engines and turbines for electricity generation. Figure 10.39 summarises the possibilities. A range of chemicals including food flavourings, specialities, resins, agri-chemicals, fertilisers, and emissions control agents can also be extracted or derived from bio-oil. At least 400 hours' operation has been achieved on a 250 kW_e specially modified dual fuel engine and experience has been gained on a modified 2.5 MW_e industrial gas turbine. As noted above, upgrading bio-oil to transportation fuels is feasible but not currently economic.

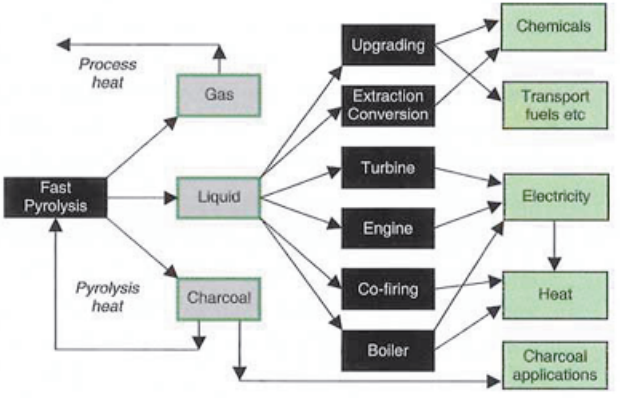


Figure 10.39 Applications of bio-oil.

A range of chemicals can also be produced from specialities such as levoglucosan to commodities such as resins and fertilisers, as summarised in Table 10.14. Food flavourings are commercially produced from wood pyrolysis products in many countries. Chemicals are always attractive possibilities owing to their much higher added value compared to fuels and energy products, and this suggests a bio-refinery concept in which the optimum combinations of fuels and chemicals are produced.

Table 10.14 Chemicals from fast pyrolysis

Acetic acid	Adhesives	Calcium-enriched bio-oil
Food flavourings	Hydrogen	Hydroxyacetaldehyde
Levoglucosan	Levoglucosenone	Preservatives
Resins	Slow-release fertilisers	Sugars

Source: Bridgwater *et al.* (2001).

10.4.17 Overall fast pyrolysis system

A fast pyrolysis system consists of an integrated series of operations starting with delivery of a roughly prepared feedstock such as whole tree chips from short rotation coppice, wood waste from furniture manufacture, energy crops such as *Miscanthus* or sorghum, or agricultural residues such as straw. Figure 10.40 shows the main steps, and a complete flowsheet for a typical 10 MW_e power plant based on fast pyrolysis might be as follows:

Reception and storage	Low capacity systems of up to around 3 t/h feed typically consist of a concrete pad for tipping delivered feed and a front-end loader to move it between reception, storage and handling steps. Larger size plants will increasingly require more sophisticated systems like those employed in pulp and paper mills. These will include weighbridge, tipping units, conveyors and bunker storage.
Feed drying	This is usually essential unless a naturally dry material such as straw is used. Sources of heat for drying include waste heat from combustion of by-product char for pyrolysis process heat, combustion of by-product gas, combustion of biomass, waste heat from engine or turbine if power generation is on site, combustion of wastes from chemicals recover or bio-oil refining. Brammer and Bridgwater (1999) have reviewed equipment and drying processes.

Comminution	Particles have to be very small to allow rapid heating and achieve high liquid yields. This is costly and reactors, such as ablative pyrolysers, that can use larger particles have an advantage.
Reactor configuration	As described above, a wide variety of configurations that show considerable diversity and innovation in meeting the basic requirements of fast pyrolysis have been tested. However, the "best" method is not yet established.
Char + ash separation	Some char is inevitably carried over from cyclones and collects in the liquid. Almost all of the ash in the biomass is retained in the char, so successful char removal gives successful ash removal. The char may be separated and exported if there is a viable market; otherwise it would be used to provide process heat either directly as in circulating fluid bed reactors or indirectly as in fluid bed systems
Liquids collection	To collect the bio-liquids, quenching with an immiscible liquid such as a hydrocarbon or a cooled liquid product would usually be used in larger scale processes. Although collection of aerosols is difficult, there has been considerable success with electrostatic precipitators. This technology is widely used on very large scales, so at least in principle this should not be a problem. Careful design is needed to avoid blockage from differential condensation of heavy ends. Light ends collection is important in reducing liquid viscosity.
Storage and transport	The bio-oil will require some local storage before local or remote use. A tank farm will provide storage and blending facilities. Both storage and transport are features unique to fast pyrolysis and permit economies of scale to be realised from building as large a conversion plant as possible as well as offering economic supplies of bio-oil for distributed or decentralised small-scale power and heat applications.

10.4.18 Status and summary

A complete integrated fast pyrolysis process for power generation consists of three major stages: feedstock reception, storage and pre-treatment; fast pyrolysis; and power generation. Table 10.15 summarises the typical components of each stage. The liquid bio-oil produced by fast pyrolysis has the considerable advantage of being

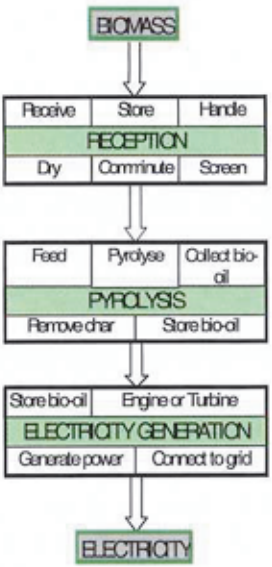


Figure 10.40 Overall fast pyrolysis process

storable and transportable, as well as having the potential to supply a number of valuable chemicals. In this respect it offers a unique advantage and its production should be considered as complementary to other biomass conversion processes. Although fast pyrolysis technologies for the production of liquid fuel have been successfully demonstrated on a small scale, and several large pilot plants or demonstration projects are in operation or at an advanced stage of construction, they are still relatively expensive compared with fossil-based energy, and thus face economic and other non-technical barriers when trying to penetrate the energy markets (Bridgwater *et al.*, 2001).

As with biomass gasification, fast pyrolysis will only be able to penetrate energy markets if completely integrated into a biomass system. Thus the innovation in practically all demonstration projects under implementation lies not only in the technical aspects of the various processes but also in the integration of pyrolysis technologies in existing or newly developed systems, where it can be demonstrated that the overall system offers better prospects for economic development. Niche markets, such as utilisation of bio-oil in a marine environment (where bio-oil is much less damaging than fuel oil in case of accidents) are likely to be the most attractive in the short term. The overall challenges include small-scale efficient and cost-effective power generation (electricity and mechanical power) and heat production systems, while also taking advantage of the more economically attractive chemicals markets.

Table 10.15 Typical components of a 10 MW_e fast pyrolysis to electricity system based on wood chip feed

Operation	Typical equipment	Specification and comments
Preparation		
Weigh	Scales × 2	Daylight hours only, 12 hours/day
Tip	Tipper × 2	
Temporary storage	Daybin × 2	Lorry load
Transport to storage	Front end loader	
Long term storage	Radial stacker	2 weeks storage
Reclaim from store	Front end loader	
Temporary store	Bunker	4 hours
Transport to dryer	Conveyor	
Magnet	Overband magnet	Ferrous metal removal
Dry	Rotary dryer	To 10% water.
Fines recovery	Cyclone + bag filter	
Fines storage	Bin	
Fines transport	Conveyor	Can be used in dryer or passed to reactor
Transport to surge bin	Conveyor	
Storage	Surge bin	4 hours. Only needed if drying used
Transport to grinder	Conveyor	
Grind	Grinder	
Transport to surge bin	Conveyor	
Temporary store	Bunker	4 hours
Transport to screen	Conveyor	
Screen(s)	Disc or vibratory	Separate undersize if not utilised in reactor 1 or 2 screens can be used

Table 10.15 cont'd.

Oversize transport	Conveyor or screw back to grinder	
Undersize transport	Conveyor	Only if not utilised in reactor
Undersize storage	Bunker	Only if not utilised in reactor
Undersize transport	Conveyor	Only if not utilised in reactor
Transport to surge bin	Conveyor	
Temporary storage	Surge bin—live bottom	4 hours
Transport to pyrolyser	Screw to feeder	
Fast pyrolysis		
Feed to pyrolyser		
Pyrolyser	Fluid bed	Range of possible configurations
Cyclone(s)		Two are recommended
Char transport	Screw	
Char store	Closed vessel	Char is pyrophoric
Char transport to combustor	Screw	Used when char provides process heat
Char combustor		
Cyclones(s)		
Heat recovery	Direct injection to dryer, or indirect via gas–gas exchange.	Possible dilution with air in direct injection for temperature control
Filter for particulates	Bag filter	
Ash recovery		Can be recycled to forest or field
Ash transport and handling		
Vapour quench	Spray or disc tower	

Table 10.15 cont'd.

Aerosol recovery	Electrostatic precipitator and/or demister(s)	Multiple de-misters may be needed if no precipitator
Liquid storage	Tank	
Liquid transport to storage	Pump	
Liquid storage	Tanks	Acid resistant material, stirred
Gas recycle	Fan or compressor	Acid resistant material
Gas conditioning	Carbon monoxide oxidation, preheating	Only for fluid bed and entrained flow reactors requiring fluidising gas
Power generation		Can be decoupled Can be local and/or remote
Liquid storage	Tanks	Acid resistant material, stirred
Liquid transport	Pumps	Acid resistant material
Liquid preparation	Mild heating for viscosity Filtration for solids	
Firing in engine(s) or turbine		
Heat recovery from turbine exhaust	HRSG for steam	
Steam turbine		
Heat exchanger	For CHP	Optional
Heat exchanger	Drying heat recovery	As steam, hot flue gas, hot air *etc.*

There is no obvious 'best' technology. Fluid beds offer robust and scalable reactors, but the provision of adequate heat transfer at large scales is not yet proved. Circulating fluid beds and transported beds may overcome the heat transfer problem but scale-up is not yet proved and there is an added problem of char attrition. Intensive mechanical devices such as ablative and rotating cone reactors offer the advantages of compactness and absence of fluidising gas, but may suffer from scaling problems and always have the problems associated with moving parts at high temperatures. There are also specific challenges facing fast pyrolysis that relate to technology, product and applications. These include:

- The cost of the bio-oil, which is 10% to 100% more than fossil fuel;
- Availability: there are limited supplies of bio-oil for testing and development of applications;
- A lack of standards for use and distribution of bio-oil. Inconsistent quality inhibits wider usage, and considerable work is required to characterise and standardise these liquids and develop a wider range of energy applications;
- Bio-oil is incompatible with conventional fuels;
- Users are unfamiliar with this material;
- Dedicated fuel handling systems are needed;
- Pyrolysis as a technology does not enjoy a good image.

The most important issues that need to be addressed are:

- Scale-up;
- Cost reduction;
- Improving product quality including setting norms and standards for producers and users;
- Environment health and safety issues in handling, transport and usage;
- Encouragement for developers to implement processes, and for users to implement applications;
- Information dissemination.

The technology and market status of bio-oil is less easy to define than for gasification owing to the absence of large-scale plants, but Fig. 10.41 provides a view.

Finally, we note that a complete bio-energy process starts with reception of biomass fuel and ends with delivery of an end product such as electricity or bio-oil to the customer. At each stage of the overall process there are potential impacts on the workers at the plant, on the surrounding population and on the environment generally. All of these must be properly considered by reference to international, national and local legislation.

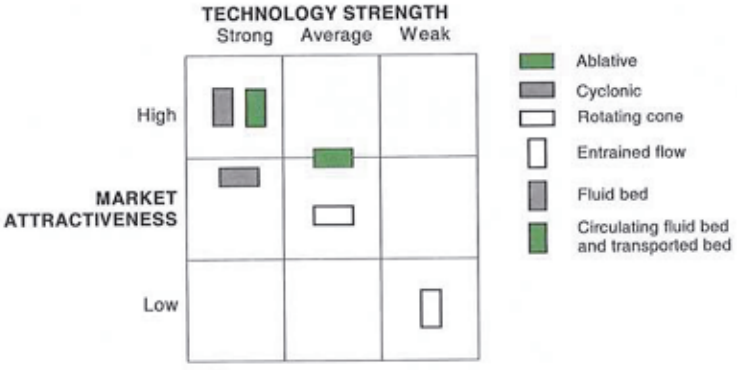


Figure 10.41 Status of fast pyrolysis systems.

10.5 Co-processing

Co-processing of biomass with conventional fuels is potentially a very attractive option that enables full economies of scale to be realised as well as reducing the problems of product quality and clean up.

Most current co-firing applications are those where the biomass fuels are first converted to a fuel gas via gasification, and this fuel gas is subsequently fed to an existing coal-fired boiler. The substitution of part of the coal by biomass reduces CO_2 emission from fossil fuels. At present, co-firing offers the best opportunities for market penetration of biomass as the overall costs are relatively low because the power cycle in the coal-fired power plant is already there. In addition, co-firing has the advantage over co-combustion (where the biomass fuels are mixed with coal before or during the combustion process), in that the biomass residual ash is not mixed with the coal ash, which has an existing market as a construction material and can thus be recycled back to the forest or field. Also the technical risks are low as the gas is utilised hot and therefore there is no tar problem. In re-burning applications, (where the fuel gas is introduced almost at the top of the coal boiler), the environmental performance of the power station is significantly improved, in addition to the replacement of fossil fuels by renewable biomass fuels (CRE Group, 2000).

There are many ways of co-processing biomass with conventional fuels, which we have summarised in Fig. 10.42 and also in bullet form below:

- Mix the biomass with a solid fuel such as coal or waste before combustion, gasification or pyrolysis;
- Gasify the biomass and fire the raw gas in:
  - a solid fuel combustor;
  - a liquid fuel combustor;
  - a gas fuel combustor.
- Pyrolyse the biomass and:
  - fire the charcoal into a solid fuel combustor, with the opportunity to store and/or transport the charcoal;
  - and/or fire the liquid into a solid fuel or liquid fuel combustor, with the opportunity to store and/or transport the liquid;
  - and/or fire the gaseous fuel into a solid fuel, liquid fuel or gaseous fuel combustor.

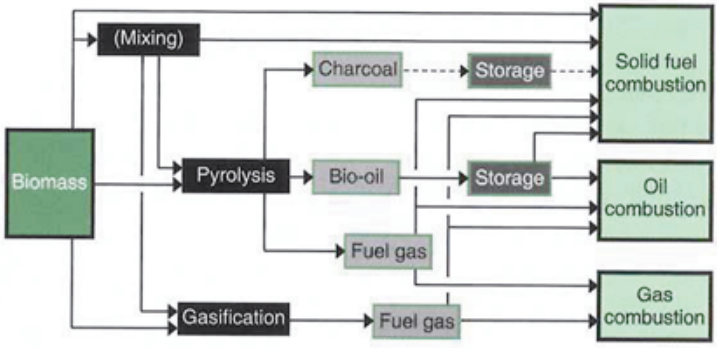


Figure 10.42 Opportunities for co-processing biomass and biofuels in conventional heat and power applications.

10.5.1 Challenges

There are a considerable number of challenges in implementing these alternative ways of co-processing biomass, and we summarise these below in bullet form as follows:

Solid biomass

Solid biomass can be premixed with a solid conventional fuel in either loose or densified form and then fired to a combustor. The challenges are:

- the uniformity of mixing;
- successful technical and economic densification methods;
- combustion characteristics;
- combustor performance and modification;
- gas cleaning;
- contamination and where contaminants report to;
- ash characteristics and behaviour.

Biomass can also be fired separately into a solid fuel combustor, and in this case the challenges are:

- combustion characteristics;
- combustor performance and modification;
- gas cleaning;
- contamination and where contaminants report to;
- ash characteristics and behaviour.

Charcoal from pyrolysis

Charcoal is normally stored and possibly transported from one or more remote production sites. Charcoal from slow pyrolysis would typically arise as a residue from waste processing, when contamination would be a problem. Charcoal from fast pyrolysis would normally be used for internal process heat and would only be exported if it had a significant value, such as for activated charcoal, and then it would not enter the fuel chain. The main challenges for charcoal are:

- charcoal characteristics and analysis;
- charcoal combustion characteristics;
- combustor performance and modification;
- gas cleaning;
- contamination and where contaminants report to;
- ash characteristics and behaviour.

Liquid fuel from fast pyrolysis

Bio-oil, the liquid fuel from fast pyrolysis, can be fired in liquid or solid fuel combustors. It may be possible to fire it in a gas-fuelled combustor. The bio-oil would normally be stored and possibly transported from one or more remote production sites. The main challenges here are:

- bio-oil physical and chemical characteristics;
- bio-oil stability;
- bio-oil combustion characteristics;
- burner design;
- combustor performance and modification;
- gas cleaning;
- contamination and where contaminants report to;
- ash characteristics and behaviour.

Gas fuel from pyrolysis and gasification

The raw gas from either high-temperature pyrolysis or gasification can be fired directly into a gas, liquid or solid fuel combustor, possibly without any clean-up. The main challenges are:

- char loading and limits, although in some cases (such as pulverised coal burners) this is not a problem since the char is burned with the coal;
- dust loading and limits;
- tar loading and limits, which is only a potential problem where the rate of tar combustion is significantly different from that of the conventional fuel;
- gas mixing;
- burner design;
- gas combustion characteristics;
- contaminants;
- contaminant analysis and characteristics;
- combustor performance and modification;
- gas cleaning;
- contamination and where contaminants report to;
- ash characteristics and behaviour.

Mixed-feed gasification and pyrolysis

In addition to firing biomass gasification or pyrolysis products into a conventional boiler, the possibility of co-gasification or co-pyrolysis of a mixture of biomass and fossil fuel can be considered, although these technologies are more difficult to implement and offer limited advantages.

10.5.2 Co-firing case studies

The Lahti Plant

The Finnish utility Lahden Lämpövoima Oy has built a Foster Wheeler CFB gasifier for its Kymijärvi Power Station in Lahtiproviding a successful example of a commercial plant that is co-processing a range of biomass fuels at an existing coal-fired power station. Figure 10.43 shows the plant. Refuse from households and industry comprises one-third of the gasifier fuel and various types of biomass (industrial waste wood, chips, fuel peat and recycled fuel) the other two-thirds (Nieminen, 1999). (Shredded tyres have recently been tested as a possible future fuel.) The gas from the gasifier is burned in the power station's steam boiler at a high flame temperature, ensuring the purity of the flue gases. The objective is to replace 50 MW_{th} of the steam boiler's 350 MW_e output by biofuel energy (Nieminen, 1999).

The product gas for combustion is led directly from the gasifier through the air preheater to two burners that are located below the coal burners in the boiler. The gas is burned in the main boiler and replaces part of the coal. Figure 10.44 shows a schematic flowsheet. When the fuel is wet, the heating value of the gas is very low. Typically, the heating value of the gas is only about 2.2 MJ Nm^{-3} at a fuel moisture content of about 50%. The combustion air for the burners is extracted from the main boiler air supply. Air is divided into primary and secondary air to ensure optimum combustion conditions. The operating temperature of the gasifier is typically 830–860 C. The gasifier contributes between 35 MW_e and 55 MW_e to output power, depending on the moisture content of the fuel and the required load. The stability of the main boiler steam cycle has been excellent. The large openings made for the low heating value gas burners have not caused any disturbances in the water/steam circulation system. Furthermore, product gas combustion has been stable even though the moisture content of the solid fuel has been mostly high and the heating value of the gas very low. Table 10.16 summarises the effect of the co-combustion of the gasifier product gas on the main boiler emissions (Kivela, 1999).

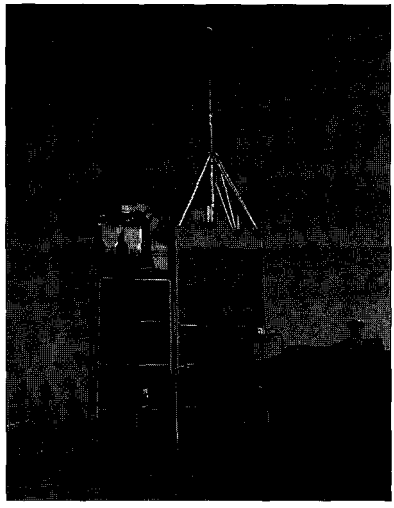


Figure 10.43 View of the Kymijärvi Power Station with gasifier in the small building.

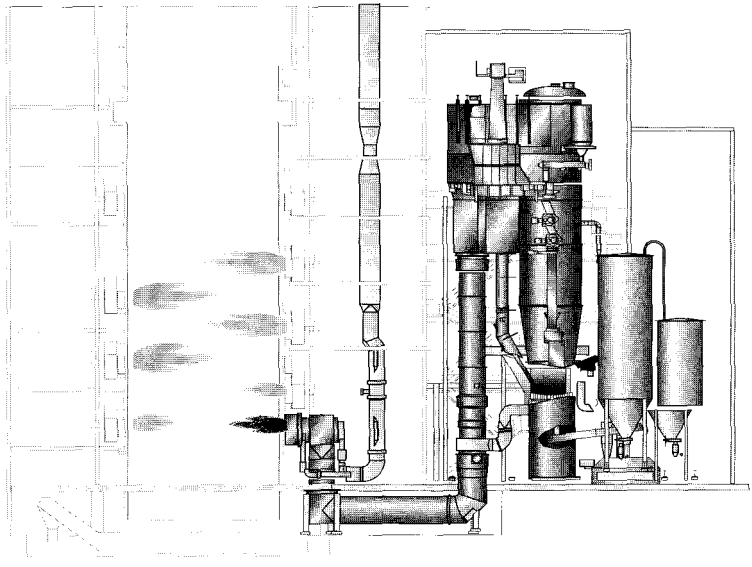


Figure 10.44 Plan of the gasifier–coal boiler system

Table 10.16 The effect of the Lahti gasifier on the main boiler emissions

Emission	Change caused by gasifier
NO_x	Decreased by 10 mg/MJ (= 5–10%)
SO_x	Decreased by 20–25 mg/MJ
HCl	Increased by 5 mg/MJ [a]
CO	No change
Particulates	Decreased by 15 mg/N m^3
Heavy metals	Slight increase in some elements, base level low
Dioxins, furans, PAH, benzenes, phenols	No change

[a] When using low-chlorine coal in the main boiler and REF + tyres in the gasifier.

The BioCoComb Plant in Zeltweg

This demonstration plant has been installed at the Zeltweg power plant in Austria operated by Draukraft (Anderl and Mory, 1999). The BioComb process is designed for preparation of biofuels for co-combustion by partial gasification and attrition by mechanical and thermal stress in a CFB reactor (Fig. 10.45). The product gas is fired in the furnace of a coal-fired power plant. The proportion of biofuels reaches about 3–5% of the total thermal input corresponding to 10 MW_{th}. The fluidising medium of the CFB is hot air, which is taken from the air preheater of the power plant. The CFB reactor operates at conditions where the biomass will be partly combusted and partly gasified at temperatures between 750 and 850 C.

The product char is ground to a fine powder by mechanical attrition and thermal stress. The attrition is maximised by optimising the operating conditions of the CFB: its cyclone is designed in such a way that only char particles that are small enough to burn completely in the coal furnace can pass through it. The fine particles are fed into the furnace together with the hot gas, which contains combustible components from the gasification. Larger char particles stay with the bed material until they are small enough or completely gasified. The fuel gas can be used as a reducing gas in the re-burning zone of the combustion chamber, thus reducing ammonia consumption and reducing or even avoiding the need for additional de-NO_x measures.

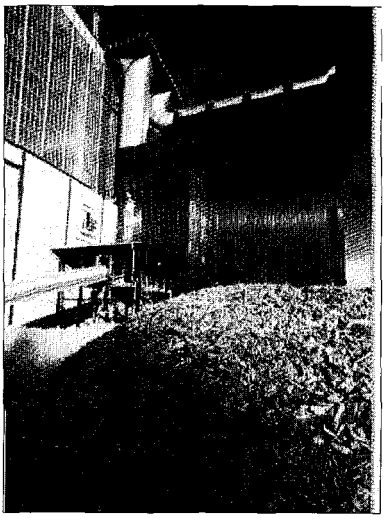


Figure 10.45 The BioCoComb plant in Zeltweg.

More than 5,000 tons of biomass and supplementary fuels have been gasified since start up. The main base fuel was spruce bark with a moisture content of about 55%, but chopped wood and sawdust have also been used. Operating experience is very positive, with gasification and combustion of the gas performing according to design. The power range of the gasifier has varied between 5 and 20 MW_{th}, the maximum output depending on the moisture content of the biomass fuel. The re-burning effects in the boiler have performed extremely well, the ammonia consumption being reduced by 10–15% when only 3% of the total thermal input came from biofuels.

The AMER project

This 29 MW_e wood gasification plant supplied by Lurgi is located at the AMER power station in the relatively new coal-fired heating unit of EPZ, Amer 9 at Geertruidenberg in The Netherlands. The aim of the project is to achieve savings of 70,000 t/y of coal, corresponding to 170,000 t/y reduction in CO_2 emissions, by using 150,000 t/y of wood waste from construction and demolition.

The gasifier (Fig. 10.46) is of the atmospheric circulating fluidised bed type, operating at temperatures of 800–950 C with the addition of bed material and possible limestone or dolomite. After the cyclone, the raw product gas is cooled in a gas cooler to about 200 C, producing slightly superheated steam of intermediate temperature.

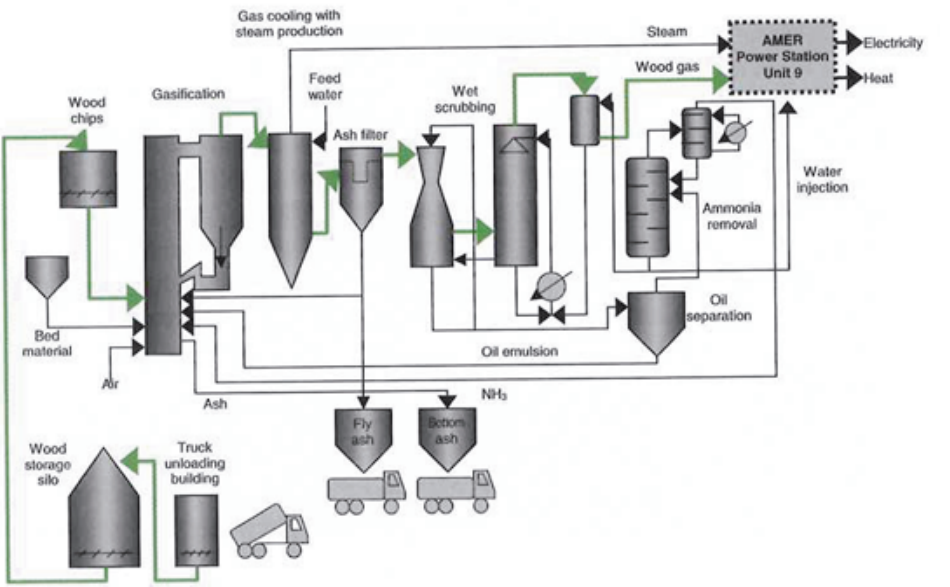


Figure 10.46 Process flow diagram of the AMER gasification plant.

The gas is de-dusted in a bag house filter at 200–250 C. The dust-free gas is then washed with water in a scrubbing section, mainly to remove ammonia. After this, the gas is reheated to ~100 C and fed to special burners in the existing coal-fired boiler of Amer unit 9. The wastewater from the scrubbing section is stripped to remove the ammonia, which is recycled to the gasifier. From the waste water system, a bleed stream is injected into the coal-fired boiler. At the time of writing, the plant had been commissioned but full-scale operation has not commenced as slug formation problems have been reported on the gas-cooling unit (Willeboer, 2000).

10.6 Economics of thermal conversion systems for electricity production

Figures 10.48 to 10.50 compare electricityproduction from biomass by combustion (Combust), atmospheric pressure gasification (GasEng), pressurised gasification in combined cycle (IGCC) and fast pyrolysis with an engine (PyrEng). Figure 10.48 shows capital costs for plants constructed now (*i.e.* first plant costs for gasification and pyrolysis and *n*th plant costs for combustion; all costs in Euros, 2000). Figure 10.49 shows the resultant electricity costs for the four systems, while Fig. 10.50 shows the benefits of learning in reducing capital costs as more plants are

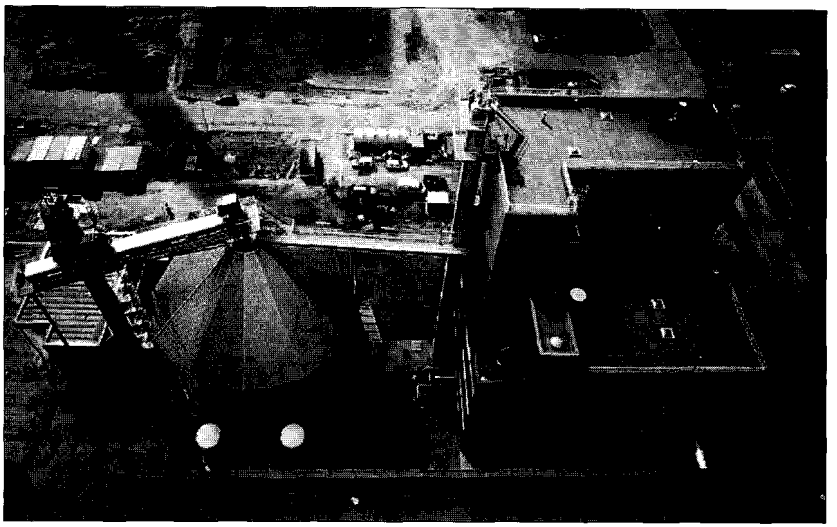


Figure 10.47 The AMER gasification plant in the Netherlands.

built. In all cases, the process is assumed to start with wood delivered as wet chips, and all steps and costs needed to produce electricity by turbine (Combust and IGCC) or engine (GasEng and PyrEng) are included. Bridgwater *et al.* (2002), from which these data are derived, give full details. Although IGCC systems are more efficient overall, there is greater process complexity, which only benefits from economies of scale at larger throughputs (Fig 10.46). While combustion capital costs and electricity costs are lower, overall system efficiencies are also lower, thus requiring more biomass, emissions are more difficult to control and significant problems have arisen with ash deposition in many Californian bio-energy plants. There is much interest in the potential for high efficiency IGCC plants of up to 50 MW_e where the logistical limitation of a diffuse biomass resource becomes very important.

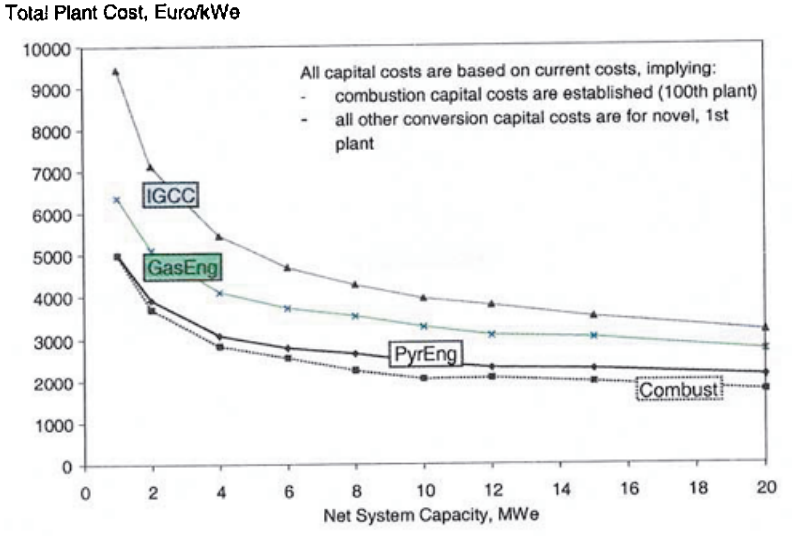


Figure 10.48 Comparison of total plant costs for four biomass-to-electricity systems on a CURRENT cost basis. The key to the technologies depicted in Figs. 10.46–10.48 is: **PyrEng**: fast pyrolysis process with one dual fuel engine accepting all the output; **GasEng**: fluid bed gasifier with a close-coupled dual fuel diesel engine accepting all the output; **IGCC**: pressure CFB gasifier with gas turbine and steam turbine utilising the waste heat in cogeneration mode; **Combust**: orthodox CFB combuster and steam turbine.

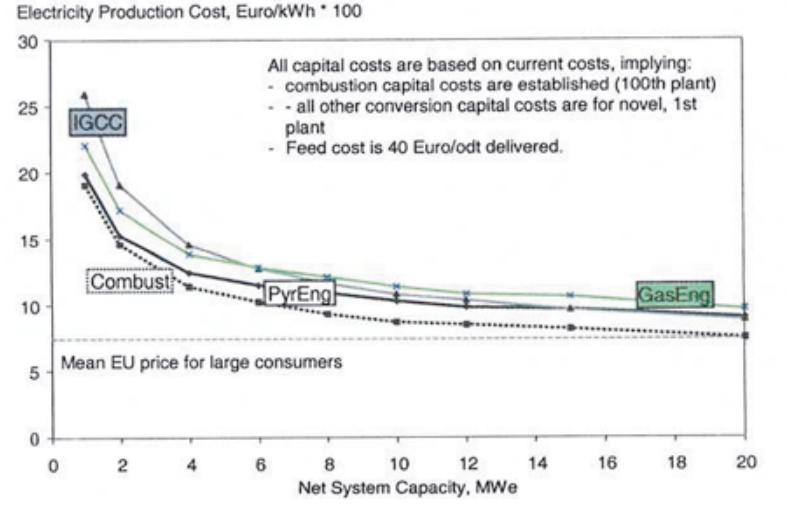


Figure 10.49 Comparison of electricity production costs for four biomass-to-electricity systems on a **current** cost basis.

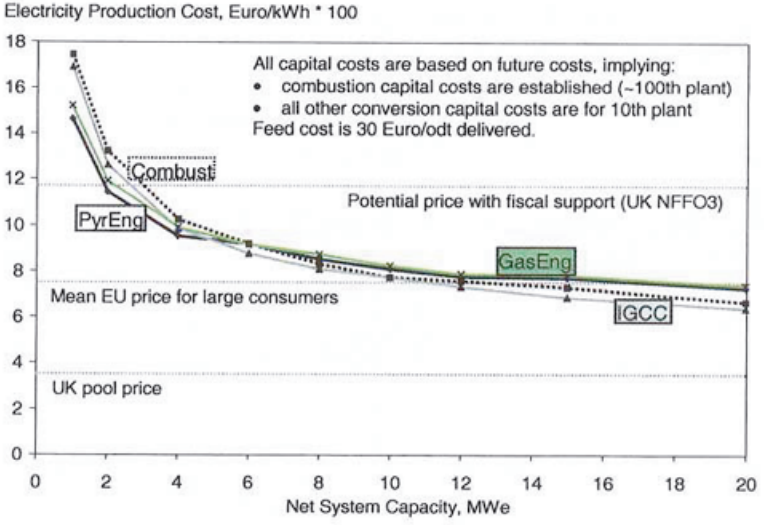


Figure 10.50 Potential electricity production costs using **future** system conditions.

10.7 Barriers

Thermal biomass conversion technologies have to overcome a number of technical and non-technical barriers before industry will undertake their commercialisation. The technical barriers have been described above, and we shall now look at some of the more important non-technical barriers.

Economics is a critical barrier, as all biofuels have to compete with fossil fuels. For those countries who have made commitments to reduce fossil fuel-derived carbon emissions, the current disincentive to implementation of bio-energy on simple cost grounds will have to be overcome—no company is going to invest in ventures that are guaranteed to lose money, regardless of the environmental benefits that may accrue. Industry will only invest in technology that has an acceptable return at an acceptable risk (Thornley and Wright, 2002). Acceptable returns will come from incentives for capital expenditure of product purchase, or to disincentives to orthodox options by, for example taxation on fossil fuels or legislation, *etc*. This is one of the major roles that governments have to play.

NIMBY attitudes present another barrier. There is widespread approval of the interest in, and move towards, renewable energy and bio-energy—as long as it is not near me! Increasing attention will need to be placed on 'selling' the idea to the

populace where the plant is to be built, and this problem could be exacerbated by the need to build smaller plants, and thus many more plants, than conventional power stations and refineries. Early plants will have the curiosity factor and may enjoy popularity as an attraction by themselves or as part of a 'green' site. However the increasing acceptance of biofuels such as pellets in many parts of Europe should lead to greater acceptance of the value and virtues of these technologies.

For industry to implement renewable energy technologies to meet greenhouse gas mitigation targets, investment has to be attractive. Without some fiscal incentives (or disincentives against fossil fuels), companies will only invest in those projects that are sufficiently profitable, and most of these will be in niche markets and special opportunities. Only governments can create the necessary instruments.

Economies of scale are a vital feature of the development of industry and technology: the larger a process can be built, the cheaper it becomes. This is particularly important in the energy and process industries that will shoulder responsibility for technology development and implementation. However, biomass is a diffuse resource that has to be harvested from large areas. A modest 10 MW_e power station operating at a modest efficiency of 35% will require about 40,000 t/year of wood on a dry basis, which will require about 4000 ha of land or 40 km^2. This could reduce to 2000 ha or 20 km^2 if the promise of high-yielding short rotation forestry is realised. Neither figure makes any allowance for non-productive land. The cost and logistics of transporting biomass to a processing plant impose an upper limit on plant size. The maximum size that has been suggested in Europe ranges from 30 to 80 MW_e in the short to medium term, and 100 to 150 MW_e in North America. This places a practical upper limit on the benefits of scale.

Investors are generally risk-averse and always prefer low-risk investments, but if risks have to be accepted, a higher return is expected. Technology developers can do much to minimise technical risk and this topic has been thoroughly described and discussed (Thornley and Wright, 2002).

The final non-technical barrier to biomass that we should acknowledge is that created by the established energy suppliers and providers, who have considerable investments in conventional energy systems and who will naturally seek to maximise the returns and maintain the competitive edge of these. Most major energy companies have their own programmes of supporting renewable energy, but there have always been concerns over the extent to which they will seek to protect their other interests in energy supply.

10.8 Conclusions

There is substantial and growing interest in thermal processing of biomass for biofuels, to make both energy and chemicals. Gasification and pyrolysis are complementary processes that have different market opportunities and should not be viewed as competitors. In both technologies, there is still considerable progress to be made in optimal interfacing of conversion and utilisation of the primary products from conversion, as well as the important interface between biomass production and conversion that has been largely left to market forces.

The main challenges lie first in bringing the thermal conversion technologies closer to the power generation or chemicals production processes, with both sides of the interface moving to an acceptable middle position, and second, in fully appreciating that, with rare exceptions, bio-energy systems will always be relatively small and must therefore be technically and economically competitive at much smaller scales of operation than the process and power generation industries are used to handling.

References

Anderl H. and Mory A. (1999), 'Operation experiences in the CFB gasification project BioCoComb for biomass with co-combustion of the gas in a PF boiler at Zeltweg power plant, Austria', in *Power Production from Biomass III, Gasification & Pyrolysis R&D&D for Industry*, Sipila K. and Korhonen M., eds., VTT Symposium 192, VTT Espoo.

Baglioni P., Chiaramonti D., Bonini M., Soldaini I. and Tondi G. (2001), 'BCO/diesel oil emulsification: main achievements of the emulsification process and preliminary results of tests on diesel engine', in Bridgwater (2001), pp. 1525–1539.

Beenackers A. A. C. M. and Maniatis K. (1997), 'Gasification technologies for heat and power from biomass', in *Biomass Gasification and Pyrolysis: State of the Art and Future Prospects*, Kaltschmitt M. and Bridgwater A.V., eds., CPL Press, Newbury, UK.

Beenackers A. A. C. M. (2001), 'Bio-energy implementation: constraints for large-scale commercialisation, conclusions of workshop', in *Proc. 1st. World Conf. and Exhibition on Biomass for Energy and Industry*, Kyritsis S., Beenackers A. A. C. M., Helm P., Grassi A. and Chiaramonti D., eds., James & James, London.

Black J. W. and Brown D. B. (1990), 'Preliminary mass balance testing of the continuous ablation reactor', in *Biomass Thermal Processing, Proc. 1st. Canada/ European Community R&D Contractors' Meeting*, Hogan E., Robert J., Grassi G. and Bridgwater A. V., eds., pp. 123–125.

Boukis I., Gyftopoulou M. E. and Papamichael I. (2001), 'Biomass fast pyrolysis in an air-blown circulating fluidized bed reactor', in Bridgwater (2001), pp. 1259–1267.

Brammer J. and Bridgwater A. V. (1999), 'A review of biomass drying technologies for thermal conversion', *Renewable and Sustainable Energy Reviews* **3**, Elsevier, pp. 243–289

Bridgwater A. V. and Evans G. D. (1993), ETSU report B/T1/00207/REP, Energy Technology Support Unit, Harwell, UK.

Bridgwater A. V. (1994), 'Catalysis in thermal biomass conversion', *Appl. Catalysis A*, **116**, 5–47.

Bridgwater A. V. (1995), 'The technical and economic feasibility of biomass gasification for power generation', *Fuel* **74**, 631–653.

Bridgwater A. V. (1996), 'Production of high grade fuels and chemicals from catalytic pyrolysis of biomass', *Catalysis Today* **29**, 285–295.

Bridgwater A. V. and Boocock D. G. B. (1997, eds.), *Developments in Thermochemical Biomass Conversion*, Blackie Academic and Professional, London.

Bridgwater A. V., Beenackers A. A. C. M. and Sipila K. (1999), 'An assessment of the possibilities for transfer of European biomass gasification technology to China', Report to EC DGXVII THERMIE Programme, 1999.

Bridgwater A. V. and Peacocke G. V. C. (1999), 'Fast pyrolysis processes for biomass', *Sustainable and Renewable Energy Reviews* **4**, 1–73.

Bridgwater A. V. (2001, ed.), *Progress in Thermochemical Biomass Conversion*, Blackwell, Oxford, UK.

Bridgwater A. V., Czernik S. and Piskorz J. (2001), 'An overview of fast pyrolysis technology', in Bridgwater (2001), pp. 977–997.

Bridgwater A. V., Toft A. J. and Brammer J. G. (2002), 'A techno-economic comparison of power production by biomass fast pyrolysis with gasification and combustion', *Sustainable and Renewable Energy Reviews* **6**, 181–248.

Corella J., Herguido, J., Gonzalez-Saiz. J., Alday F. J. and Rodriguez-Trujillo J. L. (1988), 'Fluidised bed steam gasification of biomass with dolomite and with a commercial FCC catalyst', in *Research in Thermochemical Biomass Conversion*, Bridgwater A. V. and Kuester J. L., eds., Elsevier Applied Science, pp. 754–765.

Costello R. (1999), 'An overview of the US Department of Energy's biomass power program', in *Power Production from Biomass III, Gasification and Pyrolysis*

R&D&D for Industry, Sipila K. and Korhonen M., eds., VTT Symposium 192, VTT Espoo.

Cottam M.-L. and Bridgwater A. V. (1994), 'Techno-economic modelling of biomass flash pyrolysis and upgrading systems', *Biomass and Bioenergy* **7**, 267–273.

CRE Group Ltd (2000), 'Technical review on opportunities and markets for co-utilisation of biomass and waste with fossil fuels for power generation', Report prepared for the European Commission, Brussels.

Cuevas A., Reinoso C. and Scott D. S. (1995), 'Pyrolysis oil production and its perspectives', in *Proc. Power Production from Biomass II*, Espoo, March 1995, VTT Espoo.

De Ruyck J., Maniatis K., Distlemans M. and Baron G. (1994), 'The Free University of Brussels biomass gasification CHP plant', in Bridgwater and Boocock (1997), pp. 411–422.

De Ruyck J., Allard G. and Maniatis K. (1996), 'An externally fired evaporative gas turbine cycle for small-scale biomass CHP production', *Proc. 9th. European Bioenergy Conf.*, Copenhagen, Chartier P., Ferraro G.-L., Henius U. M., Hultberg S., Sachau J. and Wiinblad M., eds., Pergamon, Oxford.

Diebold J. P. and Scahill J. (1987), 'Production of primary pyrolysis oils in a vortex reactor', in *Production, Analysis and Upgrading of Oils from Biomass* (Pyrolysis oils from biomass: producing, analyzing and upgrading), Ratcliffe C. T., Suuberg E. M. and Vorres K. S., American Chemical Society **32**, pp. 21–28.

Diebold J. P., Czernik S., Scahill J. W., Philips S. D. and Feik C. J. (1994), 'Hot-gas filtration to remove char from pyrolysis vapours produced in the vortex reactor at NREL', in Milne T. A., ed., *Biomass Pyrolysis Oil Properties and Combustion Meeting*, National Renewable Energy Laboratory, Boulder, CO, pp. 90–108.

Diebold J. P., Milne T. A., Czernik S., Oasmaa A., Bridgwater A. V., Cuevas A., Gust S., Huffman D. and Piskorz J. (1997), 'Proposed specifications for various grades of pyrolysis oils', in Bridgwater and Boocock (1997), pp. 433–447.

Diebold J. P. and Czernik S. (1997), 'Additives to lower and stabilize the viscosity of pyrolysis oils during storage', *Energy & Fuels* **11**, 1081–1091.

Diebold J. P. (2002), 'A review of the chemical and physical mechanisms of the storage stability of fast pyrolysis bio-oils', in Bridgwater A. V., ed., *Fast Pyrolysis of Biomass, A Handbook*, Vol. 2, CPL Press, U, pp. 243–292.

Ensyn website (2001), *www.ensyn.com/rtp.htm.*

EPI website (2001), *www.energyproducts.com/EPISearchPage.htm.*

ETSU for the DTI (1997), 'Development of an improved gas clean-up system for biomass gasification plant', *Renewable Energy Project Summary 414*, 1st. issue, September 1997.

ETSU (1998) B/U1/00549/22/REP, CRE Group Ltd. for ETSU.

European Commission (1997), *Communication from the Commission: Energy for the Future: Renewable Energy Sources—White Paper for a Community Strategy and Action Plan*, COM (97) 599, Final of 26.11.97, Brussels.

European Commission (2000), *Proposal for a Directive on the Promotion of Electricity from Renewable Energy Sources in the Internal Electricity Market*, COM (2000) 279, of 10.05.00, Brussels.

Fluidyne (2001), See 'Fluidyne Special Project.pdf' on website: *www.fluidynenz.250x.com/anniversary.htm.*

Graboski M. S. and Brogan T. R., 'Development of a downdraft modular skid mounted biomass/waste gasification system', in Klass, D., ed. *Energy from Biomass and Waste XI*, IGT, 1988.

Harrisson G., Fell D. A., McDonald N. M., Limbrick A. J. and Pike D. C. (1998), 'A study of market constraints on the development of power from biomass', Final Report, EC THERMIE contract STR–1125–96/UK, Green Land Reclamation Ltd. Berkshire.

Hofbauer H. and Rauch R. (2001), 'Stoichiometric water consumption of steam gasification by the FICFB–gasification process', in Bridgwater (2001), pp. 199–208.

Huffman D. R., Vogiatzis A. J. and Bridgwater A. V. (1993), 'The characterisation of RTP bio-oils', *Advances in Thermochemical Biomass Conversion*, Bridgwater A. V., ed., Elsevier.

IEA (1994), IEA policy analysis series, ISBN 92 64 142339.

IEA (2000), World Energy Outlook 2000, IEA, Paris.

IEA Bioenergy (1998), *The Role of Bioenergy in Greenhouse Gas Mitigation*, Position Paper, IEA Bioenergy, New Zealand.

IEA Bioenergy (2002), *www.ieabioenergy.com.*

Ikura M., Slamak M. and Sawatzky H. (1998), 'Pyrolysis liquid-in-diesel oil micro-emulsions', US Patent 5,820,640.

Kivela, M. (1999), Final report: 'The Lahti gasification plant', Report by Lahden Lämpövoima Oy, Lahti, Finland.

Klasson K. T., Elmore B. B., Vega J., Ackerson M. D., Clausen E. C. and Gaddy J. L., 'Biological production of liquid and gaseous fuels from synthesis gas', *Appl. Biochem. Bioengin.* **24/25**, 857–873.

Knoef H. A. M., Hunnik A. V. van, Pourkamal A. and Buffinga G. J. (2000), 'Value engineering study of a 150 kW_e downdraft gasification system', Final Report for Shell Renewables and Novem.

Knoef H. A. M. (2000), 'Inventory of biomass gasifier manufacturers and installations', Final Report to European Commission, Contract DIS/1734/98–NL, Biomass Technology Group B. V., University of Twente, Enschede *http://btgs1.ct.utwente.nl/*.

Kovac R. J. and O'Neil D. J. (1989), 'The Georgia Tech entrained flow pyrolysis process', in *Pyrolysis and Gasification*, Ferrero G. L., Maniatis K., Buekens A. and Bridgwater A. V., eds., *Elsevier Applied Science*, pp. 169–179.

Kurkela E., Ståhlberg P., Laatikainen J. and Simell P. (1993), 'Development of Simplified IGCC processes for biofuels—supporting gasification research at VTT', *Bioresource Technol.* **46**, 37–48.

Kurkela E. (1999), 'PROGAS—Gasification and Pyrolysis R&D Programme 1997–1999', in *Power Production from Biomass III, Gasification & Pyrolysis R&D&D for Industry*, Sipila K. and Korhonen M., eds., VTT Symposium 192, VTT Espoo.

Lédé J. (2000), 'The cyclone: a multifunctional reactor for the fast pyrolysis of biomass', *Ind. Eng. Chem. Res.* **39**, 893–903.

Lédé J., Panagopoulos J., Li H. Z. and Villermaux J. (1985), 'Fast pyrolysis of wood: direct measurement and study of ablation rate', *Fuel* **64**, 1514–1520.

Limbrick A. J. (2000), TASK 28 Annual Report, IEA Bioenergy, Task 28 Solid Biomass Fuels Standardisation & Classification, Rotorua, New Zealand.

Mackie K. L. (1993), IEA Bioenergy Agreement Environmental Systems Group, Interim Report, FRI, New Zealand.

Maggi R. and Elliott D. (1997), 'Upgrading overview', in Bridgwater and Boocock (1997), pp. 575–588.

Maniatis K., Baeyens J., Peeters H. and Roggeman G. (1993), 'The Egemin flash pyrolysis process: commissioning and results', in Bridgwater and Boocock (1997), pp. 1257–1264.

Maniatis K. and Millich E. (1998), 'Energy from biomass and waste: the contribution of utility scale biomass gasification plants', in *Int. Biomass Gasification Utility Scale Demonstration Projects*, Beenackers A. A. C. M. and Maniatis K., guest eds., *Bioenergy* **15**, 195–200.

Maniatis K. (1999), 'Overview of EU THERMIE gasification projects', in *Power Production from Biomass III, Gasification & Pyrolysis R&D&D for Industry*, Sipila K. and Korhonen M., eds., VTT Symposium 192, VTT Espoo.

Maniatis K. (2001), 'Progress in biomass gasification: an overview', in Bridgwater (2001), pp. 1–32.

McLellan R. (2000a), 'Design of a 2.5 MW_e biomass gasification power generation module', ETSU report B/T1/00569/REP, AEA, Harwell, UK.

McLellan R. (2000b), PyNe Newsletter No. 10, December 2000, ISSN 1470–3521, Aston University, Birmingham UK, p. 12.

Nieminen J. (1999), 'Biomass CFB gasifier connected to a 350 MW_{th} steam boiler fired with coal and natural gas—THERMIE demonstration project in Lahti, Finland', in *Power Production from Biomass III, Gasification and Pyrolysis R&D&D for Industry*, Sipila K. and Korhonen M., eds., VTT Symposium 192, VTT Espoo.

Paisley M. A., Overend, R. P. and Farris M. C. (2001), 'Preliminary operating results from Battelle/FERCO gasification demonstration plant in Burlington, Vermont, USA', in *Proc. 1st World Biomass Conference*, Elsevier, pp. 538–556.

Peacocke G. V. C. and Bridgwater A. V. (1995), 'Ablative plate pyrolysis of biomass for liquids', *Biomass and Bionergy* **7**, 147–154.

Pitcher K. and Weekes A. (2001), 'Arable biomass renewable energy (ARBRE)—the development of a biomass gasification combined cycle plant', in *Proc. 1st World Conf. & Exhibition on Biomass for Energy & Industry*, Kyritsis S., Beenackers A. A. C. M., Helm P., Grassi A. and Chiaramonti D., eds., James & James, London.

Prins W. and Wagenaar B. M. (1997), 'Review of rotating cone technology for flash pyrolysis of biomass', in *Biomass Gasification and Pyrolysis*, Kaltschmitt M. K. and Bridgwater A. V., eds., CPL Scientific Ltd., UK, pp. 316–326.

Robson A. (2001), PyNe Newsletter No. 11, June 2001, ISSN 1470–3521, Aston University, UK, pp. 1–2.

Rossi C. and Graham R. G. (1997), 'Fast pyrolysis at ENEL', in *Biomass Gasification and Pyrolysis*, Kaltschmitt M. K. and Bridgwater A. V., eds., CPL Scientific Ltd, UK, pp. 300–306.

Salo K. and Horwath, A. (1999), 'Minnesota agri-power project' (MAP), in *Power Production from Biomass III, Gasification & Pyrolysis R&D&D for Industry*, Sipila K. and Korhonen M., eds., VTT Symposium 192, VTT Espoo.

Scott D. S., Piskorz J. and Radlein D. (1985), 'Liquid products from the continuous flash pyrolysis of biomass', *Ind. Eng. Chem. Process Des. Dev.* **24**, 581–588.

Scott D. S. and Piskorz J. (1982), 'The flash pyrolysis of aspen poplar wood', *Can. J. Chem. Eng.* **60**, 666–674.

Scott D. S., Legge R. L., Piskorz J., Majerski P. and Radlein D. (1997), 'Fast pyrolysis of biomass for recovery of speciality chemicals,' in Bridgwater and Boocock (1997), pp. 523–535.

Shihadeh A. L. (1998), 'Rural electrification from local resources: biomass pyrolysis oil combustion in a direct injection diesel engine', PhD Thesis, Massachussetts Institute of Technology.

Simell P. and Kurkela E. (1997), 'Tar removal from gasification gas', in *Biomass Gasification and Pyrolysis: State of the Art and Future Prospects*, Kaltschmitt M. K. and Bridgwater A. V., eds., CPL Scientific Ltd., pp. 207–217.

Solantausta Y. O. (1998), 'Cost and performance analysis of new wood-fuelled power plant concepts', PhD Thesis, Aston University, UK.

Ståhl K., Neergaard M. and Nieminen J. (2001), 'Final report: *Värnamo demonstration programme*', in Bridgwater (2001), pp. 549–563.

The System Johanssen Gasifier (2001), *www.eskomenterprises.co.za/Casestudies/TSIcases/systemjohansson.htm.*

Thornley P. and Wright E., 'Evaluation of bio-energy projects', in *PyNe Final Report to the EC*, October 2001.

Trebbi G., Rossi C. and Pedrelli G. (1997), 'Plans for the production and utilisation of bio-oil from biomass fast pyrolysis', in Bridgwater and Boocock (1997), pp. 378–387.

UK DTI (2001), New Review No. 35, *www.dti.gov.uk/NewReview/nr35/html/gasifier.html.*

van Swaaij W. P. M., van den Aarsen F. G. , Bridgwater A. V. and Heesink A. B. M. (1994), 'A review of biomass gasification', EC DG XII, EC 1994

Vierrath H. and Greil C. (2001), 'Energy and electricity from biomass, forestry and agricultural waste', in *Proc. 1st. World Conf. and Exhibition on Biomass for Energy and Industry*, Kyritsis S., Beenackers A. A. C. M., Helm P., Grassi A. and Chiaramonti D., eds., James & James, London.

Volund website (2001), *www.volund.dk/rd2.html.*

Wagenaar B. M., Venderbosch R. H., Carrasco J., Strenziok R. and van der Aa B. J. (2001), 'Rotating cone bio-oil production and applications', in Bridgwater (2001), pp. 1268–1280.

Waldheim L., Morris M. and Leal M. R. L. V. (2001), 'Biomass power generation: sugar cane bagasse and trash', in Bridgwater (2001), pp. 509–523.

Walker M., Jackson G. and Peacocke G. V. C. (2001), 'Small-scale biomass gasification: development of a gas cleaning system for power generation', in Bridgwater (2001), pp. 441–451.

Waymand M. and Parekh S. R. (1990), *Biotechnology of Biomass Conversion*, Open University Press, Milton Keynes, UK.

Wheatley A. (1990), *Anaerobic Digestion: a Waste Treatment Technology*, Elsevier Applied Science, Oxford, UK

Whiting K. J. (1997), 'The market for pyrolysis and gasification of waste', Juniper Consultancy Services Ltd., UK.

Willeboer W. (2000), 'AMERGAS biomass gasifier starting operation', in *Proc. EU Seminar: The Use of Coal in Mixture with Wastes and Residues II*, Brem V., ed., BEO, Jülich, Germany.

Williams P. T., Horne P. A. and Taylor D.T. (1995), 'Analysis of aromatic hydrocarbons in pyrolytic oils derived from biomass', *J. Anal. Appl. Pyr.* **31**, 15–37.

Yang J., Blanchette D., de Caumia B. and Roy C. (2001), 'Modelling, scale-up and demonstration of a vacuum pyrolysis reactor', in Bridgwater (2001), pp. 1296–1311.

This page intentionally left blank

CHAPTER 11

PHOTOSYNTHESIS AND THE GLOBAL CARBON CYCLE

DAVID SCHIMEL
Climate and Global Dynamics Division, 1850 Table Mesa Drive
National Center for Atmospheric Research, Boulder, Colorado 80305, USA
schimel@ucar.edu

Per me gli augeli han canti,
I fior profumi e incanti,
L'albe il color di rose,
E palpiti le cose.

(Through me the birds have their song,
The flowers their perfumes and enchantments,
The dawn the colour of roses
And creatures their heartbeats.)

Pietro Mascagni, *Hymn to the Sun*, from *Iris*, 1898.

The global carbon cycle is a key aspect of life on Earth, linking together the living and the non-living, coupling the land, atmosphere, ocean and human spheres. Importantly, the carbon cycle also unites or integrates processes on timescales of seconds (photosynthesis itself) to the geological (formation and weathering of carbon-bearing rock and oil deposits). Because one of the phases of carbon in the Earth System, carbon dioxide, is a potent greenhouse gas, carbon plays a central role in the Earth's climate. As the backbone of most biotic compounds, carbon also plays the central chemical role in life on planet Earth. Carboniferous fossil fuels are our civilisation's primary energy source, making the carbon cycle a central feature of socio-economic processes and politics. All this makes the carbon cycle one of the great intellectual and practical subjects of science in the latter 20th and 21st centuries. In this chapter, I will review the basic structure of the carbon cycle, the contemporary carbon budget, some practical implications of the way the carbon cycle works and how it may change in the foreseeable future. I will focus on, first, the carbon cycle on timescales of seconds to a century or so, leaving the geological carbon cycle to other authors (Royer *et al.*, 2001) but not forgetting the role of fossil carbon, and second, on the role that photosynthesis plays in the carbon cycle.

11.1 The contemporary carbon cycle

Figure 11.1 is a schematic of the contemporary carbon cycle. At its heart is atmospheric CO_2, the most mobile phase of carbon, which facilitates interchange between land, ocean and fossil forms. Atmospheric CO_2 comprises a relatively small fraction of the Earth's carbon store but, through concentration-dependent chemical and biological reactions, controls much of the carbon cycle. Atmospheric CO_2 is taken up by land plants (and a few microorganisms) through photosynthesis, and released back to the atmosphere by aerobic decomposition of plant and animal tissue, fermentation in anaerobic environments (such as wetlands) and by combustion (fire or biomass burning). The difference between photosynthesis, decomposition and fire determines how much carbon is stored by photosynthesis. While photosynthesis is controlled by environmental factors such as light, temperature, water and carbon dioxide, modulated by some biotic feedbacks, such as substrate inhibition when the rate of photosynthetic assimilation A exceeds biosynthesis rates, storage depends on the types of tissue produced and their lifetime. In fast-growing annual plants, much new photosynthate is rapidly respired to support high rates of growth and no long-lived tissue is produced. Conversely, slow-growing trees with low rates of A may produce a significant amount of wood and so store appreciable amounts of carbon for decades.

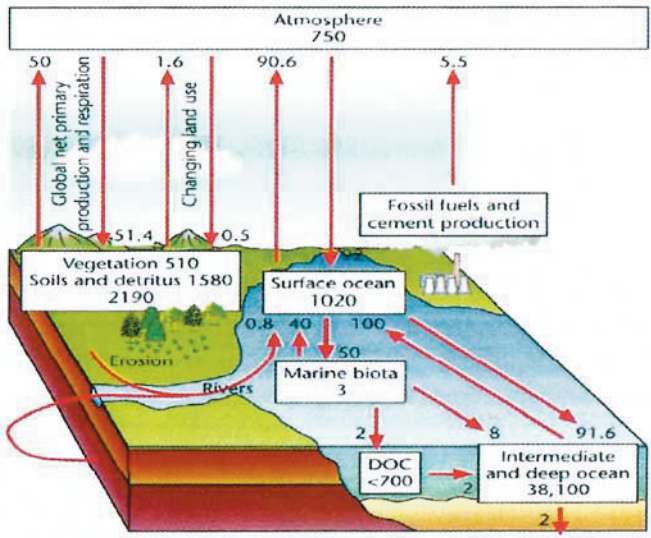


Figure 11.1 Schematic of the contemporary carbon cycle, showing mean fluxes (in GtC per year) for the 1980s and estimates of the amount of carbon (in GtC) contained in the atmosphere, vegetation and ocean. DOC = Dissolved Organic Carbon.

The surface, sunlit ocean waters (the top few hundred metres) also exchange significant amounts of carbon with the atmosphere, taking up CO_2 in cold, CO_2-depleted waters, mainly at high latitudes, and releasing it from warm, CO_2-saturated waters in the tropics (Siegenthaler and Sarmiento, 1994; Schimel *et al.*, 1996; Prentice *et al.*, 2001). CO_2-depleted waters generally occur in areas of high biological activity, reflecting high nutrient availability and abundant phytoplankton growth. Oceanic CO_2 exchange, like that with the land, depends on both biological processes (*i.e.* photosynthesis) and physical processes. However, storage in the oceans is determined more by the physical removal of dissolved organic and inorganic carbon, and also particulates, from the surface ocean into the depths where CO_2 persists in storage for decades to millennia.

These components form the bulk of the 'fast' or biogeophysical carbon fluxes. On decadal to centennial timescales, these processes were in approximate balance before the industrial age (Schimel *et al.*, 1996), with A roughly balanced by decomposition and fire: this is shown by the relative stability of atmospheric CO_2 in post-glacial times up to 100–200 years ago (Trudinger *et al.*, 1999). The human injection of fossil fuel into the biogeophysical cycle has disturbed this balance. Before fossil fuel use began, the geological carbon cycle was essentially uncoupled from carbon variations on human timescales, slowly storing and releasing carbon over millions of years with coal and oil diagenesis (formation from precursor organic matter), subduction and weather of continental carbonates. Introducing billions of tons of carbon from the geological cycle into the biogeophysical cycle has increased the amount of carbon circulating through the 'fast' cycles by an amount equivalent to ~40% of the current CO_2 content of the atmosphere. This carbon will continue to circulate until once again stabilised in geological forms such as carbonaceous sediments. The 'pulse' increase in the atmosphere will attenuate gradually, but this 'new' fossil carbon will remain in the oceanic and land ecological reservoirs for many thousands of years (Moore and Braswell, 1994). The human perturbation to the carbon cycle is thus a long-term experiment, whose unintended consequences remain unknown.

11.2 The modern carbon budget

Table 11.1 shows the estimated fluxes of carbon entering and leaving the atmosphere during the 1980s and 1990s. Some of these estimates are robust; for example, the current increase of 3.2 GtC per year in the amount of carbon in the atmosphere is well established from the measurements of global atmospheric CO_2 concentrations made by Keeler at Mauna Loa in Hawaii, shown in Fig. 11.2.

Table 11.1 Contemporary carbon budgets for the 1980s and 1990s (GtC per year)

Source/sink	**1980s**	**1990s**
Emissions (fossil and cement)	5.4 ± 0.3	6.3 ± 0.4
Atmospheric increase	3.3 ± 0.1	3.2 ± 0.1
Ocean–atmosphere flux	–1.9 ± 0.5	–1.7 ± 0.5
Land–atmosphere flux, *partitioned as*	–0.2 ± 0.7	–1.4 ± 0.7
Emissions due to land use change, mainly in tropical areas	1.7 (0.6–2.5)	assume 1.6 ± 0.8
Terrestrial uptake	–1.9 (–3.8 to 0.3)	assume –2 to –4

Source: Schimel *et al.* (2001) and Prentice *et al.* (2001). Fluxes entering the atmosphere are positive, and those leaving the atmosphere are negative. The estimates for the 1990s are based on partial data.

The partitioning of CO_2 between the land–atmosphere and ocean–atmosphere fluxes is also now well established from measurements of the change in atmospheric O_2 relative to CO_2. Photosynthesis affects CO_2 and O_2 stoichiometrically, while ocean uptake of CO_2 leaves O_2 unaffected (except for a seasonal cycle due to temperature-dependent solubility). Marine and aquatic photosynthesis do not affect dissolved O_2 exchange with the atmosphere much, and so do not affect the atmospheric O_2 budget.

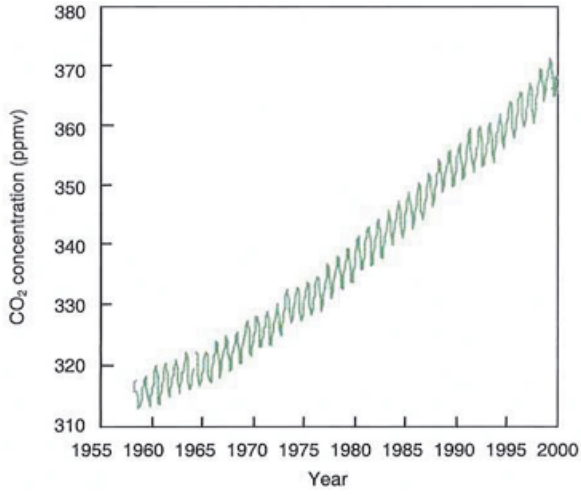


Figure 11.2 Global atmospheric CO_2 concentrations in ppmv (parts per million by volume), 1958–2000 (Keeling and Whorf, 2001). The increase over time is due to the burning of fossil fuels and deforestation. The evident seasonal oscillation arises from the metabolism of the biosphere as a whole, and is dominated by the seasonality of the Northern Hemisphere land biosphere.

Measurement of atmospheric oxygen concentrations is a relatively new capability requiring extraordinary precision (Keeling and Shertz, 1992). Unfortunately, these measurements indicate only the net flux of CO_2 to the land, that is the difference between uptake and release (*e.g.*, from land use change), so the storage and release terms cannot be separately estimated this way and direct data on land use change must be used (Houghton *et al.*, 2000). With these caveats, it is widely accepted that the increase in the CO_2 flux from atmosphere to land from 0.2 GtC per year in the 1980s to 1.4 GtC per year in the 1990s reflects a real change in terrestrial uptake, which appears to have been unusually high in the early 1990s because of climatic factors that are not well understood. Cooling due to the injection of stratospheric aerosols after the Mount Pinatubo eruption has been invoked (Schimel *et al.*, 1996; Vukicevic *et al.*, 2001), along with an aerosol-induced increase in diffuse relative to direct radiation (which allows deeper penetration of light and less shading in the canopy) and a concomitant increase in A (Roderick *et al.*, 2001).

Table 11.2 The carbon cycle over the past two centuries

Reservoir	**Source (GtC since 1800)**	**Sink (GtC since 1800)**	**Net change (GtC since 1800)**
Fossil carbon	265	0	–265
Oceans	0	115	+115
Terrestrial biosphere	140	110	–30
Atmosphere	0	180	+180

Table 11.2 shows how the sources and sinks of atmospheric carbon have changed over the past 200 years. Since 1800, the amount of carbon in the biogeophysical carbon cycle has increased (and that in the geological cycle decreased) by 265 GtC, as fossil carbon has been burned and transferred into the 'fast' cycles. The terrestrial biosphere has also been a net source of atmospheric CO_2 since 1800, as deforestation and urbanisation have reduced the carbon stored in trees, plants and soils.

The pronounced variability of terrestrial carbon exchange over both a 20-year and a 200-year timescale, revealed by Tables 11.1 and 11.2, highlights the sensitivity of carbon cycling to climate, and suggests that future climate variability and change will affect carbon storage on land.

11.3 Photosynthesis as a carbon storage process

The amount of carbon stored by terrestrial photosynthesis is an important quantity, which can be defined in various ways according to what subsequent carbon loss processes are taken into account. The amount of carbon that is "fixed" (removed from the atmospheric pool of CO_2) by photosynthesis globally or over a stand or region is termed *gross primary production* (GPP). On the smaller scale of a single leaf, the equivalent quantity is called photosynthetic assimilation A, the term and symbol already introduced in this chapter. Global terrestrial GPP has been estimated from measurements of ^{18}O uptake by plants to be ~120 GtC per year.

About half of this assimilated carbon is incorporated into plant tissues such as wood, leaves and roots, and the other half is quite quickly converted back to CO_2 by autotrophic respiration R_a (dark respiration and photorespiration in plant tissues). The amount of carbon stored by plant growth is the difference between photosynthetic assimilation and plant respiration and is termed *net primary production* (NPP).

$$NPP = A - R_a \tag{11.1}$$

Field *et al.* (1998) estimated global terrestrial NPP to be 52 GtC per year (see Chapter 6). More recently, Saugier and Roy (2000) have given the higher estimate of 60 GtC per year, but all NPP estimates still have considerable uncertainties.

Nearly all the carbon fixed by net primary production eventually returns to the atmosphere as CO_2 by one of two processes: 1) heterotrophic respiration R_h, which is respiration of plant material by external organisms (bacteria, fungi and animals) that consume photosynthetically fixed carbon for energy, and 2) fire, whether natural or deliberate. The difference between NPP and R_h is the amount of carbon gained by the ecosystem in the absence of fire or other means of removal and is termed the *net ecosystem production* (NEP).

$$NEP = NPP - R_h = A - R \tag{11.2}$$

where $R = R_a + R_h$ is the overall rate of respiration. For reasons we shall explore further in the next section, A and R are highly correlated in undisturbed ecosystems, and so tend to change together (Baldocchi *et al.*, 2001). As a result, NEP is 1% or less of NPP in 'natural' or mature conditions (Schlesinger, 1997; Schimel *et al.*, 2001). However, NEP may approach 10–20% of NPP in young or recently disturbed ecosystems (Schimel *et al.*, 2001). In such systems, a significant amount of photosynthate is allocated to long-lived plant tissue and soil carbon may also accumulate rapidly. Table 11.3 shows NEP and NPP values for three major regions.

Table 11.3 Net Ecosystem Production (NEP) and Net Primary Production (NPP) in three major regions

Region	Vegetated area ($m^2/10^{12}$)[a]	NEP (GtC/year)	NPP (GtC/year)	NEP/NPP[b]
North America	20	0.8	7	12%
Eurasia	39	1.7	11	15%
Tropics	47	0.4	36	1%

Source: Schimel *et al.* (2001).

[a]Estimates of vegetated area are from satellite measurements, uptake is from atmospheric data, productivity from model estimates. [b]The 1% value for NEP/NPP in the tropics probably reflects the high emissions in these regions rather than the actual regional ecological efficiency. The high values in North America and the Tropics probably indicate that these regions are dominated by disturbed vegetation.

NEP is not the end of the line for carbon storage because 'NEP-stored' carbon is also lost by fire, soil erosion and the removal of dissolved organic carbon (DOC) by rivers. What stored carbon remains, if any, is termed the *net biome production* (NBP). This is the difference between the amount of carbon fixed by photosynthetic assimilation and the amount lost by the oxidation or removal of plant material by all routes (Thomp son and Randerson, 1999); in other words, NBP is what the atmosphere 'sees' as carbon uptake by the land on an annualised basis. NBP would be zero in an ecosystem in steady state. However, ecosystems seldom are in a steady state. As Table 11.1 showed, the terrestrial biosphere is currently acting as a modest net sink for carbon, with NBP averaging 0.2 ± 0.7 GtC per year during the 1980s and 1.4 ± 0.7 GtC per year during the 1990s. We explore possible reasons for this in the next two sections.

11.4 Assimilation and respiration

Early in the discussion of carbon sinks, climate modellers made the reasonable assumption that carbon storage (NPP or NEP) in the terrestrial biosphere would track trends in photosynthetic assimilation A, and that A in turn would track increasing atmospheric levels of CO_2 through the phenomenon known as CO_2 fertilisation, whereby assimilation is proportionately enhanced by an increased atmospheric CO_2 concentration (Mooney and Koch, 1994; Prentice *et al.*, 2001; Poorter and Perez-Soba, 2001; Luo *et al.*, 2001). This simple assumption was incorporated into many early models of the carbon cycle (reviewed in Schimel *et al.*, 1996). However, it

gradually became clear that it was at best only partly true. Factors that uncouple the response of A to changing CO_2 levels include nutrient stress (Poorter and Perez-Soba, 2001; Schimel *et al.*, 1995), acclimation (also known as 'down regulation', meaning that the response of A to CO_2 is initially proportionate, but then tails off) and several others (Mooney and Koch, 1994).

Assimilation A and respiration R are, however, highly correlated in 'natural' ecosystems. Much of this correlation is nearly immediate, reflecting the strong dependence of plant respiration R_a on the amount of metabolic carbon produced by assimilation. Both A and R_a tend to increase with higher temperatures and wetter conditions, although A responds directly to radiation and R_a only indirectly through temperature and the adjustment of A to radiation. In the slightly longer term, as the amount of photosynthetic apparatus (chlorophyll, rubisco *etc.*) changes in response to radiation and other limiting factors, so the respiration rate changes, because the amount of photosynthetic apparatus dominates R_a (Spruegel *et al.*, 1994). On still longer time scales, heterotrophic respiration R_h is also ultimately a function of A because it depends on the amount of dead plant matter produced. Hence the strong correlation between A and R in 'natural' conditions.

However, this correlation can be upset by circumstance. For example, in young stands of trees, canopy closure leads to competition for light and new photosynthate is preferentially allocated to wood for height growth. This response may be accelerated by high CO_2 (Waring and Running, 1998). In this case, A will exceed R for substantial periods of time, leading to long periods of carbon storage (hence the emphasis in the Kyoto Protocol on creating *new* forest plantations as biological stores of 'extra' carbon). In old forests, on the other hand, most incoming photosynthetically active radiation is used at the top of the closed canopy and assimilation is capped, while respiration may continue to increase as plant matter accumulates on the forest floor and decomposes.

Dead leaves, wood and roots decompose at very different rates (Poorter and Nagel, 2000), so the allocation of new photosynthate in a plant influences the lifetime of its fixed carbon. These allocation patterns may be affected by changing environmental conditions. For example, there is evidence that some CO_2-fertilised plants allocate a significant part of their increased assimilation to labile compounds (root exudates) that are released in the root zone and readily oxidised or otherwise lost (Hungate *et al.*, 1997; den Hertog *et al.*, 1998). Luo *et al.* (2001) found the root exudate pathway to be less important than the loss of carbon via increased root turnover. In either case, much of the enhanced uptake of CO_2 would equilibrate with respiration; that is, R would quickly catch up with increased A and the beneficial effect of CO_2 fertilisation would be lost (Cao and Woodward, 1998). Thompson and

Randerson (1999) summarise forest dynamics and show that their annual carbon storage can be approximated by the product of NPP and the average residence time τ of the carbon fixed (averaging over both live and dead organic matter produced in the time period).

Nutrient availability is another factor that may disturb the correlation between A and R. Carbon is stored as organic matter, which also contains nutrients incorporating elements such as nitrogen and phosphorus, the lack of which may slow plant growth (Schlesinger, 1997). If carbon is accumulating (R < A), then nutrients will be co-sequestered in biomass and soil and nutrient limitation of plant growth may be increased (Luo and Reynolds, 1999). Storage in live biomass, especially wood, requires relatively small amounts of nutrients, as the carbon : nutrient ratios of live biomass are fairly wide (100–1000 : 1 in wood, depending on species), and this has a minimal effect on system-level nutrient availability. However, if soil carbon is accumulating, then the nutrient demand of carbon storage can be high and may eventually even reduce photosynthesis and carbon gain (Luo and Reynolds, 1999). If soil carbon is decreasing (R > A), then nutrients will be released and plant growth and biomass carbon may increase, reducing or reversing the effects of soil carbon loss on the whole system carbon budget.

11.5 CO_2 fertilisation

Given the dangers of global warming, an obvious and important question to ask is to what extent CO_2 fertilisation of photosynthesis is enhancing or could enhance net biotic carbon storage, thus sequestering some of the man-made emissions that are causing atmospheric CO_2 levels to rise and helping to mitigate any consequent climate change.

McGuire *et al.* (2001), Schimel *et al.* (2000), Cox *et al.* (2000) and Cao and Woodward (1998) give estimates of the global and regional strength of the CO_2 fertilisation effect. Most of this work suggests a significant increase in carbon uptake over the next century due to CO_2 fertilisation, albeit diminished by the negative effects of warming and drying produced by climate change. However, the quantitative findings are quite variable, and even depend on assumptions about future patterns of fuel consumption: accompanying the fossil fuel release of CO_2 is the production of reactive nitrogen (the most common limiting nutrient in natural terrestrial ecosystems) as a variety of NO_x air pollutants from inert atmospheric N_2 and O_2 (Holland *et al.*, 1999). Paradoxically, this pollution or eutrophication of the biosphere may lead to increased carbon storage through nitrogen fertilisation of assimilation, although

increasing reactive nitrogen deposition in acid rain also acts as a pollutant and probably reduces carbon uptake in at least the most severely affected areas (Holland *et al.*, 1999; Conley *et al.*, 2000).

However, a growing body of evidence suggests that much of today's sink is due not to CO_2 fertilisation but to land use recovery (Schimel, 1995; Pacala *et al.*, 2001; Schimel *et al.*, 2001), and newer modelling studies suggest that the CO_2 effect may be small compared with land use effects in the same region (Schimel *et al.*, 2000). If today's CO_2 effect is responsible for only a small part of the overall sink, it follows that it will act as a relatively weak feedback in the future, and future land use change may be more important. Much of the Northern Hemisphere mid-latitude region was extensively disturbed for agriculture, livestock husbandry and forestry in the 19th and 20th centuries (Houghton, 1999). Forest cover is currently increasing over much of this region, and carbon stocks are increasing as carbon stocks recover towards their pre-disturbance levels. This historical trend has many causes, including agricultural intensification, abandonment of marginal lands and pastures, improved forest management practices and management for multiple uses (including watersheds and recreation). Carbon is now accumulating on lands that were a source in past centuries. On the whole, the Northern Hemisphere stores less carbon than 100–150 years ago, but more than at the mid-20th century (Houghton *et al.*, 2000). The stock of terrestrial carbon stored in vegetation, soils and detritus currently stands at 2190 GtC (see Fig. 11.1). In the tropics, land conversion for agriculture, pastures, forestry and settlements remains a source of carbon to the atmosphere, although even there, abandoned clearings are a potentially significant sink of carbon (Houghton and Hackler, 2000).

11.6 Global warming and the carbon cycle

The nature of the feedbacks between global warming and the carbon cycle has been discussed for some years (Woodwell *et al.*, 1998). Some have argued that global warming will lead to a positive feedback, with more CO_2 being released from the oceans, leading to increasing warming, and so on. Others have argued for a dominant negative feedback, in which warmer temperatures and higher CO_2 levels will lead to increased biotic carbon storage, limiting the warming effects.

The literature makes it clear that the effects of temperature on carbon storage are complex (Schimel *et al.*, 1994; Trumbore *et al.*, 1996; Giardina and Ryan, 1999; Valentini *et al.*, 1999; Holland *et al.*, 2000). Temperature effects on respiration, which could lead to carbon loss from ecosystems, may be limited by the resistant nature of

soil carbon, and by nutrient feedbacks (as soil carbon is oxidised, nutrients are released, increasing productivity). While some process models are highly sensitive to temperature (Cox *et al.*, 2000), others, especially those with nutrient feedbacks, are less so (Melillo *et al.*, 1995). As importantly, disturbances such as wildfire may respond dramatically to changing climate, leading to abrupt and episodic releases of carbon to the atmosphere even in the presence of enhanced photosynthesis.

What may we expect the future trends in biotic carbon storage to be, based on current science? The balance of evidence suggests that carbon storage will be reduced in warmer, drier future climates, although considerable increases in carbon uptake could occur in warmer, wetter conditions (Melillo *et al.*, 1995; Prentice *et al.*, 2001). If disturbance increases with warmer conditions, for example, with more frequent fire in boreal, tropical or wetland regions, carbon emissions could be immense, and would not be compensated for by recovery for many decades (Cox *et al.*, 2000). Direct human effects will continue to be large, with trends in deforestation (Houghton, 1999) and reforestation/afforestation potentially causing large fluxes. Although the risk of catastrophic climate effects on ecosystem carbon storage is remote (Cox *et al.*, 2000), some human effects are likely to be inevitable. While the trend towards tropical deforestation is irreversible for the near future, developments in biofuels, carbon sequestration management and soil management can also increase biological carbon storage if new agricultural, agroforestry and forestry practices are adopted.

There are a number of ways in which ecosystems may be managed to reduce the impact of continuing fossil fuel use. These include simply increasing biotic carbon storage (sequestering carbon in long-lived forms such as wood), or substituting biomass for fossil energy; these options were discussed in the two preceding chapters. A variety of intentional modifications to the biotic carbon cycle are likely in the near future, including the introduction of more biofuels, carbon sequestration projects and conservation of natural ecosystems for their carbon storage potential. As a clearer picture of the carbon cycle and climate change emerges, management of the biogeophysical carbon cycle and its single largest flux, photosynthesis, will become increasingly important.

Acknowledgements

I thank Roger Dargaville for his help with graphics and for helpful discussions. The National Center for Atmospheric Research is sponsored by the US National Science Foundation.

References

Baldocchi D., Falge E. and Wilson K. (2001), 'A spectral analysis of biosphere–atmosphere trace gas, flux densities and meteorological variables across hour to multi-year time scales', *Agricultural Forest Meteorol.* **107**, 1–27.

Cao M. K. and Woodward F. I. (1998), 'Dynamic responses of terrestrial ecosystem carbon cycling to global climate change', *Nature* **393**, 249–252.

Conley A. H., Holland E. A., Seastedt T. R. and Parton W. J. (2000), 'Simulation of carbon and nitrogen cycling in an alpine tundra', *Arctic, Antarctic Alpine Res.* **32**, 147–154.

Cox P. M., Betts R. A., Jones C. D., Spall S. A. and Totterdell I. J. (2000), 'Acceleration of global warming due to carbon-cycle feedbacks in a coupled climate', *Nature* **408**, 750–750.

den Hertog J., Stulen I., Posthumus F. and Poorter H. (1998), 'Interactive effects of growth-limiting N supply and elevated atmospheric CO_2 concentration on growth and carbon balance of Plantago major', *Physiologia Plantarum* **103**, 451–460.

Field C., Behrenfeld M., Randerson J. and Falkowski P. (1998), 'Primary production of the biosphere: integrating terrestrial and oceanic components', *Science* **281**, 237–240.

Giardina C. P. and Ryan M. G. (2000), 'Evidence that decomposition rates of organic carbon in mineral soil do not vary with temperature', *Nature* **404**, 858–861.

Holland E. A., Dentener F. J., Braswell B. H. and Sulzman J. M. (1999), 'Contemporary and pre-industrial global reactive nitrogen budgets', *Biogeochem.* **46**, 7–43.

Holland E. A., Neff J. C., Townsend A. R. and McKeown B. (2000), 'Uncertainties in the temperature sensitivity of decomposition in tropical and subtropical ecosystems: implications for models', *Global Biogeochem. Cycles* **14**, 1137–1151.

Houghton R. A. (1999), 'The annual net flux of carbon to the atmosphere from changes in land use 1850–1990', *Tellus* **51**, 298–313.

Houghton R. A. and Hackler J. L. (2000), 'Changes in terrestrial carbon storage in the United States. 1: The roles of agriculture and forestry', *Global Ecol. Biogeog.* **9**, 125–144.

Hungate B. A., Holland E. A., Jackson R. B., Chapin F. S., Mooney H. A. and Field C. B. (1997), 'The fate of carbon in grasslands under carbon dioxide enrichment', *Nature* **388**, 576–579.

Keeling R. F. and Shertz S. R. (1992), 'Seasonal and interannual variations in atmospheric oxygen and implications for the global carbon cycle', *Nature* **358**, 723–727.

Keeling C. D. and Whorf T. P. (2001), Atmospheric CO_2 records from sites in the SIO air sampling network', *http://cdiac.esd.ornl.gov/trends/co2/sio-mlo.htm.*

Luo Y. Q. and Reynolds J. F. (1999), 'Validity of extrapolating field CO_2 experiments to predict carbon sequestration in natural ecosystems', *Ecology* **80,** 1568–1583.

Luo Y. Q., Wu L. H., Andrews J. A., White L., Matamala R., Schafer K. V. R. and Schlesinger W. H. (2001), 'Elevated CO_2 differentiates ecosystem carbon processes: deconvolution analysis of Duke Forest', *FACE Data Ecological Monographs* **71**, 357–376.

Melillo J. M., Borchers J., Chaney J., Fisher H., Fox S., Haxeltine A., Janetos A., Kicklighter D. W., Kittel T. G. F., McGuire A. D., McKeown R., Neilson R., Nemanil R., Ojima D. S., Painter T., Pan Y., Parton W. J., Pierce L., Pitelka L., Prentice C., Rizzo B., Rosenbloom N. A., Running S., Schimel D. S., Sitch S., Smith T. and Woodward I. (1995), 'Vegetation ecosystem modeling and analysis project: comparing biogeography and biogeochemistry models in a continental scale study of terrestrial ecosystem responses to climate change and CO_2 doubling', *Global Biogeochem. Cycles* **9**, 407–437.

Mooney and Koch (1994), 'The impact of rising CO_2 concentrations on the terrestrial biosphere', *Ambio* **23**, 74–76.

Moore B. and Braswell B. H. (1994), 'The lifetime of excess atmospheric carbon dioxide', *Global Biogeochem. Cycles* **8**, 23–38.

Pacala S. W., Hurtt G. C., Baker D., Peylin P., Houghton R. A., Birdsey R. A., Heath L., Sundquist E. T., Stallard R. F., Ciais P., Moorcroft P., Caspersen J. P., Shevliakova E., Moore B., Kohlmaier G., Holland E., Gloor M., Harmon M. E., Fan S. M., Sarmiento J. L., Goodale C. L., Schimel D. and Field C. B. (2001), 'Consistent land- and atmosphere-based US carbon sink estimates', *Science* **292**, 2316–2320.

Poorter H. and Nagel O. (2000), 'The role of biomass allocation in the growth response of plants to different levels of light, CO_2, nutrients and water: a quantitative review', *Austral. J. Plant Physiol.* **27**, 595–607.

Poorter H. and Perez-Soba M. (2001), 'The growth response of plants to elevated CO_2 under non-optimal environmental conditions', *Oecologia l* **29**, 1–20.

Prentice I. C., Farquhar G. D., Fasham M. J. R., Goulden M. L., Heimann M., Jaramillo V. J., Kheshgi H. S., Le Quéré C., Scholes R. J. and Wallace D. W. R. (2001), 'The carbon cycle and atmospheric carbon dioxide', in Houghton J. T., Ding Y., Griggs D. J., Noguer M., van der Linden P. J., Dai X., Maskell K. and Johnson C A., eds., *Climate Change (2001), The Scientific Basis. Contribution of Working Group I to the Third Assessment Report of the Intergovernmental Panel on Climate Change*, Cambridge University Press, pp. 83–237.

Roderick M. L., Farquhar G. D., Berry S. L. and Noble I. R. (2001), 'On the direct effect of clouds and atmospheric particles on the productivity and structure of vegetation', *Oecologia1* **29**, 21–30.

Royer D. L., Berner R. A. and Beerling D. J. (2001), 'Phanerozoic atmospheric CO_2 change: evaluating geochemical and paleobiological approaches', *Earth Sci. Rev.* **54**, 349–392.

Saugier B. and Roy J. (2000), 'Estimations of global terrestrial productivity: converging towards a single number?', in *Terrestrial Global Productivity*, Saugier B., Roy J. and Mooney H. A., eds., Academic Press, San Diego.

Schimel D. S., Braswell B. H. Jr., Holland E. A., McKeown R., Ojima D. S., Painter T. H., Parton W. J. and Townsend A. R. (1994), 'Climatic, edaphic and biotic controls over storage and turnover of carbon in soils', *Global Biogeochem. Cycles* **8**, 279–293.

Schimel D. S. (1995), 'Terrestrial ecosystems and the carbon cycle', *Global Change Biol.* **1**, 77–91.

Schimel D. S., Alves D., Enting I. G., Heimann M., Joos F., Raynaud D., Wigley T. M. L., Prather M., Derwent R., Ehhalt D., Fraser P., Sanhueza E., Zhou X., Jonas P., Charlson R., Rodhe H., Sadasivan S., Shine K. P., Fouquart Y., Ramaswamy V., Solomon S., Srinivasan J., Albritton D., Isaksen I., Lal M. and Wuebbles D. (1996), 'Radiative forcing of climate change', in Houghton J. T., Meira Filho L. G., Callander B. A., Harris N., Kattenberg A. and Maskell K., eds., *Climate Change 1995: The Science of Climate Change. Contribution of WGI to the Second Assessment Report of the Intergovernmental Panel on Climate Change*, Cambridge University Press, Cambridge, pp. 65–131.

Schimel D., Melillo J., Tian H., McGuire A. D., Kicklighter D., Kittel T., Rosenbloom N., Running S., Thornton P., Ojima D., Parton W., Kelly R., Sykes M., Neilson R. and Rizzo B. (2000), 'Contribution of increasing CO_2 and climate to carbon storage by ecosystems in the United States', *Science* **287**, 2004–2006.

Schimel D. S., House J. I., Hibbard K. A., Bousquet P., Ciais P., Peylin P., Braswell B. H., Apps M. J., Baker D., Bondeau A., Canadell J., Churkina G., Cramer W., Denning A. S., Field C. B., Friedlingstein P., Goodale C., Heimann M., Houghton R. A., Melillo J. M., Moore B., Murdiyarso D., Noble I., Pacala S. W., Prentice I. C., Raupach M. R., Rayner P. J., Scholes R. J., Steffen W. L. and Wirth C. (2001), 'Recent patterns and mechanisms of carbon exchange by terrestrial ecosystems', *Nature* **414**, 169–172.

Schlesinger W. H. (1997), *Biogeochemistry: An Analysis of Global Change*, 2nd ed., Academic Press, San Diego.

Siegenthaler U. and Sarmiento J. L. (1993), 'Atmospheric carbon dioxide and the ocean', *Nature* **365**, 119–125.

Spruegel D. F., Ryan M. G., Brooks J. R., Vogt K. A. and Martin T. A. (1994), 'Respiration from the organ level to the stand', in Smith W. K. and Hinckley T. M., eds., *Resource Physiology of Conifers: Acquisition, Allocation, and Utilization*', Academic Press, Boca Raton, pp. 255–299.

Thompson M. V. and Randerson J. T. (1999), 'Impulse response functions of terrestrial carbon cycle models: method and application', *Global Change Biol.* **5**, 371–394.

Trudinger C. M., Enting I. G., Francey R. J., Etheridge D. M. and Rayner P. J. (1999), 'Long-term variability in the global carbon cycle inferred from a high-precision CO_2 and $\Delta^{13}C$ ice-core record', *Tellus* **51**, 233–248.

Trumbore S. E., Chadwick O. A. and Amundson R. (1996), 'Rapid exchange between soil carbon and atmospheric carbon dioxide driven by temperature change', *Science* **272**, 393–396.

Valentini R., Matteucci G., Dolman A. J., Schulze E. D., Rebmann C., Moors E. J., Granier A., Gross P., Jensen N. O., Pilegaard K., Lindroth A., Grelle A., Bernhofer C., Grunwald T., Aubinet M., Ceulemans R., Kowalski A. S., Vesala T., Rannik U., Berbigier P., Loustau D., Guomundsson J., Thorgeirsson H., Ibrom A., Morgenstern K., Clement R., Moncrieff J., Montagnani L., Minerbi S. and Jarvis P. G. (2000), 'Respiration as the main determinant of carbon balance in European forests', *Nature* **404**, 861–865.

Vukicevic T., Braswell B. H. and Schimel D. (2001), 'A diagnostic study of temperature controls on global terrestrial carbon exchange', *Tellus* **53**, 150–170.

Waring R. and Running S. W. (1998), *Forest Ecosystems: Analysis at Multiple Scales*, Academic Press, San Diego, California.

Woodwell G. M, Mackenzie F. T., Houghton R. A., Apps M., Gorham E. and Davidson E. (1998), 'Biotic feedbacks in the warming of the earth', *Climatic Change* **40**, 495–518.

This page intentionally left blank

CHAPTER 12

MANAGEMENT OF TERRESTRIAL VEGETATION TO MITIGATE CLIMATE CHANGE

RICHARD TIPPER and REBECCA CARR
The Edinburgh Centre for Carbon Management, Unit 2, Tower Mains Studios, 18 Liberton Brae, Edinburgh EH16 6AE, Scotland
richard.tipper@eccm.uk.com

Is there not the earth itself, its forests and waters, and all other natural riches, above and below the surface? These are the inheritance of the human race, and there must be regulations for the common enjoyment of it. What rights, and under what conditions, a person shall be allowed to exercise over any portion of this common inheritance, cannot be left undecided. No function of government is less optional than the regulation of these things, or more completely involved in the idea of civilised society.

John Stuart Mill (1806–1873), *Principles of Political Economy*, 1848.

Concern about the economic impacts on industrial economies of severe constraints on fossil fuel consumption has led to considerable interest in the management of carbon flows associated with terrestrial vegetation. As international climate change policies developed under the UN Framework Convention on Climate Change (UNFCCC, 2002) seek to accommodate terrestrial carbon flows within the broader strategy of greenhouse gas mitigation, this chapter discusses the main technical, economic and policy issues in this area.

12.1 Potential carbon management activities in the forestry and land use sector

Around 50% of the mass of dry plant matter consists of carbon in the form of carbohydrates and other organic compounds, derived from atmospheric CO_2 through photosynthesis. Recent surveys indicate that the present stock of carbon in the terrestrial biosphere is around 2000 Gt (Malhi *et al.*, 1999). While there is considerable uncertainty as to how terrestrial carbon stocks have changed in the 18,000 years since the last glacial maximum, there is clear evidence that human activities over the past 500 years—principally the expansion of agriculture and

exploitation of forests for fuel and timber—have led to significant reductions of these stocks. According to Houghton (1996), over 100 GtC were released to the atmosphere between 1850 and 1980 through changes in land use. Current concern about rising levels of CO_2 in the atmosphere has now focussed considerable attention on both conventional and novel techniques to increase the carbon stored in terrestrial stocks. The integration of greenhouse gas considerations into other objectives of land use management has now been termed 'terrestrial carbon management'.

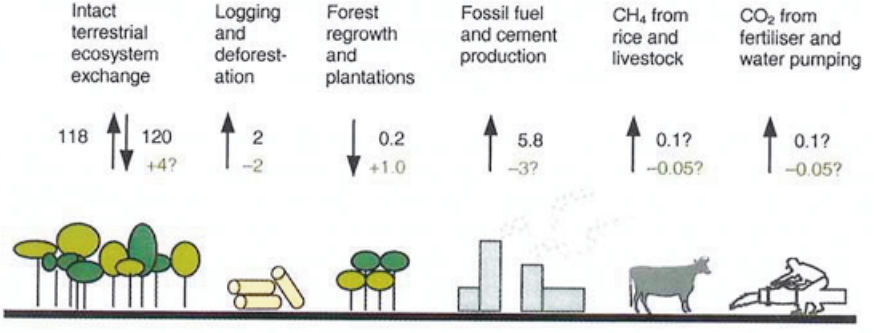


Figure 12.1 Potential change to annual carbon flows from 2020 to 2080 due to carbon management in forestry and land use sectors. The figures in black represent current annual flows, and those in green represent approximate potential for managed change (both are expressed in GtC yr^{-1}).

Figure 12.1 shows how some of the main options for increased carbon storage in terrestrial stocks relate to the carbon cycle. Forests, which account for about 80% of the flows of carbon between terrestrial vegetation and the atmosphere, have attracted most attention for potential carbon management. Intact forests may be managed to increase their uptake of carbon and conserved to reduce emissions; new forests may be established to act as carbon sinks—so-called carbon sequestration; forest products may also provide products that can substitute for fossil fuels and energy-intensive products such as cement and steel. Other land use measures, such as the adoption of zero tillage or non-burning agricultural practices, appear to offer considerable potential for stabilising or increasing durable stocks of soil carbon. Improvements in the efficiency of fertiliser and irrigation water use can have an indirect effects on CO_2 emissions by reducing fossil fuels used in the Haber–Bosch process and for pumping. Non-CO_2 greenhouse gas emissions, including methane and N_2O, can be mitigated by changes to rice cultivation, fertilisation practices and livestock feeding.

The potential of land use and forestry activities to reduce GHG emissions over the next 50–100 years appears considerable, possibly of the order of 60–120 GtC (Brown *et al.*, 1996; Kauppi and Sedjo, 2001). However, it should be stressed that terrestrial

carbon management cannot provide a comprehensive solution to greenhouse gas emissions and that stabilisation of atmospheric CO_2 concentrations below 600 ppm will also require significant reductions in emissions from fossil fuel sources. The following sections explore the evidence behind current estimates of the technical and economic potential of the main options for carbon management in the forest sector.

12.1.1 Afforestation / reforestation

Afforestation and reforestation are differentiated by the IPCC as follows: afforestation means the establishment of forests on areas that were not covered by forest for at least the past 50 years, whereas reforestation is the establishment of forests on areas that have been were deforested more recently. According to the FAO (2001), the rate of global afforestation during the 1990s was over 6 million ha per year. Over half of this occurred by natural regeneration on abandoned farmland, principally in Eastern Europe and Russia. Around 3 million ha of plantation forests were also established, mainly for timber production, with Asia seeing the most rapid expansion.

Estimates of the total land available for afforestation / reforestation (AR) are somewhat speculative given the uncertainty about future requirements for food production, urban developments and the suitability of certain soil / vegetation types for tree cover. The two most comprehensive studies of land availability (Dixon *et al.*, 1993; Brown *et al.*, 1996) were based on official statistics on land use categories planning obtained from a number of countries. These studies concluded that 345–480 million hectares could be available for AR, including agroforestry (the combined cultivation of trees and agricultural crops) over the next 50 years. They also estimated that a further 217 million hectares of degraded or damaged forest could be available for restoration in the same timeframe. Another detailed study of twelve developing countries[1] in Asia and Africa (Sathaye and Ravindranath, 1998) suggested that the global area available for afforestation / reforestation might be considerably higher, since they identified 268 million hectares available in these countries alone.

The carbon sequestration potential of any AR scheme depends on species, site conditions and management at each location, and is therefore very variable. Typical sequestration rates for forests established using conventional plantation practices in the boreal region are 0.8–2.4 tC per hectare per year; this rises to 1.7–7.5 tC per hectare per year in temperate regions and to 3.2–10.0 tC per hectare per year in the

[1] China, India, Indonesia, Mongolia, Myanmar, Pakistan, Philippines, South Korea, Thailand, Vietnam, Cameroon and Ghana.

tropics. From these ranges and information about plantation productivity in different countries, Brown *et al.* (1996) estimated that AR could sequester about 38 GtC (0.76 GtC per year) worldwide between 2000 and 2050. Figure 12.2 shows the estimated contributions to this overall figure from the main climatological regions; tropical and temperate regions are expected to have the greatest potential. The Brown and Dixon studies also estimated that an additional 11.5–28.7 GtC (0.25–0.58 GtC per year) could be sequestered through assisted regeneration of some 217 million ha of degraded forestland in the tropics.

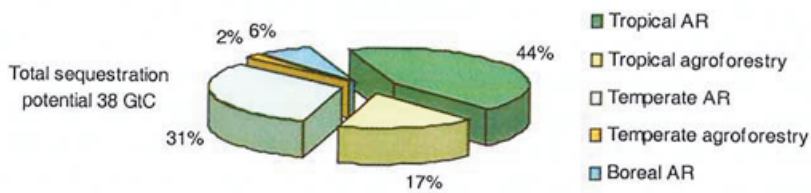


Figure 12.2 Potential contribution to global sequestration by AR and agroforestry activities 1995–2050 (data from Brown *et al.*, 1996). Note: Brown *et al.* revisited their 1996 estimates of global AR potential for the IPCC Third Assessment Report and found no significant new evidence indicating substantial change to this estimate.

Other authors, including Houghton *et al.* (1991) and Bass *et al.* (2000), have pointed out that the area of land that it would be practically feasible viable to afforest is likely to be considerably less than the technical potential when various social and economic factors are fully taken into account. Assuming that only one-third of ecologically suitable land may actually be available for AR activities, Houghton *et al.* (1991) estimated that AR activities could more realistically sequester about 0.25 GtC per year and restoration of degraded lands a further 0.13 GtC per year. Thus the quantity of carbon that could be sequestered by AR over the next 50 years could technically be as high as ~65 GtC, but is unlikely to be more than 20 GtC. The amount of CO_2 in the atmosphere is currently increasing by ~3.5 GtC per year. AR could therefore store no more than 5 to 20 years' worth of this anticipated rise in the next 50 years and its contribution to global CO_2 abatement, although useful, is quite limited. Furthermore, it is worth noting that these new forests would need to be preserved so as to prevent the subsequent reversal of this uptake.

It is very hard to estimate the cost of implementing afforestation on the scale contemplated for significant mitigation of climate change. According to forestry economists Ingles and Baguley (pers. comm., 2001), the cost of establishment for most plantations, excluding the purchase of land is US $500–1000 per ha. Most

afforestation undertaken today is either profitable, when income from timber is taken into account or represents the least-cost use of certain areas of land, when agricultural production is no longer viable. It is likely that significant expansion from current levels of afforestation could take place at costs of $5–10 per tonne CO_2 stored. This would make afforestation a relatively inexpensive CO_2 sequestration option compared with, say, storage in geological sub-strata, but only where land and opportunity costs were minimal.

12.1.2 Management and conservation of existing forests

The conservation of existing forest carbon stocks has the greatest potential for rapid mitigation of climate change. As the majority of carbon emissions from deforestation occur within a few years of forest clearance, reducing the rate of deforestation will produce a more immediate effect on global atmospheric CO_2 levels than will AR measures, in which similar volumes of carbon may be removed from the atmosphere but over a much longer period.

The estimation of benefit from the conservation of forest cover depends on the assumed baseline for deforestation (*i.e.* the amount of forest that would be lost in the absence of intervention). However, estimates of deforestation rates in the tropics are still highly uncertain. Estimates of emissions from tropical deforestation for the 1980s, based on the FAO's Forest Resource Assessment (Dixon *et al.*, 1993) were in the range of 1.2–2.2 GtC per year. However, a more recent study based on extensive sampling of satellite data produced significantly lower estimates, in the range of 0.3–0.8 GtC per year for the 1980s, increasing to 0.5–1.4 GtC per year for the 1990s (DeFries *et al.*, 2002). (This is an example of a common problem in this young field: different methodologies lead to substantially different estimates of the same quantity. It is expected that these differences will be resolved in time as measuring techniques are refined.) Theoretically, these amounts of carbon could be conserved if deforestation were completely halted. However, the experiences of governments and NGO agencies involved in forest protection have shown that projects need to address the underlying causes of deforestation and unsustainable use to achieve effective carbon conservation. Brown *et al.* (1996) estimated that a reduction in deforestation in tropical regions could feasibly conserve 10–20 GtC by 2050 at the rate of 0.2–0.4 GtC per year.

The conservation of carbon stocks in production forests[2] may be achieved through improved management practices. Potentially the most important is the use of reduced impact logging in the tropics. Conventional logging practices often result in a high level of damage to the residual stand, with up to 50% of remaining trees damaged or killed (Kurpick *et al.*, 1997). Application of reduced impact logging techniques can reduce the level of damage to the residual stand by 50% (Sist *et al.*, 1998) and hence reduce the level of carbon emission associated with logging. Nabuurs and Mohren (1993) calculated that the long-term carbon conservation that would be achieved by reduced impact logging in tropical rainforest is between 73 and 97 tC per hectare. Given that an estimated 15 million hectares of tropical forest are logged each year (Singh, 1993), the majority of which is considered to be unsustainable (Poore, 1989), the potential for increased carbon storage is considerable.

Wildfires result in large losses of carbon from forests every year. Weather conditions brought on by climate change, such as the enhanced El Niño, may increase the potential risk of wildfires. Fire management practices have the potential to conserve carbon stocks in forests. However, to be effective fire prevention and fire-fighting efforts must be combined with land use policy changes and measures to address the needs of rural populations.

Carbon uptake in many natural forests, particularly at high northern latitudes, is acutely constrained by nutrient availability. The carbon sink capacity of such areas could be significantly increased by sparing applications of fertiliser coupled with extension of the length of rotation. However, the potential for increased emissions of the powerful GHG N_2O resulting from fertiliser applications should be taken into account. There are few estimates of the potential GHG benefits of this type of carbon forestry. A recent meeting of experts (Royal Society, 2001) produced a preliminary estimate of an enhanced sink capacity of 0.3 GtC per year from worldwide forest management.

12.1.3 Substitution of fossil fuels and materials

Biofuels currently provide about 49 EJ (1170 Mtoe) of energy per year, or ~11% of global primary supply. In developing countries, biofuels account for about 20% of primary energy supply (IEA, 2000). If current biofuels were to be replaced by fossil-fuel-derived energy, an additional 1.1 GtC per year would be released into the

[2] Production forests are natural forests, such as large areas of Amazonia, Kalimantan and the Congo, which are managed for timber.

atmosphere (IPCC, 2000, Ch. 5). Sustainably produced biofuels do not result in net emissions of CO_2 to the atmosphere since the CO_2 released through the combustion of the fuel is taken up by re-growing biomass. The substitution of fossil fuels by sustainable biofuels will therefore result in a reduction of CO_2 emission directly proportional to the volume of fossil fuel replaced. Predictions of the future role of biofuels in meeting energy requirements range from 59 to 145 EJ in 2025 and 94 to 280 EJ in 2050 (Bass *et al.*, 2000; Hall *et al.*, 2000). The more optimistic scenarios for biomass development imply avoided CO_2 emissions of ~3.5 GtC yr^{-1}. The success of biomass-fuelled power generation will to a large extent depend on the technologies being developed for efficient gasification of wood products and on the logistics of transporting biomass to the point of combustion.

New biofuel plantations will also have a long-term positive sequestration effect if they replace a land use with lower carbon density. While the long-term average carbon density of forests managed for biofuels (particularly for short-rotation coppice) will be lower than a natural forest or long-rotation plantation, they store more carbon than most non-forest land uses. However, if natural forests were to be replaced with short-rotation coppice for biofuel production, the beneficial effect of fossil fuel substitution would be lost by the emissions resulting from forest conversion.

The use of wood products in place of materials that are associated with the release of large volumes of carbon dioxide (either during processing, such as cement, or through energy consumption, such as steel) could also lead to significant net CO_2 emission reductions.

12.1.4 Other land use activities

There is a range of other activities in the agricultural sector that could be used to mitigate various sources of GHG emissions. Given the huge area of land devoted to agricultural activity (1.5 Gha, according to FAO, 1999), changes to farming practices could have a significant impact on GHG emissions if they were widely adopted. Promising techniques include reduced tillage and erosion control to reverse the decline in soil organic matter, improved fertilisation methods to reduce N_2O emissions arising from denitrification and improved wetland rice cultivation methods to reduce methane and N_2O emissions. Only a few studies of the global potential GHG benefits of such practices have been made. However, Lal *et al.* (1999) estimated that conservation tillage methods could sequester 0.012–0.023 GtC per year in the

USA alone. Studies of the potential for new farming practices to reduce N_2O emissions are still in their infancy (Smith, pers. comm., 2001).

Changes to the diets of ruminant animals, whose gut bacteria produce methane in large quantities, are also being evaluated. Some results indicate that CH_4 emissions may be cut by at least half by the introduction of improved diets (Kurihara *et al.*, 1999). Grazing practices can also be altered to increase carbon uptake and storage in grasslands, particularly where soil nutrients are limited. Haynes and Williams (1992) and Schnabel *et al.* (2000) showed that fertilisation and improved stock management could lead to significant increases in biomass and soil carbon in US grazing lands.

12.2 Forests and land use in the Kyoto Protocol

The 1997 Kyoto Protocol (*www.unfccc.int*) was a landmark in international environmental policy. If ratified, the Protocol would oblige 31 industrial (so-called Annex 1) countries to constrain their emissions of a basket of six GHGs to around 95% of their levels in 1990 during a "commitment period" of 2008–2012. This is only a fraction of the total reduction necessary to stabilise atmospheric concentrations of GHGs around the 450–550 ppm thresholds mooted by organisations such as the EU Council of Ministers, the UK's Royal Commission on Environmental Pollution, WWF and Greenpeace. However, these commitments should be viewed against the background of expected economic growth that, in the absence of intervention, would probably lead to an increase in emissions of 20–30% by the middle of the commitment period.

The role of forestry and land use activities as a means of helping countries to achieve their 2008–2012 targets has been one of the most contentious aspects of the Kyoto deal. Europe and most developing countries have sought to limit the extent to which forestry and land use change activities would be eligible towards the achievement of the Kyoto targets, while the USA, Canada, Australia and Japan have sought a wider degree of flexibility, incorporating a relatively high allowance for potential 'sink' activities in these areas.

Between the tortuous intergovernmental negotiations that took place at The Hague and Bonn in the autumn of 2000 and the summer of 2001, the USA signalled its intention to withdraw from the Kyoto Protocol, followed shortly by Australia. In theory, the Protocol could still come into force, as long as countries representing 55% of global emissions complete the ratification process. As of September 2003 the fate of the Protocol depends on ratification by Russia.

The specific articles of the Kyoto Protocol referring to forestry and land use mitigation activities are 3.3, 3.4, 6, and 12:

Article 3.3 Annex 1 countries are allowed to take account of the uptake of carbon in the period 2008–2012 by forests planted since 1990 (so-called Afforestation and Reforestation or AR) and they may also take credit for any reduction in the rate of emissions from deforestation, relative to 1990.

Article 3.4 Annex 1 countries are allowed to take some account of uptake of carbon by managed forests, agricultural and grazing lands between 2008 and 2012. The precise rules governing the amount of uptake by these sinks that countries will be allowed to take credit for are complex, with different accounting rules applying to three tiers of activities (Pronk, 2001). A first tier of forest management activity, up to 30 million tCO_2 per year,[3] will receive 100% credit. A second tier of forest management activity will be discounted by 85%, to take account of the uncertainties regarding the permanence and possible indirect factors (such as climate change and nitrogen deposition) that may have contributed to carbon uptake. A third tier of carbon uptake by management of cropland, grazing land and "revegetation" [4] will be accounted for on a net–net basis, *i.e.*, the annual uptake of carbon during the commitment period minus the annual uptake during the base year (1990).

Article 6 (Joint Implementation) Annex 1 countries are allowed to undertake and share the emission reduction benefits from "Joint Implementation" (JI) projects in both energy and land use sectors in other Annex 1 countries. A wide range of forestry and land use project types are allowed under the JI framework. There are no specific limitations on the extent of land use projects other than those deriving from Article 3.3 and 3.4.

Article 12 (Clean Development Mechanism) Annex 1 countries are furthermore allowed to achieve part of their emission reduction obligations by purchasing Certified Emission Reductions (CERs) from projects in developing countries through a mechanism known as the Clean Development Mechanism (CDM). These projects are also supposed to contribute to the sustainable development objectives of the host country as well as reducing emissions or capturing carbon. The only land use activities that will be allowed under the CDM during the first commitment period will be afforestation and reforestation. The amount of CERs from forestry activities that an

[3] Various complex conditions apply, relating Article 3.3 and the first tier of Article 3.4.

[4] Revegetation is where the carbon density of land is increased through addition of woody vegetation but not to the level that constitutes 'forest'.

Annex 1 country will be allowed to purchase each year of the first commitment period will be limited to 1% of the national emissions of the Annex 1 country in 1990.

It is still too early to determine whether the Kyoto Protocol will have a significant effect on the overall level of investment in forestry and land use or in the type of activities that are undertaken in the land use sector. Many observers have noted that the proposed rules for project eligibility and accounting are complex, restrictive and open to more than one interpretation. This is perhaps not surprising given the convoluted negotiating process and the large number of stakeholders involved in setting out the proposed structures.

What does appear certain is that, for some Annex 1 countries, existing afforestation programmes and forest management activities will make significant contributions towards emission reduction obligations in the years leading up to 2010. Table 12.1 shows the expected contribution of forestry in six Annex 1 countries during the first commitment period of the Kyoto Protocol.[5]

Table 12.1 Expected contribution of forestry towards national emission reduction obligations for selected Annex 1 countries in period 2008–2012

Country	Emission reduction required 2008–2010 with respect to 1990	Emission reduction (+) or increase (–) expected from ARD[a]	Removals by managed forests and agriculture[b]	Cap on credit for removal by sinks under Marrakech Agreement
USA	117	–7.2	298.0	52.4
Canada	10	–4.3	14.1	4.9
Japan	20	–1.1	9.8	1.3
New Zealand	0	7.6	0.2	7.6
UK	16	0.6	2.5	0.9
Germany	26	–0.2	8.6	1.3

Figures in MtC per year, based on national communications to the UNFCCC, 2001.

[a] ARD = afforestation, reforestation and reduced deforestation, as reflected by the national emissions inventory.

[b] Based on national submissions by the Parties on 1 August 2000. The data are not fully comparable because Parties use different definitions for land use categories.

[5] The US government's figure for carbon uptake by its managed forests has created considerable controversy within the scientific and environmental policy community. One explanation for the magnitude of the number is the categorisation by the US Department of Environment of all forests where fire protection measures are applied as 'managed forests'.

Taking the Annex 1 countries as a whole, afforestation and reforestation may contribute up to 150 MtC per year towards the expected gap of 800 MtC between emissions and the Kyoto targets and the probable level of emissions in the absence of emission reduction interventions (IPCC, 2000). Some NGOs have asserted that allowing forestry projects into the CDM will create a further significant dent in the requirement for parties to the Convention to cut their emissions at source. However, analyses by Tipper and Bass (2001) and Tipper *et al.* (2002) of the capacity of forestry organisations to complete the project development and approval processes implicit in the CDM text suggests that forestry projects within the CDM are unlikely to deliver more than 10 million tonnes of emission reduction credits per year during the first commitment period unless significant 'free riding' of existing forestry projects into the mechanism is tolerated.

12.3 Climate change management, carbon assets and liabilities

While there is still much uncertainty about the eventual outcome of international efforts to constrain GHG emissions, it is becoming clear that activities or projects leading to reductions in emissions are increasingly regarded in business circles as potential assets, while those that lead to additional GHG emissions are regarded as liabilities. The strategies that emerge to manage these assets and liabilities could have an important effect on policies for land use and forestry. Developing countries are those with most at stake, for several reasons:

- Most of their populations are involved in or dependent on the rural economy;
- Rural carbon flows are a significant portion of their total GHG balances, particularly in countries with either high deforestation rates or high levels of consumption of woodfuel (most developing countries fall into one or other of these categories);
- Low land prices, low wages and high vegetation growth rates (at least in moist tropical areas) mean that carbon sequestration projects are relatively cheap compared with afforestation schemes in developed countries;
- The global distribution of deforestation means that most of the potential benefit of reducing deforestation is to be found in developing countries.

The potential implications for rural populations in developing countries, particularly the rural poor, are considerable and could be either positive or negative. On the positive side, there is the possibility of investment through the CDM and via voluntary carbon purchasing initiatives for the development of agroforestry systems,

farm forestry diversification, watershed protection and the rehabilitation of degraded forests. On the negative side, there may be new limitations on access to resources such as firewood and timber, displacement of agriculturalists by large-scale plantations and concentration of wealth and power in the hands of those who have access to the international markets for carbon and timber.

In the light of these opportunities and threats, international institutions such as the CDM Executive Board and the World Bank need to devise strategies to ensure the poorest elements of developing country populations are not disadvantaged by the very mechanisms of an international convention that has been designed to promote sustainable development.

Those institutions in developing countries with responsibility for the development of rural areas and the management of forests and agricultural land will need to factor carbon assets into their decision making. The following questions are likely to arise:

- Which types of rural development or land management activities could generate carbon assets?
- What are the conditions for generating *bona fide* carbon assets (*e.g.* monitoring systems; guarantees regarding the permanence of land use change)?
- Who are the owners of the assets and how are transfers handled in legal terms?
- How should the assets be monitored and verified?
- What is the added value of projects in terms of social and biodiversity benefits?

Such questions are unlikely to have simple answers, especially when the ownership of assets is divided between many small landowners or different types of rural enterprise. It will be interesting to see the types of administrative structures that develop to address these issues over the next ten years.

12.4 Experiences and issues arising from land use and forestry projects designed to mitigate greenhouse gas emissions

The first projects in the agricultural and forestry sectors specifically designed to mitigate GHG emissions were started in the early 1990s. Early projects included a reduced impact logging initiative in Sabah, Malaysia and an agroforestry scheme in Guatemala; each of these projects received partial funding from US utility companies wishing to explore the opportunities for low cost GHG abatement (Stewart and Moura Costa, 1998). During the late 1990s, the underlying conventions regarding the quantification of the GHG mitigation impacts of projects were developed. The basic approach to quantifying the mitigation impact of a project involves comparison of

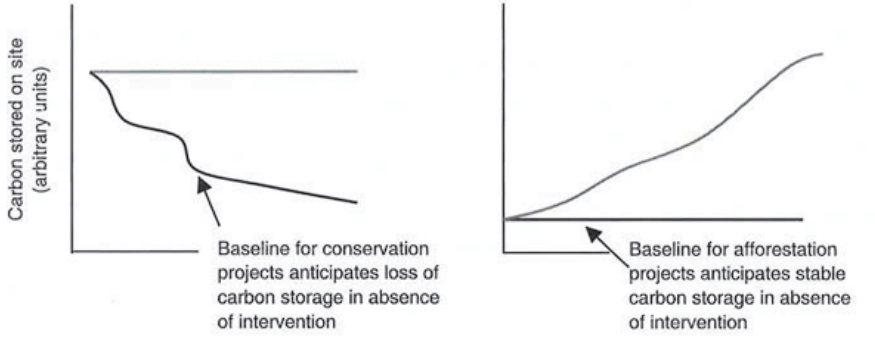


Figure 12.3 Illustrative trajectories of carbon storage under project scenario (green) and baseline scenario (black) for (a) a forest conservation project and (b) an afforestation or reforestation project.

GHG emissions from within a defined boundary[6] if the project is implemented with the emissions that would occur if the project is not implemented (the so-called reference case or *baseline* case). The emission reduction attributable to the project is the difference between these two cases. This value may then be adjusted to take account of possible *leakage* (when project activities lead to unintended but measurable increases in emissions outside the boundaries of the project) or lack of permanence. Figure 12.3 illustrates how the baseline-case and project-case trajectories for carbon storage compare in two project types. In conservation projects (Fig. 12.3a) the baseline reflects an anticipated loss of terrestrial carbon stocks in the absence of intervention. In afforestation/reforestation projects (Fig. 12.3b), the baseline reflects a continued low level of carbon storage on the site.

By the end of 2001, there were about fifty active carbon management projects in the land use and forestry sector around the world (ECCM, 2002). These varied considerably in their scale, the type of management systems being implemented and associated benefits. Most projects are still at pilot or demonstration stage and Latin America was the most active area of development. One factor common to all these projects is multi-objective management—the goals associated with carbon sequestration include biodiversity conservation, ecological restoration, fire protection, soil protection, income generation, agricultural diversification, rural energy supply, livestock improvement and sustainable timber production. Table 12.2 gives an overview of three prominent schemes currently underway, their critical issues (in terms of the effectiveness of GHG benefits) and their approximate scale.

[6] The boundaries normally define a physical space and activities, such as transport or electricity generation, which are directly affected by the proposed intervention.

Table 12.2 Three examples of carbon management projects in the land use sector

Project, Country	Summary, critical issues and web reference	GHG benefit
Noel Kempff Mercado, Bolivia	*Summary* Around 0.5 million hectares of tropical forest that would have been subject to a logging permit have now transferred to a national park. *Critical issues* How much carbon would have been lost assuming that low impact logging would have taken place? Will loggers simply move to other areas of forest? *http://nature.org/aboutus/projects/climate/work/art4253.html*	7 MtC over 25 years
Face-Profafor, Ecuador	*Summary* Up to 75,000 hectares of grassland in upland Ecuador will be afforested using pine and local species. *Critical issues* Will oxidation of soil C on these upland slopes reduce the overall carbon uptake by the plantations, and will the plantations be maintained. *www.facefoundation.nl/*	3 MtC over 40 years
Scolel Té, Mexico	*Summary* Small farmers undertake agroforestry, small-scale plantations and forest restoration activities. *Critical issues* Will farmers maintain the systems they have established for long enough to provide sustainable carbon benefits? *www.eccm.uk.com/scolelte*	0.5 MtC over 40 years

Based on the growing experience of pilot projects for carbon management in the land use sector, there are a number of common issues that arise concerning the validity and effectiveness of these initiatives. The principal issues raised are baselines, leakage, permanence and associated impacts, and we discuss each of these in turn.

Baselines and additionality A number of questions have been raised about the feasibility of making credible predictions about the future carbon balance of a particular area of land or forest. In particular, the baseline scenarios of projects that purport to reduce deforestation have come under close scrutiny. There are a number of difficulties with predicting the fate of vulnerable forests. The social and economic pressures that lead to deforestation events are both spatially and temporally varied, and may be influenced by a wide range of factors, including agricultural policies, accessibility by roads and rivers, population migrations, land tenure arrangements and demography. Because of this complexity it has frequently been argued that project developers may manipulate baseline estimates for potential projects to inflate the credit accruing to the project (Tipper and de Jong, 1998). In the case of afforestation

projects, particularly those with additional commercial benefits such as timber production, it may be difficult to demonstrate that establishment of a plantation would not have occurred in the absence of intervention, particularly if there is a ready market for the products. The screening out of so-called free-riding projects is likely to be one of the critical aspects of the regulations governing the CDM.

Leakage The GHG benefits of certain projects in the land use sector could be undermined by increases in emissions outside the boundaries of the project but which are, nevertheless, attributable to the project's activity. Potential examples are projects that aim to avoid deforestation by protecting specific forest areas as reserves; if the underlying causes of deforestation are not addressed and the actors are mobile they may simply move to other areas of forest. Project effects on the markets for timber, land and agricultural products could produce similar although less direct effects.

Permanence The potential reversibility of carbon uptake by terrestrial ecosystems has been one of the main concerns expressed by those countries and organisations wishing to constrain the use of forestry within the framework of the Kyoto Protocol. The potential for failure of management systems in the face of unforeseen future circumstances, natural disasters and climate change itself are among the dangers associated with a strategy of storing carbon in vegetation. Project developers are beginning to explore a number of ways of reducing the exposure to risk by increasing the resilience of the forest system to the effects of fires and pests. Potential actors in the carbon trading market are looking at instruments such as portfolios and insurance, and regulatory authorities are looking at strategies such as the creation of contingency reserves and the discounting of project benefits to take account of risk.

Environmental impacts While many of the non-GHG environmental impacts of carbon sequestration projects are likely to be beneficial (in particular the conservation of biodiversity), there may also be negative impacts if afforestation activities are not planned within the context of local and regional biodiversity and water management strategies. The potential for extensive areas of monoculture plantations becoming established under the Clean Development Mechanism is one of the principal concerns of environmental organisations observing the evolution of the Kyoto Protocol.

12.5 Conclusions

If the international momentum to address climate change is maintained, carbon uptake and storage by vegetation will be one of the important objectives of forest managers and landowners for the foreseeable future. However, the management of land solely

for GHG benefits is unlikely, since any market for emission reductions is unlikely to dominate the markets for conventional fibre and food products. Carbon management in the land use sector will be defined as the integration of GHG considerations into broader management goals. The development of clear and consistent guidelines for assigning GHG credits to projects in the land use sector remains a considerable challenge and there is an on-going debate as to the best methods. The opportunities and threats for developing countries are considerably greater than for industrial countries, owing to the overall importance of the rural sector within developing country economies, the large areas of land and high recent rates of deforestation. Carbon management using terrestrial ecosystems should be considered as only one aspect of a broader strategy to address climate change. The principal challenge remains the reduction of emissions associated with the use of fossil fuels.

Acknowledgement

The authors thank Alexandra Anghel for her assistance with data compilation for this chapter.

Useful websites

www.eccm.uk.com Edinburgh Centre for Carbon Management
www.cdiac.esd.ornl.gov/home.html Carbon Dioxide Information Analysis Centre
www.unfccc.int United Nations Framework Convention on Climate Change
www.cdmcapacity.org A website describing the emerging regulations of the CDM, as they apply to land use and forestry projects.

References

Bass S., Dubois O., Moura Costa P., Pinard M., Tipper R. and Wilson C. (2000), *Rural Livelihoods and Carbon Management*, IIED Natural Resources Issues Paper No.1, Institute for Environment and Development, London.

Brown S., Sathaye J., Cannel M. and Kauppi P. (1996), 'Management of forests for mitigation of greenhouse gas emissions', in *Climate Change 1995, Impacts, Adaptations and Mitigation of Climate Change: Scientific-Technical Analyses.*

Report of Working Group II, Assessment Report, IPCC, Watson R. T., Zinyowera M. C. and Moss R. H., eds., pp 773–797, Cambridge University Press, Cambridge.

DeFries R., Houghton R. A., Hansen C., Field C. B., Skole D. and Townshend J. (2002), 'Carbon emissions from tropical deforestation and regrowth based on satellite observation for the 1980s and 1990s', *Proc. Nat. Acad. Sci.* Published online, October 16, 2002, 10.1073/pnas.182560099.

Dixon R. K., Andrasko K. J., Sussman F. G., Lavinson M. A., Trexler M. C. and Vinson T. S. (1993), 'Forest sector carbon offset projects: near-term opportunities to mitigate greenhouse gas emissions', *Water Air and Soil Pollution* **70**, 561–577.

ECCM (2002), List of known carbon management projects in the land use sector, ECCM internal document, The Edinburgh Centre for Carbon Management, Scotland.

FAO (1999), *FAOSTAT Agricultural Production Database*, Food and Agriculture Organisation, Rome, Italy.

FAO (2001), *State of the World's Forests 2001*, Food and Agriculture Organisation of the United Nations, Rome, Italy.

Hall D. O., House J. I. and Scrase J. I. (2000), 'Introduction: overview of biomass energy', in *Industrial Uses of Biomass Energy*, Rosillo-Calle F. S., Bajay D. and Rotman H., eds., Taylor and Francis, London.

Haynes R. J. and Williams P. H. (1992), 'Accumulation of soil organics matter and the forms, mineralization potential and plant availability of accumulated organic sulfur: effects of pasture improvement and intensive cultivation', *Soil Biol. Biochem.* **24**, 209–217.

Houghton R. A., Unruh J. and Lefebvre P. A. (1991), 'Current land use in the tropics and its potential for sequestering carbon', in *Proc. Tech. Workshop to Explore Options for Global Forest Management*, Howlett D. and Springer L, eds., April 1991, Bangkok, Thailand. London, IIED.

Houghton R. A. (1996), 'Converting terrestrial ecosystems from sources to sinks of carbon', *Ambio* **25**, 267–272.

IEA (2000), IEA World Energy Outlook 2000—data for 1998 (*www.iea.org/statist/keyworld/p_0402.htm* and *p_0404.htm*)

Ingles and Baguley (pers. comm., 2001) are forestry economists based in Edinburgh, Scotland.

IPCC (2000), *Land Use, Land-Use Change and Forestry*, Special Report, Cambridge University Press, 377 pp.

Kauppi P. and Sedjo R. (2001), 'Technological and economic potential of options to enhance, maintain, and manage biological carbon reservoirs and geo-engineering', in *Climate Change 2001*, Third Assessment Report of the IPPC.

Kurihara M., Magner T., Hunter R. A. and McCrabb G. J. (1999), 'Methane production and energy partitioning of cattle in the tropics', *Brit. J. Nutrition* **81,** 263–272.

Kurpick P., Kurpick U. and Huth A. (1997), 'The influence of logging on a Malaysian rain forest: a study using a forest gap model', *J. Theor. Biol.* **185**, 47–54.

Lal R., Follett R. F., Kimble H. and Cole C. V. (1999), 'Managing US cropland to sequester carbon in soil', *J. Soil Water Conservation* **54**, 374–381.

Malhi Y., Baldocchi D. D. and Jarvis P. G. (1999), 'The carbon balance of tropical, temperate and boreal forests', *Plant, Cell and Environment* **22,** 715–740.

Nabuurs G. J. and Mohren G. M. (1993), 'Carbon fixation through forestry activities', IBN Research Report 93/4, Face Institute for Forestry and Nature Research, Arnhem/Wageningen, The Netherlands.

Poore D. W. (1989), *No Timber Without Trees*, Earthscan, London.

Pronk J. (2001), New Proposals by the President of COP6. Documents presented to the Parties of the UNFCCC, 9 April 2001.

Royal Society (2001), *The Role of Carbon Sinks in Mitigating Global Climate Change,* Policy Document 10/01, July 2001 (*www.royalsoc.ac.uk*).

Sathaye J. and Ravindranath N. H. (1998), 'Climate change mitigation in the energy and forestry sectors of developing countries', *Ann. Review. Energy Environment* **23**, 387–437.

Schnabel R. R., Franzluebbers A. J., Stout W. L., Sanderson M. A. and Stuedemann G. B. (2000), 'Pasture management effects on soil carbon sequestration', in *Carbon Sequestration Potential of US Grazing Lands*, Follett R. F., Kimble J. M. and Lal R., eds., Springer-Verlag, New York, pp. 194–220.

Singh K. D. (1993), 'Forest resources assessment 1990: tropical countries', *Unasylva* **44**, 10–22.

Sist P., Nolan T., Bertault J. G. and Dytstra D. (1998), 'Harvesting intensity versus sustainability in Indonesia', *Forest Ecol. Management* **108**, 251–260.

Smith K. (pers. comm., 2001), Professor of Soil Science at the Institute of Ecology and Resource Management, University of Edinburgh.

Stewart M. and Moura Costa P. H. (1998), *Climate Change Mitigation by Forestry: a Review of International Initiatives*, IIED, London.

Tipper R. and De Jong B. (1998), 'Quantification and regulation of carbon offsets from forestry: comparison of alternate methodologies with special reference to Chiapas, Mexico', *Commonwealth Forestry Review* **77**, 219–228.

Tipper R. and Bass S. (2001), *Analysis of Options for Carbon Sequestration Activities in the CDM,* Report prepared for the UK Department for International Development, UK.

Tipper R., Carr R. and McGhee W. (2002), 'Guidance on baselines methodologies and crediting periods for LULUCF projects in the CDM', ECCM Policy Paper prepared for UK DEFRA.

UNFCCC (2002), United Nations Framework Convention on Climate Change; subsidiary articles and documentation can be found at *www.unfccc.de*.

This page intentionally left blank

CHAPTER 13

BIOTECHNOLOGY: ITS IMPACT AND FUTURE PROSPECTS

DENIS J. MURPHY
Biotechnology Unit, School of Applied Sciences,
University of Glamorgan, Trefforest, Cardiff CF37 1DL, United Kingdom
dmurphy2@glam.ac.uk

For out of olde feldes, as men seith
Cometh al this newe corn fro yeer to yere;
And out of olde bokes, in good feith,
Cometh all this newe science that men lere.

Geoffrey Chaucer, *The Parlement of Foules, ca.* 1382.

13.1 Introduction

Biotechnology was originally an entirely empirical process, pursued by human societies for many millennia. This classical form of biotechnology involved the creation of new products by harnessing biological systems, most particularly microbial fermentation. The raw materials for such biotechnological conversions were either naturally growing plants or, more commonly, were obtained from agricultural crops or livestock. Animal or microbial extracts were used to convert milk from cows, sheep, goats *etc.* into foodstuffs like cheese or yoghurt, while yeasts were used to make leavened bread from wheat flour. The poor quality of drinking water, particularly in urban areas, made it desirable to consume fermented drinks as a safer alternative beverage. Extracts of any sugar-containing raw material, including most fruits, grains and animal milks, could be used in conjunction with yeasts to produce a wide range of such alcoholic liquors. For example, malted barley can be made into beer, grapes into wine, rice into saki and yak's milk into chang. During the past fifty years, biotechnology has gradually come to have a rather different meaning, namely the scientific application of modern biological techniques, such as recombinant DNA technology, for the manipulation of organisms to enhance their usefulness to mankind.

13.1.1 Microbial biotechnology

In many cases, modern recombinant DNA-based biotechnology is still applied at the microbial level, for example in the large-scale production of pharmaceuticals like insulin or blood-clotting factors in bacterial cultures. Such applications have been particularly beneficial in enabling the production of recombinant proteins in culture as an alternative to their extraction from animal organs. Indeed, for the majority of newer peptide- or protein-based therapeutic agents, microbial fermentation is now the most obvious production method of choice. This has been accentuated further by recent concerns over new diseases like BSE (bovine spongiform encephalopathy or mad-cow disease), which have led to a general distrust of many products derived from animals or humans. In addition, the contamination of some human blood stocks by pathogens including the hepatitis C virus and human immunodeficiency virus (HIV) has led to the often-fatal infection of vulnerable groups like haemophiliacs following blood transfusions. In 2000, the World Health Organisation (WHO) estimated that 80% of the world population was at risk because their national blood banks were contaminated by such pathogens (*www.wo.int/inf-pr-2000/en/pr2000-25.html*). This is now leading to widespread calls for the more rapid development of alternative recombinant forms of the many therapeutic agents that have traditionally been derived from human or animal sources. Unfortunately, although some of these recombinant drugs are already available via microbial fermentation, their high cost sometimes limits or precludes their use and has even led to a black market in the case of blood-clotting peptides. The problem is particularly acute in developing countries, most of which are unable to afford such drugs. A possible way to produce recombinant drugs more cheaply is to use plants or plant cultures as alternative expression systems to bacteria; we shall discuss this in Section 13.4.5.

Some of the newer applications of modern biotechnology are related to food production or processing. For example, in 1990 approval was granted in the UK for the use of recombinant microbial chymosin for the manufacture of vegetarian cheese. This was an alternative to extracting chymosin in the traditional manner from rennet obtained from the stomachs of slaughtered newborn calves. Chymosin breaks down the milk protein casein to paracaesin, which then combines with calcium in the milk to form the insoluble compound calcium paracaesin. The latter precipitates out as a solid mass that also includes the milk fat, hence forming the curds from which cheese is derived. The use of recombinant chymosin makes it unnecessary to slaughter calves and guarantees a source of 'vegetarian' cheese that now forms a significant proportion of all the cheese sold in supermarkets. Despite some initial ambivalence, most consumers have now accepted this GMO-based product, which, although it tastes and

costs the same as conventional cheese, has the benefit of being ethically more acceptable to many people who have concerns about the use of non-dairy animal products.

13.1.2 Agricultural biotechnology

More recently, the term biotechnology (sometimes called "agbiotech") has also been applied to the manipulation of crop plants by non-traditional methods, such as induced mutagenesis or gene transfer. The most familiar commercial manifestations of agbiotech involve modifications of input traits such as disease resistance or drought tolerance in existing crops. Manipulation of input traits may make the crop easier or cheaper to grow, but does not affect the type of products that are obtained from such crops. A more radical use of biotechnology in agriculture is for the modification of output traits, whereby a host of new products can potentially be manufactured in the plant (or animal) for edible, industrial or even pharmaceutical applications.

However, it must be stressed that biotechnology is much more than the production of genetically manipulated organisms (GMOs). For example, DNA-fingerprinting and genomic profiling are new biotechnological techniques that can be used with great effect in accelerating the selection of new crop varieties in conventional, non-transgenic breeding programmes. Biotechnology research has also given plant breeders access to other new techniques, such as embryo rescue or induced mutation, that can be used in conjunction with ever-improving tissue culture and regeneration methods to enhance crop plant variation and the use of wild relatives to improve both input and output traits, without resorting to gene transfer.

The aim of this chapter is to assess the impact of modern biotechnology on crop agriculture, particularly for non-food production, and its future potential over the next few decades. A key aspect of the chapter is a global perspective that also considers the impact of other relevant factors such as the huge increase in the world population over the next 20–30 years and the likely decline in non-renewable petroleum reserves over the same period. We shall also discuss the more general issues of public concern over biotechnology and the complex interplay of scientific, economic, commercial and political issues.

13.2 Background

13.2.1 Scientific developments

The development of modern plant biotechnology has been made possible by a series of technical breakthroughs over the past twenty years, particularly in molecular and cell biology. In the late 1970s, the discovery of the restriction enzymes, DNA polymerases and reverse transcriptases made it possible 'cut and paste' DNA fragments and also to synthesise DNA from RNA *in vitro*. This in turn enabled the construction of cDNA libraries and the isolation and copying of individual genes. This process was further facilitated in the early 1990s by the widespread use of the polymerase chain reaction (PCR) both for the isolation of specific DNA sequences and for the rapid cloning of novel genes. Some of the most dramatic and well-known achievements in applying this technology to plants have included the use of the bacterial vector *Agrobacterium tumefaciens* to transfer foreign DNA into plant tissues (Otten *et al.*, 1981) and the use of particle bombardment to deliver DNA into plants (Ye *et al.*, 1990). This was particularly useful in cases where *Agrobacterium*-mediated transfer was problematic. A further development was the isolation of gene promoters that were able to regulate the expression of the transferred gene, or transgene, so that the gene product was (apparently) only accumulated in specific tissues or at specific stages of development (Gallie, 1998).

As well as adding completely new genes to plants, transgenic methods can be used to insert extra copies of existing genes. If the extra copies of the existing gene are in the reverse, or antisense orientation, the expression of the resident gene is often reduced, probably because of the formation of complexes between the sense and antisense copies of mRNA that disrupt its translation into proteins. In principle, this technique allows us to reduce or eliminate the expression of any unwanted gene in a plant, providing we know at least part of its sequence. Another method for manipulating gene expression is to add extra copies of an existing gene in the correct, or 'sense' orientation. When additional sense copies of the resident gene are inserted, this sometimes results in a higher rate of gene expression, although sometimes a phenomenon called co-suppression occurs, which reduces gene expression as if an antisense transgene had been added. Both antisense and co-suppression have been used with great effect to reduce or eliminate the expression of undesired genes in plants. Meanwhile, newer and potentially more powerful methods of gene disruption, some involving viral vectors, are now being developed (Angell and Baulcombe, 1999).

These molecular advances have been matched by the often rather underrated achievements in cell and tissue culture that have allowed the regeneration of cell or tissue explants into adult plants; the use of mass clonal propagation, as used particularly to multiply trees crops; and the development of embryo rescue as a tool to assist wide crosses that might otherwise be sterile. Induced mutagenesis has also increasingly been used as a method for deleting the function of unwanted genes, such as those responsible for the accumulation of a toxin in the crop. Mutations can be induced by treating plants with chemicals like ethane methane sulphonate or by irradiation with X- or γ-rays. Induced mutation has been used recently to create completely new crops, such as 'linola', which was originally derived from linseed (Green and Dribnenki, 1994). In the case of linola, random chemical mutagenesis followed by selection of over 10,000 mutagenised seeds was used to delete the function of two fatty acid desaturase genes from the linseed genome. This resulted in the virtual elimination of α-linolenic acid from the seed oil and the accumulation instead of linoleic acid. This new high-linoleic oilseed crop is now marketed as "linola": it produces an edible-grade oil similar to that of sunflower. In contrast, the traditional linseed oil that is high in α-linolenic acid is inedible but has uses as an industrial drying agent and is commonly used in the manufacture of putty and linoleum. Interestingly, although new crop varieties obtained from induced mutagenesis may contain many other altered genes as well as the target genes, these crops are subject to none of the stringent regulatory regimes applied to transgenic crops.

Early attempts to produce transgenic plants relied on the use of model systems such as tobacco and tomato that were receptive to *Agrobacterium*-mediated gene transfer. One of the earliest field releases of transgenic crops were potatoes containing a bacterial marker gene, grown in the Plant Breeding Institute, Cambridge, UK in the mid-1980s. During the 1990s, the list of crops that could be transformed by *Agrobacterium* grew ever larger, until by the end of the decade even the hitherto recalcitrant cereals were added. For species where the sometimes-arcane protocols for *Agrobacterium*-mediated gene transfer have yet to be developed, particle bombardment, or biolistics, is a good alternative. By the end of the year 2001, it was possible to transform nearly all of the major annual crops by one or other of these methods and several dozen tree crops had also been transformed, at least with marker genes. Some of the transgenic trees being developed included apple, walnut, pecan and persimmon in the USA, eucalyptus in Australia and South America, and oil palm in Malaysia. The time scales for some of the transgenic tree projects are perforce very long term. For example, the new transgenic oil palm plantlets containing marker genes that were a year old in 2000 will not reach maturity until about 2007, while adult plants with agronomically relevant transgenes are unlikely to be available before 2015 or later.

Table 13.1 Accumulation of novel fatty acids by some oil-producing plants

Fatty acid[a]	Amount[b]	Plant species	Uses
8:0	94%	*Cuphea avigera*	Fuel, food
10:0	95%	*Cuphea koehneana*	Detergents, food
12:0	94%	*Litsea stocksii*	Detergents, food
14:0	92%	*Knema globularia*	Soaps, cosmetics
16:0	92%	*Myrica cerifera*	Food, soaps
18:0	65%	*Garcinia cornea*	Food, confectionery
20:0	33%	*Nephelium lappaceum*	Lubricants
22:0	48%	*Brassica tournefortii*	Lubricants
24:0	19%	*Adenanthera pavonina*	Lubricants
$18:1_{\Delta 6}$	76%	*Coriandrum sativum*	Nylons, detergents
$18:1_{\Delta 9}$	78%	*Olea europaea*	Food, lubricants
$22:1_{\Delta 13}$	58%	*Crambe abyssinica*	Plasticisers, nylons
$18:2_{\Delta 9,12}$	75%	*Helianthus annuus*	Food, coatings
$\alpha 18:3_{\Delta 9,12,15}$	60%	*Linum usitatissimum*	Paints, varnishes
$\gamma 18:3_{\Delta 6,9,12}$	25%	*Borago officinalis*	Therapeutic products
18:1–hydroxy	90%	*Ricinus communis*	Plasticisers, cosmetics
18:2–epoxy	60%	*Crepis palestina*	Resins, coatings
18:2–triple	70%	*Crepis alpina*	Coatings, lubricants
18:3–oxo	78%	*Oiticica*	Paints, inks
18:3–conj	70%	*Tung*	Enamels, varnishes
20:1/22;1wax	95%	*Simmondsia chinensis*	Cosmetics, lubricants

[a] Fatty acids are denoted by their carbon chain length followed by the number of double bonds or nature of other functionalities; [b] Percentage of total fatty acids; data are taken from Murphy (2001).

The advent of PCR-based cloning and automated DNA sequencing in the mid-late 1990s made possible the near-complete sequencing of the first higher plant genome, as announced in late 2000 (The Arabidopsis Genome Initiative, 2000). The plant in question is a small, weedy relative of the common brassicas called *Arabidopsis thaliana*. These techniques have also given biotechnologists the ability to create and sequence large cDNA expression libraries from any tissue of interest. For example, as shown in Table 13.1, there are numerous relatively obscure plants that accumulate high levels of unusual and potentially valuable fatty acids in their seeds. In order to isolate the genes responsible for the synthesis of these compounds, it is necessary to

construct and sequence a library of all the RNAs expressed in developing seeds of these plants at the stage when the fatty acids are accumulating. This strategy has been used to great effect by scientists at companies such as Dupont in order to isolate dozens of new genes encoding many different fatty acid modification enzymes. These genes can then be inserted into crops such as soybean or rapeseed in order to produce various novel fatty acids in their seed oil on an agricultural scale (Mazur *et al.*, 1999).

In the last few years, there have been several technical developments that will further accelerate the pace of progress in this already fast-moving area. These include the use of automated *in silico* methods, such as DNA chip technology, to screen arrays of thousands of genes, for example to select those genes involved in plant defence against a specific pathogen (Somerville and Somerville, 1999). Advances in computing hardware and software have vastly simplified the handling of the terabytes (10^{12}) of data now present in genome and protein databases. Automated systems are being developed for the 2D electrophoretic separation and sequencing of all the proteins present at a particular stage of cell development—the so-called 'proteome' (Ryu and Nam, 2000). Perhaps even more impressive is the automation of the process of protein overexpression in bacteria (Edwards, 2000), followed by purification and structural analysis either by X-ray crystallography (Abola *et al.*, 2000) or high-field nuclear magnetic resonance spectroscopy (Montelione *et al.*, 2000). These developments have led to the emerging discipline of structural genomics, recently reviewed by Burley (2000).

These past and present achievements have made it possible to perform manipulations of biological systems that were in the realm of science fiction only a few decades ago. The manner in which biotechnology will be applied to agriculture will to some extent be dictated by these and future scientific developments, but of equal or greater importance are the commercial, geographical and societal factors that will govern the nature and demand for agricultural resources in the 21st. century, which we will now consider.

13.2.2 Population growth and agriculture

Projections of global population growth over the next fifty years are in general agreement that there will be a gradual slowdown in the rate of growth, but that even with modest rates of increase there will be an additional 3.0±1.7 billion people by 2050, as shown in Fig. 13.1. In the next decade alone, we can confidently predict an additional 1 billion people, *i.e.* a 17% increase. Factors that curtail economic growth will, ironically, lead to even higher rates of population increase as less developed

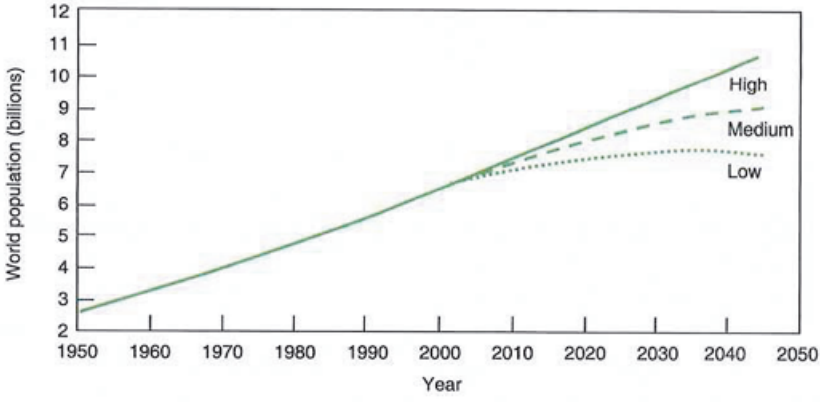


Figure 13.1 World population size: past estimates and high, medium and low fertility projections for the future. In all three scenarios, more than 97% of the population increase will take place in developing countries. Data are taken from the United Nations Department of Economic and Social Affairs (*www.popin.org/pop1998/1.htm*).

societies tend to have far higher ratios of births per woman. For example, this ratio is 5.0 in Pakistan but only about 1.5 in Europe (*www.iiasa.ac.at/Research/LUC/Papers/glch1/chap1.htm*). Virtually all of the population growth will take place in developing countries, especially in Asia and Africa. These statistics must be taken into account when planning for the future use of agricultural resources.

Obviously the primary concern of global agriculture over the next few decades should be to provide sufficient foodstuffs to sustain the increased populations. The current world agricultural production provides a modest surplus of food, although often this does not reach the most disadvantaged groups for a variety of economic or political reasons. Given the political will to distribute them, sufficient food reserves exist to cope with periodic localised catastrophic events, such as droughts, floods or warfare. The prognosis for increasing yields of food crops to cope with the inevitable future population increases is the subject of some controversy. On the one hand there are Malthusian predictions of widespread famine within the next 20–30 years in the absence of a new scientific breakthrough (enter agbiotech to save the world!) and on the other hand are more sober projections, based largely on previous yield increases, that food production will easily keep pace with population numbers. The truth probably lies between these two extremes but more towards the optimistic projections that the world will not starve. As shown in Fig. 13.2, world population has increased by 2.2-fold since 1950, but what is less well appreciated is that food production increased by 3.1-fold over the same period. This means that we now produce 40% more food *per capita* than in 1950 and most of the world's population is now

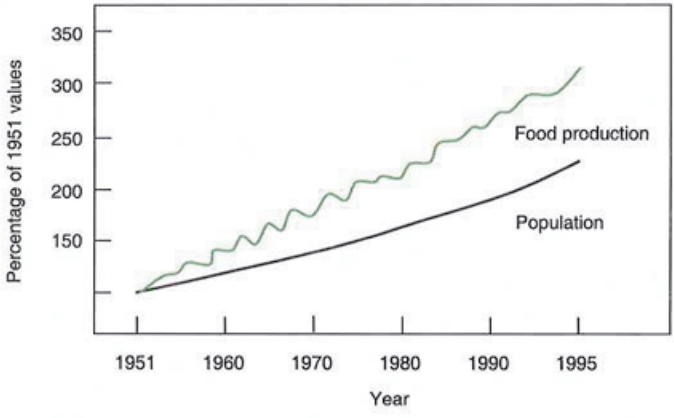


Figure 13.2 Growth in world food production and population. Over the past fifty years, world food production has consistently increased more rapidly than population, with the result that average *per capita* food consumption is now 40% more than in 1950. Data from the Food and Agriculture Organisation (*www.fao.org*).

consuming significantly more food *per capita* than previously. The *per capita* consumption of edible vegetable oils is a particularly good indicator of the economic well being of a country and this has increased even faster than general food consumption over the past few decades. During the 1990s, *per capita* vegetable oil consumption rose by 31% in Mexico, 35% in South Africa, 64% in China, 65% in Indonesia and 94% in India. In contrast, an economic slowdown may result in a decrease in the intake of such foodstuffs as shown by the 35% fall in food oil consumption in Russia between 1990 and 1994. Krattiger (2000) explores some of the complex arguments relating to the economics of demand for food and the possible role of agbiotech.

The relative glut in food production, which has been accentuated by agricultural subsidies in most developing countries, has been reflected in a steady decline in the prices of edible commodities such as palm and soybean oil, which at \$400 t^{-1} as opposed to \$2000 t^{-1} (Fry, 1998) are now at about 20% of their 1950 values. This has led to a depression of the agricultural economy in many parts of Europe and the USA and the set-aside or even the abandonment of agricultural land for food production. This means that, even if the world population increased by 40% in the next few decades (which it will not), it could still be fed by reverting towards a 1950s-like diet, *i.e.* less meat and fat and more plant carbohydrates. However, it is unlikely that we will need to alter our diet in this way because there is still considerable scope for future increases in food yields.

More food can be produced in developed countries by re-cultivating the considerable amount of land that is currently fallow due to set-aside or abandonment. Even larger increases in food yield will be possible by the better management of existing crop systems, particularly in developing countries, where the yields of many crops are only a fraction of those in the North. This does not imply the use of intensive input regimes in developing countries but it will require improved planning and management of existing crop systems to minimise unnecessary waste. Some modest increases in the yield potential will also be possible from conventional breeding. Modern biotechnology may play an important role in assisting in the necessary increases in food production but it is by no means a *sine qua non*.

An example of some of the potential for yield increases in a tropical crop can be seen with oil palm, which is the major export crop in Malaysia and is projected to become the number one source of edible vegetable oil during the next decade. Much of this oil will be used to supply the rapidly increasing populations of India and China whose 2.3 billion people already make up 37% of the global total. The current average yield of oil palm in Malaysia is about 3 t oil ha^{-1} but this can be increased to 6 t ha^{-1} simply by improving the way the crop is managed and harvested (Murphy, 2000; Tinker, 2000). We know that this is possible because some plantations already achieve these high yields by virtue of being better managed. Therefore, considerable yield improvements can be achieved in existing plantations and do not require new varieties or expensive transgene technology. A further increase to 8–10 t ha^{-1} could be realised using existing high-yield oil palm germplasm, but this will require a programme of clonal propagation and replanting of existing plantations. Finally, even higher yielding plants can almost certainly be selected using advanced screening methods and/or transgenic approaches. Given that most annual oilseed crops yield less than 1 t oil ha^{-1}, it is clear that oil palm has significant potential, not only to satisfy the increasingly demanding markets for edible oil in India and China, but also to provide a source of valuable non-food products for the oleochemicals industry (Murphy, 2000).

Similar considerations to those applying to oil palm also apply, albeit to varying extents, to many of the other major crops of developing countries. For example, in Africa the yield of maize is only 1.7 t ha^{-1} versus a global average of 4 t ha^{-1}, while sweet potato yields are 6 t ha^{-1} versus a global average of 14 t ha^{-1} (Wambugu, 1999). The reasons for this productivity gap are manifold but improvements in crop germplasm and management practices could contribute significantly to raising overall yields closer towards the global average and therefore increase food production in some of the countries that need it most.

In summary, we are about to be faced with a significant challenge to increase food yields to satisfy the rising world population. It should be possible to meet these demands by better crop management and improved breeding efforts but this will require concerted action from both public and private agencies in each country plus international bodies like the Food and Agriculture Organisation (FAO). Agbiotech methods like marker-assisted selection and clonal propagation may make a useful contribution to this goal by making it easier to breed improved cultivars of the mainly tropical and subtropical crops that are grown in the regions of maximum population growth. These efforts could be seriously jeopardised by renewed wars or civil unrest but, if these can be avoided, one can be relatively optimistic that in 2050 the world will not only be feeding its forecast 8–10 billion people but will also have sufficient agricultural capacity to produce significant amounts of renewable non-food crops.

13.2.3 Global petroleum resources

Before the late 19th century, most hydrocarbon-based products were derived from crops: for example, oilseeds provided vegetable oils for lubrication and lighting, while trees provided wood for fuel. This situation changed with the discovery of large deposits of coal, petroleum and natural gas. Now we find that much of our modern economy is based on the exploitation of fossil hydrocarbons that were originally the products of the massive radiation of terrestrial photosynthetic organisms in the Carboniferous era. These deposits took many millions of years to accumulate, but it will probably only take a few centuries for mankind to use them up (Kerr, 1998). The extent of our dependence on petroleum was underlined by the worldwide economic and political effects of the interruption of oil supplies from the Middle East in 1973.

The most versatile hydrocarbon is petroleum, which in addition to its ubiquitous use as a fuel is also the feedstock for a global petrochemicals industry that manufactures products as diverse as plastics, textiles, paints, cosmetics and pharmaceuticals. Since it is the product of past, rather than present, photosynthesis, petroleum is not renewable and stocks will eventually run out, but when? The lifetime of extractable petroleum reserves is difficult to estimate because we are not sure exactly how much petroleum exists in as yet undiscovered subterranean or submarine deposits. There are also many uncertainties in estimating future rates of consumption, even in the short term, as oil consumption is very sensitive to economic performance. As shown with the unexpected downturn in many Asian economies in the late 1990s, economic performance can be quite variable as well as notoriously unpredictable. Despite these drawbacks, there is a fairly consistent picture from estimates made by a

variety of industrial and academic experts, who agree that there is likely to be a peak in petroleum supplies at some point in the next one to three decades, followed by an irreversible decline as reserves dwindle and extraction costs escalate (Kerr, 1998; *www.geology.utoledo.edu/department/faculty/cbh/DL/Lec.htm*). Figure 13.3 shows a typical range of scenarios for future oil production. Some estimates from reputable sources are even more pessimistic than this and predict a peak in world oil production as soon as 2005–2010 (*www.oilcrisis.com/laherrere*). Given the enormous demand for petroleum at present, even the discovery of major new reserves like the Caspian basin will only add on a few extra years to the lifetime of known stocks. Indeed, it has been estimated that each additional billion barrels that is discovered will only push back the inevitable shortfall in world petroleum stocks by 5.5 days (Kerr, 1998).

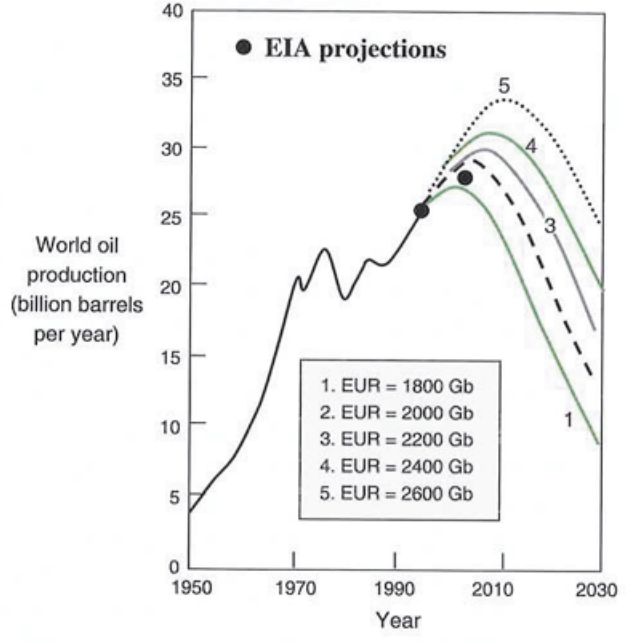


Figure 13.3 Scenarios for future global oil production. The estimated ultimately recoverable (EUR) deposits of crude oil range from 1800–2600 Gb (1 Gb = 1 billion barrels or 152 MT). Five scenarios for the future pattern of global oil production are shown. Using the most pessimistic value for EUR of 1800 Gb, the year of peak production is 2007, while with the most optimistic EUR of 2600 Gb a date of about 2020 is predicted. After this peak date in oil production, there will still be a considerable quantity of oil, but it will be an ever-diminishing resource that will inevitably become more expensive and difficult to obtain. Data from J. J. MacKenzie at the World Resources Institute (*www.wri.org/wri/climate/finiteoil/oilfutur.html*).

Once petroleum stocks become depleted, and long before they run out altogether, alternative sources of fuels and refined hydrocarbons will be required. Coal and gas will last longer than oil and may substitute for a while as fuel sources, but even they will run out at some point in the future. In the longer term, it will be necessary to use alternative fuel sources such as nuclear fission/fusion and renewables like hydro, wind or tidal power. It is unlikely that we can use bulky lignified biomass, for example from mature or coppiced trees or from straw, in a sustainable way other than for rather limited and localised power generation. Equally, the use of vegetable oil for diesel fuel is a waste, both economically and energetically, of a potentially valuable, partially refined industrial raw material. Also, it should be borne in mind that the entire 87 Mt annual production of plant oils is only about 2% of current petroleum consumption. Furthermore, even if the whole of our global agricultural production were switched to oil crops for biodiesel production, there would not be enough oil to satisfy more than a small fraction of the current demand for petroleum-derived fuels. A more appropriate non-food use of oil (and starch) crops would be as a replacement for the use of petrochemicals for the manufacture of relatively valuable oleochemicals. Indeed, in the long term, plants will eventually be the only source of hydrocarbons for refining to create the spectrum of products currently obtained from petrochemicals.

This leaves the question of what fuel(s) we will use for vehicles once petroleum reserves become sufficiently depleted as to render oil-derived fuels uneconomic. In the medium term, liquefied natural gas can probably substitute for petroleum, and longer term there are still estimated to be several hundred years of coal reserves that could be either used directly as solid fuel or converted to a more convenient liquid fuel (easily recoverable coal reserves are estimated at about 2830 Gb oil equivalents but there are another 42,000 Gb equivalents of coal reserves that are currently uneconomic to mine: this is about 20-fold more than total global oil reserves, (*http://starfire.ne.uiuc.edu/ne201/course/topics/resource_availability/oil_reserves.html*). It is also possible that, within a few decades, vehicles could be powered by renewable energy sources, for example by electricity from solar cells or by liquid hydrogen fuel produced by electrolysis of water. As non-renewable fuels become more expensive and difficult to use, and the performance and price of the renewable fuels continues to improve, there will be an inevitable shift to the latter for vehicle transportation. It is less likely that bio-derived fuels will be used extensively in transportation, as bioproducts would be better employed for food use or for the manufacture of industrial products.

13.2.4 The opportunity

The dramatic scientific advances of the past few decades, coupled with the increase in human populations and the depletion of non-renewable resources like hydrocarbons, present us with a unique opportunity to redesign the products of global photosynthesis, both food and non-food, to sustain the continued development of human populations over the coming decades. The immediate challenge will be to match the forecast population increase of 17% over the next decade with an equivalent increase in food production. As outlined above, yield improvements of this magnitude are almost certainly achievable by a combination of plant breeding and better management of existing crop systems. Modern biotechnology can contribute to non-transgenic breeding efforts by the use of molecular markers and tissue culture techniques that are now within the reach of many developing countries.

There is also the opportunity to develop a whole range of crop-based, renewable non-food products that will be required to substitute for fossil-based hydrocarbons over the next few decades. Development of such non-food crops will of course be predicated on the assumption that there will be sufficient land available in addition to that required for food production. Providing that land that is presently fallow or under-utilised is brought into cultivation and yield improvements continue, there is a reasonable likelihood that a modest percentage of global arable resources will be available for production for non-food crops. However, it is unlikely that there will be extensive cultivation of large quantities of low-value commodity non-food crops, *e.g.* for biodiesel or other fuels, as this will inevitably impinge on food production.

Nearly all of our major crops have been selected and bred primarily for food use and there are relatively few existing crops that are immediately suitable for industrial use. Therefore the development of many non-food products derived from annual or perennial crops is likely to involve either the use of transgenes to manipulate existing crops or the breeding of new crop species that already make the desired products. There are three major categories of crop-derived non-food products (Various, 2000): commodity chemicals (including fuels), specialty chemicals and materials.

Commodity chemicals are lower-value products that are normally required on a large scale. They include industrial starches, many oils, ethanol, biodiesel and (possibly) bioplastics, all of which compete directly with petroleum derivatives. The current price differentials between petroleum-based and crop-based chemicals will gradually erode as petroleum reserves become depleted. However, owing to population growth, large-scale commodity non-food crops will have to compete with staple food crops for ever more scarce arable land, and this may ultimately limit their commercialisation and favour higher-value crops instead.

Specialty chemicals include high-value fatty acids, enzymes and pharmaceuticals. They are typically produced from relatively small crop acreages and do not compete with either petroleum or food crops. However, in some instances, the production of such compounds in plants may compete directly with microbial fermentation.

Crop-derived materials include lumber, paper and wood products. There is some potential for biomaterials to be used more extensively for fuel but trees currently compete rather poorly with petroleum as a fuel source. In addition, there will be competing pressures to convert forests to arable use and to establish more carbon sinks to address the increasing levels of atmospheric CO_2 (see Sections 13.4.6 and 13.7.3). The use of trees for wood and timber production will also increase in line with the population, which will further limit the scope for using trees for fuel production.

Commercial use of the opportunities presented by modern biotechnology has hitherto been largely restricted to relatively short-term applications relating to input traits and breeding for yield improvement, as discussed below. However, the application of some of the newer discoveries relating to genomics and metabolic engineering will make possible a much more extensive application of biotechnology in the future for the manipulation of more complex traits such as crop architecture and seed quality. Regarding output traits, there are opportunities for agbiotech to contribute to the development of more nutritious food crops and also to all three categories of non-food crops, although in the short to medium term the commercial prospects for higher-value specialty chemical crops are significantly better than those for commodity- or material-producing crops.

13.3 Agbiotech: current applications

The most widespread use of agbiotech methods is in support of conventional breeding programmes, where new molecular markers and tissue culture techniques are having a considerable impact. The present commercial use of transgenic crops is almost entirely limited to the manipulation of a few input traits in a very small number of major commodity crops. There are many other examples of potential transgenic crops being developed with modified output traits, but the widespread commercial cultivation of such crops probably lies some years ahead. The Biotechnology Industry Organisation in the USA provides a list of transgenic and non-transgenic agbiotech products that are either already on the market or under development for release in the next six years (*www.bio.org/er/agri-products.asp*).

13.3.1 Marker-assisted selection

Classical plant breeding relied on the selection of favourable characters from the diverse gene pool that is present in any biological species. Often these characters were visible and easily identified, *e.g.* height or flower colour or resistance to fungal attack. In other cases, the characters were much more subtle and sometimes could only be measured by sophisticated analytical techniques, *e.g.* the amounts of certain secondary products or the fatty acid composition of the seed oil. In all of these cases, it was necessary for the breeder to grow up and analyse each new generation before it was possible to measure the character, or phenotype, and select the appropriate plants. The advent of marker-assisted selection has changed this as breeders can now select a few plants that are likely to express the required characters from amongst tens of thousands of progeny even before the plants have developed to maturity. The basis of the method is DNA-fingerprinting and is in principle no different from the methods used to great effect in medical diagnostics or in forensic science (Gill, 1985).

Molecular markers such as microsatellites, RFLPs (restriction fragment length polymorphisms) and RAPDs (random amplified polymorphic DNA) have been developed for many crops, including trees. These markers can be assembled into genetic maps that have considerable utility both in basic biological research and in commercial breeding programmes. The markers can be used to track the presence of valuable characters in large segregating populations as part of a crop-breeding programme. For example, if a useful trait like disease resistance or high oil yield can be linked with a specific marker, many hundreds or even thousands of young plantlets can be screened for the likely presence of the trait without the necessity of growing all the plants to maturity or doing costly and time-consuming physiological or biochemical assays. Although the earlier molecular markers like RFLPs were relatively expensive, new markers like microsatellites and SNPs (single nucleotide polymorphisms) and are considerably cheaper and easier to use. The use of molecular markers can decrease the timescale of crop breeding programmes by several years and reduce costs. Although largely limited to the major temperate crops at present, the same technology can be applied to assist the breeding of many crops in developing countries and even to domesticating entirely new crops. Indeed it is probably true to say that, notwithstanding all the hype about transgenic crops, marker-assisted selection is by far the most significant application of biotech in agriculture at present.

A good example of the potential for marker-assisted selection can be seen with tree crops. With their relatively long life cycles and their formidable bulk (imagine trying to grow several dozen oak trees in a glasshouse), studying the various aspects of tree biology has always been a relatively difficult undertaking. Modern breeding

methods, as used for many decades with non-tree crops, are only just beginning to be applied to trees. However, thanks to marker-assisted selection and genomics, we can now expect some significant progress in the understanding of the genetics of trees and the application of this to breeding programmes. Molecular genetic maps of several tree species are currently being compiled and the possibility has been mooted of sequencing several tree genomes, including that of oil palm, in the near future.

13.3.2 Transgenic crops: a restricted but growing list of target species

In 2001, worldwide plantings of transgenic crops exceeded 52 Mha, although they were overwhelmingly concentrated in the Americas, with the USA, Argentina and Canada accounting respectively for 68%, 22% and 6% of the total (*www.isaaa.org*). To put this figure into context, 52 Mha is equivalent to more than twice the total area of the United Kingdom. The major transgenic crop species were soybean with 68% total area, maize with 22%, cotton with 13% and rapeseed with 5%. There were also much smaller commercial plantings of transgenic potato, squash and papaya. It is estimated that this area of transgenic crops could triple in the next five years (Abelson and Hines, 1999). The development of only a few commercial transgenic crop species to date reflects the relative technical difficulties in gene transfer in some of the other major crops like wheat and barley plus a focus on the development of temperate crops by commercial companies rather than less profitable staples like rice.

As outlined above, the transformation of virtually all of the major annual crop plants has now been achieved, although in many cases this remains a time-consuming and costly technical achievement. For example, the transformation of soybean by gene transfer into cultured embryos is a relatively lengthy, inefficient and labour-intensive process. Even in some of the species like rapeseed, where transformation is relatively facile, it is often highly cultivar-dependent. This means that it may not be feasible to transform the current elite rapeseed cultivars with a gene or genes of interest but instead an older variety such as Westar, which has been optimised for transformability, must be used. In order to produce a commercial transgenic variety the Westar transformant must then be backcrossed to an elite line—a process that can take several years and add significantly to development costs.

In the past few years, the use of new strains of *Agrobacterium* plus developments in tissue culture have resulted in the successful transformation of the major cereals such as wheat and rice by this method, which is generally more reliable than the previous method of choice (biolistics). This promises to facilitate the future development of transgenic cereal crops. By the year 2002, more than 30 tree species

had been transformed with various genes, most of which were marker (40%) or herbicide-tolerance (24%) genes. Although there have been over 120 field trials of transgenic trees, none of these has yet resulted in commercial planting. The future prospects for transgenic tree crops are considered in more detail in Section 13.4.4.

13.3.3 Engineering input traits

Input traits are those characteristics of a crop that affect its cultivation and yield but do not affect the quality of products from the crop. They are typically related to agronomic inputs such as herbicides, pesticides and fungicides, as well as nitrate and mineral fertilizers. Input traits were the first commercial target of transgenic manipulation in crops for several reasons. First, the traits can often be modified by the insertion of a single gene and many of the relevant genes had already been isolated by the mid-1980s. Second, seeds harbouring the new traits would have an added value that could be captured by the company that developed them, hence rapidly offsetting the R&D costs. Third, the end result should be better yields and/or lower production costs; no changes in harvesting methods are needed and no new crop products have to be processed. This made the development of transgenic crops with enhanced yield traits an attractive short-term proposition for the seed companies who developed this first generation of genetically manipulated (GM) crops. In the 2001–2002 season, nearly all of the commercially cultivated GM crops were modified for input traits. The main commercial downside of this strategy has been that consumers have perceived no benefit from such crops, which produce the same foods at the same price as conventional crops. Indeed, many consumers (especially outside the Americas) have perceived a risk from GM-derived foodstuffs. This has delayed the adoption of transgenic crops in Europe, possibly for several years (see Section 13.5.3).

Herbicide tolerance Weeds are plants that compete with the crop of interest, reducing its yield and complicating harvesting. Crop plants themselves can be serious weeds, *e.g.* volunteer cereals in rapeseed fields and *vice versa*. Weeds are conventionally controlled by the serial application of herbicides that may be more or less specific, *i.e.* they may damage the crop to some extent as well as eliminating the weeds. The development of crops that are resistant to some of the most powerful broad-range herbicides was one of the earliest targets of transgenic research. Of the total global output of 52 Mha of GM crops in 2001, over 77% were modified for herbicide tolerance alone while a further 8% were modified for both herbicide tolerance and insect resistance.

The most widespread types of transgenic herbicide-tolerant crops are those developed by Monsanto under the trade name of "Roundup Ready" (*www.monsanto.com*). Such crops are resistant to the widely used broad-spectrum herbicide, glyphosate, which is marketed by Monsanto as 'Roundup'. Glyphosate is a toxin that inhibits the enzyme 5-enolpyruvyl shikimate 3-phosphate synthetase (EPSPS) in plants, resulting in a lethal disruption in their ability to synthesise proteins. Although all known plant versions of EPSPS are highly sensitive to inhibition by glyphosate, many bacteria have a slightly different form of the enzyme that is insensitive to the herbicide. Therefore, if a copy of a bacterial EPSPS gene is inserted into a crop plant, the resulting transgenic crop variety will be resistant to applications of glyphosate while all non-transgenic plants in the same area will be killed. The cultivation of transgenic glyphosate-resistant crops is claimed to result in significant financial benefits for farmers because of reduced overall herbicide applications and higher yields per hectare (reportedly worth \$15–28 ha^{-1}). For their part, the seed companies are able to sell a profitable package, including seeds and herbicide, to the growers.

Agrevo has developed a similar package of herbicide-resistant transgenic crops, plus the related herbicide, under the trade name of 'Liberty Link' (*www.agrevo.com/biotech/ps/psllid.htm*). In this case, the herbicide is the fungal toxin, glufosinate, which is marketed under several names including 'Basta' and 'Challenge'. This widely used compound is an inhibitor of glutamine synthase in plants, causing a disruption of photosynthesis that results in the death of the entire plant within a few days. Glufosinate is a broad-spectrum (*i.e.*, non-specific) herbicide and its use is therefore limited to total eradication of vegetation or to control weeds shortly after crop emergence. In contrast, glufosinate-resistant crops can be sprayed with the herbicide at any time, resulting in the effective elimination of all other plants from the field. Resistance to glufosinate is conferred by the addition of a gene from the soil-dwelling bacterium *Streptomyces viridochromogenes*, which encodes the enzyme phosphinothricin acetyltransferase. This enzyme is able to acetylate glufosinate, which results in the loss of its toxic activity. Transgenic plants expressing this transgene are therefore able to grow normally, even after the application of relatively large doses of the glufosinate. Transgenic glufosinate-resistant rapeseed was first grown commercially in Canada in 1995, soybean and maize were approved in 1997, and other crops such as sugar beet will soon be available.

There are concerns that in some crops like rapeseed or sugar beet, which have closely-related weed species, the herbicide-resistance trait may spread into the weed population by cross-pollination. However, non-transgenic herbicide-tolerance has also been developed in rapeseed and other crops. For example, the so-called SMART-canola varieties of Canadian rapeseed developed by American Cyanamid (now owned

by BASF), are resistant to imidazolinone herbicides but are the products of conventional (*i.e.*, non-transgenic) plant breeding. These crops have been grown since 1995 and their use is widespread in Canada (*www.bio.org/food&ag/approvedag98.html*). In principle, such non-transgenic herbicide-resistant crop varieties are as likely to outcross and transfer their resistance traits to weedy relatives as the transgenic varieties. Therefore, concerns about the spread of traits like herbicide-tolerance should not only be related to the use of transgenic crops *per se*, but rather involve more general issues of the management of any agronomic trait where its transfer to weedy relatives, or elsewhere, may have deleterious consequences.

Pest and disease resistance Crop yields are regularly reduced by herbivore attack, most notably from insects, and by diseases caused by nematodes, fungi, bacteria or viruses. Even in intensively managed agronomic systems, losses of >30% from various pests and diseases are commonplace; while in developing countries the figures are much higher. After herbicide tolerance, the second most common modification in transgenic plants in 2001 was insect resistance, which alone accounted for 8 Mha, plus a further 4 Mha where the trait was expressed in combination with herbicide tolerance (www.isaaa.org). Insect resistance in transgenic maize, cotton and potatoes was conferred by insertion of a gene encoding a protein toxin from the gram-positive soil bacterium *Bacillus thuringensis* (Bt). The use of insect-control sprays containing a live toxin-producing *Bacillus thuringensis* suspension has been common for over 30 years in organic farming but the widespread use of Bt toxins in transgenic crops is much more recent. The Bt toxins are a family of so-called crystalline (cry) proteins that are converted into their active form during digestion in the gut of a range of insect larvae, resulting in a disruption of potassium ion transport that rapidly becomes lethal (*www.nal.usda.gov/bic/BTTOX/bttoxin.htm*). Mammals do not convert the toxins into their active forms and are therefore unaffected by them. Early indications (Briggs and Koziel, 1998) suggest that transgenic Bt crops are effective in controlling insects and improving yields (by 7%), while also reducing the need for spraying with more toxic and less desirable pesticides that often affect beneficial organisms such as insectivorous birds (saving growers some \$40 ha^{-1}).

The obvious danger in relying on a single class of toxins is that it tends to establish a strong selection pressure favouring the survival of insects that are able to sequester the toxin or otherwise render it harmless. Before the mid-1990s, only a few thousand hectares of land on organic farms were sprayed annually with live Bt, but in the last few years the cultivation of transgenic Bt crops has expanded to over 11 Mha and this area is still increasing. The resultant chronic and widespread exposure of

hundreds of insect species to the Bt toxin must increase the likelihood that resistance will eventually develop in some species. Indeed, as long ago as 1995, at least two insect species had already become resistant to Bt toxins in the field with at least another ten species showing the potential for the acquisition of resistance in laboratory studies (*gophisb.biochem.vt.edu/news/1995/news95.dec.html#dec 9501*).

Another risk with the expression of xenoproteins like Bt toxin in crops is that their accumulation may be curtailed if the plants are stressed. For example, in 1996 a combination of heat and drought caused a reduction in levels of Bt toxin in Monsanto's transgenic "Nu Cotn" variety of cotton. Although deleterious to the crop, these climatic conditions actually favoured the development of the cotton bollworm, resulting in severe infestation in nearly 1 Mha of "resistant" cotton crops (Kaiser, 1996). The infestation was eventually controlled with conventional chemical pesticides, but the more serious long-term danger is that exposure of the bollworms to sublethal doses of the Bt toxin would be an ideal mechanism for the development of resistance by the insects.

To combat the development of resistance to Bt toxins in the field, growers are now advised to set aside refugia. The refugia are areas adjacent to the main transgenic crop that are sown with non-transgenic seeds of the same crop. In these areas, non-tolerant insect populations can continue to thrive and hopefully will out-compete those of their con-specifics that develop Bt tolerance. This strategy relies upon the cooperation and enforcement of good management practices by growers and can fail if it not implemented rigorously on all farms. Some of the recent experiences of breakdowns in communications between seed companies and farmers growing transgenic crops (*e.g.* the STARLink maize affair, described in Section 13.5.2) serve to underline the potential difficulties in maintaining an effective refugia policy. Another strategy to prevent or delay the acquisition of resistance by insects is the inclusion of several unrelated toxin genes in a transgenic crop, but this so-called "gene pyramiding" will be a much more expensive and much longer-term option.

Viruses, bacteria, fungi and nematodes are major pathogens of crops and there has been much research aimed at producing resistant varieties by transgenic approaches. While various fungicides and nematicides are available to help farmers control these pathogens, there are no equivalent virus-control agents, so the combating of viral diseases normally relies on the endogenous resistance of the plant. In the absence of endogenous resistance, viral infections can be particularly devastating to a crop. This has stimulated efforts to engineer viral resistance into transgenic crops. The commercial cultivation of several transgenic potato, squash and papaya varieties with virus-resistance genes has already been approved in some countries (*www.isaaa.org/frbrief8.htm*), and advanced field trials are underway in others (*www.health.gov.*

au/tga/gene/gmac/pr108.htm). These transgenic crops express complete or partial proteins from a particular virus (typically part of the viral coat protein complex), which causes the plants to become sensitised to subsequent infections with the same virus. When such plants are attacked by the viral pathogen, they mount a successful defence response in a manner that is somewhat analogous to immunisation in animals that have been injected with an attenuated virus, although the exact mechanism of viral immunity in plants remains to be explained. A recent example of a transgenic virus-resistant crop is a papaya variety developed in Hawaii and in Australia. The papaya ringspot virus is a major threat to the cultivation of papayas in tropical countries like Hawaii. Transgenic papayas that express the ringspot virus coat protein, which on its own is harmless to the plants, are considerably more resistant to infection with the active virus than are non-transgenic papayas (*www.gov.au/tga/gene/gmac/pr108.htm*).

The engineering of resistance to bacterial, fungal and nematode pathogens has been more problematic although several promising approaches have been demonstrated, at least in principle. One possibility is the expression of resistance genes like the Xa21 bacterial blight resistance gene that was recently transferred to five Chinese rice varieties (Zhai *et al.*, 2000). Anti-fungal agents like phytoalexins or chitinases have also been expressed in plants (Shah *et al.*, 1995). In another case, a gene encoding an anti-fungal protein from alfalfa was transferred to potatoes (Gao *et al.*, 2000). The transgenic potatoes were resistant to the soil-borne fungus *Verticillium dahliae*, which causes some $70–140 million in damage to potato crops each year. However, this resistance was quite specific and the potatoes were still susceptible to the serious pathogen, *Phytophthora infestans*, the late-blight fungus that caused the devastating Irish famine in the 1840s. This highlights one of the difficulties with engineering fungal resistance in crops, namely that it is very difficult to produce broad-spectrum resistance, and so to achieve this it may be necessary to transfer numerous resistance genes. Also, fungal resistance often evolves naturally and can be found in different varieties of a crop or in sexually compatible wild relatives, from which it can be transferred to an elite crop cultivar by conventional breeding. In the above case, a potato variety called Russet Burbank was transformed with an alfalfa gene so as to confer resistance to the fungus, *Verticillium dahliae*, but there is another potato cultivar called Russet Ranger that is naturally resistant to this fungus anyway.

Nematodes are non-herbivorous and are the major class of animal parasites of crops, causing over $100 billion in annual losses to world agriculture. Natural resistance to nematodes is relatively restricted, while chemical control agents like carbamates include some of the most toxic pesticides in widespread use and are both costly and environmentally damaging. The development of transgenic crops with

nematode resistance could therefore have appreciable economic and environmental benefits. Some of the research approaches include the induction of "suicide genes" in plant cells infected with a nematode or the expression in plants of protease inhibitors that inhibit nematode growth (Atkinson *et al.*, 1995).

Other input traits There are numerous additional input traits of interest to seed companies that are being developed using transgenic plants. Male sterility is desirable for the production of hybrid crops, which normally have considerably higher yields compared with their non-hybrid counterparts. Male sterility can also have an environmental benefit since the pollen will be either absent or sterile. This can be a useful trait in transgenic crops, as it will prevent cross-pollination and the spread of the transgenes into other related species.

A well-established way of producing male sterility is to prevent the formation of viable pollen grains during their development in the anther. This can be done by inserting a cytotoxic transgene under control of an anther-specific promoter into a crop such as rapeseed. Expression of the transgene is restricted to the pollen-nourishing tapetal cells of the anther and prevents the formation of mature pollen grains: this renders the male plants sterile (Mariani *et al.*, 1990). A popular cytotoxic gene of choice is *barnase*, which is obtained from *Bacillus amyloliquefaciens* and encodes a ribonuclease that destroys RNA, hence killing any cells in which it is expressed. The ribonuclease is unable to enter adjacent cells so that its lethal effects are limited to those cells in which the *barnase* gene is expressed. In a further refinement of this approach, the addition of a second gene called *barstar* causes the *barnase* toxin to be disabled, which allows the pollen grains to develop, hence restoring fertility. This provides breeders with the male-sterile and male-fertile genotypes required for the production of commercial hybrids (Peacock, 1992). Although this technology has been available for nearly a decade, it has yet to be successfully commercialised.

Another well-known transgenic sterility trait is that conferred by the unfortunately named "terminator" technology that was being developed by a company acquired by Monsanto but was (temporarily) withdrawn from use in crops late in 1999 following adverse public reaction (Niiler, 1999). This technology is a really a series of approaches that have numerous possible manifestations (as well as possible drawbacks) as usefully explained in more detail on the University of Indiana website (*www.bio. indiana.edu/people/terminator.html*). In one of the applications, a gene would be inserted into a crop such that, as the seeds germinated or the seeds from the second generation developed, a toxic protein would be produced and the seedlings or the seeds would die. The main benefit of this would to seed companies since farmers

would be unable to save seed for replanting in subsequent years (as they have traditionally done in most developing countries) but would instead have to re-purchase the seed each season from the company. An additional more general benefit would be to prevent the spread into the environment of potentially deleterious transgenic traits like herbicide tolerance that may be present in such seeds. Several major agbiotech companies are continuing to research sterile-seed technologies and in August 2001 the USDA licensed similar technology to Delta & Pine Land, albeit with the condition that it should not be used commercially before 2003.

13.3.4 Engineering output traits

The manipulation of output traits is often considerably more complex than that of most input traits. Whereas most input traits can be usefully expressed constitutively, *i.e.* throughout the plant, new output traits are normally only desired in the harvested portion of the crop, *e.g.* the seed or fruit, and may even be deleterious if expressed elsewhere. Output or quality traits are often the products of complex metabolism and may require the insertion of several transgenes to have an effect. Finally, and most importantly, the modification of an output trait will by definition result in a new crop variety with different products to non-modified varieties. Unless the transgenic crop completely replaces non-transgenic varieties, it will require complete segregation at every stage of production from seed storage and planting to harvesting and downstream processing. This can add at least 10–20% to costs and imposes considerable (and often overlooked or underestimated) management problems (see Section 13.5.2). The predominant output traits that are currently under development relate to the major seed and fruit storage products, *i.e.* starches, oils and proteins.

Carbohydrates In terms of both traded output and value, sucrose is the most important carbohydrate product of agriculture with an annual yield of 100 Mt that is worth some $50 billion. There has been some interest in modifying potatoes to prevent them from accumulating sucrose during storage (so-called cold sweetening). This sucrose production is undesirable as it reduces starch levels and also the presence of the sucrose leads to the occurrence of a Maillard reaction during frying, which causes undesirable darkening in potato products such as crisps and chips. The insertion into potatoes of an antisense copy of a gene encoding a starch granule protein of unknown function has been reported to alleviate the cold sweetening problem (*www.mpg.de/news1.htm*). Other simple carbohydrates like trehalose may also be manipulated by transgene insertion. Trehalose is most commonly extracted

from yeast and is used as an additive to improve processed and dried foods by making them taste fresher. Trehalose produced from yeast is very expensive at \$200 kg^{-1} but several biotech companies have demonstrated that it is now possible to accumulate trehalose in transgenic plants (Goddijn and Pen, 1995).

There is much more interest, however, in the complex carbohydrates, and particularly in starches, which are the major products of the principal cereal grain crops such as rice, wheat, maize and barley. It is estimated that 19 Mt starches, worth some \$5 billion, are produced annually (Goddijn and Pen, 1995). In the EU and USA, as much as 25–30% of the starch production is used for industrial purposes with the remainder being used in foods and beverages.

Unlike oils and proteins, starches are indeterminate molecules, being made up of glucose polymers of varying chain lengths and extents of branching that exhibit considerable diversity in their structure and properties. Starch grains in plants contain two principal polysaccharides, amylose and amylopectin. Both polymers are made up of chains of α(1–4)-linked glucose molecules but, whereas in amylose the chains are long and largely unbranched, in amylopectin the chain length is much shorter and they are joined by frequent α(1–6)-linkages. It is the chain length and branching that largely determine the physical properties of extracted starches, *e.g.* they may be more or less gelatinous constituents of foodstuffs; they can be incorporated into non-food products like packaging materials; or even used to make biodegradable plastics. Since different crops contain very different types of seed starch, the useful properties present in the starch form of one crop are often not present in other crops. Hence the EU imports huge amounts of maize starch for many types of food manufacture because the starches produced in its home-grown cereals, such as wheat and barley, do not have the appropriate structure for these applications.

The indeterminate nature of starches renders them more complex, compared with oils and proteins, in terms of their potential for biotech manipulation within the plant. It is possible to effect some drastic changes in starch composition, *e.g.* amylopectin levels can be reduced to almost zero by expressing an antisense copy of the granule-bound starch synthase gene in potato tubers (Visser *et al.*, 1992). However, it is much more difficult to produce a 'designer starch' with a predetermined ratio of amylose: amylopectin and therefore with predictable physiochemical properties. Many of the key biosynthetic enzymes involved in starch formation have now been characterised and their genes cloned, but such studies have served to emphasise the complexity of this process. This is true, not only at the metabolic level, but also at the cellular level of assembly of the paracrystalline starch granules within plastids, where additional proteins may be involved in various aspects of the three-dimensional organisation of the granule. These factors make it difficult to predict the consequences, in terms of

seed starch composition, of manipulating the expression of biosynthetic enzymes such as starch synthase or starch branching enzyme in transgenic plants. Efforts are now underway to understand the biosynthetic and physiochemical mechanisms of starch granule formation in model bacterial systems and until these bear fruit the use of gene transfer to redesign starches in crop plants for specific end uses will remain an essentially empirical endeavour.

Proteins Seeds accumulate specific storage proteins that are normally substantially enriched in the diamino acids, glutamine and asparagine. These proteins are an important reserve of nitrogen and amino acids for the germinating seedlings. Seed storage proteins can be divided into a small number of distinct families, each of which probably evolved from a different class of non-storage ancestral proteins, such as proteases or desiccation-related proteins (Shewry and Casey, 1999). Following their co-translational insertion into the endoplasmic reticulum, storage proteins are targeted to the vacuole where they are processed and become folded into dense, compact granules. Seed proteins are mainly used for their nutritional value, although some of them also have important physiochemical properties that are important in the manufacture of certain foodstuffs. For example, the prolamins of wheat grains play a vital role in bread making while some legume seed proteins can be spun out to produce fibres that can mimic meats in the so-called 'textured vegetable proteins'.

Interest in manipulating seed protein composition via transgene insertion has focussed on objectives like increasing the levels of essential amino acids, *e.g.* methionine, and in changing the protein structure to enhance qualities such as breadmaking ability. Many seed storage proteins are relatively deficient in the sulphur amino acids, methionine and cysteine. These amino acids are required in the human diet because they cannot be synthesised endogenously. There have even been rare cases of children reared on non-dairy vegetarian diets who developed significant deficiency symptoms due to the lack of these essential amino acids. One strategy to increase levels of sulphur amino acids in seeds is to create transgenic plants expressing a new protein that is enriched in these desirable amino acids. This was done by a group at Pioneer who expressed a Brazil nut storage protein in transgenic soybean with a resultantly satisfactory increase in the methionine content of the seeds. Unfortunately, subsequent tests showed that some people were allergic to the Brazil nut protein and therefore would also probably be allergic to all of the many dozens of the soybean-derived food products in which it could be present. Although this was depicted in the press at the time as a serious setback for agbiotech, it actually demonstrated that the quality control safeguards were effective since the problem was recognised at an early stage and further development of these transgenic seeds was

halted forthwith. Nevertheless, this episode has served as a salutary warning of the risks of generating allergens, particularly when manipulating seed proteins, which are present in considerable abundance in many staple foodstuffs.

Several initial attempts to overexpress foreign proteins in seeds were unsuccessful because the new proteins were not targeted efficiently to the storage vacuole or could not fold properly once they reached the vacuole; in both cases most of the mistargeted or misfolded proteins were degraded. It is only quite recently that some of the intricacies of storage protein targeting and assembly in the vacuole have begun to be explained (Herman and Larkins, 1999; Jiang *et al.*, 2000). In the future, the re-engineering of storage proteins will have to take into account the various signalling motifs and folding patterns that enable the endogenous proteins to accumulate to such high levels in seeds. As well as changing seed storage proteins, there has been much interest in expressing other types of protein like antibodies or commercially useful enzymes either in seeds or in other plant tissues, such as leaves or roots. Although this is relatively straightforward in principle, it has been much more difficult to achieve in practice. The expression in plants of pharmaceutically interesting peptides like enkephalin (Vanderkerkove *et al.*, 1989) or functional full-length antibodies (Hiatt *et al.*, 1989) was demonstrated more than a decade ago and may be the basis for a new generation of high-value crops produced by molecular pharming (see Section 13.4.5).

Oils In 2002, annual and perennial crops together produced an annual output of >90 Mt in traded oils that was worth about $35-40 billion. Plant-derived oils are mainly used as commodities for the manufacture of foodstuffs. Oil crops are second only to cereals as a source of calories for human societies as well as providing essential fatty acids like linoleic acid plus many of the lipid-soluble vitamins, including carotenoids (vitamin A) and tocopherols (vitamin E). Some plant oil-derived foodstuffs, such as cooking oils, margarine or chocolate are quite obviously lipidic and are referred to as visible fats. However, the vast majority of the plant oils that are consumed in the western diet are the so-called invisible fats that lurk in over half of all the food products in a typical supermarket. These invisible fats are found in nearly all processed foods including biscuits, shortenings, cakes, breads, canned foods, frozen foods, yoghurts, milk substitutes, spreads and dips, to name but a few.

About 20% of the total output of plant oils is used as a feedstock for the production of oleochemicals. Over the past century, plant oil crops were nearly all bred to provide edible products and their fatty acid compositions are therefore quite restricted, being mostly limited to C16 and C18 saturates and unsaturates, as shown in Table 13.2. There are many desirable changes that could be made to enhance both the edible and industrial uses of plant oils and the use of transgenes to effect such

modifications has been an attractive option. Indeed, the current list of transgenic crops approved for general release in the USA includes only two crops with modified seed quality traits, both with altered oil profiles that can be used for edible and non-edible applications.

Table 13.2 Percentage fatty acid composition of the 'big four' oil crops

Fatty acid[a]	Soybean	Oil palm[b]	Rapeseed	Sunflower
16:0	11	45	5	6
18:0	4	5	1	5
18:1	22	38	61	20
18:2	53	11	22	69
18:3	8	0.2	10	0.1

[a] Fatty acids are denoted by their carbon chain length followed by the number of double bonds; [b]mesocarp. Data are taken from Murphy (2001).

There are two important targets for improving the edible quality of plant oils. First, the amount of C18 polyunsaturates should be reduced substantially. This would avoid the need for chemical hydrogenation, which produces the high levels of *trans*-fatty acids that many people believe to be undesirable in the diet. The extent of *trans*-fatty acids in foods may well become more apparent if the US Food and Drug Administration (FDA) proceeds with plans for their mandatory labelling in all food products by 2002 (Anonymous, 2000d). Second, the amount of the very long chain (C20–C24) ω-3 polyunsaturates, such as docosahexenoic acid (DHA) or eicosapentenoic acid (EPA), should be substantially increased. These fatty acids are nutritionally beneficial precursors of hormones and physiological effectors such as prostaglandins, leukotrienes and thromboxanes. Fish and other marine creatures produce oils that are rich in DHA and EPA but stocks have been drastically depleted by overexploitation, leading to the virtual elimination of some fisheries like the North Atlantic cod. It is estimated by the FAO that the shortfall between the annual demand for seafood and its supply from wild fisheries will be 50 Mt by 2025: it is most unlikely that fish farms can compensate for this shortfall. The resulting decrease in availability and high prices for marine oils make it necessary to consider alternative sources of these useful fatty acids, particularly for less affluent groups.

Although food uses of oil crops are important, there has been much more interest from the agbiotech industry in the development of these crops for a wider range of non-food uses. By changing the chain length and functionality of the fatty acids it is possible, in principle, to produce oils with carbon chain lengths from C8 to C24

containing anything from 0–5 double bonds or other functionalities such as hydroxy, epoxy or acetylinic groups. Such oils can be used for the manufacture of adhesives, paints, detergents, lubricants, nylons, cosmetics and pharmaceuticals, to name but a few. As shown in Table 13.1, many oil-bearing seeds already produce some of these novel and potentially useful fatty acids, and such plants have been used as sources of genes for transfer into mainstream oil crops in the hope that the latter would accumulate the novel oils.

The first transgenic crop with a modified output trait to be approved for commercial cultivation was a lauric oil (12-carbon) rapeseed variety grown in 1995 (Murphy, 1999b). At this time there was a perception that the biochemistry of oil formation in seeds was well understood and that, as an inert storage product, its composition could be easily and radically modified without affecting other metabolic or physiological processes in the plant. Indeed, the initial results were encouraging. The insertion of a single thioesterase gene from the California bay converted rapeseed from a plant with no lauric acid in its oil to one that contained 40% lauric (Voelker *et al.*, 1992). Genes could also be down-regulated to change the oil profile. The insertion of antisense copies of a stearate desaturase gene resulted in transgenic rapeseed plants with ten times the normal levels of stearic acid in their seed oil. During the past decade, genes encoding the vast majority of the enzymes involved in specifying the chain length and functionality of plant fatty acids have been isolated. The insertion of these genes was expected to result in the accumulation of moderate to high levels of the corresponding fatty acids. However, the regulation of fatty acyl composition of oils has turned out to be more complex than was first thought, as depicted in Fig. 13.4. Indeed, very recent findings suggest that our understanding of even the basic pathway of triacylglycerol oil biosynthesis is far from complete and that there are probably multiple pathways rather than just one (see Fig. 13.8).

The consequence of these complexities of plant lipid metabolism has been that, despite many impressive achievements in isolating oil-related genes and producing transgenic plants with modified oil compositions, it has not been yet possible to achieve the kind of high levels, *i.e.* 80–90% of novel fatty acids that will make possible their widespread commercial exploitation. The lauric-oil variety of rapeseed has been improved from 40% to 60% lauric by the insertion of several additional transgenes (Voelker *et al.*, 1996) but remains far from being a commercial success.

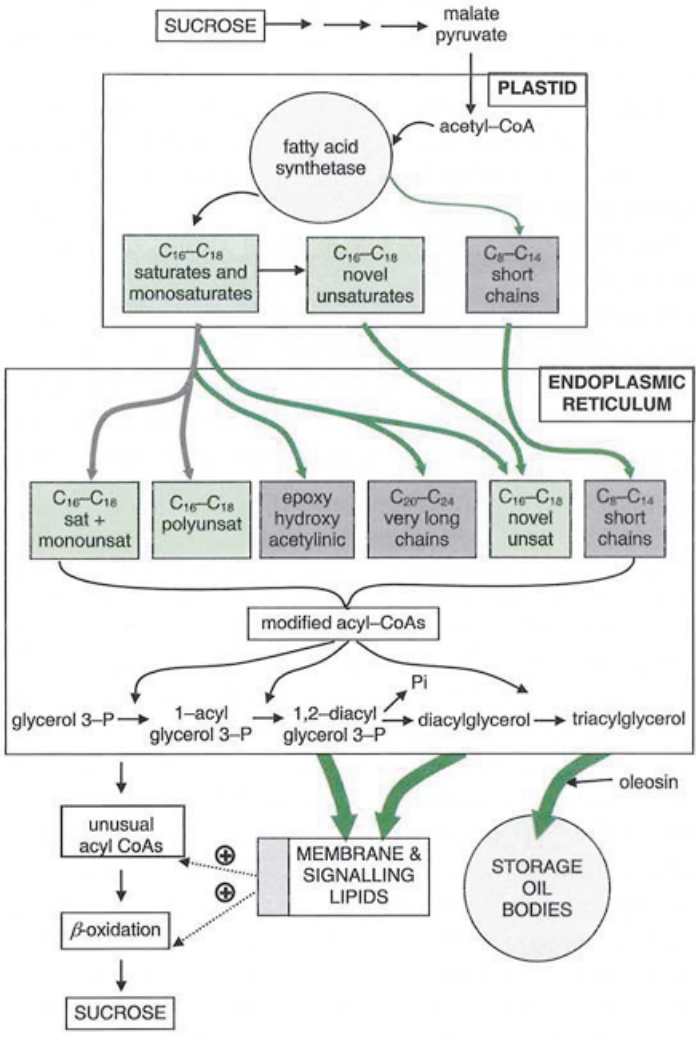


Figure 13.4 Fatty acid metabolism in oilseeds. Fatty acids are synthesised *de novo* in plastids using imported precursors such as malate and pyruvate that are derived from sucrose. Most of the C16 and C18 fatty acids (shown in green) can be readily incorporated into either membrane/signalling lipids or storage lipids. However, many of the more 'exotic' fatty acids (shown in grey) destabilise membranes and can only be safely accommodated in storage lipids. Plants that normally synthesise such unusual fatty acids are able to channel them efficiently towards the storage lipids, but in some oil crops like rapeseed this channelling is less efficient. In transgenic rapeseed, the synthesis of exotic fatty acids can sometimes trigger the induction of genes involved in fatty acid breakdown and re-conversion to sucrose. This may be a protective response to prevent the accumulation of these fatty acids in membrane lipids. The net result is a futile cycle that may limit accumulation of the novel fatty acid of interest (see also Murphy, 1999b).

The availability of many genes involved in fatty acid modification and the good progress in transforming the main oil crop species will doubtless encourage further efforts to resolve the challenge of low levels of novel fatty acid production but even if such efforts are successful the commercial success of transgenic oil crops will remain problematic. It will be necessary to identify or develop robust markets for their products—simply substituting for petroleum-derived products is unlikely to be economic for several decades (see Sections 13.2.3 and 13.7.4). The additional costs of identity preservation preclude the use of such transgenic oils as large-scale commodities in competition with conventional plant oils, even for industrial applications. In summary, transgenic oil crops have a lot of potential promise for the long-term future but their commercial prospects over the next few years remain uncertain.

Bioplastics Virtually all of our conventional plastics are made from non-renewable petroleum-derived products such as adipic acid and vinyl chloride. An alternative is to harness the ability of soil bacteria like *Ralstonia eutrophus* that are able to accumulate up to 80% of their mass in the form of non-toxic biodegradable polymers called polyhydroxyalkanoates (PHAs). The PHAs are made up of β-hydroxyalkanoate subunits that are synthesised from acetyl-CoA via a relatively short pathway involving as few as three enzymes for the most common PHA, polyhydroxybutyrate (Steinbüchel *et al.*, 1998). During the 1980s and 1990s, the UK-based company, ICI, developed a fermentation process to produce PHB and other PHAs in transgenic *E. coli* cultures expressing PHA genes obtained from bacteria such as *Ralstonia eutrophus*. However, the price of the resulting plastic was ten-fold higher than that of conventional plastics. Despite the enormous environmental benefits of these biodegradable plastics (they can be composted into soil and degrade completely in a few months), their high cost has rendered them uneconomic for large-scale production. Interestingly, there is a small but lucrative niche market for biodegradable plastics as the framework of artificial tissues. Following their insertion into the body, the PHAs are gradually broken down and the body reassembles the natural tissue in the same shape as the original PHA template. In such a specialised medical application, the price of this kind of PHA product is obviously not as important as for lower-value materials like plastic toys, pens or bags, *i.e.* high-value applications tend to relatively be price-elastic whereas commodities are not.

The cost of PHAs could be considerably reduced if they were produced on an agricultural scale in transgenic crops. This prospect led Monsanto to acquire rights to PHA production from ICI/Zeneca in the mid 1990s and to transfer the bacterial genes into transgenic rapeseed plants. Providing the PHAs accumulate in the plastids, and not in the cytosol, it is possible to obtain modest yields of the polymer from either

leaves or seeds (Valentin *et al.*, 1999). A major and as yet unresolved technical hurdle is how to extract the polymer from the plant tissue in an efficient and cost-effective manner. Another complexity is that polyhydroxybutyrate, which is the most widespread PHA, is a rather brittle plastic and is not suitable for most applications. The best plastics are co-polymers of polyhydroxybutyrate with other PHAs like polyhydroxyvalerate, and the production of such co-polymers in transgenic plants is considerably more difficult than that of single-subunit polymers. As of early 2000, these perceived difficulties coupled with its own cash-flow problems prompted Monsanto to offer its transgenic PHA business for sale. Although there are several other small ventures attempting to make PHAs in plants (including one in oil palm) it is sadly unlikely that these environmentally friendly products will be commercially available for many years to come.

A second category of bioplastics is the polylactides (PLAs), which are based on lactic acid. The lactic acid feedstock can be obtained by fermenting starch from any crop with a sufficiently high starch content and there is no need to use transgenic plants. A joint venture company called Cargill Dow Polymers (*www.cdpoly.com*) has recently developed a low-cost method of heating up lactic acid monomers to form PLAs with the appropriate mixture of D- and L-isomers to confer the optimal physical properties. The result is a glossy transparent thermoplastic with properties similar to polycarbonates and polyesters that can also be used to make acrylic-like fibres for clothing. The so-called 'Nature Works™' PLA products will be fully biodegradable and compostable. In late 2001, the first full-scale production facility for Nature Works™ PLA opened in Blair, Nebraska, with plans to build a second unit in Europe at a later date. The Nebraska factory uses maize as the feedstock (sugar beet can also be used) and it is planned to produce 140,000 t yr^{-1} of PLA. There has been some scepticism about whether PLA can truly compete against petroleum products economically or whether it will pass the test of a rigorous life-cycle analysis (*www.ends.co.uk/report/jan00_2_tx.htm*). However, this is an interesting alternative way of making biodegradable plastics that could be used in medium-value markets like textiles and do not come from transgenic plants. One of the first applications of Nature Works™ fibres is for the manufacture of performance fabrics aimed at the outdoor and sportswear markets. Despite the reservations from some quarters, PLAs and related bioplastics certainly merit further investigation.

Storage/shelf life Many losses of crops occur because of spoilage during storage or transportation. Often such losses are reduced by harvesting relatively unripe crops, especially fruits, and ripening them when required by applying agents such as the plant hormone ethylene. A drawback of this process is that the unripe fruits have often

not developed their full range of flavours and these are not always induced by artificial ripening. One has only to taste the thick-walled, long shelf-life tomatoes from supermarkets, particularly in the USA, and compare them with shorter lasting but much more highly flavoured home-grown produce. The main reason for the deterioration of soft fruits during storage and transport is a decline in firmness, which increases the chances of injury and infection of the fruits. The fruits become softer by cell wall breakdown and one of the key enzymes in this process is polygalacturonase.

One approach to slowing down the softening of fruits is to decrease the activity of polygalacturonase during ripening. This could allow fruits such as tomatoes to remain for longer on the vine in order to develop more flavour, but without the risk that they would lose the firmness necessary for them to be harvested, transported and stored without damage. The isolation of the polygalacturonase gene in the 1980s allowed this approach to be adopted by Calgene in the USA and Zeneca in the UK. In both cases, an antisense (*i.e.* back-to-front) copy of the gene was inserted into tomatoes.

The result was the first food crop to be produced by transgenic manipulation, *i.e.* the FLAVR SAVR™ tomato, which was released for human consumption by Calgene, in 1994. The FLAVR SAVR™ tomatoes were claimed to taste better and last longer in storage than conventional tomatoes and initially sold well in supermarkets in the USA. Ironically, FLAVR SAVR™ tomatoes were not a success for rather mundane reasons that are unrelated to GMO technology but are a useful lesson for future attempts to commercialise such crops. The variety of tomato used for the gene transfer did not grow as well as some conventional varieties under the commercial cultivation conditions that were used. This meant that, although FLAVR SAVR™ tomatoes had some modest advantages over non-transgenic fruits regarding their taste and shelf life, these were more than offset by the better agronomic performance and lower cost of the conventional varieties. The take-home message is "ensure that your transgene(s) is in an elite variety that can compete effectively with conventional cultivars before you release it".

In 1995, the UK-based company Zeneca released a related transgenic food product. This was a tomato paste from tomatoes in which the enzyme polygalacturonase had also been down regulated so that the paste was claimed to be appreciably thicker and better tasting. Unlike the FLAVR SAVR™ fresh tomatoes, the Zeneca tomato paste was a modest commercial success until it was overwhelmed in the anti-GM sentiment following the Pusztai affair in the UK and it was withdrawn from sale by supermarkets early in 1999. Unlike the crops with transgenic input traits, these two examples of transgenic tomatoes with modified output traits were segregated from non-transgenic foodstuffs and were distinctively labelled as GMOs. Since they were perceived as beneficial by many consumers, *i.e.* they tasted better or lasted longer,

each of the transgenic tomato products was popular with shoppers; indeed when they were eventually withdrawn from sale it was not due to their unpopularity but rather to external factors unrelated to the products themselves.

Nutritional value About half of all food products in developed countries are nutritionally enhanced to some degree. Examples include fibre-enriched foods, sugar substitutes, vitamin D milk, low-fat or no-fat meat, yoghurt and spreads, fortified vegetables and sterol margarines. The market sector was valued at $58 billion in 2000. It is therefore not surprising that transgenic approaches are currently being used to produce several crops with enhanced nutritional value.

Probably the best-known example is the development of the transgenic 'golden rice' by a Swiss-based group (Ye *et al.*, 2000). The grains of this rice are yellow because of the accumulation of β-carotene (provitamin A), which is normally absent from rice grains. The transgenic rice contains three inserted genes encoding the enzymes responsible for conversion of geranyl diphosphate to β-carotene. The consumption of this rice may alleviate vitamin A deficiency that currently afflicts some 124 million children worldwide. Efforts are also underway to produce transgenic staple crops like rice that are enriched in iron. Iron-deficiency anaemia is estimated to affect as many as 1.4 billion women, the vast majority in developing countries. Approaches include increasing iron content by expressing ferritin or metallothionein transgenes or making the existing iron more available for digestion by reducing levels of the iron-sequestering protein phytase (Goto *et al.*, 1999).

Margarines enriched in phytosterol extracted from (non-transgenic) wood pulp or vegetable oils have recently been marketed and, despite an appreciable price premium compared to conventional margarines, they have enjoyed modest commercial success. The appeal of the sterol-enriched margarines is based on evidence that they may help to reduce blood cholesterol levels and hence combat heart disease (Moreau *et al.*, 1999). Such products could be made more cheaply if more of the phytosterols were synthesised in the same seeds as the oil from which the margarine is derived and efforts are underway to up-regulate phytosterol pathways in transgenic plants.

The boundary between nutritional and therapeutic effects of some of these products is becoming blurred. Indeed, whereas phytosterol-enriched margarines were readily approved for sale in some European countries, they faced more challenges in the USA. In the case of Benecol, in keeping with some of the prominently advertised health claims, the distributor wished to market the margarine as a dietary supplement. However, the FDA ruled that Benecol must be regarded as a basic food, which means the phytosterols would be regarded as food additives that must have further regulatory approval.

Another interesting example of such functional foods is some newly developed brassica vegetables. Many vegetables of the brassica family produce isothiocyanates, which have been shown in animal and human cell culture model systems to exert a protective role against certain carcinogens (Tawfig *et al.*, 1995; Zhang *et al.*, 1994). Such studies confirmed long-established folk traditions and more recent epidemiological evidence concerning the role of certain fruits and vegetables in cancer protection (Block *et al.*, 1992, London *et al.*, 2000). New isothiocyanate-enriched varieties of vegetables such as broccoli have recently been produced by conventional breeding and are marketed as part of a health lifestyle choice. Transgenic approaches to modify isothiocyanates and other nutritionally relevant secondary products are underway, but are unlikely to be pursued commercially in the present anti-GMO climate, which particularly affects many of the target consumers of so-called 'lifestyle foods'.

The increasing interest in the development and promotion of these and other nutritionally enhanced products raises the question of when do we stop considering them as mainstream foods and instead regard them as supplements like evening primrose oil or even as therapeutic agents like taxol®. Indeed, the agbiotech industry is now interested in a new generation of 'nutraceuticals', which are foodstuffs that may contain enhanced levels of known or supposed nutrients or even potent therapeutic agents such as vaccines or antibodies. In a recent survey, 74% of all US consumers were found to use dietary supplements and the market is valued at $14 billion yr^{-1}. An interesting comment from a director of a pharmaceutical company was that, whereas it can cost $600 million for FDA approval of a drug defined as a pharmaceutical, the procedure for clinical tests on a nutraceutical, *i.e.* a food product, would only cost $100,000 to $30 million (Fitzpatrick, 2000).

13.4 Transgenic crops: the future

As we progress beyond the first generation of transgenic crops, it may be useful to take stock of the potential for the development of new types of transgenic crops over the next decade or so. The emerging developments in genomics, proteomics and bioinformatics may allow for some radical modifications of plant architecture, growth habit and composition, which we shall consider in this section.

13.4.1 Complex traits

Many traits of agricultural importance in crop plants appear to be regulated by a large number of genes and therefore do not segregate into simple Mendelian ratios, as would be expected if only one or two genes were involved. Examples of such complex traits include height, branching, seed oil and protein yield and flowering time. During the past few years, however, the use of more sophisticated genetic tools has shown us that, although dozens of genes may be involved in the expression of such complex traits, much of the variation in their expression can be regulated by a small number of key genes. These regulatory genes can now be mapped using molecular markers and eventually isolated and sequenced. Research along similar lines in humans has revealed that some of the classical traits that were hitherto regarded as very complex, such as height, may be regulated by one or a few genes. For example, a gene called *phog* that is located on the human X-chromosome is reported to explain 70% of all height differences in children at the age of 15 (Nance *et al.*, 1998). Like many such regulatory genes, *phog* encodes a transcription factor homeodomain protein that controls the expression of a whole series of other genes that in this case are involved in determining human growth rates. Identification of these and other genes in humans is beginning to be used as part of DNA testing of developing foetuses *in utero* in order to predict future characteristics ranging from serious congenital disorders to gender. While many of these applications remain controversial in humans, there is little doubt that the use of similar genomic techniques in plants will considerably facilitate the selection of agronomically favourable characters like yield or seed retention or disease resistance with the assistance of molecular markers.

Genes regulating many agronomically relevant complex traits in model plants like *Arabidopsis*, and also some crops like maize, are now being isolated at an ever-faster pace. Examples include height (Peng *et al.*, 1999), flowering time (Pineiro and Coupland, 1998), vernalisation (Johanson *et al.*, 2000), shattering of seed pods (Liljegren *et al.*, 2000) and stem branching (Doebley *et al.*, 1997). Like the human *phog* gene described above, many of these plant genes also encode transcription factor proteins that in turn regulate the expression of large sets of other genes. For example, transcription factors can switch on entire metabolic pathways or patterns of cell division, resulting in the formation of new tissues or organs and the accumulation of new storage products (Murphy, 1998).

The isolation and mapping of important regulatory genes from a model plant species like *Arabidopsis* or rice will soon enable the equivalent gene, in terms of both sequence and chromosomal location, to be isolated from other plants, including most

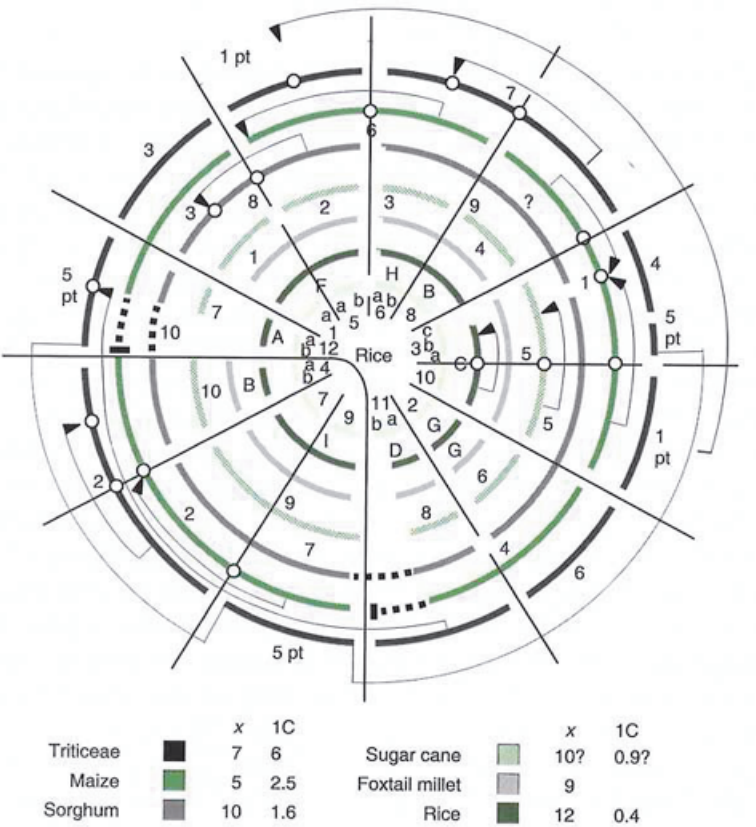


Figure 13.5 Alignment of the genomes of six major cereal crops, showing the 12 haploid chromosomes of the rice genome as the innermost circle. The other five cereal genomes are shown as concentric circles extending outwards in the order: foxtail millet, sugar cane, sorghum, maize and wheat. Within each sector, as defined by the radiating lines, the genomic segments are highly conserved with respect to the order of their component genes. For example, if a gene of interest is located in the middle of chromosome 10 in rice, its equivalent is likely to be located in the segments of the other genomes that are intersected by a line drawn from the centre of the circle through the middle of rice chromosome 10 and radiating outwards.

of the major crop species. This is due to a striking feature of many plant genomes, namely their high degree of similarity to one another in terms of the structure of their genes and the order in which they occur on chromosomes. As shown in Fig. 13.5, this gene order, or synteny, is particularly well conserved in monocotyledonous plants, which include all of the cereal crops (Moore *et al.*, 1995). Very recent evidence suggests that there is also appreciable genome synteny within the dicotyledons, which include the important model plant *Arabidopsis* as well as major crops like soybean, rapeseed and tomato (Grant *et al.*, 2000; Casci, 2000).

The long-term implications of these developments are profound. They open up the prospect of being able to manipulate some of the most basic features of crop plants. In annual crops, the height and branching could be adjusted to optimise harvestability and the capacity of the plant to bear and retain its seed. The seeds could be engineered to accumulate the exact mixture of carbohydrate, protein, oil and fibre required for a particular end use. Flowering time could be adjusted to suit particular climatic conditions. For example, the development of earlier flowering varieties of some crops in a location like the Canadian prairies would allow them to set seed and be harvested before the onset of early frosts that can otherwise halt seed development before it is completed. When early frosts hit a crop like rapeseed, the seed ceases its growth while it is still green. These green seeds cannot be sold and, even worse, if they become mixed with normal seeds the entire batch may be condemned. A slight advancement of flowering time could also dramatically improve yields of rice in some tropical and subtropical regions where the current growing season is just over six months. If the length of the growing season could be reduced to less than six months, the farmers in such regions could grow two rice crops in each year (Moffat, 2000). The development of shorter varieties of cereal grain crops in the 1960s and 1970s led to such huge increases in yields that it was dubbed the Green Revolution. However, some crop varieties, including the highly prized Basmati rice, have remained recalcitrant to efforts at introducing dwarf traits by conventional breeding. The ability to alter height by gene insertion is therefore an attractive prospect (Peng *et al.*, 1999).

13.4.2 Environmental stress

Environmental stresses already seriously affect crop performance in the field. If short-term (*i.e.* within a few decades) global climate change is indeed a reality (which remains to be demonstrated conclusively), the incidence of all forms of biotic and abiotic stresses in agricultural systems is likely to increase significantly. There are already anecdotal accounts of the increased ability of many pests to overwinter following the relatively mild series of mild winters in Western Europe during the 1990s. Even if there are no major climatic changes over the next 50 years, environmental stresses are increasingly affecting agriculture. In some regions, changing patterns of land and water use are causing increased salinisation of agricultural areas, while elsewhere there are serious problems of mineral toxicity. Drought and salinisation are already the most common natural causes of famine in arid and semi-arid regions and are the most significant threats to agriculture in many parts of the world. It has been estimated that as much as 30% of arable land may be lost to

salinisation over the next 25 years (Altman, 1999). The resulting conflicts over water resources at both local and international levels could exacerbate food shortages still further in the affected regions.

Drought tolerance arises from a complex set of traits that may have evolved as separate mechanisms in different plants. In view of the likelihood of there being more arid regions, it is surprising that there have been relatively few attempts to produce transgenic drought-tolerant crops, even by publicly funded organisations. An alternative approach to transgenesis is to use advanced breeding methods to improve the agronomic performance of existing arid-region crops, such as pearl millet, which is grown on over 40 Mha in Africa. The synteny of the pearl millet genome with the other major cereals (see Fig. 13.5) gives some hope that drought-tolerance traits will eventually be introduced into local varieties by conventional breeding with the help of some of the newer and less expensive molecular marker maps.

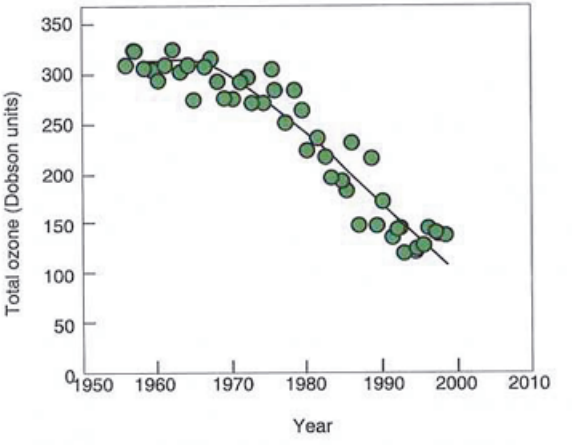


Figure 13.6 Depletion of atmospheric ozone layer over Antarctica from 1957–2000. Data are mean ozone concentrations as sampled at the Halley experimental station of the British Antarctic Survey. They show a greater than 50% reduction in ozone levels over this period. Similar data have been collected from other north and south polar regions. For latest ozone measurements and further information, see *www.nerc-bas.ac.uk/public/icd/jds/ozone*. Data are reprinted with permission from J. Shanklin, BAS.

Over the past fifty years, scientists have observed an ever-deepening 'ozone hole' that is currently located mainly around the polar regions, as shown by the Antarctic data in Fig. 13.6. However, decreases in atmospheric ozone concentrations have also been observed to be spreading towards the major temperate crop-growing areas. Although some ameliorative measures including prohibitions on the use of chlorofluorohydrocarbons (CFCs) have been taken, the damage to the ozone layer and

the consequent increases in ground-level irradiation may take a considerable time to reverse. Already, ozone depletion has started to affect Australia where levels of UV radiation in Sydney have increased by 6% in the past decade. A reduction in the thickness of the ozone layer will increase the amount of radiation-associated (mostly UV) stresses in crops. Plants subjected to increased irradiation will need either to develop more efficient energy-quenching mechanisms or risk free-radical generation and oxidative damage, particularly to the photosynthetic apparatus. An increased investment in photoprotective mechanisms, even if successful, would probably be at the expense of overall productivity and crop yields could therefore decline. Increased UV irradiation also causes cell wall abnormalities, probably by cross-linking some of the cell wall components, which in turn leads to morphological changes that are deleterious to plant growth. There would also be an increased rate of radiation-induced mutations in such plants. To date there have been very few studies on the effect on crops of ozone depletion, but some preliminary findings indicate that half of the common crops suffer yield depression in response to elevated UVB irradiation, with maize showing a 28% decline and barley and oats also seriously affected (*www.umich.edu/~gs265/society/ozone/htm*). Given the number and wide range of effects of a decreased ozone layer, it seems unlikely that transgenic plants could provide a ready solution. Even if plants could be engineered to be more resistant to UV, *e.g.* by having higher levels of carotenoids or antioxidants, they might still be prone to cell wall damage. The best contribution that biotech can make is probably as part of a marker-assisted conventional breeding programme to develop new UV-tolerant cultivars.

Commercial transgenic crops resistant to various biotic stresses such as insects and viruses have already been developed (Section 13.3.3) and there are numerous new varieties in the pipeline. There has been less attention paid to developing tolerance to abiotic stresses in commercial crops, but some studies are beginning to show the potential of such an approach. One example is a transgenic tobacco line, expressing an *E. coli* mannitol-1-phosphate dehydrogenase gene, that accumulates elevated levels of mannitol and is therefore better able to withstand high salinity (Tarczynski *et al.*, 1992). Laboratory and small-scale field studies have shown that the accumulation of other compounds, including betaine or trehalose, in transgenic plants may also enhance salt tolerance (Nuccio *et al.*, 1999). However, it is not clear whether such relatively simple modifications will lead to a sustained effect on crop yields in the much more complex real-life cropping systems where osmotic stress is often linked with a combination of periodic aridity, mineral/salt build up and/or erosion.

Some plants are able to withstand relatively high levels of mineral toxins, such as heavy metals. The metals are often absorbed by these plants but are then chelated to specific proteins or other compounds, which allows them to be sequestered in a non-toxic form. The ability to express such traits in crop plants could extend their range of cultivation or may allow them to be used for reclaiming polluted land. Another intriguing idea is to use such plants for 'bioprospecting'. This could involve the cultivation of a metal-sequestering crop in an area that contains valuable mineral deposits that are not sufficiently abundant to justify direct mining. The possibilities of such an approach are illustrated by a report that a brassica species has been used to hyper-accumulate gold (Brooks *et al.*, 1998). One of the best-studied classes of proteins associated with heavy-metal tolerance is the metallothioneins, which can bind metals such as Zn, Cd and Cu. Transgenic rapeseed or tobacco plants expressing mammalian metallothioneins can tolerate elevated levels of heavy metals (Prasad and de Oliveira Freitas, 1999). More recently there have been efforts to overexpress another class of glutathione-derived metallothioneins, termed phytochelatins, by transferring a bacterial γ-glutamylcysteine synthase gene into poplar trees (Arisi *et al.*, 1997). Preliminary data indicate that the transgenic trees contain higher levels of glutathiones, but it is not yet clear whether this leads to increased phytochelatin accumulation or tolerance to heavy metals.

Although these approaches are yielding promising early results, it will be necessary in future to carry out a thorough analysis of the performance of such transgenic plants in order to assess possible pleiotropic effects. This is particularly relevant to the overexpression of metallothioneins because the precise biological function(s) of these proteins is still unclear and they may be involved in numerous processes other then metal tolerance. Recent studies have shown that metallothioneins are highly expressed during fruit ripening (Moriguchi *et al.*, 1998), leaf senescence (Buchanan-Wollaston and Ainsworth, 1997), wounding and viral infection (Choi *et al.*, 1996) and fungal and bacterial infection (Butt *et al.*, 1998). Indeed it is quite possible that the primary role of metallothioneins in plants is related to a process such as redox regulation rather than metal sequestration. This is a useful example of how our emerging but still imperfect knowledge of many aspects of plant physiology and biochemistry is revealing that some of the assumptions that have informed strategies for plant manipulation by transgenesis may require revision (see Section 13.5.1).

13.4.3 Pathway engineering

In addition to the products of primary metabolism, such as proteins, lipids and carbohydrates, plants produce a host of secondary products, many of which are highly sought after for various uses, ranging from drugs to beverages. For example, even after over a century of synthetic pharmaceutical production, more than a quarter of all prescribed drugs contain one or more the ingredients of natural (normally plant) origin. Some of the milder-acting plant products are ingested as crude extracts of plant tissues, as in coffee, teas and other infusions. In the case of many of the more potent pharmaceuticals, the active product is normally first purified from the plant before being used for therapeutic purposes, as with the anti-cancer drug taxol® obtained from the Pacific yew or the anti-malarial agent quinine extracted from the yellow cinchona. Alternatively, precursor compounds may be extracted from plants and then converted chemically into desired products like codeine and ascorbic acid (Vitamin C). Many of these useful secondary metabolites are only produced in plants that are difficult to cultivate or accumulate in relatively small quantities. Given the very high value of some secondary products, it is not surprising that there is a great deal of interest in engineering their biosynthetic pathways to make more of the desired product in the original plant or in transferring some of the genes into higher-yielding crop plants. The difficulties involved in this endeavour are formidable as most of the pathways concerned are tortuously complicated and are often subject to a high degree of regulation.

One of the better-studied plants is *Catharanthus roseus*, the Madagascan periwinkle, which accumulates the two high-value anticancer drugs, vincristine and vinblastine. These indole alkaloids are synthesised as intermediates in an intricate series of interconnected pathways involving dozens of enzymes. Attempts have been made to elevate the amount of vincristine and vinblastine in transgenic periwinkle by increasing levels of the enzymes involved in their immediate biosynthesis or by decreasing levels of enzymes responsible for their conversion into other compounds. To date these efforts have had only limited success and it is likely that a more thorough understanding of the regulation of indole alkaloid production will be required before such complex manipulations will be achieved on a commercial scale. Slightly more success has been forthcoming in less complex systems such as the atropine-producing medicinal plants. The insertion of a hyoscyamine-6-hydroxylase (*h6h*) gene into *Atropa belladonna* resulted in the production of the sedative scopolamine in the transgenic plants (Yun *et al.*, 1992).

A potentially interesting alternative to growing transgenic plants is to develop transgenic cell or tissue culture systems that synthesise the product of interest under

controlled laboratory conditions. Transgenic root cultures of the plant *Hyoscyamus mutinous* expressing the *h6h* gene accumulated 100-fold more scopolamine than controls (Oksman-Caldentey, 2000). These data and those from other studies with transformed root cultures indicate that they often give better yields of bioactive secondary metabolites than intact plants or conventional cell cultures. Therefore, in the case of very high-value products like pharmaceuticals, where only relatively small quantities are required to satisfy medical requirements, transformed cell cultures may be a viable and more easily contained alternative production system to transgenic plants. Also, the more secure containment of transgenic cell cultures, as compared with whole plants, will doubtless make it easier to obtain regulatory approval for their use and may make such *in-vitro* methods of producing high-value products significantly more acceptable to the general public than producing them via conventional agriculture in the open environment.

13.4.4 Protein engineering

Useful plant products are the result of biosynthetic pathways involving the action of a series of enzymes. In addition to manipulating these pathways, as described above, some of the important individual enzymes can be modified using the emerging techniques of protein engineering. This means that it may be possible to redesign enzymes to make entirely new compounds that have never been produced before, either chemically or biologically. The rational re-design of enzymes requires detailed knowledge about their 3D structures, their interaction with substrates and cofactors, and their catalytic mechanisms. This kind of information is not available for most enzymes but may be forthcoming during the next decade, following recent advances in the determination of protein structures. Not only is it getting easier to determine structures to very fine resolution using X-ray crystallography and high-field NMR spectroscopy, it is also becoming much easier to assign predictive structures based on known folding patterns (Burley, 2000). Once the detailed structure of the enzyme–substrate complex is known, it may be possible to redesign the enzyme to accept different substrates and to convert them into different products.

An example relevant to crop biotechnology is the engineering of fatty acid desaturases. The soluble Δ-9 stearate desaturase of plants converts stearic into oleic acid by inserting a double bond into the carbon chain at the Δ-9 position between C9 and C10. This was the first desaturase to be crystallised and its structure was resolved by X-ray diffraction analysis to 2.4 Å (Lindqvist *et al.*, 1996). As shown in the model in Fig. 13.7, the desaturase protein has an extended internal cleft to hold its long-chain

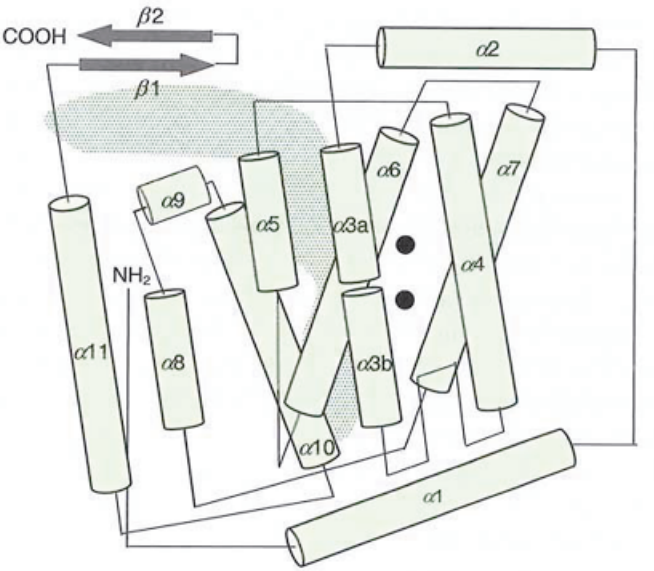


Figure 13.7 Topological model of one subunit of the stearoyl–ACP desaturase homodimer. The protein consists of eleven α-helices, nine of which form an antiparallel helix bundle. The location of the di-iron active site, which is associated with ligands from four of the α-helices in the bundle, is indicated by the two black circles. The putative hydrophobic substrate cleft into which the fatty acyl chain is inserted is indicated by the green shaded area. Figure adapted from Lindqvist *et al.* (1996).

C18 fatty acid substrate with the active site for double bond insertion positioned half way along the cleft, *i.e.* immediately opposite the Δ-9 position. Preliminary experiments indicate that shortening the cleft at its base by engineering in more bulky amino acid side chains does indeed have the predicted effect of changing the substrate preference from C18 to the shorter C16 fatty acids and can also shift the site of double bond insertion from the Δ-9 to the Δ-6 position (Cahoon *et al.*, 1997). Perhaps even more exciting is the discovery that many other fatty-acid-modifying enzymes like epoxidases, acetylinases and hydroxylases are just slightly modified desaturases. This opens up the prospect of being able to engineer a whole family of desaturase-like proteins so that they can insert double bonds, triple bonds or other interesting functionalities into any desired position on an acyl chain of any desired length. In this way it may be possible to biosynthesise many novel organic compounds that cannot be made chemically, *e.g.* those with specific chiral properties, and then to produce those that have particularly useful properties on a large scale in transgenic crops (Murphy, 1999a and 1999b).

Another less direct but possibly more effective way to engineer enzymes is to perform random mutagenesis of certain selected amino acid residues and then to screen the resulting mutant library by complementation of an auxotrophic strain of a suitable microorganism. This method has recently been applied to the engineering of the Δ-9 stearate desaturase of plants in combination with an *E. coli* mutant that is auxotrophic for unsaturated fatty acids (Cahoon and Shanklin, 2000). Four amino acid residues that were predicted to be important in determining acyl chain-length specificity of the Δ-9 stearate desaturase were selected for saturation mutagenesis, *i.e.* they were each converted into all of the 19 other possible alternative amino acids. The resulting mutagenised proteins were expressed in thousands of lines of the auxotrophic mutant of *E. coli* and screened for ability to grow on C14 or C16 fatty acids. Only those bacteria expressing a mutagenised desaturase that could use C14 or C16 fatty acids would grow under such conditions. The screen resulted in the isolation of a new form of the desaturase that had a 15-fold higher specific activity towards C16 fatty acids compared with the wild-type enzyme. This kind of complementation/ screening approach has significant potential for the isolation of novel forms of many types of enzyme of biotechnological interest.

13.4.5 Molecular pharming: the expression of high-value products

Molecular pharming can be regarded as the cultivation of transgenic crops or cultures to accumulate medium- to high-value products ranging from industrial enzymes to pharmaceuticals. Some of these may be low-molecular-weight products derived from pathway engineering, as described above, while others may be peptides or proteins that are direct products of an introduced gene. The worldwide annual sales of peptide drugs alone are substantially in excess of $1 billion (Kelley, 1996). Given the high-value, low-quantity relationship of the target products of molecular pharming (see Fig. 13.8 and Section 13.7.4), there is a great deal of interest in this potentially very profitable technology, which in many ways is more akin to microbial fermentation than to conventional large-scale agriculture. A distinct advantage of a eukaryotic protein-production system, such as plants, is that they do not suffer from many of the problems encountered with the expression of xenoproteins in bacterial cells. Such problems include self-toxicity, the lack of post-translational processing, the insolubility of many proteins and their accumulation in inclusion bodies. Plants also have the distinct advantage that their major inputs, CO_2 and light, are free, while other inputs like fertiliser and herbicides are relatively cheap. Plants also require little maintenance compared with fermentation units and production can be scaled up or

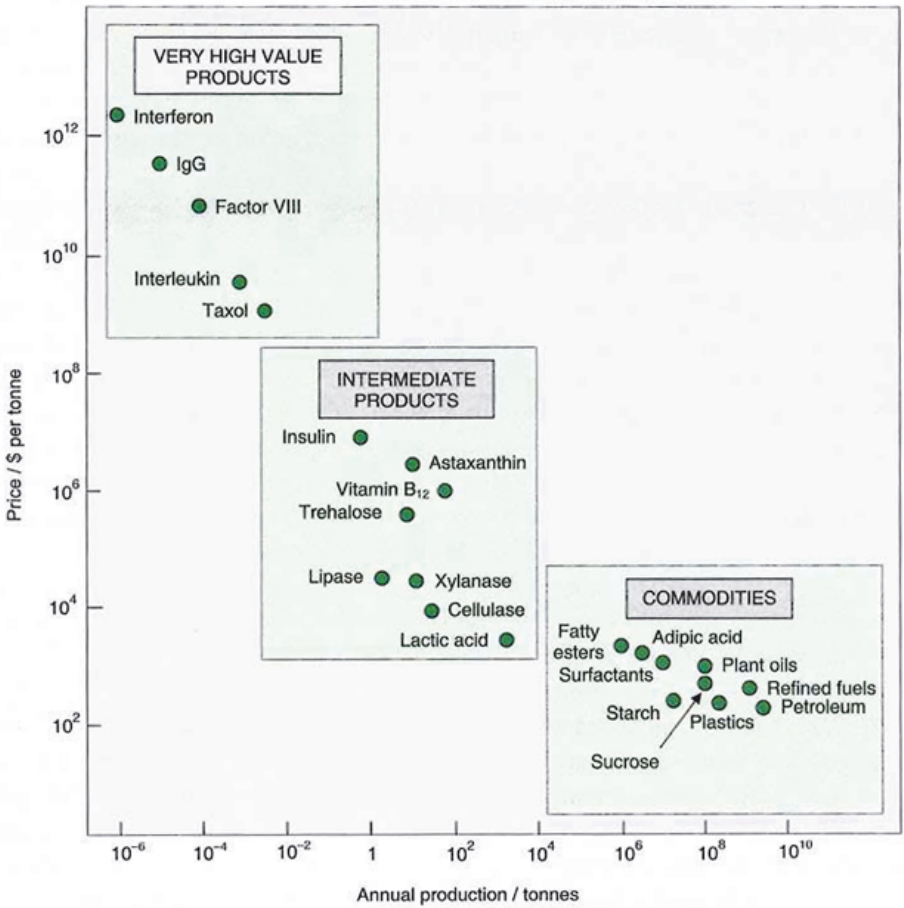


Figure 13.8 Relationship between annual market size and price for various products. In general, there is an inverse relationship between the price per tonne of a product and its annual sales volume. At the bottom end of the market there are the commodities like petroleum, plastics, plant oils and starches, which are traded in bulk (>10^4 t) at low unit cost ($100–2000 t^{-1}). The intermediate products range from fine chemicals and industrial enzymes up to vitamins and some of the cheaper therapeutics like insulin, with annual sales of 1–5000 t but at much higher prices ($\$10^3$–$10^7$ t^{-1}) than the bulk commodities. Finally, there are the very high-value pharmaceutical products that are made in small quantities (<10 kg) but command extremely high unit prices ($\$10^8$–$10^{13}$ t^{-1}). The use of plant systems for molecular pharming may enable some of the very high cost pharmaceuticals to be made more cheaply.

down simply by cultivating more or less land, rather than the costly process of constructing or decommissioning fermentation facilities. Finally, in most of the transgenic plant systems the recombinant protein is accumulated in the seed. In this

form, the protein is inert and can be stored for many years in a convenient compact configuration. Recombinant seed proteins have been extracted in fully active form after more than five years of storage and it is possible that even longer storage periods can be achieved.

Of course, there is already another alternative recombinant protein expression system of proven utility, namely transgenic mammals. Various body fluids, including milk, blood and urine, have been used as convenient media for expression and access to such proteins (van Cott *et al.*, 1997; Kerr *et al.*, 1998). However, the pharmaceutical industry is now also becoming more interested in plant-based molecular pharming, at least for some products, because of several perceived advantages over animal systems: first, plants could be more economic production systems; second, plants are unaffected by contamination by animal microbial pathogens or by other infectious agents like prions; and third, the use of transgenic plants may be more acceptable to some groups, as it avoids ethical issues related to the creation of transgenic animals in general or religious issues involving the use of a particular animal species.

Products of molecular pharming Over the past decade, a large number of mainly protein products have been investigated for expression in various plant systems. For example, there are at least 15 cases of recombinant vaccines targeted at either animal or human diseases that have been produced in transgenic plants (Hood and Ma, 1999). Antibodies were first produced in plants in 1989 (Hiatt *et al.*, 1989) and can be used as part of an input trait manipulation to combat viral diseases (Section 13.3.3) or as products targeted against human diseases including tooth decay (Ma *et al.*, 1995) and herpes (Potera, 1999). Some of the general issues relating to the industrial scale production of antibodies in plants have been reviewed by van der Logt *et al.* (1998).

Food crops have also been proposed as possible vehicles for the expression of edible vaccines that would be both inexpensive and readily available even to isolated rural populations. A popular image is that of a transgenic banana being happily consumed by a child, who thereby becomes immunised against a myriad of devastating diseases (Langridge, 2000). In addition to being a lot more pleasant to administer, such orally delivered vaccines would have an advantage over injected vaccines in that they could elicit both mucosal and systemic immunity. This would enable their use against intestinal pathogens, such as diarrhoea-causing agents (still a major cause of child mortality), as well as against internal body pathogens like the hepatitis B virus, or even to suppress the autoimmune response that leads to type I diabetes (Arakawa *et al.*, 1998a; Carter and Langridge, 2001). Such technology is still in its infancy and would need to be very carefully regulated, *e.g.* access to the plants

or fruits would need to be restricted to trained personnel and dosages may be difficult to calculate if the plants exhibit their normal wide range of variation, both genetic and environmental. Nevertheless, it is an intriguing idea that is being seriously pursued. Table 13.3 shows a selection of some of the many pharmaceutically active or other high-value peptides and proteins already produced in transgenic plants in research laboratories.

As yet, very few plant-produced animal or microbial proteins have been developed for commercial production. Two of the rare examples of such proteins are avidin and β-glucuronidase (GUS), both produced in transgenic maize. Avidin is used as a biochemical reagent for research and diagnostics and may also be developed as a biopesticide (McGraw, 2000). Conventionally, avidin has been obtained from chicken egg whites, where the cost of the starting material is $\$1000\,t^{-1}$, while sufficient transgenic maize to yield the same amount of avidin costs only \$20 (Hood *et al.*, 1997, 1999). GUS is a bacterial protein that is widely used in research labs as a marker enzyme that can be detected in highly sensitive cytochemical, spectrophotometric and fluorimetric assays. When expressed in transgenic maize, GUS is significantly cheaper than the GUS purified from bacteria. Both avidin and GUS are now produced as recombinant plant proteins and marketed as research biochemicals by Sigma-Aldrich (McGraw, 2000). Success in the commercial production of these two very different animal and bacterial proteins demonstrates the versatility of plants as expression systems for proteins. Avidin is a small, 17 kDa glycosylated, basic eukaryotic protein whereas GUS is a relatively large, 68 kDa non-glycosylated, acidic bacterial protein and yet both were correctly processed and folded into biologically active forms when expressed at high levels in plants. Although these two proteins are only produced on a small scale for niche markets, they may be the harbingers of a much more extensive use of plants as vehicles for molecular pharming in future.

Molecular pharming Molecular pharming is a completely different type of venture to conventional agriculture; Table 13.4 shows the contrast between the two systems. Of course, some non-transgenic high-value low-acreage crops are cultivated already, but the advent of molecular pharming could result in dozens or even hundreds of new transgenic high-value crops. This raises the question of containment. Although they are potentially excellent production systems, transgenic crop plants currently face the challenge that their containment is not as straightforward as with transgenic animals or microbes. However, while physical containment of plants grown in the field may be problematic, the possibility of using biological mechanisms like pollen or seed sterility is being studied.

Table 13.3 Selection of peptides and proteins expressed in transgenic plants

Class of protein/peptide	Reference
Peptide	
Hirudin	Parmenter *et al.*, 1995
Lytic peptides	Garbabino *et al.*, 2000
Antibody	
Streptococcus mutans	Ma *et al.*, 1998
Single-chain Fv	Tavladoraki *et al.*, 1993
Immunoglobulin	Cabanes-Macheteau *et al.*, 1999
Herpes simplex monoclonal	Zeitlin *et al.*, 1998
Colon cancer monoclonal	Verch *et al.*, 1998
Antigen	
Cholera toxin B subunit	Arakawa *et al.*, 1998b
E. coli enterotoxin	Mason *et al.*, 1998
Foot-and-mouth virus	Carillo *et al.*, 1998
Rabies virus	Modelska *et al.*, 1998
S aureus toxin	Brennan *et al.*, 1999
Insulin-cholera toxin	Arakawa *et al.*, 1998a
Industrial enzyme	
α-Amylase	Pen *et al.*, 1993a
Cellulase	Ziyu *et al.*, 1998
Cerebrosidase	Cramer *et al.*, 1996
β-Glucanase	Ziegler *et al.*, 1999
Lipase	Gruber *et al.*, 1998a
Xylanase	Liu *et al.*, 1997
Phytase	Pen *et al.*, 1993b
β-glucuronidase	Hood *et al.*, 1997, 1999
Others	
Avidin	Hood *et al.*, 1997, 1999
Human epidermal growth factor	Hooker *et al.*, 1998
Collagen	Gruber *et al.*, 1998b
Haemoglobin	Dieryck *et al.*, 1998
Human lactoferrin	Salmon *et al.*, 1998
Interleukin	Magnuson *et al.*, 1998
Interferon	de Zoeten *et al.*, 1989

It is likely that these or other scientific solutions to the containment issue will be developed in the next 5–10 years. However this issue is resolved, it will still be advantageous, both from a management and a public acceptability perspective, if the plants chosen as the production vehicles for molecular pharming are not mainstream food crops. Meanwhile, the limited area required for cultivation of molecular pharming crops means they can often be grown within monitored compounds located in geographically isolated areas. For example, the field trials in Canada of transgenic rapeseed expressing recombinant pharmaceutical peptides were located in a remote area of British Columbia that was separated by many hundreds of kilometres and by the barrier of the Rocky Mountains from the nearest conventional rapeseed crops.

Table 13.4 Molecular pharming *vs.* conventional farming

Characteristic	**Pharming**	**Farming**
Production scale (per crop)	1–3000 ha	40.1–10 Mha
Cost of inputs ($ ha^{-1})	3-600	3-600
Value of inputs ($ ha^{-1})	1–5 M (industrial enzymes) 10–200 M (pharmaceutical proteins)	3–800
Profitability ($ ha^{-1})	Many millions	1–400

The most common production vehicle for molecular pharming in plants is the seed, but others include tubers from potatoes (Tacket *et al.*, 1998) and leaves from tobacco (Cramer *et al.*, 1998). Irrespective of the tissue of origin, the production of high-value recombinant proteins in plants faces a similar range of challenges to microbial fermentation systems. These include the level of expression, solubility, ability to fold appropriately, fidelity of processing (*e.g.* glycosylation and phosphorylation), stability, extractability and ease of purification. There are some indications that the pattern of N-glycosylation of some recombinant proteins in plants may be different from that in animals. Expression of two mouse antibody genes in tobacco resulted in proteins that were less stable than those made from the same genes when they were expressed in mouse hybridoma cells (Stevens *et al.*, 2000). Another finding from the same study was that the levels of the recombinant protein fluctuated significantly according to the developmental and physiological status of the transgenic plants. Thus plants may not be suitable vehicles to express all animal proteins. Also, it may be necessary to optimise plant growth conditions to obtain the best levels of protein expression for a given species. Finally, as seen with the transgenic cotton expressing a Bt toxin (see Section 13.3.3), climatic factors can dramatically affect levels of xenoproteins in crops that are grown in the open.

Given that typical expression levels for recombinant proteins in plants are less than 1% total tissue protein, a high priority for future development of plant pharming will be either to increase the expression and/or to have a rapid, inexpensive and effective purification system. There are several interesting methods that are currently at the research stage. One idea is to let the plants pre-purify the protein by secreting it, *e.g.* from the roots of hydroponically grown plants (Borisjuk *et al.*, 1999), from cultured root cells (Wongsamuth and Doran, 1997) or even from the guttation fluid of leaves (Komarnytsky *et al.*, 2000). An alternative method that has now been developed to pilot-plant scale is to bind the recombinant protein to oil bodies within seeds and then purify the protein–oil body complex by flotation, as first described in 1995 (van Rooijen and Moloney, 1995).

This latter technology, as pursued by the Canadian biotech company SemBioSys, involves the expression of the protein/peptide of interest as a fusion protein with oleosin. Oleosin is a highly abundant protein in oil-rich seeds that is targeted exclusively to oil bodies. Since they are less dense than water, the oil bodies, along with their cargo of recombinant peptide, can be readily purified from the remainder of the seed material by flotation following centrifugation of a seed extract. The recombinant peptide can then, in principle, be cleaved away from the oil body using a pre-engineered protease recognition site at the junction of the oleosin and the fusion protein. If successful on a larger scale, this approach could be used for the commercial production of a variety of relatively low-cost easily purified peptides or proteins. The method has already been used successfully on a lab scale with several oilseed brassica species to produce active recombinant peptides including the anticoagulant hirudin from the leech *Hirudo medicalis* (Parmenter *et al.*, 1995) or large proteins such as bacterial GUS (Chaudhary *et al.*, 1998) and a fungal xylanase (Liu *et al.*, 1997). Further development of this oleosin technology is now being pursued in collaboration with the US agrosciences company, DowElanco (*www.sembiosys.ca*).

13.4.6 Transgenic tree crops

Tree crops are big business. The current turnover of unprocessed timber alone in the industrial forestry sector is \$400 billion yr^{-1}. The area of tree plantations in developing countries doubled between 1980 and 1995 and is set to double again by 2010. Trees are therefore an increasingly important, if often overlooked, component of world agriculture. Tree crops vary considerably in their morphology, cropping systems and the types of products that are harvested. They range from relatively small

shrubby plants like cocoa or coppice willow to large long-lived species like oil palm or redwood. Their products range from fruits like citrus or coconut to industrial materials like paper or timber. Trees can be grown in small orchards or larger plantations from which their products may be harvested regularly or they may be clear felled and replanted over much longer intervals—sometimes extending over many decades. Such diversity makes it difficult to treat tree crops as a single category but they are clearly distinct from annual crops in terms of their management and some of the challenges to the application of biotech methods to their improvement.

There are several traits that tree growers are attempting to manipulate using transgenic approaches. Plantation managers are interested in trees that become established more rapidly, have faster growth rates and lay down wood fibre that is both more uniform and of higher quality. There is also interest in the range of input traits that are being manipulated in annual crops (Section 13.3.3) and in various types of stress tolerance (Section 13.4.2). As with annual crops, input traits have been an attractive early target both because of the perceived commercial success of some existing GMO crops and because of the estimated profitability of such traits in trees. It is estimated that the introduction of a herbicide-tolerance trait would reduce the cost of new forestry plantations by \$188 million yr^{-1} in the USA and \$975 million yr^{-1} worldwide (Sedjo, 1999) and most of the current batch of field trials of transgenic trees involve this trait. Disease and pest control are the next most common targets and there are also some recent patents for transgenic sterility in trees. The latter trait would preclude the spread of transgenes out of plantation areas and would also protect the owners from theft of the germplasm.

The kind of output traits of interest in fibre tree crops mainly relate to lignin composition. In the case of softwoods and other tree crops destined for pulping, *e.g.* for paper manufacture, most efforts are directed at reducing the lignin content of wood. This reduces the cost of chemical processing at pulping mills and can have significant environmental benefits by cutting down on pollution caused by the pulping effluent. Lignin accumulation has been reduced in some of the more easily transformed tree crops like poplars (Lapierre *et al.*, 1999) and aspen (*http://users.ox.ac.uk/~dops0022/conference/wood.htm*). This has been achieved using gene-silencing methods to down-regulate enzymes, such as cinnamyl alcohol dehydrogenase or caffeic acid O-methyltransferase, that are involved in the biosynthesis of monolignols, the precursors of lignin. Reduction in lignin content was normally accompanied by a compensatory increase in cellulose deposition and an overall faster growth rate. There are some concerns that low-lignin trees may be more susceptible to disease or that the transgene could spread to wild relatives and field trials to address these issues will be desirable before any commercial releases are sanctioned. As well

as manipulating the amount of lignin, it may also be possible to change its quality. When the gene encoding caffeic acid O-methyltransferase was almost completely silenced in transgenic poplars, the wood contained more highly condensed lignin and gave a 10% better pulp yield than untransformed controls (Jouanin, 1999). This holds out the prospect that in the future it may be possible to effect rational manipulations of lignin quality in tree crops (Merkle and Dean, 2000).

Ironically, while there are intense efforts to reduce lignin in trees, there is also interest in increasing the lignin content of trees that are destined to be used as biomass for energy generation or as carbon sinks to alleviate the increasing levels of atmospheric CO_2. It has been estimated that the replanting of about 600 Mha of tropical forest, which represents one third to a half of the area destroyed over the past 50 years, would remove sufficient atmospheric CO_2 to correct the rise from 260 ppm to 360 ppm that has taken place over the past two centuries (Shanks and Teh, 1999). Several oil companies and automobile manufacturers, including Shell and Toyota, have made large investments in agroforestry and are pursuing both transgenic and non-transgenic approaches. An example of a non-transgenic biotech strategy to increase wood biomass in forest plantations is the Toyota tetraploidy project (*http://forests.org/archive/general/gmtechsc.htm*). In this case, chemical treatments are used to double the chromosome number in the cells of tissue explants from a tree crop. This converts the cells from the normal diploid condition (two copies of each chromosome) to tetraploid (four copies). The trees are then regenerated in culture and propagated by clonal methods. Tetraploid plants are often larger and grow more rapidly than diploids and should therefore be more effective carbon sinks. However, when commercial growers producing ornamental shrubs tried this method in the past, they found that the tetraploids suffered from weaknesses in their wood that made them unsuitable for cultivation. It remains to be seen whether either the transgenic or the tetraploid approaches to increasing the effectiveness of trees to act as carbon sinks will succeed. The issue of carbon sinks is discussed further in Section 13.7.3.

Despite the progress made in producing several dozen species of transgenic trees, the application of the technology to many of the major softwood crop species is not yet possible. This is because most pines cannot at present be propagated vegetatively, and this is a prerequisite for the production of large numbers (often millions) of transgenic seedlings. However, given the emerging commercial interest and investment in agroforestry, it is likely that improved tissue culture methods will eventually be developed for the high-yield softwoods, enabling transgenic varieties to be to be propagated on a large scale.

13.4.7 Microalgae

The cultivation and manipulation of marine and fresh-water microalgae as sources of valuable products is an emerging area of modern biotechnology. There are over 30,000 species of unexploited microalgae that synthesise a diverse array of natural products including proteins, enzymes, fats, sugars, vitamins and unusual bioactive compounds. These algae are the fastest growing plants on earth; they can double their weight each day and can increase their biomass one hundred times faster than trees. In some cases it is even possible to harvest a new crop of microalgae each day. Like terrestrial plants, some of the key inputs for the cultivation of microalgae, notably energy and carbon, are free and they are now being developed as an alternative to yeast or bacterial-based fermentation systems for the manufacture of some high-value products and also for bioremediation. More general information about microalgae and their uses can be found in Chapter 9.

Several commercial biotech ventures to cultivate, harvest and process microalgae and their products have been set up in locations ranging from inland deserts to mid-oceanic islands. One of the more favourable locations is Hawaii, with its high rate of insolation, moderate temperatures and ready access to the cool deep waters needed for some of the production processes. At present, the major products from microalgae are naturally occurring compounds such as β-carotene, γ-linolenic acid and xanthophylls. The compound attracting some of the greatest interest is the pigment astaxanthin, which is produced by the freshwater microalga *Haematococcus pluvialis* (Lorenz and Cysewski, 2000). Although it can be synthesised from petroleum, astaxanthin may be more cheaply derived from microalgae. Astaxanthin has an established \$150 M yr^{-1} market as a dye that is used to accentuate the colour of the rather pallid shrimp and fish that result from rearing in pens on fish farms; it could also find uses as a colour enhancer for poultry and other farm livestock. However, an emerging and potentially much more lucrative market for astaxanthin is as a dietary supplement or nutraceutical. These applications are based upon the powerful antioxidant properties that are exhibited by astaxanthin formulations, which have been shown in human and animal models to have efficacy against several important conditions, including macular degeneration (the leading cause of blindness in the USA), some cancers and stomach ulcers (Jyonoucki *et al.*, 2000; Wang *et al.*, 2000).

The biotechnological exploitation of microalgae is very much in its infancy and is still the realm of small start-up companies that are often spin-offs from academic labs. Descriptions of the activities of two such ventures can be found on the websites of Cyanotech (*www.cyanotech.com/index.htm*) or Aquasearch (*www.aquasearch.com/business.html*). The current first generation of biotech products from microalgae does

not involve transgenesis and, while transgenic techniques are being developed at the research level, it is unlikely that they will be applied commercially until the industry has matured sufficiently to attract larger investment or has a reliable revenue stream. As well as making valuable chemicals, microalgae could also be usefully exploited for bioremediation, including removal of chemical toxins from flooded workings or cleaning up oil spills, although development of such products will require significant additional investment in microalgal research. Looking further into the future, the exploitation of this hitherto largely overlooked resource has enormous potential, alongside agriculture, agroforestry and molecular pharming, to supply renewable products as alternatives to microbial fermentation or synthesis from petrochemicals.

13.5 Challenges for transgenic crops

Transgenic crops from agriculture, forestry or aquaculture face significant challenges before they can become part of the mainstream global food and non-food supply chains. In addition to the well-known and complex problem of public acceptability, there are also unresolved scientific and management issues that should be addressed.

13.5.1 Scientific issues

Manipulating metabolic pathways The rapid pace of development in molecular biology over the past twenty years has not always been matched by our understanding of many of the basic physiological processes or biochemical pathways that we are attempting to manipulate. This is equally true for animal, plant and microbial systems. While many transgenic manipulations do indeed have the predicted effect, this is not always the case and in the past few years there have been several sobering reminders of our ignorance of some of the key metabolic pathways that are targets for transgenic modification. Bailey (1999) has pointed out the difficulties involved in trying to manipulate even relatively simple organisms like *E. coli*. To give just one example, a mutant strain of *E. coli* was made in which the pyruvate kinase gene was deleted. Quite unexpectedly, in view of the metabolic importance of pyruvate kinase, the bacteria grew completely normally. This was because the mutants were able to synthesise pyruvate from phosphoenolpyruvate via the tricarboxylic acid cycle and malic enzyme, which according to conventional biochemical wisdom should not have been possible.

Two further examples of surprising discoveries about what were believed to be well-known metabolic pathways in plants relate respectively to terpenoid and triacylglycerol biosynthesis. Terpenoids are of great interest as plant defence compounds and as sources of pharmaceuticals like paclitaxel (taxol®), vincristine and camptothecin (Kutchan, 1995). Recent studies indicate that a newly discovered plastidial pathway, the glyceraldehyde-3-phosphate (GAP) pathway, contributes significantly to monoterpene and sesquiterpene biosynthesis, even though the latter had hitherto been regarded as a cytosolic activity (reviewed by McCaskill and Croteau, 1998). In the opinion of two senior workers in this field "it is extraordinary that a widely distributed pathway, central to the biosynthesis of such a wide variety of essential natural products in plants, should have gone unrecognised until very recently" (McCaskill and Croteau, 1998). Of course, the discovery of such new phenomena is the essence of scientific research, but the point here is that up to now the attempts to manipulate this biological process, *i.e.* terpenoid biosynthesis, in transgenic plants have been based on an inaccurate description of the pathway, and the same is probably also be true for other areas of agbiotech.

The second example concerns the biosynthesis of triacylglycerols, which are the main component of most plant oils. According to textbooks, triacylglycerols in plants are formed by so-called 'Kennedy pathway'. This pathway involves the sequential acylation of glycerol-3-phosphate by three acyltransferases, the last of which adds acyl-CoA to diacylglycerol and is termed diacylglycerol acyltransferase or DGAT. Since the previous enzymes in the pathway can also contribute to membrane lipid biosynthesis, the enzyme DGAT was regarded as the committed step to triacylglycerol oil formation and as such was a key target for biotech manipulation, *e.g.* to increase flux of metabolites to oil accumulation in order to produce higher oil yields in crops. However, these assumptions have been questioned in the past few years with the discovery of at least two alternative acyl-CoA-independent routes for triacylglycerol biosynthesis (Dahlqvist *et al.*, 1999; Stobart *et al.*, 1997). This means that the conversion of diacylglycerol to triacylglycerol in plants can potentially proceed by one or more of the three different routes shown in Fig. 13.9. At present, the relative contribution of each of these three routes (or indeed whether there are any additional routes) is unknown. At best, this level of metabolic redundancy will complicate efforts to manipulate triacylglycerol biosynthesis by transgene insertion and at worst it could make such an approach unfeasible. Interestingly, similar multiple redundancies have now been described for triacylglycerol biosynthesis in yeast, where even the knocking out of no less than five acyltransferase genes reduced but failed to halt the process (Oelkers *et al.*, 2000).

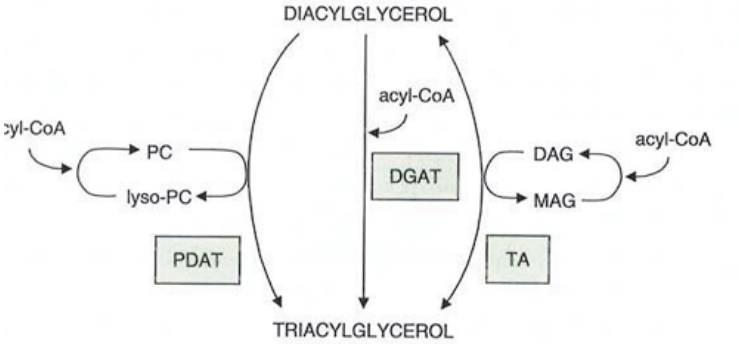


Figure 13.9 Three alternative pathways for triacylglycerol accumulation in plants. The conventional route for triacylglycerol (TAG) biosynthesis is by the transfer of an acyl group from acyl-CoA to diacylglycerol (DAG) via the enzyme, diacylglycerol acyltransferase (DGAT). Recently, two new routes for TAG synthesis have been discovered. Firstly, a reversible transacylase (TA) can transfer an acyl group from one DAG to another, resulting in formation of one molecule of TAG plus one of monoacylglycerol (MAG). Secondly, an acyl group can be transferred from phosphatidylcholine (PC) to DAG by the enzyme phosphatidylcholine: diacylglycerol acyltransferase (PDAT). Such metabolic redundancy means that attempts to manipulate the pathway of oil formation in plants may be more difficult than was first thought.

It is clear from these two examples that a truly rational approach to the manipulation of many metabolic pathways will not be possible until more progress is made in the full description of each of these pathways and the connectivities between them, not to mention their higher-level regulation under different developmental and environmental conditions. The recent sequencing of the *Arabidopsis* genome has highlighted still further the issue of metabolic redundancy in higher plants. While *Arabidopsis* appears to contain a similar gene complement to the far simpler photoautotrophic cyanobacterium *Synechocystis*, the big difference is that most enzymes in *Synechocystis* are encoded by a single gene, whereas in *Arabidopsis* there may be many genes encoding a given enzyme (The Arabidopsis Genome Initiative, 2000). Not all members of these multigene families may be functionally redundant, *e.g.* they may be expressed in different tissues, but these genome data do underlie the metabolic complexity of even one of the simplest of higher plants—and the genomes of most crop species are far larger than that of *Arabidopsis*, from 20-fold larger in maize to over 120-fold larger in wheat (Murphy, 1998).

Pleiotropic effects of transgenes Before they are approved for commercial release, transgenic plants must be tested in field trials to monitor their agronomic performance, as well as factors such as transgene stability and environmental impact. However, it is also important that the behaviour of transgenic plants is analysed

thoroughly for so-called pleiotropic effects, *i.e.* unforeseen phenotypic consequences of transgene insertion that are apparently unrelated to the intended primary effect of the transgene. For example, a transgene may appear to have the desired effect in changing the seed storage oil composition, but may have other unexpected effects elsewhere in the cell. This was found to be the case when Calgene developed a transgenic rapeseed variety with enhanced levels of the saturated fatty acid stearic acid in its seed oil (Knutzon *et al.*, 1992). Later analysis revealed that the stearic acid was present, not only in the storage oil as expected, but had also 'leaked' into the membrane lipids of the developing seeds. The presence of more than tiny amounts of this fatty acid in membranes reduces their fluidity and can severely affect their biological function. This meant that the stearate-rich seeds tended to have relatively poor germination rates, resulting in a gradual erosion (over several years) of the high stearic phenotype when transgenic plants were grown on a field scale (Thompson, 1996). A subsequent transgenic variety of high-stearic rapeseed, also developed by Calgene but using a different transgene, apparently does not suffer from this poor-germination phenotype and also accumulated a lot more stearic acid in its seed oil (Facciotti *et al.*, 1999). This demonstrates how much variation there can be in transgenic phenotypes even when the same character is being manipulated in two different ways.

This example also illustrates the importance of segregating novel fatty acids towards accumulation in storage oil bodies and away from membrane lipids. This is because membrane lipids have very specific fatty acid profiles and if these are altered by the incorporation of novel (*e.g.* very long, very short or saturated) fatty acids, the function of the cell membranes may be seriously perturbed. Plants that naturally accumulate exotic fatty acids in their seed oils have mechanisms to prevent 'leakage' of these into membrane lipids, but some oilseed crops, such as rapeseed, appear to lack such mechanisms, at least for some fatty acids (Wiberg *et al.*, 1997). There are several reported instances of the presence of novel fatty acids in transgenic plants inducing their own breakdown by β-oxidation, which is apparently a protective response to prevent their accumulation in cell membranes (see Fig 13.4 and Murphy, 1999b).

Another interesting example of an unexpected and unwelcome pleiotropic effect of metabolic manipulation has been described for a class of toxins. Although the data relate to aflatoxins in fungi rather than to plants, they serve as a useful cautionary tale that could also apply to plants. Aflatoxins, which are produced by several filamentous fungi, are potent toxins that can cause liver cancer in humans or animals that eat nuts and seeds that have been infected by such fungi. During a study of aflatoxin metabolism, a protein called FadA was found to inhibit the formation of aflatoxins

and other carcinogens in the fungi. This was an interesting finding as it suggested a strategy for eliminating a whole class of fungal toxins that contaminate many important crops. However, further analysis of the fungi in which aflatoxins had been eliminated showed that they had responded to this absence of one of their main defence compounds by synthesising another even more potent toxin, namely the antibiotic penicillin (Tag *et al.*, 2000). Although it is one of our most important antibiotics, penicillin is not exactly the kind of molecule that one would wish to be released into the environment on any scale. While this may be an interesting observation on fungal adaptivity, it also illustrates how any organism may respond to a perturbation in its metabolism in a completely unexpected way. In the case of crop plants, the elimination of an unwanted class of toxins like alkaloids or glucosinolates could potentially lead the plant to respond by accumulating another equally or even more noxious agent. This could be an immediate response, as with the aflatoxin-producing fungi, or it could take place as a result of natural selection over several generations. Either way, this observation highlights the need for the thorough monitoring, and ideally the metabolic profiling, of transgenic crops destined for human or livestock food chains.

Tissue-specificity of transgenes One of the most important attributes of many transgenic plants is that the transgene is expressed in a tissue-specific manner. The inappropriate spatial or temporal expression of any transgene may be deleterious to the plant and, in some instances like molecular pharming, it could have much wider consequences. Therefore when attempting to alter seed traits like starch or oil quality, companies would be expected take great pains to express transgenes under the control of seed-specific promoters. Indeed, various groups have patented the use of several gene promoters for seed-specific expression of transgenes. However, there are now several reports that some of the most commonly used 'seed-specific' gene promoters may also direct the expression of transgenes in other parts of the plant, *e.g.* in the root tips of rapeseed and tobacco plants (Baumlein *et al.*, 1991; Murphy *et al.*, 2001). There are other unpublished observations of such 'seed + root tip' expression patterns that were assumed to be artefacts or simply ignored. In many cases, it may not matter very much to the plant if a transgene is active in root tips as well as seeds, but this will not always be true. These results show that we need a better understanding of tissue-specific gene promoters before we assume that they are only directing expression in the expected tissues. They also show how important it is to perform a comprehensive analysis on the full range of different plant tissues at different developmental stages for possible side effects of transgene insertions.

Improving transformation technology The widely used *Agrobacterium* vectors for the delivery of transgenes into plants were developed almost two decades ago. It is therefore surprising that this method of gene insertion has undergone relatively little subsequent refinement. Transformation of plants is still fairly crude and inefficient compared with, for example, the transformation of microbes or animals. Transgenes in plants are inserted randomly into the genome, their copy number can range from one to a dozen or more, and the introduced gene constructs are frequently modified in the process. For example, the constructs may lose part of one or both of their border regions, or they may be cleaved and the fragments inserted into different parts of the genome. Both the position of insertion in the genome and the copy number of a transgene can dramatically affect its expression in the resulting plant. The presence of multiple copies of a transgene can result in instability of its expression in succeeding generations as copy number tends to be reduced with time. Finally, transgenes are inserted into the recipient genome as part of a gene construct that also contains regulatory elements and a selectable marker, often an antibiotic or herbicide-resistance gene. There is already public concern about the use of antibiotic resistance markers, and many researchers and breeders are also concerned that, as transgenic crops become more widespread, they will inevitably be subject to many further rounds of transformation as additional genes are inserted in order to keep improving the crop. The accumulation of many different selectable marker genes would soon present problems as breeders ran out of new benign markers for further rounds of transformation. The continued use of the same marker can lead to co-suppression and the loss of expression of the transgene itself, and also of genes with related sequences.

These drawbacks to existing transformation technology highlight what should be a major research priority in the future (see also Gelvin, 1998). It is striking that compared with the enormous investment in all the new "-omic" technologies (genomics, proteomics, metabolomics) there has been relatively little attention paid to the possibly less glamorous, but certainly fundamentally important, technologies involved in gene insertion into plants. A key goal should be to develop a method in plants for the site-specific insertion of genes, as exists already for animals. Extraneous DNA, *e.g.* antibiotic-resistance genes, should be removed from constructs after they have been inserted into the plant genome Several methods, such as the *cre-lox* system (Dale and Ow, 1991), have been already developed for this purpose, but these need to be refined and made easier to use. Another potentially promising development is the FLP-recombinase system of yeast (USDA server: *www.nal.usda.gov/pgdic/Probe/v4n3_4/theflp.html*). Several new selectable marker systems, such as the threonine dehydratase/deaminase from *Arabidopsis thaliana* (Purdue University Server. *www.otc.purdue.edu/Lsbio.htm#agP-97029*) or phospho-mannose isomerase (Joersbo,

2001) are also being developed. An alternative to selectable markers is the use of 'scoreable markers', which encode enzymes not normally present in the plant and whose activity can easily be measured. An example is the recent development of a transgenic sunflower where the marker was oxalate oxidase, which can be detected by a simple and sensitive assay (Coughlan *et al.*, 1999). The development of plastid trasformation should also contribute to site-specific gene insertion and greatly reduce the chances of gene flow in transgenic plants (Daniell *et al.*, 2002).

13.5.2 Management and segregation of transgenic crops

A transgenic crop producing a novel product will, by definition, require segregation from non-transgenic commodity crops and from other transgenic varieties of the same species that accumulate different products. This is a formidable task given the complexity of the supply chain from breeder to grower to crusher to processor and so on, all the way to the retailer and ultimately to the consumer. The difficulties in ensuring strict segregation of otherwise indistinguishable transgenic crops have been pointed out (Murphy, 1996) but have consistently been underestimated by many in the industry. However, several well-publicised failures in the segregation of transgenic crops over the past two years have thrown this issue into much sharper focus.

The first incident was reported early in 2000, when a consignment of conventional edible-quality rapeseed, produced in Canada by the seed company Advanta and sent to European growers, was found to contain an admixture of about 1% of seeds from a transgenic herbicide-tolerant variety of rapeseed. After considerable public furore in several countries, this led to the eventual destruction of many of the affected crops (which had already been planted) in hundreds of farms and the payment by Advanta of significant compensation to all the farmers affected. The farmers were concerned that unless they could guarantee that their crops were GMO-free they would be unable to sell them and maybe even future crops grown in the same fields. A contamination level of even 1% was deemed unacceptable in this case, even though the UK organic food industry normally accepts contamination by non-organic produce of up to 5%. What is even more telling about this example is that the contaminating rapeseed was only modified for an input trait and therefore the oil and feed products of the crop would have been indistinguishable from those of conventional rapeseed. Interestingly, in February 2000, the Japanese government proposed guidelines whereby a level of up to 5% of transgenic soybeans would be allowable in soybeans marketed as GMO-free. Clearly some degree of international standardisation will ultimately be required for what may become the inevitable cross-contamination of different seed types.

An even more serious contamination event occurred later in 2000 when it was found that taco shells on sale in US supermarkets contained the *cry9c* protein (Kaiser, 2000). This is a member of the Bt family of insecticidal toxins that had been engineered into a transgenic maize variety by Aventis and called STARLink. Pending further trials to test whether the STARLink maize caused allergies in humans, the Environmental Protection Agency (EPA) in the USA only authorised use of STARLink maize in animal feed formulations; so in theory the *cry9c* protein should never have entered the human food chain. In September 2000, Kraft recalled over 2 million boxes of taco shells, but it then emerged that many other foods were affected and, by December 2000, well over 300 additional food brands had been recalled. Food products such as baking powders that were exported to Japan and Taiwan were also contaminated with STARLink maize and Aventis admitted that as much as 12 M bushels of STARLink maize was unaccounted for, raising the possibility that some or all of it had entered the human food chain. Although Aventis quickly recalled all the seed it could trace, it now appears that STARLink maize already in grain elevators and silos could remain in the human food chain for another four years (Kaiser, 2000). When Aventis asked the EPA for a special 4-year exemption to the ban on STARLink maize in human foods, a specially convened panel judged that, although the *cry9c* protein had a low probability of allergenicity, the evidence was not conclusive enough to waive the ban.

The third incident was a reported finding late in 2001 of transgene-containing maize, in a traditional variety growing far away from any authorised transgene releases, at two sites in the remote Sierra Norte de Oaxaca in Southern Mexico (Quist and Chapela, 2001). This report was later challenged for some of its methodology and conclusions (Metz and Futterer, 2002; Kaplinski *et al.*, 2002), although it was widely accepted that the "escape" of transgenic maize may have occurred and moreover in the long term that such escapes were almost inevitable. Meanwhile, the incident only served to fuel concerns about the management and containment of GM crops.

These incidents are bound to damage further an already shaky public confidence in agbiotech. They could also affect the annual $1.5 billion worth of maize products exported from the USA to Japan and other countries in the Far East. Perhaps more seriously in the longer term, these events illustrate how difficult it is to maintain segregation in a widely grown commodity crop. They also demonstrate that the future cost of implementing more stringent IP (identity preservation) procedures may be higher than originally estimated. In the STARLink episode, the owners of the technology, Aventis, licensed eleven different seed companies to produce and sell the transgenic maize seed to thousands of farmers, often via dozens of middlemen. Many farmers were apparently unaware of the need to segregate the seed, and mixed it with

non-transgenic maize. Indeed, some growers have now filed a class-action suit, claiming that they were not warned about mixing STARLink maize with other varieties (Kaiser, 2000). Even if the STARLink maize was segregated correctly on the farm, there were evidently further incidents of mixing with conventional maize or maize flour during milling or food production, which led to the inappropriate inclusion of STARLink maize in hundreds of food brands in several different countries. Some of the complexities of this supply chain and more details about STARLink can be seen on the websites *www.purefood.org/ge/starlinkeverywhere.cfm* and *www.ift.org/resource/new/newhome.shtml*, while the Aventis position on the issue can be found on *www.us.cropscience.aventis.com/AventisUS/CropScience/stage/html/starlinkcorn.hm*.

It is therefore important to prevent cross-contamination of different transgenic and non transgenic crop varieties, not only because of the resultant production of what could be a useless or even potentially dangerous mixture of products, but also because of the public perception problems created by such events. The costs of enforcing both the seed and crop segregation necessary for true identity preservation are unlikely to be commercially feasible unless the new transgenic varieties have a sufficiently high value to pay for all the necessary quality control measures. In the extreme case of molecular 'pharming', where very high-value pharmaceuticals are produced, it may be possible to grow the world supply of a particular drug on just a few dozens or hundreds of hectares of strictly-contained land, possibly even in glasshouses. The new interest in small-scale segregated speciality crops and identity-preserved regional crops is beginning to be reflected in the development of much smaller commercial processing systems that typically handle seed batches of 5–50 t day^{-1} rather than the existing mills and refineries that can only handle quantities of 300–2500 t day^{-1} (Carlson, 2000). This in turn is driving improvements in processing technology that will allow for developments such as hexane-free extraction and membrane filtration as an alternative to chemical treatments during the extraction of oils and proteins from grain crops. Such innovations will be crucial for the creation of a favourable infrastructure for economically viable segregation of identity-preserved crops.

For somewhat lower-value crops with larger acreages it may be necessary to introduce geographical zoning of transgenic and non-transgenic crop cultivation, whereby whole regions would be assigned to different crop varieties. This would require all farmers in a specific region to be contracted to grow just one GMO variety and not to save seed. To make such an option attractive for the growers, guaranteed minimum prices for the crop would probably be desirable, so in turn the market demand would also need to be assured for the industrial producer. It would also be necessary to establish large-scale batch processing of the different types of seed,

something that the high-capacity refineries and silos characteristic of present large-scale agriculture were not designed to achieve. Finally, monitoring mechanisms involving biochemical, molecular or other assays would be needed to test seeds (or other tissues expressing the transgenic compound) and their downstream processed products for purity. Acceptable contamination levels for different crops and products would also need to be agreed at international level; whereas 5% may be an acceptable level for a modified input trait, it may be unacceptable for an output trait. An example would be an edible oil, where a contamination level of even 1–2% with an industrial-grade oil could well make the oil inedible.

The overall costs of rigorous identity preservation measures are likely to be at least 10–20% of current crop values and may be much higher. Such costs will have to be reflected in an increased price for the end products of the transgenic crops. The cultivation of transgenic crops with radically modified output traits is therefore probably more suitable for the manufacture of non-commodity medium-to-high value materials where the additional costs of identity preservation can be more than offset by the value of the end product. Some of the business issues raised by the requirements for labelling and segregation (often referred to as identity preservation or IP) of transgenic crops are discussed on *www.extension.iastate.edu/Pages/grains/pubs/buspub/9901/gind.htm.*

13.5.3 Addressing public concerns

There is an unfortunate tendency among some sections of the biotech industry, and even some scientists, to dismiss public concerns about agbiotech as completely irrational and unfounded. In fact, most people exercise their judgements and choices about any course of action following an often unconscious but nevertheless quite sophisticated analysis based on the information that they receive, the perceived benefit to themselves and the likely risks involved. It is a fallacy to think that people strive to live in a risk-free world; indeed people are willing to take quite large risks as long as they perceive that there is a corresponding benefit. People will also modify their behaviour in the light of new information, providing the source is deemed to be trustworthy. For example, information about the harmful effects of smoking was slowly disseminated from the 1950s and onwards (and often initially denied by the tobacco industry and their associated scientists). The response was a considerable decline in the number of adult smokers in developed countries where there was ready access to the health information. However, it is also noteworthy that some smokers have persisted with their habit because they judged that the physiological benefits of

continuing, or the pain of withdrawing, outweighed the known very high but rather long-term risks of serious illness.

The public will often embrace new products of known or possible risk with considerable enthusiasm, as long as the benefits are sufficiently high. Therefore despite a lack of understanding of how they are made or the risks (if any) involved in their use, GMO-derived recombinant drugs of all descriptions are in great demand. Also, despite a vigorous (and somewhat confusing) scientific debate about potential brain damage from frequent use of mobile (cellular) telephones, and even attempts by some schools in the UK to limit their use by young children (who are said to be most at risk), sales continue to boom and their use is now ubiquitous among all age groups. The conclusion is that, although there is a spectrum of risk taking, one can generalise that everybody will take risks if necessary and that the acceptable level of risk tends to rise as the perceived rewards increase.

So why are so many people, especially in Europe, getting so upset about GMO crops? One problem is that the current generation of agbiotech GMO products are staple foods that were not segregated to allow consumer choice. These GMO foods result from altered crop input traits so the food itself is almost or entirely indistinguishable from equivalent non-GMO foods. Therefore the GMO foods do not taste better and neither are they cheaper than before, so the consumer perceives no benefit (Trewavas, 1999). On the other hand, the public is informed by the media, by relatively well-trusted environmental groups and even by some scientists, that there may be a risk from GMOs in the food chain, however uncertain and unquantifiable.

We should also remember the recent European experiences of food-related scandals such as the spread of BSE from cattle to cause the new variant form of Creutzfeld-Jacob Disease (CJD) in humans plus the regular, often fatal, outbreaks of food poisoning caused by new virulent strains of *E. coli* and *Salmonella*. This has been compounded by disastrous outbreak of foot-and-mouth disease in the UK during 2001, which led to the slaughter of over 4 million animals and dealt a serious blow to the rural and tourist economies of the country as well as costing UK taxpayers some $4–5 billion (National Audit Office, 2002). Although they do not in any way involve GMOs, these episodes have sensitised the public to any perceived contamination of the food chain, irrespective of the source. They have also alerted the public to the adverse consequences of certain practices involved in intensive agriculture, such as feeding animal remains back to animals, or the increased opportunities for the evolution of and rapid spread of new pathogen strains under conditions of intensive livestock rearing and the widespread (mis)use of antibiotics. It is also unfortunate that government regulatory agencies are deemed to have failed, sometimes catastrophically as with BSE/CJD (*www.bseinquiry.gov.uk/report/contents.htm*), both in

preventing such scandals and in informing the public about the risks. As recently as December 2000, the previous refusal of some EU governments to ban animal remains from cattle feed, despite the urging of the European Commission, was believed to have contributed to the recent and much underestimated spread of BSE throughout Western Europe (for recent data relating to France, see Donnelly, 2000). It is not surprising that there has been a consequent sharp decline in consumer demand for beef in countries like France and Germany. This combination of events has also resulted in a greatly diminished credibility among the general public for many government agencies, ag-related industries, and even some of the publicly funded scientists associated with them. Indeed, the decision to abolish the UK Ministry of Agriculture Fisheries and Food (MAFF) in mid 2001 and to replace it with a new Department of Environment, Food and Rural Affairs (DEFRA) was largely regarded as an attempt to correct the deep mistrust of MAFF that was, rightly or wrongly, felt by large sections of the public, including many professionals.

This is the context in which the public has judged GMO foods and weighed up their relative benefit (none) and their perceived risk (maybe none but maybe a lot), as based on the available information (mostly sensational media stories) and their recent grim experience of serious food scares. The result is an entirely rational decision by many people to make what is a completely cost-free decision to reject GMOs. That is the situation as we enter 2002 with the current generation of input trait-modified GMO foods. All of this may change if/when the next generation of GMOs with modified output traits gradually becomes available. Some of the new food crops, such as the vitamin A-enhanced 'golden rice' have great, if sometimes exaggerated, potential to benefit populations in developing countries, and there is also huge potential for the production of renewable, biodegradable industrial products and new pharmaceuticals from non-food crops. If their development is allowed to proceed, such products could provide sufficient immediate benefits to many consumers to mitigate the perceived risks and may therefore become more widely acceptable.

In order to progress from the present situation, it is vitally important for independent scientists to engage more in constructive debate about all the issues raised by agbiotech. We live in very different times to the era when nuclear technology was developed during the Manhattan project. As succinctly put recently by the CEO of Monsanto, there "has been a movement from a "trust me" society to a "show me" society" (*www.monsanto.com/monsanto/media/speeches/new_pledge_speech.html*). Scientists are now held to be accountable, at least in some degree, for their use of public funds for research and the uses to which it is put. The debate about the first generation of GMO food crops, about which many scientists were rather ambivalent anyway (Trewavas, 1999), has already been lost in Europe but there is

now an opportunity to explain the potential for the new generation of potential agbiotech products. Independent scientists can now play a useful constructive role, in between the vested interests of the agbiotech companies and the sometimes rather fundamentalist anti-GMO groups, to provide unbiased information and advice to the general public.

Another important priority is to improve the quality of scientific education, both for adults (especially teachers) and children. One of the reasons that many people (including many politicians, journalists, teachers, doctors and other professionals) have heeded simplistic and even contradictory media scare stories about GMOs is that they lack even the rudiments of the technical knowledge and the ability or confidence to assess conflicting claims that would enable them to analyse the issues more critically. Scientists can also assist in this process by communicating more with schools in their communities so as to demystify research in general and biotech in particular. The compulsory science in core curricula for high school students in the USA and UK have been criticised for being "overstuffed and undernourished" (Nelson, 1998) and the Office for Standards in Education (OFSTED) has reported that "*Most pupils acquire a sound factual knowledge of the material in the Programme of Study but their understanding of the underlying scientific concepts often remains fragmentary...as the content of science becomes conceptually more demanding, there is a progressive polarisation of pupils' achievement, with the least able often becoming confused and holding incorrect ideas*" (OFSTED, 1996). Similar conclusions have been forthcoming from more recent OFSTED reports on both primary and secondary science education in the UK (OFSTED, 2000) The conclusion is that public education must focus as much on training in concepts and the development of critical faculties as much as the mere memorising of factual information. This is particularly important as the public has now access to the huge information resources available over the Internet, but little training in how to sift useful data away from less reliable data or even misleading propaganda (on all sides of the arguments). Improved education along these lines should help to empower the public to make better-informed choices, not only about GMOs but also about many of the other important technology related issues that now confront us all.

13.6 Developing new crops

Most of our global agricultural production relies on a very limited number of widely cultivated staple crops, such as wheat, rice, maize and soybean. The introduction of transgenic varieties of these major crops is likely to increase their dominance further.

This may be particularly true if output traits are modified so that the crops accumulate new products and so require even larger acreage. In some cases, the transgenic crop may produce a compound currently obtained from a minor crop, resulting in displacement of the latter. These trends may be undesirable for ecological reasons as they increase the tendency towards extensive monocultures that may be more susceptible to pests or diseases. However, a more compelling argument against a proliferation of transgenic mainstream crops with novel output traits is the problem of segregation (see Section 13.5.2). A feasible alternative strategy is to develop new crops as sources of the novel products. As well as being more easily segregated, the new crop species will already synthesise the products of interest without the need for convoluted manipulations of biochemical pathways and the possible side effects that may ensue. Oil crops provide a useful example of the way such new crops might be developed.

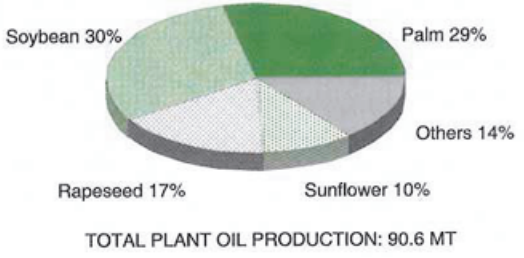


Figure 13.10 Contribution of the 'big four' oil crops to world production. These four crops together make up 86% of the total world output of plant oils. If current trends continue, the dominance of the 'big four' oil crops will become even more marked in the coming decade.

As shown in Fig. 13.10, almost 86% of world production of traded plant oils is derived from only four major oil crops: soybean, oil palm, rapeseed and sunflower (Gunstone, 2000). Each of these crops currently produces mainly edible-grade oils and they are not optimised for the production of novel and unusual fatty acids (Table 13.2). The use of transgenic methods to increase the diversity of fatty acid content in these major crops has made great strides in the past fifteen years but significant challenges remain. However, there is also an enormous diversity of naturally occurring plant species, many of which already produce a wide range of industrially useful fatty acids. Some of these plants can already accumulate over 90% of a single exotic fatty acid in their seed oil, as shown in Table 13.1. Therefore nature provides us with a ready made source of almost any type of fatty acid from C8 to C24, and containing a huge variety of functionalities, only some of which are shown in Table 13.1 (a more comprehensive list of novel oilseeds is available on the USDA new crops database on *www.ncaur.usda.gov/nc/ncdb*). Such plants also have an

advantage over some of the newer transgenic crops in that they are already adapted to accumulate these exotic fatty acids only in their storage oil—these fatty acids are hardly ever found in cell membranes or any other lipids where their presence could be damaging. A further advantage of these novel oilseed crops is that the seed oils already contain accessory stabilising agents, such as antioxidants, which prevent the breakdown of some of the more highly reactive fatty acids such as conjugated polyunsaturates and those containing acetylenic bonds.

There are many other examples of new crops that could be used as alternatives to major crops or to substitute for plants, like the Pacific yew, that are in danger of being over-exploited. Some of the potential new oil crops include cuphea, meadowfoam, coriander and *Euphorbia*. New fibre crops include kenaf and miscanthus, while possible new starch crops include quinoa and amaranth. Many issues relating to new crops are discussed in the book edited by Janick (1999) or on the Purdue University new crops website (*www.hort.purdue.edu/newcrop*).

13.6.1 Challenges for new crops

Although many of the potential new crops may already be excellent sources of useful products, such as novel fatty acids, they are often not yet suitable for large-scale agriculture. The reason for this is simple: these plants have not been optimised for agronomic performance over centuries or even millennia, as have some of our more familiar crops. They suffer from the usual characteristics of wild plants; for example, they tend to flower asynchronously throughout the summer and therefore do not produce their seed at a single time, which makes harvesting very difficult. They often produce seedpods that are prone to shatter before or during harvest, resulting in a loss of many of the seeds. Often, the canopy architecture of the plant is not suitable for existing harvesting machinery. They may be susceptible to a variety of diseases or pests, including fungi and insects. Finally, in the case of oilseeds, although they may contain as much as 90% of a novel fatty acid in their seed oil, the overall oil yield in tonnes per hectare may be relatively low.

The improvement of these important agronomic characters requires the manipulation of numerous complex traits. Companies are often dismayed by the prospect of domesticating new species, citing the example of major crops such as wheat, which is still being improved after more than 10,000 years of domestication. Nevertheless, we can now be more optimistic about the prospects for crop domestication. Many of our newer crops have been improved by scientific breeding techniques over the past 50 years much more rapidly than wheat. Examples of such crops include hybrid

maize, rapeseed, sunflower and soybean, which have only been grown as mainstream crops for a century or less. There is also now the prospect of using biotech methods to accelerate the development of new crops.

13.6.2 Using biotechnology to develop new crops

The introduction of new crops will be greatly assisted by the application of advanced biotech methods to accelerate breeding programmes aimed at improving agronomic performance and to enable faster and more reliable multiplication of seeds or plantlets for dissemination to growers. In the case of new tree crops, a significant recent advance is the development of somatic embryogenesis techniques that will soon make it possible to use mass clonal propagation as a way of rapidly multiplying a tree variety of choice (Merkle and Dean, 2000). This method could also be used to rescue and multiply endangered species, even if only one or two individuals were left. New tree crops can also be developed as an alternative to the over-exploitation of more vulnerable species. For example, the Pacific yew *Taxus brevifolia* is found in old-growth forests of the Pacific Northwest of the USA in a habitat shared with many endangered species. Between 1960 and 1982 the National Cancer Institute screened some 35,000 plant samples for anti-cancer activity. The bark of this yew tree was found to produce the compound taxol® (paclitaxel), which is an extremely useful drug for treatment of ovarian and metastatic breast tumours. Demand for taxol® has soared, but the harvesting the bark from yews kills the trees and disrupts further their already endangered habitat.

An alternative to this destructive and unsustainable use of the Pacific yew is to develop new tree crops from different species of yew that can be propagated vegetatively and grown as shrubs. These other yew species do not produce taxol® itself but their needles contain a precursor of paclitaxel from which taxol® can be synthesised by pharmaceutical companies. Some 25 million of the new types of yew shrubs are now being grown to provide a renewable source of this important drug without the need to encroach on an endangered habitat (*www.science.org/newcr_cs.htm*). Moreover, since the paclitaxel precursor can be harvested from the needles rather than the bark, there is no damage to the rest of the tree and repeated harvesting is possible. It is interesting that in this case, where high profit margins were immediately apparent, there was no hesitation by the companies concerned in embarking on the rapid (and apparently successful) development of several entirely new crops. Moreover, these crops were tree species, which are far more difficult to propagate and cultivate than are annual crops.

Some of the recent advance in genomics and its use in the manipulation of complex traits have clear applications to new crops. For example, many agronomic traits hitherto regarded as relatively complex have now been resolved to just a few genes. This means that supposedly complex characters such as height, oil yield, pod shattering and flowering time may be regulated by only one or two genes each in a given crop. Furthermore, the genes regulating a trait such as height or oil yield in one crop appear to be very similar to those regulating the same character in all other crops. Researchers in the UK have already used this knowledge to alter characters such as height and flowering time in a variety of crop and non-crop species (Peng *et al.*, 1999; Pineiro *et al.*, 1998). In the near future this approach will also be applied to elucidating and manipulating characters such as pod shattering and oil yield in crops (Knapp and Crane, 1999). These manipulations do not necessarily require the insertion of transgenes into the crops. It is possible to use molecular markers (*i.e.* DNA-fingerprinting) in order to select such desirable characters as part of a conventional non-transgenic breeding programme. The use of molecular markers can also considerably speed up the development of new varieties of crops.

These advances mean that it is possible to envisage the domestication of relatively wild plants so that they can be brought into mainstream agricultural production within just a few decades. This may seem like a relatively long time but it is comparable with the timescale for the introduction of a new pharmaceutical product into general clinical medicine following the various stages of research and development, clinical trials, regulatory approval *etc.* This timescale will also allow us to have ready a new portfolio of oil crops to serve as sources of renewable oleochemicals at a time when non-renewable petrochemicals become increasingly scarce and expensive. The domestication strategy is also attractive because it generates diversity, not only at the product level, but also at the primary production level. The encouragement of on-farm biodiversity is an explicit objective of European Union policy as well as being supported by a wide range of environmental groups throughout the world. Increasing the number of crop species also gives an additional buffer against the effects of pests and diseases, to which our current monoculture-based agriculture is so susceptible.

13.7 Future directions for agricultural biotechnology

Agbiotech began the 1990s with a great deal of promise plus no small measure of hyperbole. However, the events of the past few years have illustrated a sharp dichotomy between the pace of scientific progress and the commercial uptake and

public acceptance of the technology. The impact of commercial, political, scientific and economic factors on the future of agbiotech will be crucial.

13.7.1 Commercial background

GMO-based agricultural biotechnology, or agbiotech, is already a large global business with annual sales estimated at over $3 billion in 2000. These sales are projected to rise to $8 billion in 2005 and to $25 billion by 2010 (Anonymous, 2000a). However, agbiotech is not yet a very profitable business: in the first ten months of 2000, an index of medical biotech firms showed a 58% rise in value while those involved in agbiotech showed a decline of 15% (Anonymous, 2000b).

During the mid-1990s, several large pharmaceutical and chemical companies bought up or merged with agbiotech companies to create 'life sciences' companies that were assumed to have synergies based on common biotechnologically based research strategies aimed at the development of drug, nutritional, chemical and agricultural products. These consolidations created some large multinational conglomerates such as Agrevo, Aventis, Novartis, Monsanto, Zeneca and Dupont. This strategy has largely unravelled in recent years, for several reasons. First, the economics and scales of production of the agriculture and pharmaceutical sectors are totally different, with the former remaining largely a low-margin, high-volume, commodity-based venture while the latter is overwhelmingly a very high-value, low-volume niche market operation. Second, whereas public acceptance of medical biotech, based on microbial production of recombinant drugs, has remained firm; there has been a significant backlash against agbiotech, particularly in Europe.

The adverse public reaction to GMO foods has had serious consequences for several agbiotech companies, as respected financial institutions such as Deutsche Bank have advised investors to avoid the sector. One UK company, Axis Genetics, which was developing some promising recombinant vaccines in plants, was forced out of business in 1999 as it was unable to raise finance in this climate. In other cases there were significant falls in the value of major agbiotech stocks, which played a role in driving several of the conglomerates to sell off or demerge their agbiotech divisions and to focus on their core pharmaceutical businesses. For example, late in 2000, Astra/Zeneca and Novartis spun off their agbiotech groups to form a separate company called Syngenta, although the initial market capitalisation of the new company was rather disappointing; Pharmacia, which acquired Monsanto in 1999, is selling off parts of its agbiotech interests and is expected to completely divest by 2002; and finally, Aventis (formed in 1999 from the merger of Hoechst and Rhone-

Poulenc) announced late in 2000 its intention to sell off its agbiotech business (Aventis Crop Science) by the end of 2001 (Anonymous, 2000c) and in October 2001 it confirmed that a sale had been agreed with Bayer AG (www.aventis.com).

The short-term outlook for commercial agbiotech is therefore not very positive and many within the industry recognise that the large-scale release of commercial GMO crops outside the Americas is unlikely for at least five years, and possibly a lot longer. Nevertheless, the industry remains fundamentally strong and prospects for the medium to long term are somewhat brighter, providing the sector takes significant steps to allay public concerns and is able to exploit emerging new technologies to improve output traits in order to deliver superior products that directly benefit consumers. In this context, it was interesting to see the speech entitled "A new pledge for a new company" that was made late in November 2000 by Hendrik Verfaillie, the new CEO of Monsanto. He admitted, amongst other things, that in recent years the company had been seen as "arrogant" and "blinded by our own enthusiasm", and he pledged that in the future the company would be more committed to transparency and public dialogue (full text of the speech is on *www.monsanto.com/monsanto/media/speeches/new_pledge_speech.html*). There is clearly still a great deal that needs to be done before public trust is regained, but we may now be seeing a start to such a process.

13.7.2 Public versus private research

In parallel with the recent loss of confidence in commercial agbiotech and the retrenchment of much of the industry, there has been an increasing trend for the larger companies to contract out many aspects of their agbiotech-related research either to smaller biotech companies, particularly in the areas of genomics and proteomics, or to publicly funded universities or research institutes (Kolodziejczyk and Fedec, 1999). Some of these investments by major US and European chemically-based companies have been of the order of $10–20 million each and aim to allow the companies to catch up with cutting-edge technologies being developed in many publicly funded research labs, especially in universities. Inevitably, this input of funding will to some extent affect the future direction and progress of publicly funded research.

A further trend that has affected both public and privately funded agbiotech research has been the focus on genomics and bioinformatics. Casual perusal of most job sites demonstrates the increasing demand for researchers to fill vacancies created by the proliferation of new start-up companies or university groups in these areas. In the USA alone, several dozen companies dedicated to data mining or genomics/

proteomics have been established in the last few years, and many now have strategic alliances with each other and/or some of the larger agbiotech companies (Anonymous, 2000a). Underlying these trends are the expected rewards to be gleaned from genomics *etc.*, and the information from such studies—providing it becomes publicly available—will undoubtedly lay the foundations for a much better understanding of many basic biological processes in plants.

However, a potentially adverse consequence of the focus on genomics, proteomics and bioinformatics is that there may be relatively less funding for the sort of biochemical, cell biological and physiological research that is required to fill the many gaps in our knowledge of how plants function. The importance of additional research on metabolic pathways and their regulation is highlighted by the examples in Section 13.5.1, where just in the last few years it has been found that several key pathways that are the targets for transgenic manipulation in plants were found to operate in very different ways than had been believed. In view of its complexity and generic nature, this area would be an appropriate target for public funding initiatives. The same is probably true for the development of new crops, which is unlikely to be pursued seriously by companies who tend to regard it as being too long term and having uncertain rewards—except of course in cases like the yew trees described in Section 13.6.2, where the rewards were both obvious and substantial.

13.7.3 The political dimension

As well as being an exciting scientific endeavour and a growing global business, all forms of modern biotechnology are of enormous public interest. This is reflected by the almost daily stories in the news media of new advances in biotech research and their potential ramifications. Some aspects of medical biotech have raised unprecedented ethical dilemmas, such as the use of embryo stem cells in research or as sources of organs for transplantation, and the whole issue of testing for genetically related diseases. Such issues often have had direct consequences for the use of health-care funding and for wide areas of legislation from insurance to patenting. The impact of agbiotech, while not as great as medical biotech, has also been widespread. It has affected the law, the availability of certain foods and even relationships between countries and trading blocks. It has also raised ethical and moral concerns about potential impacts on the environment and developing countries (Robertson, 1999; Flavell, 2000; Straughan, 2000).

In such circumstances, it is hardly surprising that both medical biotech and agbiotech have become potent political issues and therefore subject to all the

manipulation and distortion that this entails. Political parties and pressure groups have used emotive GMO issues as part of their strategies to pursue public appeal and hence strengthen their influence. Public opinion can have an immense impact, but is itself often orchestrated by media that are prone to sensationalism and by different political or other groups that each have their own diverse agendas. Witness the rapid public backlash against GMO foods in Europe in 1998/99, which led most supermarkets and restaurants in the UK to withdraw (precipitately but voluntarily) all such products from sale, even before there were any consumer boycotts or other such protests. Even in the USA, where GMO foods have been much less controversial, it has been argued that this was one of the issues that gave the anti-agbiotech campaigner Ralph Nader nearly three million votes in the 2000 US Presidential elections and tipped the balance of the Presidency in favour of the Republican party.

Sometimes, global political issues such as the response to increasing atmospheric CO_2 levels can create or deny new opportunities for agbiotech. In the conferences held in Kyoto in 1997 and The Hague in 2000, world governments addressed the issue of climate change and possible remedial actions. Some of their decisions have indirectly had a considerable effect, on agroforestry in particular. These relate to the use of forests as carbon sinks that are able to sequester some of the atmospheric CO_2 caused by emissions from vehicles, factories or power plants. The protocols adopted at the Kyoto meeting allowed countries to trade their carbon emissions against the carbon sinks. This means that a country with a high level of emissions can offset these by either establishing or purchasing carbon sink equivalents. The same procedure could operate at the level of companies, whereby a company with a factory that produces high carbon emissions would be subject to a carbon tax, but could gain credits by planting forests. This has established a new and lucrative market for forests that act primarily as carbon sinks, and several companies have been set up to exploit such opportunities (see, for example, *www.co2forestsinks.com.au/aco02.htm*). In turn, there is an increased incentive for companies to maximise the biomass productivity of their forests by using biotech approaches.

The collapse of the climate change conference at The Hague in Nov 2000, which was meant to formalise the Kyoto protocol, and the subsequent uncertainty about the US attitude towards the protocol has been a setback for the concept of extensive commercial carbon sinks; indeed, carbon sinks were one of the major causes of disagreement at The Hague meeting. Nevertheless, the widespread desire for a global treaty to address climate change makes it likely that there will eventually be an agreement that will recognise the carbon sink concept in some form or other. This is one (but not the only) reason for the strong interest in agroforestry and biotech by the automobile and petroleum industries (see Section 13.4.6). In essence, a protocol on

global climate change has begun the process of establishing a new artificial carbon economy that is one of the factors behind some of the substantial new investments into tree biotechnology; this is a good example of politics, with all of its uncertainties, playing an important but inconstant role in determining the direction of one aspect of scientific endeavour.

13.7.4 Economics: problems of scale and value

The present global agricultural economy is overwhelmingly dominated by high-volume, low-margin commodity products with rather limited scope for the capture of added value. As Fig. 13.7 shows, the products of mainstream agriculture, such as oils and starch, fall decisively into the same category as other bulk materials like plastics and fuels. Agbiotech has the potential to allow many of these non-food bulk materials to be produced in crop plants. Examples include the breeding of oilseeds to produce short-chain fatty acids to be used to make nylon-based polymers or biofuels or to make biodegradable plastics. In all cases, these are simply replacements for existing petroleum-derived products and at present they cannot compete economically with the latter (see also Hitz, 1999). Even when oil prices begin to climb substantially, possibly in the next decade or so, the sheer volume of these bulk markets means that ag-based products could not substitute for more than a fraction of current demand. For example, global demand for petroleum-based hydrocarbons is ~200 Mt yr^{-1} and that for fuel oils is ~2,100 Mt yr^{-1}, but total plant oil production is less than 90 Mt yr^{-1}.

If we consider just one of these products—plastics—we can examine whether it is possible to make enough bioplastics to come anywhere near the present demand. If enough oilseed-based biodegradable plastics were produced to supply a mere 10% of the current 25 Mt yr^{-1} demand for plastic products in the USA, this would require the cultivation of an additional 6 Mt oil equivalents from crops (this assumes the production of a plastic like PHB at 40% of oil yield in transgenic plants). The additional area needed to produce even this amount of bioplastics would be more than treble the entire acreage of rapeseed currently grown in Canada and the USA.

Clearly, it is difficult to see how crops can compete economically for such very high-volume markets. Even when petroleum starts to run out, oil crops can probably only substitute for a small fraction of its present market, simply due to their limited scale of production and what will be increasing competition with edible crops for any available land. In such circumstances, it is likely that alternative materials will be developed for many of the present high-volume uses of plastics and that plastics will shift to being much lower-volume speciality products. In such a market, plant-based

plastics could have considerable economic potential. This brings us to what may be the optimum market for non-food crops, *i.e.* the intermediate range of products such as industrial enzymes and fine chemicals (see Fig. 13.7). The market volumes for such products range from 1 t yr^{-1} to 2,000 t yr^{-1} each, the high end of which would economically favour agriculture over fermentation. The land area required for such crops may still be significant. If 20–30 t yr^{-1} of an industrial enzyme were produced from transgenic rapeseed expressing it at about 0.5 kg t^{-1} seed (Hood *et al.*, 1997, 1999), this would require 40,000–60,000 ha of land. As long as such a crop could be grown under strictly segregated conditions, the value of the product at $\$10^4$–$10^5$ t^{-1} should cover all identity preservation and other costs while returning a healthy profit.

The third category of product where agbiotech may play a role is in the production of very high-value products such as therapeutics from molecular pharming (see Fig. 13.7 and Table 13.3). Here, the economics of ag-based production versus fermentation or the use of transgenic animals are not as clear as with the intermediate-value products. However, in some cases, plant products may have advantages over microbials (*e.g.* for ethical or religious reasons), as discussed in Section 13.4.5. The containment issue for crop plants expressing active drugs may prove too problematic if they were grown in the open air but, with sales volumes of only 1–500 g yr^{-1}, some of these drugs could be produced from only about 40–100 m^2 transgenic plants that could all be grown under glass. Another even more secure option for the very high-value products is to use plant cultures, which have the advantages that there would be no containment risk, no concerns about using GMOs (cultures are not organisms) and, as higher eukaryotic production systems, they may be superior to bacteria and yeast for some recombinant proteins. For example, at a plant biotech conference in 2001 it was reported that cell cultures derived from the yew tree could be grown successfully on a scale of 70,000 litres in a commercial production plant in order to produce the important anti-cancer drug, taxol (Venkat, 2001). Should this be confirmed, it could point the way to the more widespread use of plant cell cultures as safe and contained vehicles for the production of many other high value compounds.The extremely lucrative potential returns for plant-based molecular pharming has generated much excitement but must be weighed against the well-established competition from microbial and animal systems. In the end, it is likely that all three of these production routes will become generally available for the biosynthesis of different products, with the eventual system of choice depending on the nature of the particular drug and its market.

Finally, plants or plant cultures could be used to drive down the production costs of some of the current generation of recombinant drugs, so much that their markets will expand and they will move into the category of intermediate-value products.

There is already much concern about the cost of recombinant or synthesised drugs, particularly when this puts them beyond the reach of most people in developing countries. In 2000, more than three million people died of AIDS-related illnesses, most of them in Africa. Although some pharmaceutical companies have made certain anti-AIDS drugs available more cheaply, such medicines are often still too expensive for patients in the worst affected sub-Saharan countries. Recombinant vaccines or other drugs could be produced cheaply and on a large scale in plants or plant cultures and a modest profit could still be recovered because of their high production volumes, providing they were made available to the growing populations of the developing nations. While this does not fit in with the present *modus operandi* of the major pharmaceutical companies, it would be a venture worth exploring as a public/private partnership, possibly starting with drugs that are off-patent or licence-free.

13.8 Conclusions

During the past two decades, there has been a series of significant advances in the biological sciences that have the potential to be applied to human welfare through biotechnology. These scientific developments are already having a real impact on many aspects of medicine and their commercial applications in agriculture are also beginning to be explored. Some of the myriad potential products of agbiotech have been discussed in this chapter, but it is a mistake to believe that, just because science shows us how to do something, then we will necessarily do it. The public reaction to some of the initial manifestations of agbiotech should serve as a warning to scientists, companies and governments that they must engage in a serious debate about how and where the technology can be best applied. It should be borne in mind that there have been technological developments in the recent past that were initially heralded as being of great promise, but were eventually rejected by public opinion.

One of the most striking examples of public rejection of an important technology is nuclear power. Shortly after World War II, this technology promised cheap, clean energy for all and was initially relatively popular. The theoretical science underpinning the technology was sound but nevertheless it failed for more mundane reasons, mostly relating to the treatment and disposal of highly toxic and long-lived waste products and the safety of operating nuclear reactors. The last straw for many was the disaster at Chernobyl in 1986, which made it difficult for politicians to sanction the construction of nuclear power plants during the 1990s. Interestingly, the early years of the 2000s have seen a revival of the debate over nuclear power and the possibility of building new reactors is once again being mooted more seriously.

Another example of at least a partial rejection of a new technology is the irradiation of food. There are striking analogies between the tenor and content of much of the debate about food irradiation and the controversy surrounding GMOs. A flavour of some of the issues relating to food irradiation can be found on the following websites: *www.hi-tm.com/Documents/irradref.html*, *www.pure-food.com/food.htm*; *www.dainet.de/bfe/Bfe-Englisch/Information/englisch/bstrfaqe.htm* and *www.consumersinternational.org/campaigns/irradiation/irrad.html*. These examples should serve as reminders that there is nothing inevitable about the widespread use of agbiotech in the future.

If we are to realise the potential benefits of agbiotech as well as assessing its possible risks, it will be important for scientists and governments to engage more meaningfully in decisions relating not only to what research is done, but also to its downstream application. In 2000, representatives of the national scientific academies of Brazil, China, India, Mexico, UK and the USA published a report addressing these issues (*www.nap.edu.html/transgenic*). Regarding the balance of public/ private sector research, the report made the following points:

"Whereas fundamental research is still being carried out in the public sector, the strategic application, in sharp contrast to the "Green Revolution", takes place largely in the private sector where much of the intellectual property is controlled. In these circumstances, research priorities are driven by market forces (*e.g.*, price signals). Companies produce products whose costs are recoverable in the marketplace. There are also goods that benefit society as a whole rather than individuals and whose costs cannot be recovered in the marketplace (so-called public goods). Public sector funding is needed for such public-good work (Stiglitz, 1993). A classic example of a public good would be an improved plant that can be propagated by farmers with little deterioration, as with self-pollinated (*e.g.*, wheat and rice), or vegetatively propagated (*e.g.*, potatoes) crops. If such crop improvement research were left to normal markets for private provision, then it would be systematically under-supplied."

Some of these issues are also explored in recent reviews (Herrera-Estrella, 2000; Leisinger, 2000). It is manifestly true that if the application of agbiotech were to remain an entirely market-driven enterprise, then it would tend to benefit those with the strongest purchasing power, *i.e.* the relatively rich consumers in developed countries, while the more numerous but economically almost invisible populations of other countries would exert little or no influence on the market (Krattiger, 2000). There is much that could be achieved in public/private partnerships, not only in the more widespread application of agbiotech (both transgenic and non-transgenic), but also in improving some of the technical aspects of the still rather crude process of gene transfer in plants. More serious attention should also be paid to the introduction and improvement of alternative crops, which might substitute for a proliferating series of monocultures of a few major crops with novel transgenic output traits.

In developed countries, agbiotech may continue for some time in the present direction as a way of modifying input traits and maybe some output traits in mainstream commodity crops. In the longer term, there is much potential for the application of agbiotech in the form of molecular pharming for the relatively inexpensive production of a wide range of intermediate and high-value drugs, enzymes, bioplastics and more. Such applications carry the promise of delivering much cheaper medicines that could be of huge benefit to developing countries. One eminently worthy target (perhaps for public sector support) would be to produce anti-AIDS drugs in transgenic plants or plant cultures to treat the tens of millions of HIV-infected people in developing countries who cannot afford even a single dose of the currently available medications. It will be this kind of obviously beneficial use of agbiotech that will help to regain the public confidence necessary for the long-term future of this as-yet fledgling technology.

References

Abola E., Kuhn P., Earnest T. and Stevens R. C. (2000), 'Automation of X-ray crystallography', *Nature Structural Biol.* **9** suppl., 973–977.

Altman A. (1999), 'Plant biotechnology in the 21st. century: the challenges ahead', *Electronic J. Biotechnol.* **2**, no.2, available on the web: *www.ejb.org/content/vol2/issue2/full/1/.*

Anderson C. W. N., Brooks R. R., Stewart R. B. and Simcock R. (1998), 'Harvesting a crop of gold', *Nature* **395**, 553–554.

Angell S. M. and Baulcombe D. C. (1999), 'Technical advance: potato X amplicon-mediated silencing of nuclear gene', *Plant J.* **20**, 357–362.

Anonymous (2000a), 'Agricultural biotechnology', *Nature Biotechnol.* **18** suppl. IT59–IT61.

Anonymous (2000b), 'Biotechnology. Dry season', *Economist*, 4 November 113–114.

Anonymous (2000c), 'Life sciences. Green and dying', *Economist* 18 November, 125.

Anonymous (2000d), 'Mandatory *trans* labelling may come in 2002', *Inform* **11**, 173.

Arakawa T., Yu J., Chong D. K., Hough J., Engen P. C. and Langridge W. H. R. (1998a), 'A plant-based cholera toxin B subunit–insulin fusion protein protects against the development of autoimmune diabetes', *Nature Biotechnol.* **16**, 934–938.

Arakawa T., Chong D. K. and Langridge W. H. R. (1998b), 'Efficacy of a food plant-based oral cholera B subunit vaccine', *Nature Biotechnol.* **16**, 292–297.

Atkinson H. J., Urwin P. E., Hansen E. and McPherson M. J. (1995) 'Designs for engineered resistance to root-parasitic nematodes', *Trends Biotechnol.* **13**, 369–374.

Bailey J. E. (1999), 'Lessons from metabolic engineering for functional genomics and drug discovery', *Nature Biotechnol.* **17**, 616–618.

Baumlein H., Boerjan H., Nagy I., Bassuner R., van Montague M., Inze D. and Wobus U. (1991), 'A novel seed protein gene from *Vicia faba* is developmentally regulated in transgenic tobacco and *Arabidopsis* plants', *Mol. Gen. Genet.* **225**, 459–467.

Block G., Patterson B. and Suber A. (1999), 'Fruit, vegetables and cancer prevention —a review of the epidemiological evidence', *Nutrition and Cancer* **18**, 1–19.

Borisjuk N. V., Borisjuk L., Logendra S., Petersen F., Gleba Y. and Raskin I. (1999), 'Production of recombinant proteins in plant root exudates', *Nature Biotechnol.* **17**, 466–469.

Brennan F., Bellaby T., Helliwell S., Jones T., Kamstrup S., Dalsgaard K., Flock J. and Hamilton W. (1999), 'Chimeric plant virus particles administered nasally or orally induce systemic and mucosal immune responses in mice', *J. Virol.* **73**, 930–938.

Briggs S. P. and Koziel M. (1998), 'Engineering new plant strains for commercial markets', *Curr Opinion Biotechnol.* **9**, 233–235.

Buchanan-Wollaston V. and Ainsworth C. (1997), 'Leaf senescence in *Brassica napus*: cloning of senescence-related genes by subtractive hybridisation', *Plant Mol. Biol.* **33**, 821–834.

Butt A., Mousley C., Morris K., Beynon J., Can C., Holub E., Greenberg J. T. and Buchanan-Wollaston V. (1998), 'Differential expression of a senescence-enhanced metallothionein gene in *Arabidopsis* in response to isolates of *Peronospora parasitica* and *Pseudomonas syringae*', *Plant J.* **16**, 209–221.

Cabanes-Macheteau M., Fitchette-Laine A. C., Loutier-Bourhis C., Lange C., Vine Ma K. C., Lerouge P. and Faye L. (1999), 'N-Glycosylation of a mouse IgG expressed in transgenic tobacco plants', *Glycobiol.* **9**, 365–372.

Cahoon E. B., Lindqvist Y., Schneider G. and Shanklin J. (1997), 'Redesign of soluble fatty acid desaturases from plants for altered substrate specificity and double bond position', *Proc. Nat. Acad. Sci. USA* **94**, 4872–4877.

Cahoon E. B. and Shanklin J. (2000), 'Substrate-dependent mutant complementation to select fatty acid desaturase variants for metabolic engineering of plant seed oils', *Proc. Nat. Acad. Sci. USA* **97**. 12350–12355.

Carillo C., Wigdorovitz A., Oliveros J. C., Zamerano P. I., Sadir A. M., Gomez N., Salinas J., Escribano J. M. and Borca M. V. (1998), 'Protective immune response

to foot-and-mouth disease virus with VP1 expressed in transgenic plants', *J. Virol.* **72**, 1688–1690.

Carlson K. (2000), 'Rising market interest in small processing systems, *Oils and Fats International*', Jan. 2000, pp. 27–28.

Carter J. E. and Langridge W. H. R. (2001), 'Plant-based vaccines for protection against infectious and autoimmune diseases', *Critical Rev. Plant Sci.*, in press.

Casci T. (2000), 'Genome evolution', *Arabidopsis* 4, tomato 1, *Nature Rev. Genet.* **1**, 4–5.

Chaudhary S., Parmenter D. L. and Moloney M. M. (1998), 'Transgenic *Brassica carinata* as a vehicle for the production of recombinant proteins in seeds', *Plant Cell Rep.* **17**, 195–200.

Choi D., Kim H. N., Yun H. K., Park J. A., Kim T. W. and Bok S. H. (1996), 'Molecular cloning of a metallothionein-like gene from *Nicotiana glutinosa L.* and its induction by wounding and tobacco mosaic virus infection', *Plant Physiol.* **112**, 353–359.

van Cott K. E., Lubon H., Russel C. G., Butler S. P., Gwazdauskas F. C., Knight J., Drohan W. N. and Velander W. H. (1997), 'Phenotypic and genotypic stability of transgenic pigs expressing recombinant human protein C', *Transgenic Res.* **6**, 203–212.

Coughlan S., Hasting C., Hazebroek J. and Scelonge C. (1999), 'Production of transgenic sunflower with altered fatty acid profiles', *Proc. 1999 Symposium on Biochemistry and Molecular Biology of Plant Fatty Acids and Glycerolipids*, Lake Tahoe, California, p. 25.

Cramer C. L., Weissenborn D. L., Oishi K. K., Grabeau E. A., Bennett S., Ponce E., Grabowski G. A. and Radin D. N. (1996), 'Bioproduction of human enzymes in transgenic tobacco', *Ann. New York Acad. Sci.* **792**, 62–71.

Cramer C. L., Boothe J. G. and Oishi K. K. (1998), 'Transgenic plants for therapeutic proteins: linking upstream and downstream strategies', in Hammond J., McGarvey P. and Yusibov V. (1998, eds.), *Current Topics in Microbiology and Immunology. Plant Biotechnology. New Products and Applications*, Springer, Berlin, pp. 95–118.

Dahlqvist A., Stahl U., Lenman M., Banas A., Lee M., Sandager L., Ronne H. and Stymne S. (2000), 'Phospholipid: diacylglycerol acyltransferase: an enzyme that catalyses the acyl–CoA-independent formation of triacylglycerol in yeast and plants', *Proc. Nat. Acad. Sci. USA* **97**, 6487–6492.

Dale E.C. and Ow D. W. (1991), 'Gene transfer with subsequent removal of the selection gene from the host genome', *Proc. Nat. Acad. Sci. USA* **88**, 10558–10562.

Daniell H., Khan M. S., Allison L. (2002), 'Milestones in chloroplast genetic engineering: an environmentally friendly era in biotechnology', *Trends Plant Sci.* **7**, 84–91.

Dieryck W., Pagnier J., Poyart C., Marden M. C., Gruber V., Bournat P., Baudino S. and Merot B. (1998), 'Human haemoglobin from transgenic tobacco', *Nature* **386**, 29–30.

Doebley J., Stec A. and Hubbard L. (1997), 'The evolution of apical dominance in maize', *Nature* **386**, 485–488.

Donnelly C. A. (2000), 'Likely size of the French BSE epidemic', *Nature* **408**, 787–788.

Edwards P. M., Arrowsmith C. H., Christendat D., Dharamsi A., Friesen J. D., Greenblatt J. F. and Venadi M. (2000), 'Protein production: feeding the X-ray crystallographers and NMR spectroscopists', *Nature Structural Biol.* **9** suppl. 970–972.

Facciotti M. T., Bertain P. B. and Yuan L. (1999), 'Improved stearate phenotype in transgenic canola expressing a modified acyl acyl-carrier protein thioesterase', *Nature Biotech.* **6**, 593–597.

Fitzpatrick K. (2000), 'The current nutraceutical health sector', *Inform* **11**, 517–523.

Flavell R. B. (2000), 'Plant biotechnology. Moral dilemmas', *Curr. Opinion Plant Biol.* **3**, 143–146.

Fry J. (1998), 'Implication of recent developments in Asian economies and in the global economy for the oil palm industry', in Angga Jatmita *et al.* (1998, eds.), *Proc. 1998 International Oil Palm Conf.*, Indonesian Oil Palm Research Institute, Medan, 28–35.

Gallie D. R. (1998), Controlling gene expression in transgenics, *Curr. Opinion Plant Biol.* **1**, 166–172.

Gao A. G., Hakimi S. M., Mittanck C. A., Wu Y., Woerner B. M., Stark D. M., Shah D. M., Liang J. and Rommens C. M. (2000), 'Fungal pathogen protection in potato by expression of a plant defensin peptide', *Nature Biotechnol.* **18**, 1307–1310.

Garbabino J., Jaynes J. and Belknap W. (2000), 'Plants producing lytic peptides', *US Patent* No. 6084156.

Gelvin S. B. (1998), 'The introduction and expression of transgenes in plants', *Curr. Opinion Biotechnol.* **9**, 227–232.

Gill P., Jeffreys A. J. and Werrett D. J. (1985), 'Forensic application of DNA "finger prints" ', *Nature* **318**, 577–579.

Goddijn O. J. M. and Pen J. (1995) 'Plants as bioreactors', *Trends Biotechnol.* **13**, 379–387.

Goto F., Yoshihara T., Shigemoto N., Toki S. and Takaiwa F. (1999), 'Iron fortification of rice seed by the soybean ferritin gene', *Nature Biotechnol.* **17**, 282–286.

Grant D., Cregan P. and Shoemaker R. C. (2000), 'Genome synteny in dicots: Genome duplication in *Arabidopsis* and synteny between soybean and *Arabidopsis*', *Proc. Nat. Acad. Sci. USA* **97**, 4168–4173.

Green A. G. and Dribnenki J. C. P. (1994), 'Linola—a new premium polyunsaturated oil', *Lipid Technol.* **6**, 29–33.

Gruber V., Bournat P. and Merot B. (1998a), 'Pancreatic lipases and/or recombinant colipases and derived polypeptides produced by plants, methods for obtaining them and use thereof', *Patent Applic.* WO 98/17807.

Gruber V., Exposito J. V., Ruggiero F., Comte J., Garrone R., Merot B. and Bournat P. (1998b), 'Recombinant collagen and derived proteins produced by plants, methods for obtaining them and uses', *Patent Applic.* WO 98/27208.

Gunstone F. (2000), '2000–01 oilseed production should surpass demand', *Inform* **11**, 1083–1085.

Herman E. M. and Larkins B. A. (1999), 'Protein storage bodies and vacuoles', *Plant Cell* **11**, 601–613.

Herrera-Estrella L. R. (2000), 'Genetically modified crops and developing countries', *Plant Physiol.* **124**, 923–935.

Hiatt A. C., Cafferkey R. and Bowdish K. (1989), 'Production of antibodies in transgenic plants', *Nature* **342**, 76–78.

Hitz B. (1999), 'Economic aspects of transgenic crops which produce novel products', *Curr Opinion Plant Biol.* **2**, 135–138.

Hood E. E., Witcher D. R., Maddock S., Meyer T., Baszczynski C., Bailey M., Flynn P., Register J., Marshall L., Bond D., Kulisek E., Kusnadi A., Evangelista R., Nikolov Z., Wooge C., Mehigh R. J. Hernan R., Kappel W. K., Ritland D., Li C. P. and Howard J. A. (1997), 'Commercial production of avidin from transgenic maize: characterisation of transformant, production, processing, extraction and purification', *Mol. Breeding* **3**, 291–306.

Hood E. E., Kusnadi A., Nikolov Z. and Howard J. (1999) 'Molecular farming of industrial proteins from transgenic maize', in Shahidi F., Kolodziejczyk P., Whitaker J. R., Munguia A. L. and Fuller G. (1999, eds.), *Chemicals via Higher Plant Bioengineering*, Kluwer/Plenum, New York, pp. 127–148.

Hooker B. S., Dai Z., Gao J., Kimgsley M. T. and Weller R. E. (1998), 'Method of producing human growth factors from whole plants or plant cell cultures', *Patent Applic.* WO 98/21348.

Janick J. (1999, ed.), *Perspectives on New Crops and New Uses*, ASHS Press, Alexandria, Virginia.

Jiang L., Phillips T. E., Rogers S. W. and Rogers J. C. (2000), 'Biogenesis of the protein storage vacuole crystalloid', *J. Cell Biol.* **150**, 755–769.

Joersbo M. (2001), 'Advances in the selection of transgenic plants using non-antibiotic marker genes', *Physiol. Plant.* **111**, 269–272.

Johanson U., West J., Lister C., Michaels S., Amasino R. and Dean C. (2000), 'Molecular analysis of FRIGIDA, a major determinant of natural variation in *Arabidopsis* flowering time', *Science* **290**, 344–347.

Jouanin L., Goujon T., De Nadai V., Martin M. T., Mila I., Vallet C., Pollet B., Yoshinaga A., Chabbert B., Petit-Conil M. and Lapierre C. (1999), 'Lignification in transgenic poplars with extremely reduced caffeic acid O–methyltransferase activity', *Plant Physiol.* **123**, 1363–1374.

Jyonouchi H., Sun S., Iijima K. and Gross M. D. (2000), 'Antitumor activity of astaxanthin and its mode of action', *Nut. Cancer* **36**, 59–65.

Kaiser J. (2000), 'Panel urges further study of biotech corn', *Science* **290**, 1867.

Kaiser J. (1996), 'Pests overwhelm Bt cotton crop', *Science* **272**, 423.

Kaplinski N., Braun D., Lisch D., Hay A., Hake S. and Freeling M. (2002), 'Maize transgene results in Mexico are artefacts', *Nature* **416**, 601–602.

Kelley W. S. (1996), 'Therapeutic peptides: the devil is in the details', *Bio/Technology* **14**, 28–31.

Kerr D. E., Liang F., Bondioli K. R., Zhao H., Kleibich G., Wall R. J. and Sun T. T. (1998), 'The bladder as a bioreactor: urothelium production and secretion of growth hormone into urine', *Nature Biotechnol.* **16**, 75–79.

Kerr R. A. (1998), 'The next oil crisis looms large—and perhaps close', *Science* **281**, 1128–1131.

Knapp S. J. and Crane J. M. (1999), 'Breeding advances and germplasm resources in meadowfoam: a very long chain oilseed', in Janick J. (1999, ed.), *Perspectives on New Crops and New Uses*, ASHS Press, Alexandria, Virginia.

Knutzon D. S., Thompson G. A., Radke S. E. and Johnson W. B. (1992), 'Modification of *Brassica* seed oil by antisense expression of a stearoyl–acyl carrier protein desaturase gene', *Proc. Nat. Acad. Sci. USA* **89**, 2624–2628.

Kolodziejczyk P. P. and Fedec P. (1999) 'Recent progress in agricultural biotechnology and opportunities for contract research and development', in Shahidi F., Kolodziejczyk P., Whitaker J. R., Munguia A. L. and Fuller G. (1999, eds.), *Chemicals via Higher Plant Bioengineering*, Kluwer/Plenum, New York, pp. 5–20.

Komarnytsky S., Borisjuk N. V., Borisjuk L. G., Alam M. Z. and Raskin I. (2000), 'Production of recombinant proteins in tobacco guttation fluid', *Plant Physiol.* **124**, 927–933.

Krattiger A. F. (2000), 'Food biotechnology: promising havoc or hope for the poor?' *Proteus* **17**, 38–44.

Langridge W. H. R. (2000), 'Edible vaccines', *Scientific American* **283**, 66–71.

Lapierre C., Pollet B., Petit-Conil M., Toval G., Romero J., Pilate G., Leple J. C., Boerjan W., Ferret V., De Nadai V. and Jouanin L. (1999), 'Structural alterations of lignins in transgenic poplars with depressed cinnamyl alcohol dehydrogenase or caffeic acid O–methyltransferase activity have an opposite impact on the efficiency of industrial kraft pulping', *Plant Physiol.* **119**, 153–164.

Leisinger K.M. (2000), 'The political economy of agricultural biotechnology for the developing world', *Novartis Foundation for Sustainable Development*, available on the web at *www.foundation.novartis.com/political_economy_agricultural_biotechnology.htm.*

Liljegren S. J., Ditta G. S., Eshed Y., Savidge B., Bowman J. L. and Yanofsky M. (2000), 'SHATTERPROOF MADS–box genes control seed dispersal', in *Arabidopsis Nature* **404**, 766–770.

Lindqvist Y., Huang W., Schneider G. and Shanklin J. (1996), 'Crystal structure of a delta–9 stearoyl–acyl carrier protein desaturase from castor seed and its relationship to other diiron proteins', *EMBO J.* **15**, 4081–4092.

Liu J. H., Selinger L. B., Cheung K. J., Beauchemin K. A. and Moloney M. M. (1997), 'Plant seed oil bodies as an immobilisation matrix for a recombinant xylanase from the rumen fungus', *Neocallimastix patriciarum*, *Mol. Breeding* **3**, 463–470.

van der Logt C. P. E., Sidebottom C.S. and Davis P. J. (1998), 'Antibody production in plants', in Shewry P., Napier J. A. and Davis P. J. (1998, eds.) *Engineering Crop Plants for Industrial End Uses*, Portland Press, London, pp. 17–32.

London S. J., Yuan J. M., Chung F. L., Gao Y. T., Coetzee G. A., Ross R. K. and Yu M. L. (2000), 'Isothiocyanates, glutathione–S–transferases, and lung cancer risk: a prospective study of men in Shanghai', China, *Lancet* **356**, 724–729.

Lorenz R. T. and Cysewski G. R. (2000), 'Commercial potential for *Haematococcus* microalgae as a natural source of astaxanthin', *Trends Biotechnol.* **18**, 160–167.

Ma J., Hiatt A., Hein M., Vine N., Wang F., Stabila P., van Dolleweerd C., Mostov K. and Lehner T. (1995), 'Generation and assembly of secretory antibodies in plants', *Science* **268**, 716–719.

Ma J. K., Hikmat B. Y., Wycoff K., Vine N. D., Chargelegue D., Yu L., Hein M. B. and Lerner T. (1998), 'Characterization of a recombinant plant monoclonal secretory antibody and preventative immunotherapy in humans', *Nature Medicine* **4**, 601–606.

Ma J. K., Lehner K. C., Stabila P., Fux C. I. and Hyatt A. (1994), *Eur. J. Immunol.* **24**, 131–138.

Magnuson N. S., Linzmaier P. M., Reeves R., An G. H., Hayglass K. and Lee J. M. (1998), 'Secretion of active human Interleukin–2 and interleukin–4 from genetically modified tobacco cells in suspension culture', *Prot. Expr. Purific.* **13**, 45–52.

Mariani C., De Beuckeleer M., Truettner J., Leemans J. and Goldberg R. B. (1990), 'Induction of male sterility in plants by a chimaeric ribonuclease gene', *Nature* **347**, 737–741.

Mason H. S., Haq T. A., Clements J. D. and Arntzen C. J. (1998), 'Edible vaccine protects mice against *Escherichia coli* heat-labile enterotoxin (LT): potatoes expressing a synthetic LT–B gene', *Vaccine* **16**, 1336–1343.

Mazur B., Krebbers E. and Tingey S. (1999), 'Gene discovery and product development for grain quality traits', *Science* **285**, 372–375.

McCaskill D. and Croteau R. (1998), 'Some caveats for bioengineering terpenoid metabolism in plants', *Trends Biotechnol.* **16**, 349–355.

McGraw L. (2000), 'Avidin: an egg-citing insecticidal protein in corn', *Agric. Res. Mag.* **48**, 8–9.

Merkle S. A. and Dean J. F. (2000), 'Forest tree biotechnology', *Curr. Opinion Biotech.* **11**, 298–302.

Metz M. and Futterer J. (2002), 'Suspect evidence of transgenic contmination', *Nature* **416**, 600–601.

Modelska A., Dietzschold B., Sleysh N., Fu Z. F., Steplewski K., Hooper D. C., Koprowski H. and Yusibov V. (1998), 'Immunisation against rabies with plant-derived antigen', *Proc. Nat. Acad. Sci. USA* **95**, 2481–2485.

Moffat A. S. (2000), 'Can genetically modified crops go greener?', *Science* **290**, 253–254.

Montelione G. T., Zheng D., Huang Y. J., Gunsalus K. C. and Szyperski T. (2000), 'Protein NMR spectroscopy in structural genomics', *Nature Structural Biol.* **9** suppl., pp. 982–985.

Moreau R. A., Norton R. A. and Hicks K. B. (1999), 'Phytosterols and phytostanols lower cholesterol', *Inform* **10**, 572–577.

Moriguchi T., Kita M., Hisada S., Endo-Ingaki T. and Omura M. (1998), 'Characterisation of gene repertoires at mature stage of citrus fruits through random sequencing of redundant metallothionein-like genes expressed during fruit development', *Gene* **211**, 221–227.

Murphy D. J. (1996), 'Engineering oil production in rapeseed and other oil crops', *Trends Biotechnol.* **14**, 206–213.

Murphy D. J. (1998), 'Impact of genomics on improving the quality of agricultural products', in: Dixon G. K., Copping L C. and Livingstone D., eds., *Genomics: Commercial Opportunities from a Scientific Revolution*, SCI Press, Cambridge, pp. 199–210.

Murphy D. J. (1999a), 'Manipulation of plant oil composition for the production of valuable chemicals, in Shahidi F., Kolodziejczyk P., Whitaker J. R., Munguia A. L. and Fuller G. (1999, eds.), *Chemicals via Higher Plant Bioengineering*, Kluwer/Plenum, New York, 21–35.

Murphy D. J. (1999b), 'Production of novel oils in plants', *Curr. Opinion Biotechnol.* **10**, 175–180.

Murphy D. J. (2000), 'Oils and fats—meeting the demands for food, materials, energy and nutrition in the new millennium', *Inform* **11**, 1301–1303.

Murphy D. J. (2001), 'New oil crops for the 21st. century. What is in the pipeline and what is coming in the future', *Proc. Oils and Fats International Congress, 2000*, Malaysia.

Murphy D. J., Hernandez-Pinzon I. and Patel K. (2001), 'Roles of lipid bodies and lipid–body proteins in seeds and other tissues', *J. Plant Physiol.* **158**, in press

Nance W. E., Pandya A., Maes H., Ellison J. W., Blanton S. H. and Schieken R. M. (1998), 'The PHOG gene on Xpter–p22.32 appears to be a QTL for stature in adolescent twins', *9th. Int. Congr. Twin Studies* (*www.kate.pc.Helsinki.fi/twin/980605.html*).

National Audit Office (2002), 'The 2001 outbreak of foot-and-mouth disease', The Stationary Office, London or *www.nao.gov.uk/publications/nao_reports/01-02/0102939.pdf.*

Nelson G.D. (1998) 'Science literacy for all: an achievable goal?' available online at *www.project2061.org/newsinfo/research/articlesopa.htm.*

Niiler E. (1999), 'Terminator technology temporarily terminated', *Nature Biotechnol.* **17**, 1054.

Nuccio M. L., Rhodes D., McNeil S. D. and Hanson A. D. (1999) 'Metabolic engineering for osmotic stress resistance', *Curr. Opinion Plant Biol.* **2**, 128–134.

Oelkers P., Tinkelenberg A., Erdeniz N., Cromley D., Billenheimer J. T. and Sturley S. L. (2000) 'A lecithin cholesterol acyltransferase-like gene mediates diacylglycerol esterification in yeast', *J. Biol. Chem.* **275**, 15609–15612.

OFSTED (1996), *Subjects and Standards*, HMSO, London.

OFSTED (2000), 'Ofsted subject reports 1999–2000, primary science' and 'Ofsted subject reports 1999–2000, secondary science', available online at *www.ofsted.gov.uk.*

Oksman-Caldentey K. M. (2000), 'Metabolic engineering of plants for production of pharmaceuticals', *Proc. Int. Conf. on Biotechnology: Practice in Non-Food Products*, 93–96.

Otten L., De Greve H., Hernalsteens J. P., van Montagu M, Schnieder O., Straub J. and Schell J. (1981), 'Mendelian transmission of genes introduced into plants by the Ti plasmids of *Agrobacterium tumefaciens*', *Mol. Gen. Genet.* **183**, 209–213.

Parmenter D. L., Boothe J. G., van Rooijen G. J. H., Yeung E. C. and Moloney M. M. (1995), 'Production of biologically active hirudin in plant seeds using oleosin partitioning', *Plant Mol. Biol.* **29**, 1167–1180.

Peacock J. (1992), 'Twenty-first century crops', *Nature* **357**, 358.

Pen J., van Ooyen A. J. J., van den Elzen P. J. M., Quax W. J. and Hoekema A. (1993a), 'Efficient production of active industrial enzymes in plants', *Ind. Crops Products* **1**, 241–250.

Pen J., Verwoerd T. C., van Paridon P. A., Beudeker R. F., van den Elzen P. J. M., Geerse K., van der Klos J. D., Versteegh H. A. J., van Ooyen A. J. J. and Hoekema A. (1993b), 'Phytase-containing transgenic seeds as a novel feed additive for improved phosphorus utilization', *Bio/Technology* **11**, 811–814.

Peng J. R., Richards D. E., Hartley M. M., Murphy G. P., Davos K. M., Flintham J. E., Beales J., Fish J. L., Worland A. J., Pelica F., Sudakar D., Christou P., Snape J. W., Gale M. D. and Harberd N. P. (1999), ' "Green revolution" genes encode mutant gibberellin response modulators', *Nature* **400**, 256–261.

Pineiro M. and Coupland G. (1998), 'The control of flowering time and floral identity in *Arabidopsis*', *Plant Physiol.* **117**, 1–8.

Potera C. (1999), 'EPIcyte produces antibodies in plants', *Gen. Eng. News*, Feb. 15.

Prasad M. N. V. and de Oliveira Freitas H. M. (1999), 'Feasible biotechnological and bioremediation strategies for serpentine soils and mine spoils', *Electronic J. Biotechnol.* **2**, no.1, available on the web: *www.ejb.org/content/ vol2/issue1/full/5/.*

Quist D. and Chapela I. H. (2001), 'Transgenic DNA introgressed into traditional landraces in Oaxaca, Mexico', *Nature* **414**, 541–543.

Robinson J. (1999), 'Ethics and transgenic crops: a review', *Electronic J. Biotechnol.* **2**, no.2, available on the web: *www.ejb.org/content/vol2/issue2/full/3/.*

van Rooijen G. J. H. and Moloney M. M. (1995), 'Plant seed oil bodies as carriers for foreign proteins', *Bio/Technology* **13**, 72–77.

Ryu D. D. and Nam D. H. (2000), 'Recent progress in biomolecular engineering', *Biotechnol. Prog.* **16**, 2–16.

Salmon V., Legrand D., Slomianny M. C., El Yazidi I, Spik G., Gruber V., Bournat P., Olagnier B., Mison D., Theison M. and Merot B. (1998), 'Production of human lactoferrin in transgenic tobacco', *Prot. Expr. Purific.* **13**, 127–135.

Shah D. M., Rommens C. M. T. and Beachy R. N. (1995) 'Resistance to diseases and insects in transgenic plants: progress and applications to agriculture', *Trends Biotechnol.* **13**, 362–368.

Shanklin J. (2000) 'Exploring the possibilities presented by protein engineering', *Curr. Opinion Plant Biol.* **3**, 243–248.

Shanks I. and Teh M. M. (1999), 'GM trees and global cooling', *Sci. Public Affairs* Oct. issue, pp. 16–18.

Somerville C. and Somerville S. (1999), 'Plant functional genomics, *Science* **285**, 380–383.

Steinbüchel A. and Fuchtenbusch B. (1998), 'Bacteria and other biological systems for polyester production', *Trends Biotechnol.* **16**, 419–427.

Stevens L. H., Stoopen G. M., Elbers I. J. W., Mollthoff J. W., Bakker A. C., Lommen A., Bosch D. and Jordi W. (2000), 'Effect of climate conditions and plant developmental stage on the stability of antibodies expressed in transgenic tobacco', *Plant Physiol.* **124**, 173–182.

Stiglitz J. E. (1993), *Economics*, W.W. Norton, New York.

Stobart K., Mancha M., Lenman M., Dahlqvist A. and Stymne S. (1997), 'Triacylglycerols are synthesised and utilised by transacylation reactions in microsomal preparations of developing safflower (*Carthamus tinctorius* L) seeds', *Planta* **203**, 58–66.

Straughan R. (2000), 'Moral and ethical issues in plant biotechnology', *Curr. Opinion Plant Biol.* **3**, 163–165.

Tacket C., Mason H., Losonsky G., Clement J., Levine M. and Arntzen C. (1998), 'Immunogenicity in humans of a recombinant bacterial antigen delivered in a transgenic potato', *Nature Med.* **4**, 607–609.

Tag A., Hicks J., Garifullina G., Ake C., Phillips T. D., Bermand M. and Keller N. (2000), 'G–protein signalling mediates differential production of toxic secondary metabolites', *Mol. Microbiol.* **38**, 658–665.

Tarczynski M. C., Jensen R. G. and Bonhert H. J. (1992), 'Expression of a bacterial *mtlD* gene in transgenic tobacco leads to production and accumulation of mannitol', *Proc. Nat. Acad. Sci. USA* **89**, 2600–2604.

Tavladoraki P., Benvenuto E., Trinca S., de Martinis D., Cattaneo A. and Galeffi P. (1993), 'Transgenic plants expressing a functional single-chain Fv antibody are specifically protected from virus attack', *Nature* **366**, 469–472.

Tawfig N., Heaney R., Plumb J., Fenwick G., Musk S. and Williamson G. (1995), 'Dietary glucosinolates as blocking agents against carcinogenesis: glucosinalate breakdown products assessed by induction of quinone reductase activity in murine hepa 1c1c7 cells', *Carcinogenesis* **16**, 1191–1194.

'The Arabidopsis Genome Initiative (2000), 'Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*', *Nature* **408**, 796–815.

Thompson G. A. and Li C. (1997) 'Altered fatty acid composition of membrane lipids in seeds and seedling tissues of high-saturate canolas', in Williams J. P., Khan M. U. and Lem N. W., eds, *Physiology, Biochemistry and Molecular Biology of Plant Lipids*, Kluwer, Dordrecht, Netherlands, pp. 313–315.

Tinker P. B. (2000), 'The future research requirements for the oil palm plantation', in Pushparajah E. (2000, ed.), *Plantation Tree Crops in the New Millenium: the Way Ahead, Proc. Int. Planters Conf.*, The Incorporated Society of Planters, Kuala Lumpur, pp. 3–40.

Trewavas A. (1999), 'Much food, many problems', *Nature* **402**, 231.

Valentin HE, Broyles DL, Casagrande LA, Colburn SM, Creely WL, DeLaquil PA, Felton HM, Gonzalez KA, Houmiel KL, Lutke K, Mahadeo DA, Mitsky TA, Padgette SR, Reiser SE, Slater S, Stark DM, Stock RT, Stone DA, Taylor NB, Thorne GM, Tran M and Gruys KJ. (1999) 'PHA production, from bacteria to plants', *Int. J. Bio.l Macromol.* **25**, 303-6

Vanderkerkove J., Van Damme J., Van Lijsebettens M., Botterman J., De Block M., Vandewiele A, De Clerq A., Leemans J., Van Montagu M. and Schell J. (1989), 'Enkephalins produced in transgenic plants using modified 2S seed storage proteins', *Bio/Technology* **7**, 929–932.

Various (2000), *Biobased Industrial Research Products: Priorities for Research and Commercialisation*, National Academy Press, Washington D.C.

Venkat K (2001) 'Natural medicines from plants', in Kuokka A., Nuutila A. M. and Oksman-Caldenty K. M., eds., *Plant Biotechnology—Better Products from Better Plants*, VTT Biotechnology, Helsinki.

Verch T., Yusibov V. and Koprowski H. (1998), 'Expression and assembly of a full-length monoclonal antibody in plants using a plant virus vector', *J. Immunol. Meth.* **220**, 69–75.

Visser R. G. F., Somhorst I., Kuipers G. J., Ruys N. J., Feenstra W. J. and Jacobsen E. (1992), 'Inhibition of the expression of the gene for granule-bound starch synthase in potato by antisense constructs', *Mol. Gen. Genet.* **225**, 289–296.

Voelker T. A., Worrell A. C., Anderson L., Bleibaum J., Fan C., Hawkins D. J., Radke S. E. and Davies H. M. (1992), 'Fatty acid biosynthesis redirected to medium chains in transgenic oilseed plants', *Science* **257**, 72–73.

Voelker T. A., Hayes T. R., Cranmer A. M., Turner J. C. and Davies H. M. (1996), 'Genetic engineering of a quantitative trait: metabolic and genetic parameters influencing the accumulation of laurate in rapeseed', *Plant J.* **9**, 229–241.

Wambugu F. (1999), 'Why Africa needs agricultural biotech', *Nature* **400**, 15–16.

Wang X., Willen R. and Wadstrom T. (2000), 'Astaxanthin-rich algal meal and vitamin C inhibit *Helicobacter pylori* infection in BABL/cA mice', *Antimicrob. Agents Chemother.* **44**, 2452–2457.

Wiberg E., Banas A. and Stymne S. (1997), 'Fatty acid distribution and lipid metabolism in developing seeds of laurate-producing rape (*Brassica napus* L.)', *Planta* **203**, 341–348.

Wongsamuth R. and Doran P. M. (1997), 'Production of monoclonal antibodies by tobacco hairy roots', *Biotechnol. Bioeng.* **54**, 401–415.

Ye G. N., Danielle H. and Sanford J. C. (1990) 'Optimisation of delivery of foreign DNA into higher plant chloroplasts', *Plant Mol. Biol.* **15**, 809–819.

Ye X., Al-Babili S., Kloti A., Zang J., Lucca P., Beyer P. and Potrykus I. (2000), 'Engineering the provitamin A (β–carotene) biosynthetic pathway into (carotenoid-free) rice endosperm', *Science* **287**, 303–305.

Yun D. J., Hasimoto T. and Yamada Y. (1992), 'Metabolic engineering of medicinal plants: transgenic *Atropa belladonna* with an improved alkaloid composition', *Proc. Nat. Acad. Sci. USA* **89**, 11799–11803.

Zeitlin L., Olmsted S. S., Moench T. R., Co M. S., Martinell B. J., Paradkar V. M., Russell D. R., Queen C., Cone R. A. and Whaley K. J. (1998), 'A humanised monoclonal antibody produced in transgenic plants for immunoprecipitation of the vagina against genital herpes', *Nature* **16**, 1361–1364.

Zhai W., Li X., Tian W., Zhou Y., Pan X., Cao S., Zhao Y., Zhao B., Zhang Q. and Zhu L. (2000), 'Introduction of a blight-resistance gene, Xa21, into five Chinese rice varieties through an *Agrobacterium*-mediated system', *Science in China, Series C* **43**, 361–368.

Zhang Y., Kensler T. W., Cho C. G., Posner G. H. and Talalay P. (1994), 'Anti-carcinogenic activities of sulforofane and other structurally related synthetic norbornyl isothiocyanates', *Proc. Nat. Acad. Sci. USA* **91**, 3147–3150.

Ziegler M., Thomas S. and Danna K. (1999), 'Accumulation of a thermostable endo–1,4-β-D-glucanase in the apoplast of *Arabidopsis thaliana*', *Mol. Breeding* **5**, 53–59.

Ziyu D., Hooker B. S., Queensberry R. D. and Thomas S. R. (1998), 'Over-expression of cellulase in transgenic tobacco whole plants', *Amer. Soc. Plant Physiol. Annual Meeting*, Abstract 337.

de Zoeten G. A., Penswick J. R., Horisberger M. A., Ahl P., Schultze M. and Hohn T. (1999), 'The expression, localisation and effect of a human interferon', *Virology* **172**, 213–222.

APPENDIX I
Conversion Factors

$1239.8/(\lambda/\text{nm}) = U/\text{eV}$
kT at 298.15 K = 0.02569 eV
kT/q at 298.15 K = 0.02569 V
(ln 10) kT/q at 298.15 K = 0.05915 V

1 eV = 1.6022×10^{-19} J = 96.488 kJ mol^{-1} = 8065.6 cm^{-1} = 2.4180×10^{14} Hz
1 cm^{-1} = 1.9864×10^{-23} J = 11.963 J mol^{-1} = 0.12398 meV = 2.9979×10^{10} Hz
1 kWh = 3.6 MJ
1 cal (thermochemical) = 4.184 J
1 Quadrillion = 1 Quad = 1.055 EJ
1 British thermal unit (thermochemical) = 1 Btu = 1.054 kJ
1 horse power = 0.7457 kW

1 tonne oil equivalent (net, low heat value) = 1 toe = 41.87 GJ = 11.63 MWh
1 barrel of oil = 1 bbl = approx 0.136 tonnes = 5.71 GJ = 1.59 MWh
1000 m^3 of natural gas (standard, LHV) = 0.857 toe = 36 GJ = 10.0 MWh
1 tonne coal equivalent (standard, LHV) = 1 tce = 0.697 toe = 29.3 GJ = 81.4 MWh
1 tonne fuelwood = 0.380 toe
1 tonne uranium (thermal reactors) = 10^4 toe
1 tonne uranium (breeder reactors) = 0.5 Mtoe

1 Å = 10^{-8} cm
1 mile = 1.6093 km
1 hectare = 10^{-2} km^2 = 2.471 acre = 3.861×10^{-3} $mile^2$
1 acre = 4.047×10^{-3} km^2 = 0.4047 ha = 1.5626×10^{-3} $mile^2$
1 cubic foot = 2.830×10^{-2} m^3
1 litre = 1 dm^3 = 0.26417 US gallon = 0.21997 UK gallon

1 atm = 1.01325×10^5 Pa
1 torr = 1/760 atm

1 lb = 0.45359 kg
1 tonne (1 metric ton) = 10^3 kg
1 ton (UK) = 1 long ton (US) = 1.0160 tonne
1 ton (US) = 1 short ton (US) = 0.90718 tonne

APPENDIX II
Acronyms and Abbreviations

A	assimilation (of carbon dioxide through photosynthesis)
AA	arachidonaic acid
ADP	adenosine diphosphate
AEC	Arable Energy Coppice
ATP	adenosine triphosphate
AM	Air Mass
BChl	bacteriochlorophyll
BECs	biomass and energy crops
BPh	bacteriopheophytin
bp	before present
Da	dalton
DNA	deoxyribonucleic acid
DHA	docosahexanoic acid *or* dehydroascorbate
DHAP	dihydroxyacetonephosphate
dm	dry mass
dw	dry weight
CAM	Crassulacean acid metabolism
$[CH_2O]$	carbohydrate
Chl	chlorophyll
CFB	circulating fluidised bed
E4P	erythrose–4–phosphate
ELIPs	Early Light Induced Proteins
EPA	eicosapentanoic acid
ETBE	ethyl tertiary butyl ether
FBP	fructose–1,6–bisphosphate
Fd	ferredoxin
G3P	glyceraldehyde 3–phosphate
Ga	giga-annum (10^9 years)
GAI	Growth Area Index (ratio of the area of photosynthetically active crop tissue to land area)
g Nm^{-3}	grammes per N(ormal) cubic metre, *i.e.* at standard T (25C) and P (1 bar) conditions
GPP	Gross Primary Productivity
GtC	gigatonnes of carbon

$GtCO_2$	gigatonnes of carbon dioxide
His	histidine
HSPs	heat-shock proteins
IGCC	Integrated Gasification Combined Cycle
IP	identity preservation
IR	infrared radiation
kDa	kilodalton
LHI, LHII	light-harvesting complex I or II of purple photosynthetic bacteria
LHCI, LHCII	light-harvesting complex I or II of the peripheral antennae of higher plants
LSW	leaf specific weight
Ma	mega-annum (10^6 years)
MDHA	monodehydroascorbate
MPR	Mehler peroxidase reaction
MSW	Municipal Solid Waste
NAD^+	nicotinamide adenine dinucleotide, oxidised form
NADH	nicotinamide adenine dinucleotide, reduced form
$NADP^+$	nicotinamide adenine dinucleotide phosphate, oxidised form
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
NBP	Net Biome Productivity
NEP	Net Ecosystem Productivity
NPP	Net Primary Productivity
NUE	nutrient use efficiency
OAA	oxaloacetate
odt	oven-dry tonnes
OECD	Organisation for Economic Cooperation and Development
ppb	part per billion (by volume)
ppm	part per million (by volume)
PAR	photosynthetically active radiation
PCA	photosynthetic carbon assimilation
PCO	photosynthetic carbon oxidation
PEP	phosphoenolpyruvate
PET	photoinduced electron transfer
PGA	3–phosphoglycerate
P_i	inorganic phosphate
PSI	Photosystem I
PSII	Photosystem II
PSU	photosynthetic unit

PUFA	polyunsaturated fatty acids
R	(photosynthetic) respiration
RC	reaction centre
RME	rape methyl ester
RNA	ribonucleic acid
rDNA	ribosomal deoxynucleic acid
rubisco	ribulose–1,5–bisphosphate carboxylase–oxygenase
RUE	radiation use efficiency
R5P	ribose–5–phosphate
Ru5P	ribulose–5–phosphate
RuBP	ribulose–1,5–bisphosphate
S7P	sedoheptulose–7–phosphate
SBP	sedoheptulose–1,7–bisphosphate
SRC	Short Rotation Coppice
tdm	tonnes of dry matter
TFC	Total Final Consumption (of energy)
Thr	threonine
TPES	Total Primary Energy Supply
Tyr	tyrosine
UV	ultraviolet radiation
WT	wild-type
WUE	water use efficiency
Xu5P	xylulose–5–phosphate

APPENDIX III
List of Symbols

Symbol	Preferred Unit	Description
a		absorptance (absorptivity)
A		electron acceptor
c_i	mol dm^{-3}	molar concentration of species i
D		electron donor
D_i	$cm^2\ s^{-1}$	diffusion coefficient of species i
$E_{Ox,\,Rd}$	V *vs.* ref.	electrode potential for the reaction $Ox + ne^- \leftrightarrows Rd$ *vs.* reference electrode
E°	V *vs.* ref.	standard electrode potential (based on activities)
E_m	V *vs.* ref.	midpoint electrode potential
G	eV $molecule^{-1}$ or kJ mol^{-1}	Gibbs free energy
H	eV $molecule^{-1}$ or kJ mol^{-1}	enthalpy
j_λ^S	photons $m^{-2}\ s^{-1}$	spectral photon flux with respect to wavelength
q	C	unsigned charge on electron (1.602×10^{-19} C)
p	atm or Pa	pressure
T	K	absolute temperature
T		transmittance (transmissivity)
U	eV $molecule^{-1}$ or kJ mol^{-1}	internal energy
U_g	eV $molecule^{-1}$ or kJ mol^{-1}	'bandgap' or threshold energy for reaction
V	m^3	volume
V	V	voltage
α	cm^{-1} or m^{-1}	optical absorption coefficient
α_λ	cm^{-1} or m^{-1}	optical absorption coefficient at wavelength λ
ϵ_{op}		optical dielectric constant
ϵ_s		static dielectric constant
η_{PS}		photosynthetic energy-storage efficiency
λ	nm	wavelength
λ	eV $molecule^{-1}$	reorganisation energy (of an electron-transfer reaction)

Symbol	Preferred Unit	Description
λ_{in}	eV molecule^{-1}	inner reorganisation energy (of an electron-transfer reaction)
λ_{out}	eV molecule^{-1}	outer or solvent reorganisation energy (of an electron-transfer reaction)
μ_i	eV molecule^{-1}	chemical potential of species i
ν	Hz	frequency
$\overline{\nu}$	cm^{-1}	wavenumber
ϕ		quantum yield

INDEX