ADVANCES IN IMMUNOLOGY

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Immunology

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PREFACE

The pace of immunologic research has quickened as the problems and potentialities of immunology have appealed to investigators with widely differing backgrounds. The important subjects reviewed in the twelfth volume of this serial publication represent the contributions of chemists and biologists as well as immunologists. With the increasing scope of immunology, thoughtful, authoritative summations of knowledge in rapidly developing areas have become essential if those working in this field are to stay familiar with its overall progress. We are indebted to the authors of this volume for making this possible by taking the time to share with us their expertise.

The first article deals with the recently observed molecular uniformity of antibodies to bacterial carbohydrate antigens. Dr. Krause, who has initiated and carried out much of this research, discusses the practical aspects of the production of uniform antibodies and points out their potential usefulness. These molecules will play an important role in the study of the amino acid composition and topography of the antibody combining site, and by virtue of their allotypic homogeneity also should aid in the definition of the location and character of the various molecular determinants of allotypic specificity. Early sequence data from uniform rabbit antibodies already indicate significant homology in the variable regions of the human kappa and rabbit light chains, suggesting a common ancestral relationship. Finally, because of their ready detectability by physical means, uniform antibodies provide an additional means of analysis of antibody responses and the cellular and/or genetic events involved.

In the second chapter Dr. Metzger discusses in depth the physical, chemical, and biological aspects of γM antibodies, a subject to which he has contributed much. The physical characteristics, chemical composition, and subunit structure of the typical circular γM pentomers are presented, and the relationship of these molecules to low molecular weight γM-like proteins is considered. The characteristics of the interaction of γM antibodies with antigens, i.e., the nature and number of antigen combining sites, and the interaction of γM antibodies with the complement system are compared to the corresponding properties of γG antibodies. Finally, the biosynthesis and metabolism of γM antibodies and their peculiar role in the immune response are considered.

Perhaps the major barrier to rapid achievement in homotransplanta-

tion today is the paucity of our knowledge of the histocompatibility antigens. These antigens, until recently, have resisted attempts at isolation and concentration so that little could be learned of their structure or metabolism. In the third chapter, Drs. Reisfeld and Kahan, who are responsible for much of the recent progress in this field, provide a critical evaluation of the various procedures currently employed to extract these antigens and to characterize them chemically and physically. The various biological activities of the histocompatibility antigens and their use in assay systems for antigen and antibody detection and quantitation are also described.

One of the most active areas of immunologic research involves the participation and interactions of various cell types in the immune response. The recognition of the requirement for cell interaction in the antibody response has greatly complicated the problem of interpretation of much experimental data. The next two chapters in this volume complement each other admirably in presenting the current status of this rapidly developing subject. Drs. Abdou and Richter give a detailed account of the evidence supporting the roles of multiple cell types in the immune response. In addition to defining what is known of the origins and functions of macrophages, antigen reactive cells, and antibody forming cells, they indicate apparent species differences in the sources and roles of these cell types. They also point out those critical areas in which evidence is still needed in order to allow the formulation of a reasonably complete scheme for an immune response. Drs. Talmage, Radovich, and Hemmingsen deal with the same subject as it relates to our basic concepts of the immune response. Their discussion centers on the kind of interaction between the two different universes of immunocompetent cells and the probable nature of the immunologic specificity and function of each.

Recent investigations into the nature and functions of lysosomes indicate that these intracellular organelles may have the first and last words in many immunologic encounters. In the sixth chapter, Drs. Weissmann and Dukor present an authoritative summary of current information relating lysosomes both to the initial processing of antigens, a responsibility chiefly of macrophages, and to the phlogogenic events in immunologic injury, a responsibility chiefly of granulocytes. The macrophages via their lysosomal digestive capabilities appear to be not only the initial regulators of the magnitude of an antigenic stimulus by degrading much potentially immunogenic material but also the processors and perhaps conveyors of the final effective immunogen. The tissue injury which develops after the interaction of antigen, antibody, and complement or of antigen with sensitized cells is dependent to a large

extent upon the hydrolytic degradation of extracellular and intracellular macromolecules by the lysosomal enzymes.

Clarifying the relationship between the biological properties of immunoglobulins and their organization at the several levels of protein structure is one of the most exciting problems in molecular biology today. In the last chapter, Drs. Dorrington and Tanford discuss this relationship as it concerns the higher levels of organization—size, shape, and internal folding—of immunoglobulin molecules. While available measurements cannot yet define with certainty the three-dimensional configuration of immunoglobulins, they do suggest reasonable working models which fit antigen binding characteristics, electron microscopic appearances, and physical-chemical properties of these molecules.

As always, it is a pleasure to acknowledge the cooperation and assistance of the publishers, who have done much to ensure the quality of this series of volumes.

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The Search for Antibodies with Molecular Uniformity[1]

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I. Introduction

Within the last decade there has been a remarkable advance in the knowledge on the chemical structure of the immunoglobulins. The achievements are all the more remarkable because of the magnitude of immunoglobulin heterogeneity. Nevertheless, the immunoglobulins had sufficient structural features in common so that R. R. Porter (1959), Fleischman *et al.* (1963), Edelman and Gall (1969), Edelman and Poulik (1961), and others were able to determine the four-chain structure, consisting of one pair of light chains, and one of heavy chains. Because all of the molecules share this basic structure, heterogeneity is a function of variation in amino acid sequence. It was for this reason that a direct attack on the amino acid sequence of the two antigenic binding sites has been delayed, even though it was known that each of the two sites of IgG was localized to the variable portion of the molecule.

[1] Supported in part by a grant from the National Institute of Allergy and Infectious Diseases, Grant No. AI 08429, and by a Grant-in-Aid from the American Heart Association.

Once it was clear that a myeloma protein possessed molecular homogeneity and represented a single species of immunoglobulins, the way was open to employ the myeloma proteins to examine in much greater detail the chemical structure of the immunoglobulins. Indeed, the amino acid sequence of one myeloma protein is now completely elucidated (Edelman and Gall, 1969), and there is much information on vast stretches of many others. Despite such success, in all of this there is a nagging reservation. Is a myeloma protein, the product of malignant cells, a genuine representative of an antibody? The question has been answered only in part, even though a limited number of mouse and human myeloma proteins possess reactivity for certain well-defined chemical antigens. As long as ambiguity surrounds this fundamental nature of the myeloma proteins, any exploration of the topography of the antigen binding site and a scrutiny of the structure-function relationships between antigens and antibodies will require homogeneous antibody populations. It is for this and other reasons that immunologists have sought specific antibodies with molecular uniformity. It has been their goal to procure at will, and in a reproducible and predictable fashion, homogeneous antibodies to specific and defined antigenic determinants. But, the prospects for achieving such a goal, until a few years ago, appeared bleak.

A number of excellent reviews have recently been published on the subject of heterogeneity (Fleischman, 1966; Haber, 1968; Franklin and Frangione, 1969). In this series of volumes alone, at least four previous reviews have described the structural heterogeneity of the immunoglobulins and the molecular and functional heterogeneity of specific antibodies (Fahey, 1962; Cohen and Porter, 1964; Cohen and Milstein, 1967; Grey, 1969). It is not the purpose of this review to recapitulate the evidence for molecular heterogeneity of the immunoglobulins as well as most specific antibody preparations.

The review is the first of this series to deal exclusively with the search for antibodies with molecular uniformity. It will be confined to the history of this quest, and will not review in detail the chemical structure of the immunoglobulins or the evidence for molecular and functional heterogeneity of immunoglobulins and specific antibodies to a wide range of different antigens. The references cited will be selective and representative rather than exhaustive, and the final selection will undoubtedly represent the unintentional bias of the author. The focus will remain centered on the occurrence of antibodies with uniform properties following specific antigenic stimulation. Human and mouse myeloma proteins or paraproteins with antibody activity will be included because use of these proteins remains an alternative approach to procure anti-

bodies with molecular uniformity. No attempt will be made here to include all aspects of this work.

The review has been divided into several sections. First a brief summary is presented of the hallmarks of immunoglobulin heterogeneity and the criteria that must be used to judge the restricted heterogeneity or molecular uniformity of an antibody preparation are considered. Then human antibodies to certain selected antigens which appear to possess less heterogeneity than normal γ-globulin are discussed. A brief account of these human antibodies is important because their occurrence has sparked effort to procure antibodies with molecular uniformity in experimental animals. Next the experimental approaches to generate antibodies with limited heterogeneity in animals are described. Emphasis is placed on the evidence for the molecular uniformity of rabbit antibodies to bacterial carbohydrate antigens. The section after that deals with human and mouse myeloma proteins and paraproteins which have reactivity for specific antigenic determinants. The Discussion and Summation section comments about the potential usefulness of homogeneous antibodies for examining the structure–function relationships of antigens and antibodies and for probing the genetic machinery which is responsible for antibody diversity.

II. Immunoglobulin Heterogeneity and Antibody Properties Indicative of Limited Heterogeneity

It is self-evident that a specific antibody is not a homogeneous protein unless it lacks all of the manifestations of immunoglobulin heterogeneity. These manifestations include: isotypic variation, charge heterogeneity, functional heterogeneity, and allotypic variability. Specific antibodies that are generated in response to most antigenic stimuli are heterogeneous as judged by all of these manifestations or by any combination of them. Individual antigenic specificity or idiotypy refers to the antigenic individuality of a myeloma protein or a uniform antibody population. Heterogeneous immunoglobulins lack this special characteristic.

A. Immunoglobulin Classes, Subclasses, and Light-Chain Types

Five major classes of immunoglobulins have been recognized in the human (IgG, IgA, IgM, IgD, and IgE), and their distinguishing features have been recently reviewed (Franklin and Frangione, 1969; Grey, 1969). Subclasses are described for IgG, IgA, and IgM. Each of these classes and subclasses possess either κ or λ light chains, but the distinguishing characteristic of the class or subclass is the chemical structure

of the heavy chain. These characteristic heavy chains have been designated γ, α, μ, δ, and ϵ for IgG, IgA, IgM, IgD, and IgE, respectively. It is obvious that an antibody is not uniform or homogeneous unless it belongs to only one class and subclass of immunoglobulin and that its light chains are either κ or λ. In some cases, it is possible to isolate a specific antibody from an antiserum that consists of only one class. But, an antibody preparation, although all of one class, is commonly a heterogeneous mixture as judged by other criteria.

B. Immunoglobulin Charge Heterogeneity

The disperse distribution of the γ-globulins by electrophoresis is an indication of charge heterogeneity. All the classes exhibit this heterogeneity. In contrast, each myeloma protein possesses a marked uniformity of charge which results in an extremely restricted electrophoretic mobility. Most purified antibodies exhibit charge heterogeneity, although some preferential selection has been observed by Nussenzweig and Benacerraf (1967) for certain antibodies in guinea pigs and by Sela (1967) for rabbit antibodies to synthetic polypeptides. In neither case, however, was this restriction in charge as narrow as that seen for the myeloma proteins, and in both instances the antibodies were heterogeneous by other criteria. Disc electrophoresis in acrylamide gel of reduced and alkylated immunoglobulins and of most of the specific antibody preparations reveals a number of distinct fractions for both the light and the heavy chains (Reisfeld and Small, 1966). These individual fractions have been isolated, and analysis revealed differences in amino acid composition (Reisfield, 1967).

Charge heterogeneity of the immunoglobulins facilitates their separation into fractions by chromatography. Feinstein (1964) separated rabbit IgG into four chromatographic fractions; each successive fraction from the column had an electrophoretic mobility greater than the previous one. The light- and heavy-chain mobilities for the four chromatographic IgG fractions were also compared. Each possessed heavy chains with identical mobility, but the light-chain mobility was parallel to that of the intact IgG chromatographic fraction from which it was derived. Studies, to be described later, for specific rabbit antibodies are largely in agreement with these findings.

Taken altogether, these studies indicate that the charge heterogeneity of an antibody is an indication of molecular heterogeneity. It is unlikely that an antibody is uniform unless the light chains migrate as one major component by disc electrophoresis. This certainly appears to be the case for rabbit antibodies to streptococcal carbohydrates (Eichmann *et al.*, 1970a). The one qualification of this generalization stems from the work

of Awdeh *et al.* (1967). Bence-Jones proteins isolated from cells gave a single band on starch gel electrophoesis, whereas this protein incubated in serum gave several bands. Presumably, some alteration of the protein occurred in the serum which resulted in charge heterogeneity. Furthermore, charge heterogeneity of mouse myeloma light chains is related to the sialic acid content (Melchers *et al.*, 1966).

Although the light chains of a myeloma protein will commonly migrate in one major band on polyacrylamide disc electrophoresis, the pattern is more complex with respect to the heavy chains. The heavy chains migrate in at least several bands (Dorner *et al.*, 1969). Such heterogeneity may be a reflection of variability in carbohydrate content and not due to heterogeneity in the amino acid sequence. The heavy-chain pattern of the myeloma proteins is less complex than that of normal γ-globulin, however. Normal heavy chains migrate in at least ten bands.

C. Heterogeneity of Antibody-Combining Sites

Antibodies against simple antigens or haptens exhibit heterogeneity in the characteristics of the combining sites. Such heterogeneity can be detected in two different ways. First, the antibodies vary in the affinity for the antigen and, second, they vary in the size of the antigenic determinant to which they bind. Affinity heterogeneity has been studied in detail for rabbit antibodies to 2,4-dinitrophenyl (DNP) (Eisen, 1966). When DNP groups on protein substances are employed as antigens, the antibodies exhibit a wide range of binding energy to DNP. Furthermore, there appears to be combining site heterogeneity as a function of time after immunization (Eisen and Siskind, 1964). The average binding constants revealed a progressive increase from about 10^5 L/M for antibodies recovered 2 weeks after a small immunizing dose to about 10^8 L/M for antibodies recovered 8 weeks after this dose. This change in affinity remains to be explained.

Variability in the binding of antibody to haptenes of different sizes has been shown for human antidextran and anti-blood-group substances. For example, antidextran antibody was recovered from a Sephadex column by sequential elution with oligosaccharides of increasing length (Schlossman and Kabat, 1962). Precipitation of dextran by the antibody eluted with the larger hapten was inhibited by the larger oligosacchardies, whereas di- and trisaccharides were less effective. Similar studies have been reported recently for human antibodies to blood group A substance (Moreno and Kabat, 1969). In this case, the antibody was recovered from an immunoabsorbent column by elution with *N*-acetylgalactosamine and by a pentasaccharide. *N*-Acetylgalactosamine did not inhibit precipitation between the blood group A substance and the anti-

body recovered with the pentasaccharide. On the other hand, *N*-acetylgalactosamine did inhibit the precipitation when the antibody employed was that recovered by *N*-acetylgalactosamine elution. It is suggested that the antibodies eluted by *N*-acetylgalactosamine possess a combining site which is smaller in size than that of those antibodies eluted with the pentasaccharide.

D. Immunoglobulin Allotypes

Allotypic variation of the immunoglobulins is due to intraspecies antigenic differences which are inherited in a simple Mendelian manner (Oudin, 1966). In certain cases, these antigenic differences have been shown to be associated with variations in the amino acid sequences for both the light and heavy chains and were first described by Oudin (1956) in rabbit, by Grubb (1956) in man, and by Herzenberg (1964) in mouse. The localization of these allotypic antigenic markers to certain specific amino acid sequence regions of each chain has been a subject of much interest because the outcome has ramifications in regard to genetic theories of antibody formation. All of this and the associated controversy have been reviewed elsewhere (Herzenberg, 1964; Hood *et al.*, 1967; Baglioni *et al.*, 1968; Natvig *et al.*, 1968; Koshland, 1968; Wilkinson, 1969; Prahl *et al.*, 1970; Hood and Talmadge, 1970; Vice *et al.*, 1969; Dubiski, 1969).

The allotypic markers of rabbit IgG and the localization of each on either the light or the heavy chain are identified in Fig. 1. Such a diagram has been constructed from the information in the references cited above. As a general rule, specific antibodies recovered from heterozygous rabbits possess both allotypes for each of the groups for which they are heterozygous, although a modest to a marked selective shift in the proportion of the markers has been observed (Catty *et al.*, 1969).

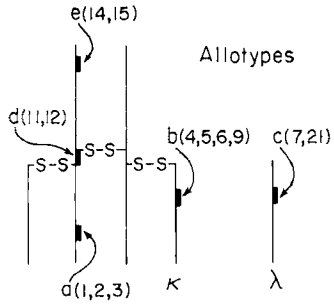

Fig. 1. Schematic diagram of γ-globulin and the approximate location of the allotypic markers. (Modified after Kindt, 1970.)

E. Immunoglobulin Individual Antigenic Specificity

Individual antigenic specificity was initially described by Kunkel (1965) and his colleagues as a property of the myeloma proteins. This property is a reflection of the antigenic individuality of the variable region of the protein and is, therefore, confined to the Fab portion. Because of this individuality, the myeloma protein induces an anti-antibody in a heterologous species which after absorption with pooled human Fraction II reacts specifically with the myeloma employed for immunization (Grey *et al.*, 1965). One interpretation of these studies is that each antigenically distinct myeloma protein is representative of a normal γ-globulin molecule and that the total pool of normal γ-globulin in the serum is the summation of a very large number of different molecules or species each bearing one of these unique antigenic specificities (Pernis, 1968). Although examination of many human paraproteins has revealed only occasional instances of cross-specificity (Williams *et al.*, 1968), most theoretical estimates suggest that normal serum contains γ-globulin of such antigenic diversity that the number of different molecules, each with a distinctive individual antigenic specificity, exceeds several thousand (Pernis, 1968; Kunkel, 1970). Such a large number is consistent with the possibility that there are between 1000 and 10,000 different heavy chains, each with distinctive features in the amino acid sequences, and an equally large number of light chains. The many possible combinations of these light and heavy chains to form a complete molecule is a very large number.

Parallel studies on antigenic specificity have also been done on a selected small number of human antibodies which, by several critiera, exhibit restricted heterogeneity (Kunkel *et al.*, 1963). Such antigenic individuality is also a manifestation of a specific antigenic determinant associated with the variable region of the molecule.

Antigenic individuality of specific antibodies has been detected in a somewhat different way by Oudin and Michel (1963) and by Kelus and Gell (1968; Gell and Kelus, 1967). In these studies, the anti-antibodies are raised in rabbits that have the same allotypy as the donor rabbit. Such antigenic individuality has been termed "idiotypy." Perhaps the most troublesome obstacle for an unambiguous interpretation of these studies on idiotypy is that clearly defined and isolated antigens and antibodies have not been employed in many of these experiments (Oudin and Michel, 1969a,b). Recently, however, idiotypy has been described by Daugharty *et al.* (1969) for antibenzoate antibodies in rabbits. In this case, the phenomenon of idiotypy has been demonstrated with isolated and well-defined antigens and antibodies. As was noted above,

there has been a good deal of speculation on the biological significance of individual antigenic specificity, and similar discussion surrounds idiotypy. Setting aside these theoretical considerations, from the practical point of view, the demonstration that all of the molecules in an antibody preparation possess the same individual antigenic specificity or idiotypy is probably the single most useful criterion which is indicative of molecular uniformity short of amino acid sequence analysis.

F. Immunoglobulin Amino Acid Sequence

Final, irrefutable proof for either heterogeneity or homogeneity of immunoglobulins and specific antibodies is obtained from amino acid sequence data. It must be admitted, however, that appreciable degrees of heterogeneity may escape detection because the methods employed for complete amino acid sequence analysis are not quantitative. Despite such reservations, much has been learned about the degree of homogeneity of antibodies and myeloma proteins through an examination of either partial or complete sequences. The magnitude of the labor to obtain a complete sequence of a homogeneous protein has spurred on the employment of indirect approaches to determine the degree of heterogeneity of an immunoglobulin or antibody preparation. It is far easier, for example, to determine the κ- and λ-chain composition by serological means than by amino acid sequence of the C-terminal portion of the light chains.

N-terminal amino acid analysis of the light chains of normal rabbit IgG and human Bence-Jones proteins leaves no question about the heterogeneity of the former and the homogeneity of the latter (Hood *et al.*, 1969). Typical, quantitative, Edman analysis data are presented in Table I. Tabulated here are the results of the quantitative Edman analysis for the first three N-terminal residues of the light chains of normal IgG recovered from a single rabbit and a human Bence-Jones protein. The amount of each amino acid at each position has been determined from a HCl hydrolyzate of the PTH[1a] amino acids (Van Orden and Carpenter, 1964). The per cent yield has been calculated from the nanomoles of the specific amino acid and the total nanomoles of all the amino acids recovered at a particular sequence step. The per cent recovery at each position has been calculated from the nanomoles of all the amino acids at each sequence step and the nanomoles of the dry weight protein used for the Edman procedure. It is obvious that for each of the first three N-terminal positions, several major amino acid alternatives exist for the nonimmune IgG of a single rabbit, whereas one major amino acid is present for each position in the Bence-Jones protein. Studies of a qualitative nature, but similar in design, by Doo-

[1a] Phenylthiohydantoin.

TABLE I

AMINO ACID RESIDUES AT N-TERMINAL SEQUENCE POSITIONS 1, 2, AND 3 FOR THE LIGHT CHAINS OF PREIMMUNE RABBIT γ-GLOBULIN AND FOR THE CONTROL BENCE-JONES PROTEIN (Hackney)[a,b]

	Position 1		Position 2		Position 3	
Light chains	Yield (%)	Recovery (%)	Yield (%)	Recovery (%)	Yield (%)	Recovery (%)
Rabbit R22-85 preimmune	Ala 54 Asp 17 Val 7 Ile 6	70	Asp 34 Val 28 Gly 15 Glu 5	18	Val 28 Asp 19 Ile 18 Met 5	39
Bence-Jones protein (Hackney) analysis 1	Glu 100	33	Ile 89 Gly 6	27	Val 82 Gly 5	50
Bence-Jones protein (Hackney) analysis 2	Glu 96 Gly 2 Asp 2	63	Ile 67 Gly 17 Glu 6	47	Val 85 Gly 7	55

[a] Quantitative Edman procedure employed. The per cent *yield* of each amino acid at each position has been calculated from the nanomoles of the specific amino acid and the total nanomoles of all the amino acids recovered at that particular sequence step. The per cent *recovery* at each position has been calculated from the nanomoles of all amino acids at each sequence step and the dry weight of protein used for the Edman procedure. Except for position 1, residue yields of 4% or less are not listed.

[b] From Hood *et al.*, 1969.

little (1965) have shown that there are just as many amino acid alternatives for each of the first six N-terminal positions for specific isolated, rabbit antibodies to DNP as for rabbit IgG. Such findings are an indication that specific antibodies can have a heterogeneity indistinguishable from normal IgG. Similar evidence will be reviewed later which suggests that the light chains of certain antibodies have a single amino acid present at each of the first three N-terminal positions and in this respect resemble the Bence-Jones proteins.

It is not within the scope of this review to explore in greater detail the structural and functional heterogeneity of antibodies or to speculate on the biological significance of heterogeneity. It is conceivable, of course, that the excessive effort expended to achieve antibody heterogeneity is, in fact, an affidavit that life will be preserved. As Eisen (1966) has pointed out, diversity of ligand-binding sites within an antibody population assures a wide range of cross-reactivity for antibody molecules of a particular specificity. Thus, the more heterogeneous the immune response to viruses and bacteria, the more broadly based the spectrum of acquired resistance. A heterogeneous immune response to one set of virus antigens, for example, may broaden the acquired im-

munity against related viruses and against new viruses arising from the natural unrelenting mutability of such microbes.

III. Human Antibodies with Restricted Heterogeneity

Cited in the literature are numerous examples of specific antibodies that exhibit much less heterogeneity than the total complement of the immunoglobulins in the antiserum from which the antibodies are isolated. This literature has been included in reviews on immunoglobulins by Fleischman (1966) and Haber (1968) and such documentation need not be repeated here.

Early in the 1960's, studies on human antibodies to various carbohydrates suggested that carefully selected human antibody populations might be less heterogeneous than had been commonly supposed. Individual antigenic specificity was demonstrated by Kunkel *et al.* (1963) for antihuman blood group A, anithuman blood group B, antidextran, antilevan, and antiteichoic acid antibodies isolated from human sera. A typical experiment is depicted in Fig. 2. The antibody to blood group A reacted specifically with its absorbed anti-antiserum. No cross-reaction occurred with four γ-globulin preparations of different types or with seven heterologous isolated anti-A antibodies. Subsequent studies revealed that the light chains of some of these antibodies migrated in one major band in 8 *M* urea starch gel electrophoresis (Edelman and Kabat, 1964). The distribution of the γ-globulin genetic factors, Gm (a), Gm(b), and Inv (a), was studies for the isolated antibodies against

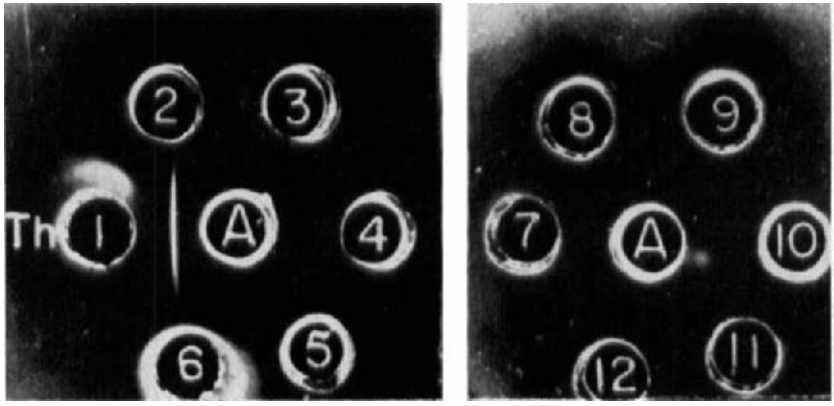

FIG. 2. Agar plate analysis showing the specific reaction of isolated anti-A antibody, Th, with anti-Th antiserum (A). Peripheral wells 2–5 contain γ-globulin preparations of different types, and wells 6–12 contain heterologous isolated anti-A antibodies. The antiserum (A) was absorbed with 5 mg. Fraction II per milliliter of serum. (From Kunkel *et al.*, 1963.)

dextran, levan, teichoic acid, and blood group A substances (Allen *et al.*, 1964). Although the majority of these antibodies contained all of the genetic factors determined in the donor's whole γ-globulin, in a number of the antibodies these factors were present at very different concentrations. Most important, in a few instances, specific factors were not detected despite their presence in the individual's whole γ-globulin. In these cases the distribution of the genetic factors appeared to approach the selective occurrence of these factors in myeloma proteins.

These studies on human antibodies were consistent with findings of a similar nature reported somewhat earlier by Gell and Kelus (1962). They observed the absence of one and possibly two allotypes in an antihapten antibody isolated from a rabbit, and Rieder and Oudin (1963) demonstrated alterations in the normal ratios of various allotypic markers in isolated antibodies to bovine serum albumin and DNP–bovine γ-globulin. Taken together, such findings raised the hopeful question, "Are specifically purified antibodies of an individual, like pathological proteins, discernably less heterogeneous than his total γ-globulin?" (Edelman and Kabat, 1964). If such proves to be the case, Edelman and Kabat went on to comment, "then the analogy of these antibodies to myeloma proteins and Bence Jones proteins may have useful application in detailed chemical studies of antibodies" and, in particular, may prove of value for the "purpose of determining the amino acid sequence of the structure of their combining sites." Such objectives have sustained the search for the means to procure in a reproducible and predictable fashion, antibodies with molecular uniformity.

Recent studies by Yount *et al.* (1968) indicate that the human anti-levan referred to above (Allen *et al.*, 1964) is a remarkably uniform antibody. It consists exclusively of γG_2 heavy chains and κ light chains and exhibits, as detected by quantitative assays, a selective absence of genetic markers.

It is also clear from these studies of Yount *et al.* (1968) that an antiserum may possess several sets of antibodies against a single antigen. For example, the degree of homogeneity of antibodies to dextran recovered from the serum of one individual was progressively enhanced by subfractionation of the antibodies on the basis of antibody specificity for the glycosidic linkage or combining site size rather than by physicochemical methods. An isomaltohexose eluate of dextran γG_2 antibody from two subjects was primarily κ, and isomaltotriose eluate was primarily λ.

The impression which is gained from all of these studies is that human antibodies to carbohydrates may possess, to a surprising degree, properties which are indicative of restricted heterogeneity. This raised the possibility that antibodies to carbohydrates, produced in experi-

mental animals, might have much less heterogeneity than the antibodies to the commonly employed synthetic haptens. The use of rabbits was prompted by the knowledge that they may respond with high levels of antibodies to carbohydrate antigens following intravenous immunization with bacterial vaccines, most notably streptococcal and pneumococcal vaccines. A major consideration is the supply of antibody which can be obtained from animals. If homogeneous antibodies are to be recovered from experimental animals in sufficient yield for structural studies, immunization must lead to antibody concentrations which are similar to the concentrations of the myeloma proteins in man.

IV. Experimental Generation of Antibodies with Restricted Heterogeneity

A. Rabbit Antibodies to Streptococcal Carbohydrates

It is apparent from the discussion thus far that immunization with any one of several bacterial antigens might provoke antibodies with restricted heterogeneity. The idiotypy of antibodies in the antisera of rabbits immunized with salmonellae suggests, for example, that these antibodies have less heterogeneity than the nonimmune γ-globulin. Use of the streptococci, however, to generate a restricted immune response has several advantages over the salmonellae. First, the immunochemistry of the streptococcal carbohydrate antigens is less complex than that of salmonellae. Sceond, antisera of rabbits immunized with streptococcal vaccines had been known to contain in some instances very high concentrations of antibody to the carbohydrate. Initial studies indicated that in certain rabbits, antibodies to the carbohydrates had an electrophoretic uniformity and a serum concentration in many ways as remarkable as that of the myeloma proteins (Osterland *et al.*, 1966). Recent evidence from other laboratories suggests that this is also the case for rabbit antibodies to Type III and Type VIII pneumococcal capsular polysaccharide (Haber, 1970; Pincus *et al.*, 1970a,b).

Considerable detailed information is now available on the rabbit immune response to streptococcal carbohydrates, and a condensed review of this work will be presented here. The points to be emphasized include: the method of immunization; the immunochemistry of the streptococcal carbohydrate antigens; the immune response; and evidence for the molecular uniformity of the antibody.

1. Method of Immunization

A good deal of experience, much of it empirical, indicates that the highest levels of antibodies to the streptococcal carbohydrate are achieved in the rabbits by intravenous immunization with a vaccine of

whole heat-killed streptococci (Lancefield, 1928). Peritoneal immunization is much less effective. Little or no antibody is formed if the soluble carbohydrates alone are employed as antigens.

Depicted in Fig. 3 are schematic representations of a living Group A *Streptococcus* and a heat-killed pepsin-treated vaccine of this organism (Krause, 1963, 1970).[2] The living *Streptococcus* possesses an outermost

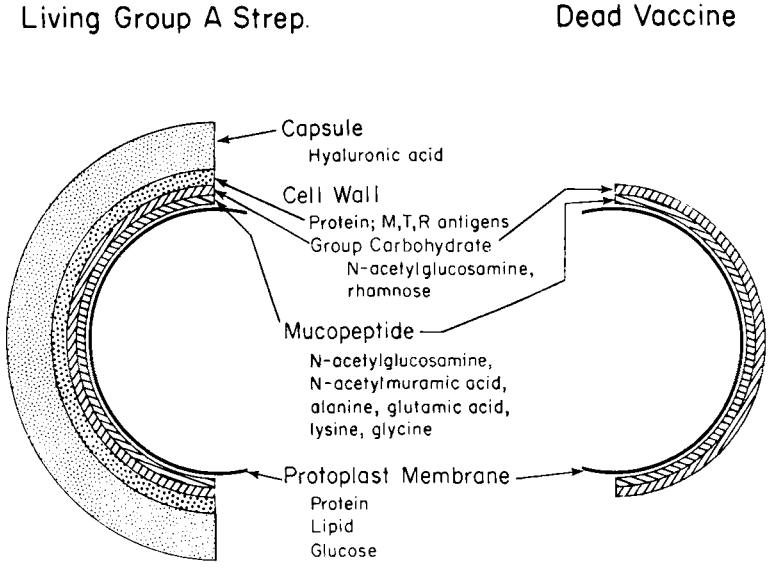

FIG. 3. Schematic diagram of living Group A streptococci and the dead vaccine prepared from them. (From Krause, 1970.)

[2] There is no recent readily available description of R. C. Lancefield's current method to prepare group-specific vaccines. For this reason, it is included here.

Vaccines are prepared from Group A streptococci, strain J17A4; Group A-variant streptococci, strain A486 (M-); and Group C streptococci, strain C74. The strains are from the collection of Dr. R. C. Lancefield, Rockefeller University, New York. A large batch of vaccine is prepared as follows: 1500 ml. of overnight Todd–Hewitt culture is collected by centrifugation and the cells washed several times with sterile saline. The bacterial sediment, resuspended in 30 ml. of sterile saline, is heat-killed at 56°C. for 45 minutes. The bacteria are again collected by centrifugation, resuspended in approximately 30 ml. of saline, adjusted to pH 2 with HCl and 75 mg. of pepsin is added. This mixture is incubated for 2 hours at 37°C., neutralized with NaOH to pH 7, centrifuged, and the supernatant discarded. The bacterial pellet is washed by resuspension in 30 ml. of sterile saline, and centrifuged. The supernatant is discarded. This washing procedure is repeated an additional time, and the final bacterial pellet is resuspended in 90 ml. of sterile saline. The final product is cultured to check for sterility. The dead streptococci in 1 ml. of this vaccine contain approximately 0.4 to 0.6 mg. of group-specific carbohydrate.

hyaluronic acid capsule, but this is washed away during the preparation of the vaccine. Pepsin removes the bulk of the surface protein antigens, M, T, and R (Lancefield, 1962). What remains and what is injected intravenously is intact heat-killed streptococci with a superficial cell wall surface of carbohydrate antigen which masks the underlying mucopeptide matrix (Krause and McCarty, 1961). Less than 10% of the dry weight of the vaccine consists of carbohydrate. The remainder consists of all the other cellular elements which have not been eliminated during vaccine preparation. Yet, it is a remarkable fact that as much as 95% of the γ-globulin in immune antisera is antibody to the outermost carbohydrate antigen (Braun *et al.*, 1969).

Rabbits are immunized intravenously 3 times a week for 4 weeks. With each injection, 0.5 ml. of vaccine is given the first week; thereafter 1 ml. is injected. Maximum antibody levels are seen between the fourth and eighth day after the last injection, as determined by quantitative precipitin tests with the purified Group C carbohydrates (Fleischman *et al.*, 1968; Braun *et al.*, 1969). Although excellent antibody levels may be achieved with the primary immunization, many rabbits require a second series of injections after an interval of 3 to 5 months. The second series of injections is similar to the first, except that maximum antibody levels are usually achieved with only 3 weeks of injections.

2. *Immunochemistry of Streptococcal Carbohydrate*

The immunochemistry and chemistry of the carbohydrate of Group A streptococci has been studied in details by McCarty and others, and

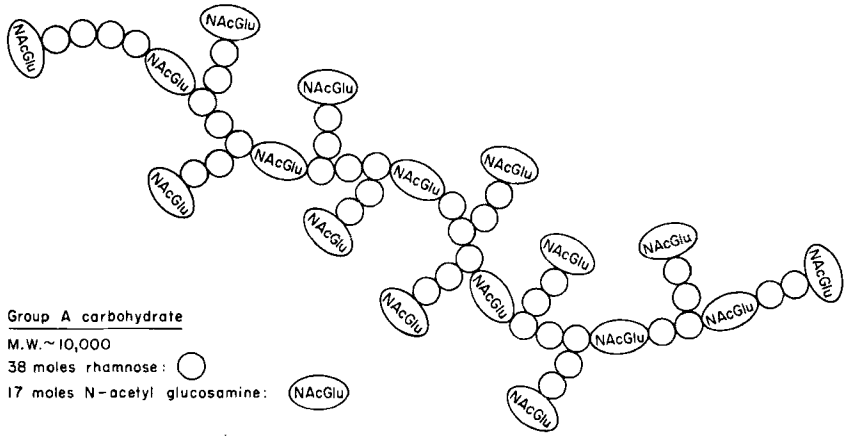

FIG. 4. Schematic diagram of the chemical structure of the Group A carbohydrate antigen extracted from Group A streptococci. (From Krause, 1970.)

this work has been reviewed elsewhere (McCarty and Morse, 1964). The picture which emerges for the structure of this carbohydrate is depicted in Fig. 4. The antigen extracted from the cell wall by chemical means is undoubtedly heterogeneous with respect to size, but, certainly, a portion of the extracted material has a molecular weight in the range of 10,000. A mole of such antigen possesses 17 moles of *N*-acetylglucosamine and 38 moles of rhamnose. Eleven of the 17 moles of *N*-actylglucosamine can be selectively stripped off with *β*-*N*-acetylglucosaminidase, leaving a residual polymer which is predominantly rhamnose. McCarty (1958) has shown that the terminal *β*-*N*-acetylglucosaminide residues are the immunodominant determinants of specificity. Companion carbohydrates to this one of Group A have been isolated from Group A-variant (McCarty, 1956) and Group C streptococci (Krause and McCarty, 1962). Each of these antigens are isolated from either the whole bacteria or cell walls by methods described by Krause and McCarty (1961) and modified by Krause (1967). The evidence suggests that a similar rhamnose polymer is shared by each, but, in the case of the Group C carbohydrate, terminal *N*-acetylgalactosaminide residues confer antigenic specificity; whereas, in the case of the

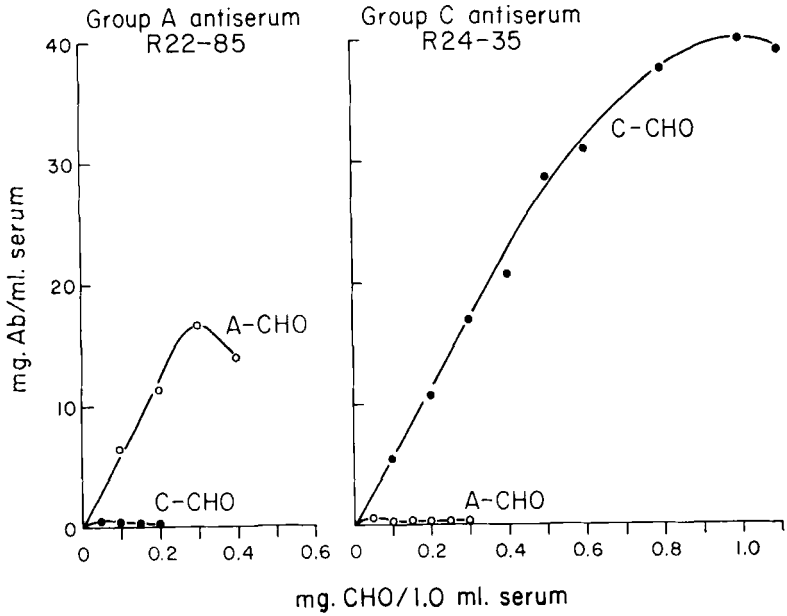

FIG. 5. Quantitative precipitin reactions between Groups A and C streptococcal carbohydrates and the homologous and heterologous group-specific antisera. (See Braun *et al.*, 1969, for details of the method.)

Group A-variant, the antigenic specificity is dependent upon the rhamnose moiety itself. The antigenic specificity of this rhamnose moiety for both Groups A and C carbohydrates is masked by the terminal amino sugar residues.

The specificity of the streptococcal antisera for the homologous carbohydrates is illustrated by the quantitative precipitin data in Fig. 5. Group A antibody gives no appreciable cross-reaction with Group C antigen and vice versa; Group C antibody gives no significant cross-reaction with the Group A antigen (Krause and McCarty, 1962). Recent evidence from Kabat and co-workers (1970) and from Greenblatt and

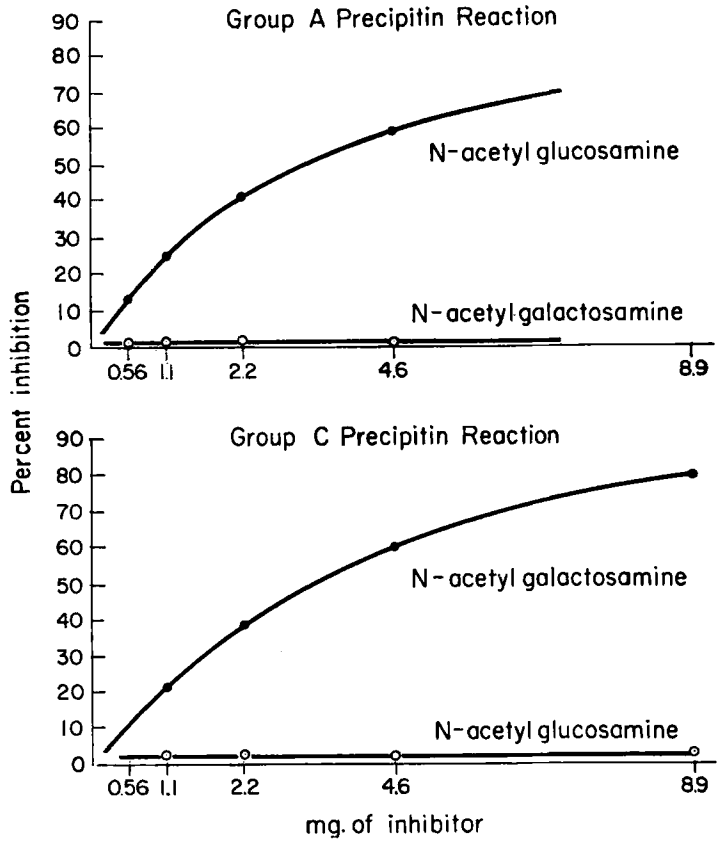

FIG. 6. Inhibition of the precipitin reaction between Groups A and C carbohydrates and their antisera with *N*-acetylglucosamine and *N*-acetylgalactosamine, respectively. The results are expressed as per cent inhibition. All reactions were performed at equivalence of antigen and antibody. (From Krause and McCarty, 1962.)

Krause (1970) indicate that the terminal *N*-acetylgalactosaminide residues of the Group C carbohydrate are α-linked to the subterminal rhamnose—a feature which probably accounts for the remarkable lack of cross-reactivity between Groups A and C carbohydrates. If the terminal *N*-acetyl amino sugars were linked in a similar fashion in both cases (i.e., either both α or both β), it is probable that greater cross-reactivity would be detected.

The streptococcal carbohydrate precipitin reactions are readily inhibited by the characteristic *N*-acetyl amino sugar. Inhibition tests, depicted in Fig. 6 (Krause and McCarty, 1962) were carried out at antigen–antibody equivalence. With these particular antisera, 50% inhibition was achieved with approximately 3 mg./ml. of *N*-acetylglucosamine in the case of Group A precipitin reaction and no inhibition was observed with *N*-acetylgalactosamine. Likewise, 3 mg./ml. of *N*-acetylgalactosamine inhibited Group C precipitin reaction and no inhibition was achieved with *N*-acetylglucosamine. Such a striking inhibition of the precipitin reaction with a monosaccharide has served as the basis for recovery of specific antibody from immunoabsorbents by elution with *N*-acetyl amino sugars. This procedure will be described in a later section.

3. *Evidence for Restricted Heterogeneity of Antibodies to Streptococcal Carbohydrates*

The initial observations of Osterland *et al.* (1966) suggesting that antibodies to streptococcal carbohydrates may have a remarkable molecular uniformity are noted in Figs. 7 and 8. In Fig. 7 are depicted the microzone electrophoretic patterns of serum from a rabbit prior to immunization and an antiserum collected from the same rabbit after 4 weeks of immunization with Group A-variant vaccine. A sharp narrow band is seen in the γ-globulin region. The protein in this band is predominantly antibody to the group-specific A-variant carbohydrate. Im-

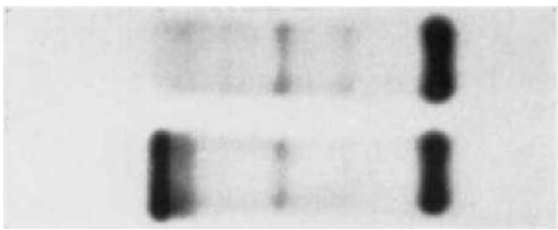

FIG. 7. Microzone electrophoresis of a rabbit serum collected prior to immunization (top frame) and of an antiserum collected after completion of 4 weeks of intravenous immunization with Group A-variant streptococci. (From Osterland *et al.*, 1966.)

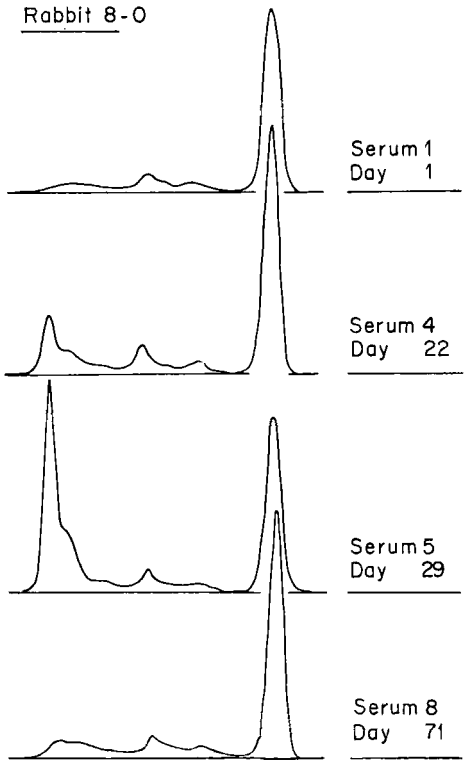

FIG. 8. Tracings of the densitometric scans of microzone electrophoretic patterns on sera of a rabbit collected prior (serum 1), during (serum 4), just after completion (serum 5), and 42 days after completion (serum 8) of 4 weeks of intravenous immunization. Sera 1 and 5 were depicted in Fig. 7. (From Osterland *et al.*, 1966.)

munoelectrophoresis reveals that it is entirely IgG. It is a 7 S protein as determined in the analytical ultracentrifuge.

The transient nonmalignant nature of this response is indicated by the immunoelectrophoretic patterns on serial sera collected well after immunization. As is noted in Fig. 8, during the interval between day 29 and day 79, at which time no vaccine was administered, the antibody peak receded and the γ-globulin concentration approached the preimmune level.

Initial evidence that antibodies in an antiserum such as this possessed restricted heterogeneity included the monodisperse electrophoretic distribution of the light chains in alkaline urea starch gel and polyacrylamide disc electrophoresis. Subsequent to these initial observations, several antibodies have been examined in much greater detail for

evidence of molecular uniformity (Eichmann *et al.*, 1970a; Hood *et al.*, 1969; Miller *et al.*, 1967; Davie *et al.*, 1968; Fleischman *et al.*, 1968; Braun and Krause, 1968). Observations on one of these antibodies will be reported in detail here.

Depicted in Fig. 9 are microzone electrophoretic patterns of a preimmune serum and a Group C antiserum R27-11 before and after absorption with Group C carbohydrate (Eichmann *et al.*, 1970a). The sharp monodisperse component contained 36 mg./ml. of γ-globulin; 93% of this is precipitable with the C carbohydrate. This monodisperse antibody component was isolated from the antiserum by preparative agarose electrophoresis. Such a preparative run is also depicted in Fig. 9. Only the values for the protein eluted from the block in the region of the

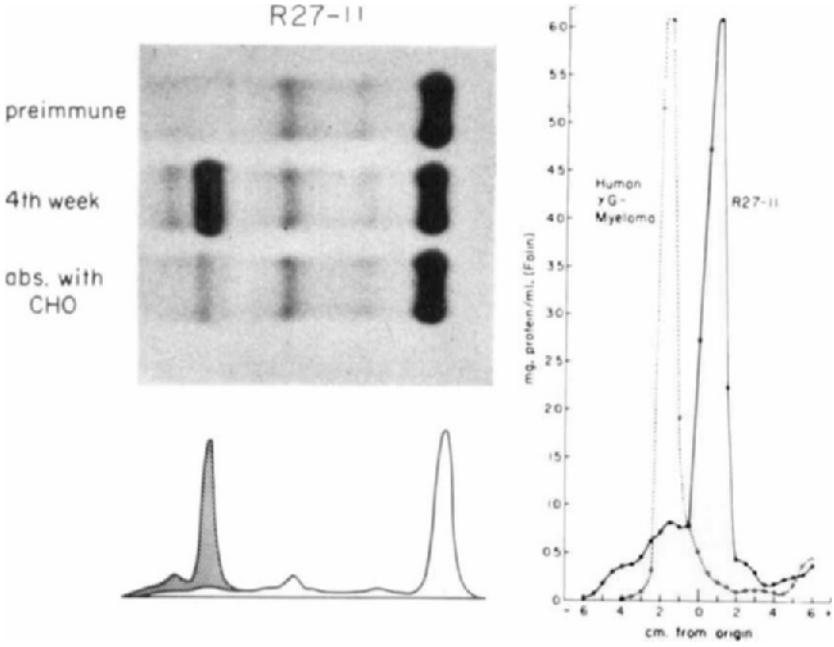

FIG. 9. On the left, microzone electrophoretic patterns of preimmune serum and antiserum from rabbit R27-11 after 4 weeks immunization with Group C vaccine. The pattern of this serum after absorption (abs) with Group C carbohydrate (CHO) is also shown. Below the microzone patterns are the densitometric patterns of the immune serum before and after absorption with carbohydrate. The shaded area in the lower diagram represents the antibody absorbed from the antiserum. On the right, preparative agarose electrophoresis of antiserum R27-11 (solid line) and, for comparison, the same electrophoresis of a human serum from a patient with multiple myeloma. (Adapted from Eichmann *et al.*, 1970a.)

γ-globulin are shown. For comparison, there is shown also the pattern of serum of a patient with multiple myeloma. Fractions of the block in the region of the peak component were pooled and concentrated. This material was employed for subsequent studies which indicate molecular uniformity.

In Fig. 10 are the 9.4 *M* urea disc electrophoretic patterns of the following partially reduced and alkylated proteins. [These gels were specially prepared to resolve the light chains (Reisfield and Small, 1966).] Gel 1 contains normal rabbit γ-globulin; gel 2 contains a Group C antibody for which there was evidence for only partial restriction in heterogeneity; gel 3 contains the very uniform antibody R27-11 isolated by preparative electrophoresis as described in Fig. 9; gel 4 contains human Bence-Jones protein. The densitometric tracings of these gel patterns are depicted in Fig. 11. Normal rabbit light chains are usually distributed in eight to ten bands. The antibody with only partial restriction of heterogeneity yields light chains that resolve into four distinct bands. The very restricted antibody R27-11, depicted in Fig. 10, has one major band which contains at least 90% of the total light-chain protein.

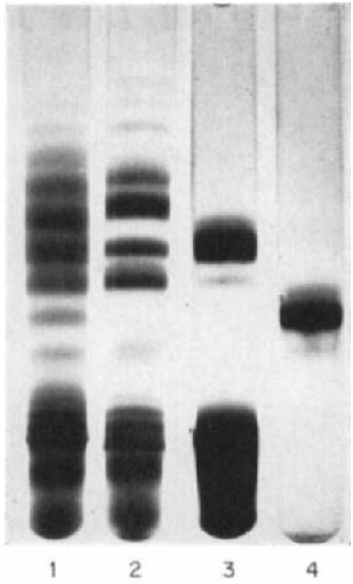

FIG. 10. Light chain patterns. Polyacrylamide gel disc electrophoresis, pH 6.74, in 9.4 *M* urea of reduced and alkylated γ-globulin preparations: gel 1, normal rabbit γ-globulin; gel 2, a Group C antibody with only partially reduced heterogeneity; gel 3, peak component of antiserum R27-11; gel 4, a human Bence-Jones protein. The direction of migration is from the bottom to the top. (From Eichmann *et al.*, 1970a.)

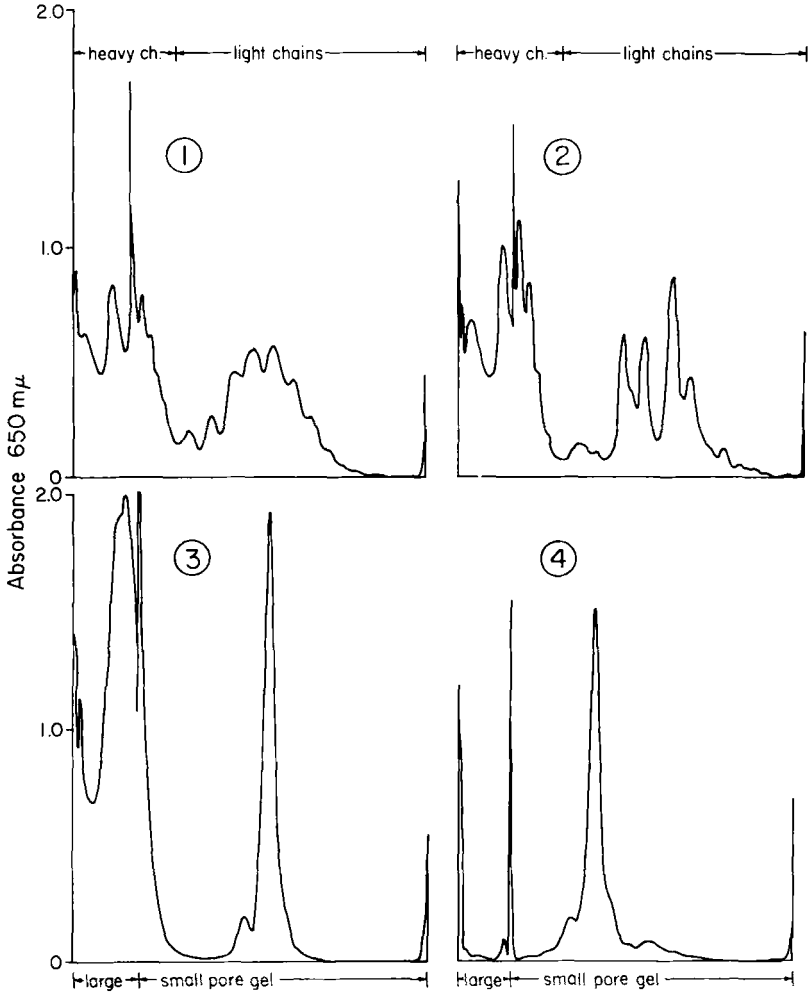

FIG. 11. Densitometric tracings of the disc electrophoretic patterns depicted in Fig. 5. Gel 1, normal rabbit γ-globulin; gel 2, a Group C antibody with only partially reduced heterogeneity; gel 3, peak component of antiserum R27-11; gel 4, a human Bence-Jones protein. The direction of migration is from the left to the right. (From Eichmann *et al.*, 1970a.)

This pattern is indistinguishable from that obtained with 40 μg. of a human Bence-Jones protein.

The disc electrophoretic gels, constructed to resolve the heavy chains, for two partially reduced and alkylated antibodies are depicted in Fig. 12. In these gels, the light chains have migrated beyond the gel

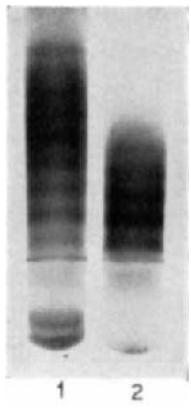

FIG. 12. Heavy chain patterns. Polyacrylamide disc electrophoresis, pH 6.74, in 9.4 *M* urea of reduced and alkylated γ-globulin preparations: gel 1, a Group C antibody with only partially reduced heterogeneity; gel 2, peak component of antiserum R27-11. The direction of migration is from the bottom to the top. (From Eichmann *et al.*, 1970a.)

and are not seen in the photograph. Heavy chains of a heterogeneous antibody were resolved into ten bands, whereas the heavy chains of the very restricted antibody R27-11, described above, were confined to only four bands. Two of these bands appear prominent. Similar patterns may be seen with the heavy chains of a myeloma protein (Dorner *et al.*, 1969).

The case for uniformity of streptococcal antibodies which are monodisperse by electrophoresis is further supported by the demonstration that they have individual antigenic specificity. Experiments similar to that depicted in Fig. 2, which demonstrated individual antigenic specificity of human antibodies to blood Group A substance were also performed with the streptococcal antibodies (Braun and Krause, 1968). In all of these studies, the anti-antibodies have been prepared in goats.

The view that the antigenic specificity of these streptococcal antibodies is associated with the Fab fragment was substantiated by immunoelectrophoretic analysis which employed papain digests of a monodisperse antibody isolated by preparative agarose electrophoresis. Such an analysis is depicted in Fig. 13. The papain digest of rabbit pooled Fraction II gave two arcs with unabsorbed specific anti-antiserum. The precipitin arc toward the cathode is formed by the Fc fragment, and the arc toward the anode is formed by the Fab fragment. The situation is reversed for the precipitin arcs of the Fab and Fc fragments of the streptococcal antibody employed here. This is because this antibody exhibited a slow migration in electrophoresis and traveled a greater

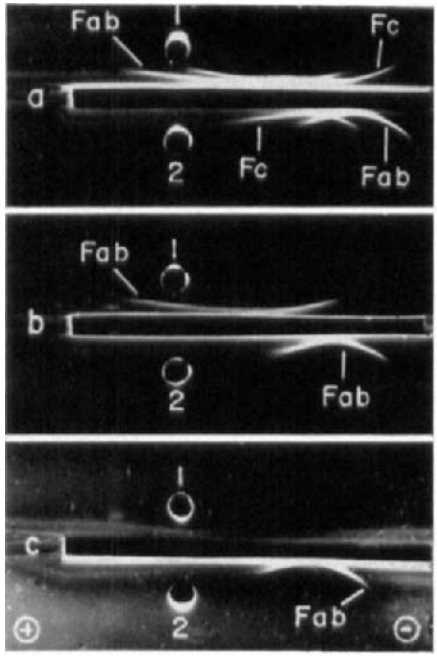

FIG. 13. Immunoelectrophoresis analysis of Fab and Fc fragments of a Group A-variant antibody with very restricted electrophoretic heterogeneity isolated from antiserum by preparative electrophoresis. Well 1, papain-digested pooled rabbit Fraction II; well 2, papain-digested isolated Group A-variant antibody. Trough a, unabsorbed goat anti-antibody to the Group A-variant antibody; trough b, the same anti-antibody absorbed with Fc fragments from pooled rabbit Fraction II. Trough c, the same antibody absorbed with Fraction II. (From Braun and Krause, 1968.)

distance toward the cathode than the bulk of the normal γ-globulin in pooled Fraction II. This interpretation was clarified by the results of immunoelectrophoresis in which either anti-antibody absorbed with Fc of pooled Fraction II or anti-antibody absorbed with pooled Fraction II was added to the troughs. When the anti-antiserum absorbed with Fc was added to the trough, only the Fab fragments of both the antibody and Fraction II gave precipitin arcs. When anti-antiserum absorbed with pooled Fraction II was added to the trough, the Fab fragments of only the antibody gave a precipitin arc. No reaction occurred between the Fab fragment of pooled Fraction II and this absorbed anti-antiserum. Taken together, such experiments indicate that individual antigenic specificity is a feature of the Fab fragment of this streptococcal antibody. Absence of precipitin reactivity between the light chain of this streptococcal antibody and the anti-antibody absorbed with Fraction II in-

dicates that the light chain alone does not determine the individual antigenic specificity. Similar results have been achieved with several other antibody preparations isolated by preparative electrophoresis.

Among the indirect criteria that have been used to judge the structural uniformity of antibodies, individual antigenic specificity is perhaps the one which is most indicative of a homogeneity similar to that observed for the myeloma proteins. Such a consideration stems from the fact that the antigenic site of antigenic individuality includes the hypervariable region of the γ-globulin molecule.

The case for individual antigenic specificity as a criterion for the molecular uniformity of an antibody is considerably strengthened when it can be shown that all, or at least a very major portion, of the antibody molecules react with the specific anti-antibody. Such a result has been shown in the following experiment. Aliquots of several antibody preparations, isolated by preparative electrophoresis, were labeled with ^{125}I and precipitated with an excess amount of the homologous anti-antiserum which had been absorbed at equivalence with Fraction II. Controls employed two absorbed heterologous anti-antisera. The proportion of the radioactive antibody in the precipitates and the supernatants were calculated as per cent of the total radioactive antibody used in the test. The results for antibody fractions from antisera R23-61, R22-79, and R27-11 are shown in Fig. 14 (Eichmann *et al.*, 1970b). The antibody from antiserum R27-11, already discussed in detail above, showed an exceptional degree of uniformity as judged by several criteria including distribution of genetic markers (Kindt *et al.*, 1970c) and N-terminal amino acid sequence of the light chain (Eichmann *et al.*, 1970a). Of this antibody preparation, 89% is precipitated by its individual anti-antiserum. A similar result is achieved with the fast fraction of antiserum R22-79. Since these antibody preparations were obtained by preparative electrophoresis alone, they contain approximately 10 to 15% nonspecific γ-globulin, which does not react with the absorbed anti-antibody. Only 70% of the slow fraction of antiserum R22-79 was precipitated by the anti-antiserum to the slow fraction. This result is consistent with other experimental data pointing to a certain degree of heterogeneity of this antibody component. It is apparent that experiments such as this afford an additional means to estimate the uniformity of an antibody preparation.

An important characteristic of myeloma proteins is the selectivity they show with respect to gene expression. Studies with the human allotypic markers (Gm and Inv types) have shown further that only one of two possible alleles for both the L and the H chains are expressed in the myeloma proteins in individual heterozygotes with respect to these markers (Martensson, 1961). Finally, only one of the many possible

The Proportion of an Antibody Preparation Precipitated by Homologous and Heterologous Anti-antisera

Goat anti-antibody absorbed with pooled rabbit Fraction II	Per cent antibody recovered in precipitate and supernatant							
	Antiserum R 22-79 (Fast Fx / Slow Fx)				Antiserum R 23-61 (Slow Fx)		Antiserum R 27-11 (Fast Fx)	
	Ppt. %	Supt. %	Ppt. %	Supt. %	Ppt. %	Supt. %	Ppt. %	Supt. %
R 22-79 Anti-fast Fx	91	8	2	97	-	-	-	-
R 22-79 Anti-slow Fx	4	98	70	31	4	95	2	99
R 23-61 Anti-slow Fx	-	-	3	95	80	21	1	99
R 27-11 Anti-fast Fx	2	100	-	-	1	99	89	8

FIG. 14. Per cent of rabbit antibody recovered in the precipitate (ppt.) and the supernatant (supt) calculated from the radioactivity. Each antibody preparation was labeled with ^{125}I. All precipitin tests done with an excess of anti-antibody. (From Eichmann *et al.*, 1970b.)

variants of each of these chain types is expressed (Hilschmann and Craig, 1965). A similar selectivity of expression was observed in some of the steptococcal antibodies formed in response to immunization with the streptococcal vaccine.

The allotype of the homogeneous antibody R27-11, which has been described in considerable detail thus far, is shown in Table II (Kindt *et al.*, 1970c). This rabbit was homozygous with respect to both the group a and group b allotypes. In the purified antibody, the H chain is selected from the population not possessing the allotypic marker from group a. In effect, no purified antibody was precipitated by antisera to the a1 marker. Oudin (1961) has shown that rabbits normally contain some γ-globulin molecules lacking the group a allotypic specificities. David and Todd (1969) have shown that it is possible to obtain rabbits devoid of immunoglobulin with the group a allotype by embryo transfer into does that produce antibody to the allotypic specificities genetically present in the transferred embryo. The absence of group a allotypic specificities in this uniform streptococcal antibody suggests that it is possible to obtain specific antibodies selected from this population which lack the allotypic marker just as individual myeloma proteins are believed

TABLE II

PRECIPITATION OF RADIO-IODINATED STREPTOCOCCAL GROUP C ANTIBODY AND IgG BY ALLOTYPIC ANTISERA[a]

Rabbit	Allotype	Sample	Precipitation by antisera (%)[b] a1	b4	Fc
R27-11	a1, b4	Preimmune IgG	62	93	97
		Group C antibody			
		Electrophoresis preparation[c]	10	99	92
		Purified antibody[d]	1	99	98
		Nonantibody IgG[e]	62	97	96

[a] From Kindt *et al.* (1970c).

[b] Determined by counting ^{125}I in the precipitate.

[c] Isolated as depicted in Fig. 9.

[d] Recovered from the electrophoretic antibody preparation by means of a Sephadex G-200 column which serves as a specific immunoabsorbent for this antibody.

[e] The nonantibody IgG in the electrophoretic antibody preparation which is not reactive with Sephadex in the group-specific C carbohydrate.

to result from enhanced production of an otherwise normal γ-globulin molecule. In additional studies with other purified streptococcal antibodies, which were isolated from a double heterozygous rabbit, a selective absence of a marker at both the a and b loci was observed. For example, from a rabbit which was typed a1, a3, b4, b5, A11, A12, the isolated Group C antibody was a1, b4, A12 (Kindt *et al.*, 1970a). This is consistent with previous work which has shown that group a allotypes share chains with A11 and A12 determinants (Prahl *et al.*, 1970), and these two groups of allotypes are linked genetically (Kindt *et al.*, 1970b; Zullo *et al.*, 1968). Recently, Rodkey *et al.* (1970) have isolated antibodies to streptococcal carbohydrate by electrofocusing. These antibodies, although isolated from a heterozygous rabbit, had a single allotypic specificity at both the a and b loci.

The case for molecular uniformity of antibody R27-11 is considerably strengthened by the amino acid N-terminal analysis of the light chains. In Table III are presented the results of the quantitative three-cycle Edman analysis of the amino acid alternatives at the first three N-terminal positions. The amino acid data from antibody R27-11 were compared to those of the preimmune γ-globulin of the same rabbit. Calculation of per cent yield and per cent recovery are also given in Table III. The data for preimmune γ-globulin and antibody R27-11 were derived from the amino acid analyzer chromatograms of the hydrolyzed PTH amino acids. In the case of antibody R27-11, the yields of a single predominant amino

TABLE III

AMINO ACIDS AT N-TERMINAL SEQUENCE POSITIONS 1, 2, AND 3 FOR THE LIGHT CHAINS OF PREIMMUNE RABBIT γ-GLOBULIN AND FOR ANTIBODY TO GROUP C STREPTOCOCCAL CARBOHYDRATE FROM RABBIT 27-11[a,b]

	Position 1		Position 2		Position 3	
Light chains	Yield (%)	Recovery (%)	Yield (%)	Recovery (%)	Yield (%)	Recovery (%)
Preimmune γ-globulin	Ala 43 Asp 14 Ile 10 Leu 10 Glu 7 Gly 5	46	Val 31 Asp 18 Glu 17 Tyr 8 Gly 7 Ala 5	25	Val 36 Asp 17 Glu 15 Ileu 11 Leu 11 Gly 10	8
Group C antibody	Ala 95 Asp (2)	48	Asp 85 Val 4 Leu 4	26	Val 92 Gly 8	10

[a] Quantitative Edman procedure employed. See footnote *a* to Table I for discussion of the calculation.

[b] From Eichmann *et al.*, 1970a.

acid at each of the first three N-terminal positions resembles those for the Bence-Jones protein (Hackney) which were given in Table I. It is obvious that there are multiple amino acid alternatives at the first three N-terminal positions in the preimmune γ-globulin light chains. Recently, the light chains of Group C antibody R27-11 have been examined in the sequenator. A single unambiguous amino acid residue was obtained at each of the first fifteen N-terminal positions, and the sequence is listed in Table IV (Hood *et al.*, 1970). Comment on the sequence data on the light chain of antibody from rabbit R24-61 will be reserved for the Discussion.

The case for molecular uniformity for the antibody in antiserum R27-11 can be summarized as follows: monodisperse distribution of the antibody by microzone and preparative zone electrophoresis; monodisperse distribution of the light chains by disc electrophoresis; allotype exclusion; and a single amino acid sequence for the first fifteen N-terminal residues of the light chains. In all respects by these various methods of examination, this antibody is as homogeneous as a myeloma protein. It remains to be determined if a single amino acid sequence is observed in the hypervariable region of the light chains. Such studies are now under way.

TABLE IV

COMPARISON BETWEEN N-TERMINAL AMINO ACID SEQUENCE OF ALLOTYPE b4 LIGHT CHAINS OF RABBIT ANTIBODIES TO STREPTOCOCCAL CARBOHYDRATES AND THE AMINO ACID RESIDUE ALTERNATIVES IN THE κ LIGHT CHAINS IN MAN AND MOUSE[a]

Rabbit and antibody preparation	Allo-type	N-Terminal position															
		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
R27-11; peak component	b4(k)	Ala	Asp	Val	Val	Met	Thr	Glu	Thr	Pro	Ala	Ser	Val	(Ser)	Glu	Pro	Val
R24-61																	
Fast component	b4(k)	( )	Ala	Phe Ile	Glx Val	Met	Thr	Glu	Thr	Pro	Ala	Ser	Val	Ser	Ala	Pro	Val
Slow component						Met		Met									
	b4(k)	( )	( )	Ile	Val	Val	Thr	Glu									
Man		Glu	Asp Glu Lys	Ile Val Met	Gln Val Leu	Met Leu	Thr	Gln	Ser Thr	Pro	Ala Ser Leu Gly Thr Asx	Ser Thr Phe	Leu	Ser Pro	Ala Leu Val Met	Ser Thr	Val Pro Leu
Mouse			Asp Glu	Ile Val Thr	Val Gln Thr Leu	Met Val Ile Leu	Thr	Gln	Ser Thr	Pro	Ala Ser Thr Leu	Ser Thr	Leu	Ser Ala	Val Ala Met	Ala Ser Thr	Ala Leu Ile

[a] Adopted from Hood *et al.*, 1970.

B. Rabbit Antibodies to Pneumococcal Capsular Polysaccharides

Intravenous immunization of rabbits with formalized pneumococci has been employed by Haber (1970) and Pincus *et al.* (1970a,b) for the generation of large quantities of antibodies with restricted heterogeneity. Rabbits immunized with the whole bacteria produce predominantly antibodies to the capsular polysaccharide. Pneumococci Types III and VIII were used for these studies. Immunochemical considerations dictated this choice. These are long-chain polymers and the sequence of the repeating sugars is known. These sequences are depicted in Fig. 15. Antibodies are

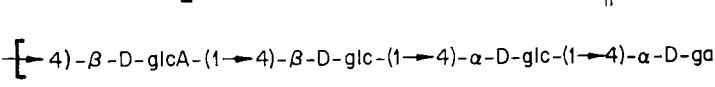

Fig. 15. Sequence of sugars in repeating subunits of pneumococcal polysaccharides. Types III (SIII) and VII (SVIII); glc = glucose; glcA = glucuronic acid; gal = galactose. (From Haber, 1970, adapted from Heidelberger, 1967.)

directed against a limited portion of such a sequence. Octasaccharides of these polysaccharides, for example, readily inhibit the precipitin reaction, and when such fragments are tritium-labeled, they can be used for binding studies to determine association constants (Pappenheimer *et al.*, 1968).

A primary, secondary, and tertiary immunization schedule, similar to that used for streptococci, was employed for pneumococcal immunization of rabbits. Approximately 6 to 8% of the rabbits had a predominant major antibody component in the antiserum. One of the Type VIII antibodies, isolated by immunoabsorbent methods to be described in the next section, has several of these properties of a myeloma protein which are indicative of uniformity. The light chains are distributed in one major band by disc electrophoresis; and a single amino acid sequence is observed for the first eleven N-terminal residues of the light chains (Waterfield *et al.*, 1970).

The binding of the tritium-labeled octasaccharides derived from the Type VIII carbohydrate to the antibody to Type VIII was examined by equilibrium dialysis, and the data are depicted in the Fig. 16 (Haber, 1970). The association constant is 2.5×10^5, a range commonly seen for antibodies to carbohydrates and for the antibodies to pneumococcal capsular polysaccharides (Pappenheimer *et al.*, 1968). The Sips plot (Nisonoff and Pressman, 1958) yields a value for the heterogeneity index which is within experimental error of unity. These homogeneous binding data are not necessarily indicative of homogeneous antibody as judged by other criteria. Other antibodies to pneumococcal polysaccharides

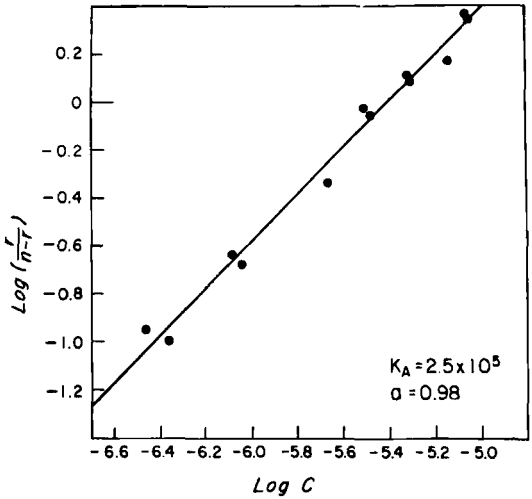

FIG. 16. Logarithmic plot of equilibrium dialysis data. Moles ligand bound per mole of antibody assuming a molecular weight of 150,000; N, number of binding sites per mole, assumed to be 2; C, concentration of per ligand; Ka, association constant; and a, heterogeneity index as derived from a Sips analysis employing an SDS 940 computer (program by Marcia Stone). (From Haber, 1970.)

which are heterogeneous by electrophoretic criteria, for example, give evidence of homogeneous binding (Pincus *et al.*, 1968). One possible explanation for such data is that heterogeneous antibodies consist of a limited selected number of sets of antibodies, all of which, however, have a similar binding site.

C. Isolation from Antisera of Antibodies to Bacterial Carbohydrates

The recovery of antibodies with molecular uniformity from rabbit antisera has been achieved by use of immunoabsorbents and preparative electrophoresis or a combination of both. The method to be employed is dictated by the electrophoretic character of the antibodies in an antiserum. If all of the antibody is confined to a single major M-type component, similar in appearance to a myeloma protein on electrophoresis, then preparative electrophoresis can be used to isolate the antibody, just as this method can be used to isolate myeloma proteins. Most commonly, however, the problem is more complex than this. It is for this reason that techniques of antibody isolation must be considered in some detail here.

In Fig. 17 are depicted representative examples of the kinds of

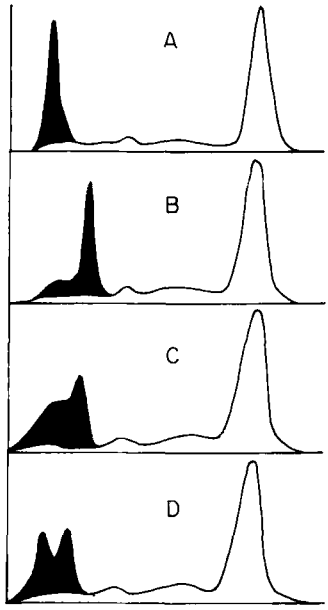

FIG. 17. Representative examples of rabbit antisera to bacterial carbohydrates. Precipitating antibody is indicated by the shaded area. a, An example of antiserum with all of the antibody in a predominant M component; b and c, examples of antisera with antibody in a monodisperse component and in a shoulder, broad component; d, an example of an antiserum with antibody distributed in two distinct components.

electrophoretic patterns of antisera to bacterial carbohydrates from which antibodies are isolated. Such patterns have been seen for the antisera of rabbits immunized with either streptococci or pneumococci. In each example, the precipitable antibody is indicated by the shaded area in the region of the γ-globulin. To be noted, in each instance, is a small portion (usually about 10 to 15%) of γ-globlin represented by the unshaded area, which is not precipitable by the soluble antigen. This nonprecipitable γ-globulin is the nonspecific γ-globulin in the serum. Therefore, recovery of the γ-globulin from such an antiserum by means of preparative electrophoresis alone will not yield an antibody population clearly devoid of nonantibody IgG. Nevertheless, recovery, by preparative electrophoresis, of a peak component when it is a major predominant one from an antiserum such as example a, yields a remarkably homogeneous antibody preparation which is adequate for most purposes. Many of the studies reported in the previous section were done with antibodies isolated in this way.

Immunoabsorbents are another powerful tool for the isolation of the

antibodies from these antisera. Clearly, in the case of antiserum a (Fig. 17), either use of immunoabsorbents or recovery of antibody from a specific immune precipitate would yield an antibody preparation that was essentially homogeneous because in such an antiserum all of the antibody is located in one monodisperse component. But, immune responses, such as the one represented by antiserum A are uncommon. More common are antisera b, c, and d which have more than one antibody component. Clearly, in these instances, reliance on immunoabsorbents alone for the recovery of an antibody would yield heterogeneous antibody preparations. In the case of antisera a and b, there is now a good deal of data which indicate that the predominant monodisperse antibody component contains antibody with molecular uniformity, whereas, the antibody in the broad shoulder component is heterogeneous. In such a case, recovery of *all* of the antibody from the antiserum would yield a heterogeneous antibody population. Finally, in the case of antiserum c, in which there are two distinct antibody components (and, on occasion, more than two), there is considerable evidence that each peak has its own individual antigenic specificity (Braun and Krause, 1968) and a distinct amino acid sequence (Hood *et al.*, 1970). Therefore, recovery of the antibody from such an antiserum by means of an immunoabsorbent would result in an antibody preparation which is clearly heterogeneous.

The conclusion to be drawn from these remarks is that the method selected to isolate antibody from a particular serum depends upon a careful evaluation of the number of major antibody components in the antiserum as judged by microzone electrophoresis. It is obvious that a combination of both preparative electrophoresis and immunoabsorbents has the potential of yielding antibody that has a very marked restriction in heterogeneity and devoid of nonantibody γ-globulin.

Several different immunoabsorbents have been successfully employed to recover the antipolysaccharide antibody from antisera. The specific immunoabsorbents for the antibodies to Type III and VIII pneumococcal polysaccharides, as described by Haber (1970), were synthesized by a two-step procedure. The polysaccharide was coupled to bovine serum albumin by the method of Avery and Goebel (1931) and the amino groups of the protein were then reacted with bromacetylcellulose (Robbins *et al.*, 1967). By use of the specific immunoabsorbent, 95% of either the Type III or VIII polysaccharide antibody was recovered from the antisera. Recovery was achieved by a batch process in which all of the absorbed antibody was eluted. This method for isolation of the antibody has the advantage over preparative electrophoresis in that all of the recovered protein is antibody to the carbohydrate. There is no

residual nonspecific γ-globulin in the isolated antibody as is the case when preparative electrophoresis is employed. However, this immunoabsorbent method of preparing uniform antibody is applicable only to antisera such as example a depicted in Fig. 17. Obviously, batch recovery by immunoabsorbents of all the antibody in antisera, such as examples b, c, and d (Fig. 17), will yield antibodies with varying degrees of heterogeneity, and, in the case of example d, the antibody preparation will have at least two distinct antibody populations.

Two recent innovations suggest technical procedures for circumventing the difficulties described thus far in isolating from an antiserum a single antibody population devoid of nonspecific γ-globulin. Recently, Parker and Briles (1970) and Eichmann and Greenblatt (1970) have described the fractionation of antibodies from streptococcal antisera by means of affinity chromatography.

The antipolysaccharide antibodies were purified from a streptococcal Group A antiserum and simultaneously fractionated into electrophoretically distinct components on the basis of their affinity for the phenyl-β-*N*-acetylglucosaminide group. β-*N*-Acetylglucosamine is the terminal immunodominant determinant of Group A polysaccharides. The immunoabsorbent was prepared by coupling *p*-aminophenyl-β-*N*-acetylglucosaminide to Sepharose activated with cyanogen bromide (Axen *et al.*, 1967; Porath *et al.*, 1967). Essentially all of the antipolysaccharide antibodies in the antiserum were absorbed on a column of this immunoabsorbent, while other serum components passed through. Specifically absorbed antibodies were eluted with a gradient of *N*-acetylglucosamine from 0.0 to 0.3 *M* at neutral pH. Two distinct antibody components were recovered each at a different amino sugar concentration, and each possessed a distinct electrophoretic mobility. Light chains from these two antibody components had one major band on disc electrophoresis.

An immunoabsorbent column for the antibodies to Group C carbohydrate were prepared by coupling the whole carbohydrate to the Sepharose instead of the *p*-aminophenyl-α-*N*-acetylgalactosaminide (Eichmann and Greenblatt, 1970). The whole carbohydrate was employed because of the difficulty in preparing the α-*N*-acetylgalactosaminide compound for use in a specific Group C immunoabsorbent, similar to the one for Group A. In order to couple the Group C carbohydrate to activated Sepharose, the antigen was partially deacetylated (Axen *et al.*, 1967; Porath *et al.*, 1967; Kristiansen *et al.*, 1969). A column, employing this immunoabsorbent, completely removed the precipitating antibody to the Group C carbohydrate from the antiserum. By elution with a pH and salt gradient starting with 0.1 *M* phosphate buffered saline at pH 7.2 and ending with 1 *M* sodium chloride–0.5 *M*

acetic acid at pH 2.5, several distinct antibody components were eluted. Each component possessed a distinct electrophoretic mobility. Such an experiment is depicted in Figs. 18 and 19.

A microzone electrophoretic pattern of a Group C antiserum, prior to absorption by an immunoabsorbent is shown in Fig. 18. The antiserum after removal of the antibody by passage through an immunoabsorbent column described above is also shown. Clearly, the bulk of the γ-globulin is specific antibody and has been absorbed onto the column. The elution

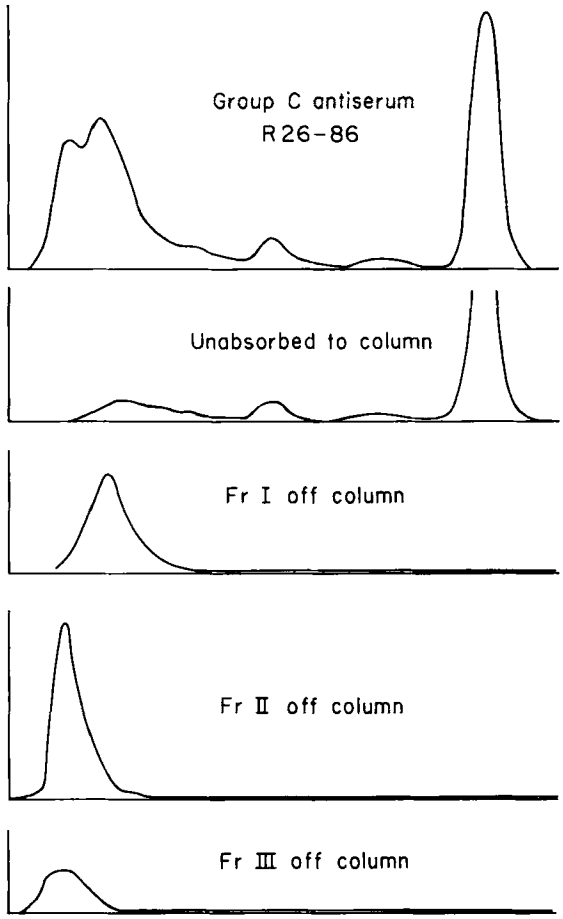

FIG. 18. Microzone electrophoretic patterns of a Group C antiserum before and after absorption by an immunoabsorbent column described in the text. The lower three patterns are antibody fractions eluted from the column by means of a pH gradient (shown in Fig. 18). Fraction I (Fr I), Fraction II (Fr II), and Fraction III (Fr III) refer to the corresponding fractions in Fig. 18.

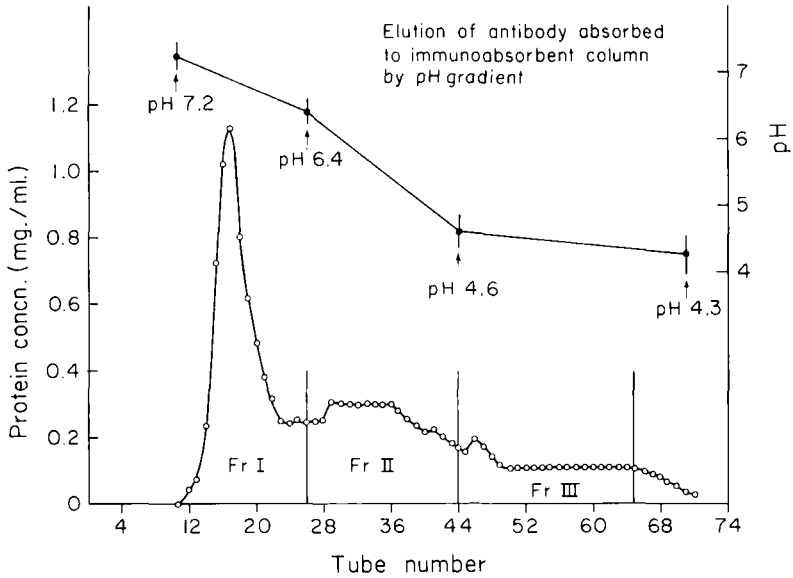

FIG. 19. Elution of antibody fractions from the Group C antibody absorbed onto a specific immunoabsorbent column by means of a pH gradient. Fraction I, which was eluted with a slight lowering of pH, has the most rapid electrophoretic mobility (see Fig. 18), whereas Fraction II, which was eluted with a greater lowering of pH has a slower mobility (see Fig. 18).

of the antibody from the column by a pH gradient is shown in Fig. 19. Three protein fractions, Fraction I, II, and III, were resolved. The tubes containing each fraction were pooled, and the pools, after concentration, were examined by microzone electrophoresis. The microzone patterns of these fractions are also shown in Fig. 18. This microzone analysis was performed simultaneously with the unabsorbed and absorbed antiserum. It is, therefore, possible to identify an isolated fraction as a particular one of the multiple antibody components in the unabsorbed antiserum. Antibodies have been recovered from more than six antisera, and, in each case, the antibody with the most rapid electrophoretic mobility is eluted with the least fall in the pH of the buffer, whereas, the antibody component with the slowest mobility is eluted with the greatest fall in buffer pH.

Fraction II recovered by the gradient elution from the immunoabsorbent column is very restricted in electrophoretic mobility and the light chains migrate in one prominent band by disc electrophoresis. Although much remains to be done to determine the efficacy of these immunoabsorbents and the resolving power of the gradient elution

technique, it would appear now that at least one, and in some cases two or three, restricted antibody components can be recovered from a single antiserum by these methods. As a consequence, antisera such as examples b, c, and d (Fig. 17) may be just as useful as sources for antibodies with uniform properties as is the much more rare "monoclonal" antiserum, example a.

A special case of affinity chromatography has been particularly useful for the isolation of uniform antibodies from occasional Group C streptococcal antisera. This is based on the fortuitous observation that the antibodies to Group C carbohydrate in these antisera bind weakly to Sephadex (Kindt *et al.*, 1970c). Because of this affinity, the specific antibody is sufficiently retarded on the column so that it can be separated from the nonantibody components that pass through more readily. A high degree of purification of an antibody was achieved with a Sephadex column, and the results of the procedure are depicted in Fig. 20. A Group C antibody preparation was recovered by preparative electrophoresis from antiserum R27-11 (Fig. 9). Such a preparation contained approximately 85 to 95% specific antibody and 10 to 15% nonspecific γ-globulin. This preparation was passed through the Sephadex G-200 column. The nonspecific γ-globulin was eluted in the same effluent in which radiolabeled γ-globulin was recovered and had no antibody activity for the

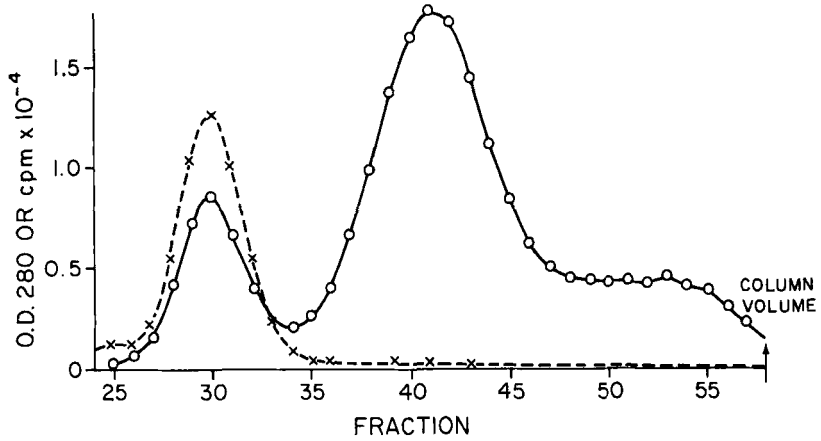

FIG. 20. Isolation of Group C antibody from rabbit R27-11 by chromatography with a Sephadex G-200 column. A 5.0-ml. sample containing 0.10 mg. of radioiodinated ^{125}I antibody isolated from antiserum by electrophoresis was mixed with 25 mg. of normal IgG and was applied to a column (90 × 2.5 cm.) of Sephadex G-200 in 0.1 *M*, pH 6.8, potassium phosphate buffer. (×- - -×) O.D., 280; (○- - -○) radioactivity. Fraction size was 8.0 ml.

Group C carbohydrate. All antibody activity was in the retarded material. The quantitative allotypic data recorded in Table II indicate that the antibody eluted in the retarded volume is devoid of the a1 marker, whereas, about 10% of the antibody preparation obtained by electrophoresis alone, prior to passage through the Sephadex column, was precipitated by anti-a1 antiserum.

D. Factors Influencing the Occurrence of High Antibody Responses with Restricted Heterogeneity

The occurrence of one prominent monodisperse antibody component in the antisera of rabbits that have been immunized with bacterial vaccines has in large measure remained unexplained. Undoubtedly, a number of variable factors, as yet undetermined, influence this occurrence. Factors which undoubtedly play a role include: the route of immunization, the physical state of the antigen, prior sensitization, and the genetic background of the rabbit.

There is little or no published information on the most effective composition of the vaccine for the production of potent antisera for streptococcal grouping. Clearly, the size of the particles in the vaccine may be important. Whole streptococci with the carbohydrate as the outermost cell wall element appear to stimulate a greater immune response than isolated cell walls which on the dry weight basis are one-fifth the size of intact streptococci (McCarty, 1970). Furthermore, the purified soluble carbohydrate which has a molecular weight between 8,000 and 10,000 is not antigenic in rabbits (Lancefield, 1970).

The possible advantages and disadvantages in alternative routes or methods of immunization with bacterial vaccines has not been examined in detail, at least not in recent years. The early impressions acquired by Lancefield (1970) were that alternative routes and the use of adjuvants were not as effective and, perhaps, were much less effective, than intravenous immunization with vaccines composed of heat-killed streptococci. It is possible that the potential advantage which can be achieved with frequent intravenous injections has been overlooked because of the magnitude of the effort to administer antigen in this way. But, to seasoned clinicians who recall patients with subacute bacterial endocarditis, prior to the days of antibiotics, who had prolonged persistent bacteremia, the profound hypergammaglobulinemia in the rabbits immunized intravenously comes as no surprise.

Histological examinations have been done on rabbits killed at the time the serum contained high concentration of uniform antibodies (Haber, 1970; Braun and Krause, 1969). Intense proliferation of plasma cells is seen in spleen, lungs, and lymph nodes. By use of immunofluores-

cence, Haber (1970) observed that the majority of the plasma cells in the spleen of rabbits immunized with Type VIII pneumococci were making antibodies to the Type VIII carbohydrate. Although much remains to be learned about the cellular events that occur with intravenous immunization, it would appear that this is a very effective way to stimulate plasma cell proliferation and, as a result, achieve high antibody responses. Additional information on antibody formation in rabbits immunized with pneumococcal vaccines can be found in Humphrey and Sulitzeanu (1957) and in Askonas and Humphrey (1957, 1958).

Rabbits which do not respond with a uniform population of antibodies or with a high antibody level after primary immunization, may do so after second immunization. In some cases, the increase in hypergammaglobulinemia after second immunization can be striking. As an example, the antibody level in one rabbit after first immunization was only 3 mg./ml. After a second immunization, the antibody level was 50 mg./ml., and in this case there was evidence for molecular uniformity of the antibodies. Although the explanation for this phenomenon is obscure, it is conceivable that intense immunization over a prolonged period selects a restricted population of cells which undergoes proliferation. In this connection, there is an intriguing parallel between these uniform populations of antibodies in rabbits after second immunization and the development of a myeloma-like condition in mink with Aleutian disease. It was observed that late in the course of Aleutian disease, some mink showed a transition from a heterogeneous hypergammaglobulinemia to a homogeneous myeloma-like hypergammaglobulinemia. Such a finding suggests the ascendancy of a few predominant clones of plasma cells (D. D. Porter *et al.*, 1965).

It remains to be clarified with certainty why some rabbits have a high immune response following intravenous immunization with streptococcal vaccines, whereas the majority respond poorly or with only modest levels of antibody. The possibility that such a response is under some form of genetic control was investigated by selective breeding of high-response and low-response breeding pairs. The immune response of the offspring was measured after immunization similar to that administered to the parents (Braun *et al.*, 1969). Reported in Table V are the precipitin levels to Group C carbohydrate in primary and secondary response antisera for both parents and their offspring. The data suggest that the magnitude of the immune response is genetically transmitted. One breeding pair had a relatively low antibody response and so did their offspring. The other breeding pair had a relatively high antibody response and their offspring had similar responses. Although there is some evidence to suggest that the antibody in the high responders is more likely to have restricted heterogeneity than the antibody in the low responders, this

TABLE V

CONCENTRATION OF PRECIPITINS TO GROUP C CARBOHYDRATE IN THE ANTISERA OF PARENTAL HIGH RESPONSE AND LOW RESPONSE RABBITS AND THEIR OFFSPRING IMMUNIZED WITH GROUP C VACCINE[a]

Conc. of precipitins (mg. ab/ml. of antiserum)	Low response breeding pair[b]				High response breeding pair[b]			
	Parents		Offspring		Parents		Offspring	
	1°	2°	1°	2°	1°	2°	1°	2°
1								
2			2				1	
3		F	3	1			1	
4	F							
5	M		1				2	
6							4	
7							3	
8							1	1
9		M		2			3	
10				1			2	
11							1	
12				1			1	1
13							1	
14							1	
15					F		1	1
16							1	
17							1	1
18								
22								1
23					M			1
25								2
29								1
30								2
31								2
32								
35						M		
37								1
38								1
42							1	
45								1
48								1
55								1
62								1
			—	—			—	—
			6	5			25	19

[a] Not all offspring survived the interval between primary and secondary immunization. Therefore, the total number for second immunization is less than the total for the first immunization. Primary response antisera collected at the end of 4 weeks of immunization. Secondary response antisera collected at the end of 3 weeks of immunization. Interval between primary and secondary, 4 months.

[b] F = female; M = male; 1° = primary immunization; 2° = secondary immunization.

matter is not yet settled. Solutions to these genetic questions will be more readily achieved if responses of this type can be reproduced in inbred mice. Such studies are now under way.

No attempt will be made here to review the literature on the influence of genetic factors on the immune response. This subject has recently been covered by McDevitt and Benacerraf (1969) in the preceding volume of this series.

E. Antibodies to Myoglobin and Angiotensin

Selective recovery of a subpopulation of antibodies to the specific C-terminal sequence of myoglobulin has been achieved by selective elution from specific immunoabsorbents. Myoglobulin is a complex protein, and rabbits immunized with a Freund's adjuvant preparation of this protein produce antisera that contain multiple antibody populations with specificities for different structural features of the molecule. Givas *et al.* (1968) isolated antibodies to the C-terminal heptapeptide of myoglobulin by elution of the antibody from the immunoabsorbent column with a synthetic heptapeptide identical to the C-terminal heptapeptide of myoglobulin. The antibody eluted with the synthetic heptapeptide was monodisperse by disc electrophoresis, and this is in contrast to the polydisperse character of the total myoblobin antibody component in the rabbit antisera. Yield of such restricted antibody, however, was low.

These studies on the restricted heterogeneity of antibodies to the C-terminal heptapeptide of myoglobin are reminiscent of the restricted heterogeneity of antibody to angiotensin which has been examined by Haber *et al.* (1967). Angiotensin is an octapeptide. The immunizing antigen was a branched chain polymer of angiotensin on poly-L-lysine. The peptide was coupled via its carboxyl terminus to the ϵ-amino groups of the poly-L-lysine. The results of binding experiments with nonfractionated antiserum and iodinated angiotensin gave points that fell on a straight line plot. The Sips analysis yielded an association constant of 2.64×10^8 L/M and a heterogeneity index within experimental error of unity. Such data point to a uniform antibody with respect to binding affinity, but there are no data available to indicate if such antibodies have structural homogeneity.

The disadvantage in these methods of obtaining antibody with molecular uniformity, by employing immunization with natural materials other than the bacterial polysaccharides, is that yields of antibody are small and the quantity is insufficient for extensive examination of amino acid sequence. These studies with antibodies to angiotensin have buttressed the notion, however, that an important cause of antibody heterogeneity is antigenic heterogeneity and that reducing the heterogeneity

of an antigen reduces the functional and molecular heterogeneity of the antibody.

F. Antibodies to Synthetic Antigens

In the past several years, evidence has been accumulating from a number of sources that antibodies with restricted heterogeneity may be generated in response to immunization with synthetic antigens. Both optimism and enthusiasm were sparked by the observation of Nisonoff *et al.* (1967) that a single rabbit produced a remarkably homogeneous anti-*p*-azobenzoate antibody. The rabbit had been immunized with *p*-aminobenzoic acid coupled to bovine γ-globulin, emulsified in complete Freund's adjuvant. The specific precipitin antibody level reached 6 mg./ml. of antiserum and the antibody, purified from a specific immune precipitate, crystallized out at a concentration of 60 mg./ml. in NaCl borate buffer, pH 8, ionic strength 0.16. There was a selective expression of the allotypic markers in the antibody. The whole antiserum was allotype a1, a2, b4; the dissolved antibody crystals were simply allotype a1, b4. It would appear that this remarkable antibody is the result of an uncommon event because such antibody was not seen in numerous other rabbits immunized with this antigen. However, it is possible, that scrutiny of many of these antibenzoate antisera by microzone electrophoresis and subsequent fractionation of antibody components from them by the methods described above would have yielded antibody preparations which approached the uniformity of the one crystallizable antibody from the one rabbit. This interpretation receives some support from the subsequent studies of Daugharty *et al.* (1969) on the idiotypic characteristics of the antibenzoate antibodies in many different antisera. The findings that only a portion of an antibody preparation is immunogenic in allotypically matched rabbits and that the same subfraction of the antibody is immunogenic in different rabbits sugest that the immunogenic population is composed of a limited number of homogeneous groups of antibody molecules.

The studies of Roholt *et al.* (1970) also suggest that antibodies of limited heterogeneity may occur in antisera of rabbits immunized with azo-*p*-benzoate–bovine γ-globulin. Rabbits were immunized intravenously over a period of several months to 2 years, and antisera were obtained throughout this time. Antibody concentrations ranged from 0.5 to 2 mg./ml. The antibodies from 2 of 9 rabbits have several properties which indicate restricted heterogeneity. The light chains were resolved into one band by polyacrylamide disc electrophoresis. The antibodies were homogeneous with respect to their binding constants, with heterogeneity

indices of 1.0, whereas an antibody with polydisperse light chains showed gross heterogeneity of binding constants.

Taken together, these several reports on rabbit antibodies to azobenzoate groups suggest that they, too, may be a useful source of antibodies with limited heterogeneity. The obstacle at the moment would appear to be the relatively low concentration of these antibodies in the serum. It is conceivable, however, that the immune response can be enhanced through manipulation of the immunization procedures and selective breeding of the rabbits.

One final approach which is employed to generate antibodies with restricted heterogeneity will be discussed here. This is concerned with a reduction in the degree of DNP antigen heterogeneity. In most studies with hapten–protein conjugates, the molecules are more or less attached randomly to the variable surface of the protein. With the thought that such random attachment of the determinants may contribute significantly to the heterogeneity of the antibody response, Brenneman and Singer (1968) constructed a special antigen in which the DNP was attached to a single characteristic site of a molecule. Papain was selected so that advantage could be taken of the single SH group per molecule. The SH group, available by activation of the enzyme, was reacted with α-(*N*-iodoacetyl) ϵ-(*N*-2,4-dinitrophenyl)-lysine. The final product had one DNP lysine group per molecule of papain. Rabbits and mice have been immunized with this antigen. Although the yields of antibodies have been very low, 5–10% of the animals produce antibodies which, by two criteria, have restricted heterogeneity. These antibodies are resolved into one major band by polyacrylamide electrofocusing. Normal γ-globulin resolves into thirty-five or forty bands (Trump and Singer, 1970). The light chains are distributed in one major band by polyacrylamide disc electrophoresis. The antibodies used in these techniques were radio-iodinated so that the distribution of the protein could be detected.

One possible explanation for the uniformity of these antibodies is that they are recovered from animals in which the magnitude of the immune response is relatively feeble. It is possible that only a few cells have been stimulated, and, as a consequence, the total antibody product exhibits restricted heterogeneity. A corollary of this argument is that with a heightened antibody response, there is a recruitment of a larger number of cells and as a consequence the total antibody population is heterogeneous. Such a suggestion stems, in part at least, from the studies of Little and Counts (1969). A homogeneous antigen DNP-lysyl-insulin was synthesized and characterized, and, in this case, the attachment site of the DNP was regulated and not random. The antibody response to this antigen was as heterogeneous as is usually the case when DNP is

coupled in a random fashion to other proteins. At present, there is no satisfactory resolution of the dilemma presented by results with the DNP–lysyl–papain and the DNP–lysyl–insulin, except to say that the designs of the experiments differ and that the protein carriers are not the same in each case. Such experimental variables may lead to opposing data.

V. Myeloma Proteins and Paraproteins with Antibody Activity

Since the first observation 10 years ago that a human paraprotein had rheumatoid factor activity (Kritzman *et al.,* 1961), a growing number of myeloma proteins and paraproteins from man and myeloma proteins from BALB/c mice have been identified which react with a variety of antigenic determinants. These reactive proteins have recently been reviewed by Metzger (1969). An extensive bibliography was cited and this need not be reproduced here.

Plasma cell tumors are now readily induced in BALB/c mice by intraperitoneal injection of mineral oil or other suitable irritants (Potter and Boyce, 1962; Potter, 1968a). These tumors arise in the peritoneal region 3–12 months after the oil injection. The majority produce IgA immunoglobulins (Potter, 1968b)—a finding which points to the preferential occurrence of the neoplastic event in immunocytes in the vicinity of the gastrointestinal tract.

The human myeloma proteins and Waldenström macroglobulins have activity for a variety of antigens, including γ-globulin, red cells in the cold, streptolysin O, heparin, lipoprotein, cardiolipin, and dinitrophenyl ligands. Mouse myeloma proteins have been identified which react with dinitrophenyl, the C-polysaccharide of the pneumococcus, dextran, and several other substances. These findings have raised the exciting possibility that the procurement of uniform antibodies is readily at hand by the simple expedient of screening with a panel of ligands a large number of myeloma proteins for specific activity. In principle, at least, this approach is a reasonable one, because most investigators would now agree that the myeloma proteins appear to be similar in all respects to normal γ-globulin, even though they are the products of malignant cells (Putnam and Udin, 1953; Metzger, 1969). Therefore, the unique amino acid sequence of the antigen-binding site should be identical for both an induced antibody and a myeloma protein if they both have the same specificity and if both are drawn from the same subpopulation of γ-globulin.

The myeloma proteins with antibody activity will not be discussed in the same detail as was the case for the antibody responses described above. This is because the theoretical and operational problems which arise in the use of these materials differ in many respects from a similar

consideration of uniform antibodies stimulated by specific immunization. For example, it is assumed, for all practical purposes, that the myeloma proteins are homogeneous, and heterogeneity need not be as rigorously excluded as is the case for antibodies stimulated by immunization. For the myeloma proteins, it is only the question of antigenic specificity which must be clarified. On the other hand, questions about the specificity of antibodies are secondary, whereas the accumulation of evidence for substantiating the uniformity of the antibody is the primary consideration.

One human IgG myeloma protein (Eisen *et al.*, 1967) and several mouse IgA myeloma proteins which bind DNP ligands have now been examined in great detail (Eisen *et al.*, 1968; Schubert *et al.*, 1968). These antibodies bind ligand selectively and binding is confined to the Fab fragment. Such data and fluorescence quenching by bound ligand parallel the behavior of antibody which occurs after stimulation with DNP protein conjugates, and, for these reasons, these myeloma proteins are regarded as antibody. It remains to be clarified, however, if these proteins are in all respects identical to the antibody which occurs in response to a specific antigenic stimulus. In other words, are these myeloma proteins really prototypes of the antibody to DNP which would have occurred if the animal or man had been immunized? The argument is speculative and at the moment, perhaps, circular. The problem remains that these proteins are, in fact, the products of cells programmed to make γ-globulin with a specificity which can only be determined at present by a screening procedure which uses a battery of antigens. If by fortuitous chance, the myeloma protein reacts selectively with one antigen in such a battery, this does not necessarily indicate that the myeloma protein is a product of cells initially programmed to make antibody with this specificity. This caution arises from several considerations which have been reviewed by Eisen *et al.* (1970). Most important of these considerations is the unexpected and unexplained high incidence of myeloma proteins with DNP activity.

Over 350 mouse myeloma sera have been screened for antinitrophenyl activity (Eisen *et al.*, 1968; Schubert *et al.*, 1968), and approximately 6% of these were reactive. However, these proteins varied enormously in their affinity for ϵ-2,4-DNP-L-lysine. Some reacted so weakly and in such an anomolous way that they had little or no resemblance to antinitrophenyl antibodies which are obtained by routine immunization. Eisen *et al.* (1970) have plotted the cumulative frequency with respect to affinity for ϵ-DNP-lysine for fifteen mouse IgA myeloma proteins. These are shown in Fig. 21. Only two of the proteins, 315 and 460, had moderate to high affinity for ϵ-DNP-lysine. These two exhibited most of the other characteristics of conventional antinitrophenyl antibodies. This would

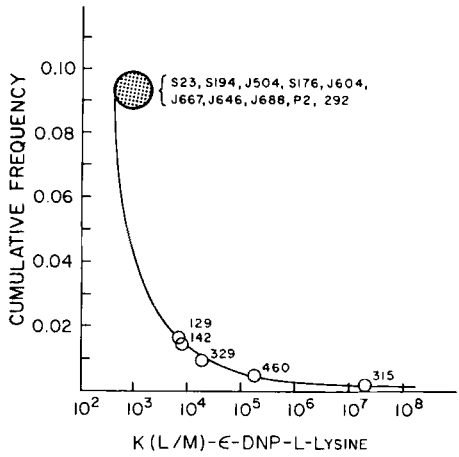

FIG. 21. Cumulative frequency for affinity (K) for binding ϵ-DNP-L-lysine (at 4°C.) by some of the antinitrophenyl mouse myeloma proteins found in a population of about 350 tumors. (From Eisen *et al.*, 1970.)

indicate, if this trend continues, that about 1% have an affinity which exceeds 10^5 L/M and are likely, therefore, to have combining sites, as measured by affinity, analogous to conventional antibodies to nitrophenyls. But, as Eisen points out, this frequency of 1% or even 0.01% is still unexpectedly high if the transformation of plasma cells is a completely random process, and the nitrophenyls, as a group, are but one of many thousands of non-cross-reactive antigenic determinants. One possibility for such a high incidence is that the activity is fortuitous and due to nonspecific binding. Such a possibility, however, seems to have been ruled out (Eisen *et al.*, 1970).

Antigenic cross-reactivity forms the basis for an alternative explanation for the high frequency of mouse myelomas which bind DNP. It is conceivable that an unknown antigen which cross-reacts with DNP has forced a numerical prejudice from among the total immunocyte pool in favor of immunocytes that synthesize antibody against the unknown antigen. As a consequence, at a subsequent time when the malignant transformation occurs, a higher proportion of myelomas arise with cross-reactive DNP activity than would have been anticipated by chance alone. Several lines of evidence suggest this might be the case, but direct proof is still lacking. It has been learned, for example, that mouse myeloma 315 reacts with naphthoquinones—a series of compounds widespread in nature which includes vitamin K. These types of compounds are common

in the intestinal tract. Under such circumstances, it might be anticipated that there would be an increase in the number of intestinal wall immunocytes which produce IgA specific for naphthoquinones. As a consequence, when the malignant event occurs among the cells producing IgA, a relatively high proportion of the tumors produce a myeloma protein which is reactive with a naphthoquinone but is cross-reactive with a DNP ligand.

The difficulty of identifying the precise specificity of a mouse myeloma protein is well exemplified by a consideration of those that react with pneumococcal C polysaccharide (Potter and Leon, 1968; Cohn *et al.*, 1969). This is a complex polysaccharide which contains two polymers—ribitol teichoic acid and a polymer of *N*-acetylgalactosamine phosphate (Gotschlich and Liu, 1967; Brundish and Baddiley, 1968). Other components of the C polysacchride include a diamino sugar, glucose, and choline. Conventional antibodies, raised by immunization of rabbits with pneumococci, commonly develop specificity for the *N*-acetylgalactosamine phosphate polymer of this complex carbohydrate. This does not appear to be the case for the mouse myeloma proteins which react with C carbohydrate. Leon has shown that these proteins appear to be specific for the choline of the C carbohydrate (Leon, 1970). In an extension of these studies, it has been observed that these myeloma proteins also have specificity for phosphorylcholine and they also agglutinate red blood cells coated with lecithin and lipoprotein (Leon, 1970). This raises the possibility that these myeloma proteins are, in fact, analogous to autoantibodies with specificity for cellular membrane determinants.

It should not go unnoticed that a number of the myeloma proteins, as well as the Waldenström macroglobulins, have autoantibody activity. Although the reason for this is not clear, there are several intriguing and speculative possibilities. One possibility is that in each individual who develops these proteins there are areas of focal inflammation with destruction of tissue and exposure of cellular antigenic sites. As immunocytes accumulate and proliferate in these inflammed areas, it is possible that a sizable proportion are converted to the production of antibodies against a variety of "self" substances. Under such a circumstance, the selective and unopposed proliferation of one of these cells would lead to synthesis of a paraprotein or myeloma protein with autoantibody activity. Osserman and Takatsuki (1965) have stressed the possible importance of chronic inflammation as the stimulus for the *in situ* occurrence of multiple myeloma.

Potter (1970), more than anyone else, has systematically considered the possibility that "the precursors of neoplastic plasma cells are actively forming antibodies to immunogens that originate in micro-organisms that inhabit the gastrointestinal tract." He has conducted an extensive search

to identify the polysaccharides of bacteria derived from the normal enteric flora of the mouse which react with IgA mouse myeloma proteins. Eighty-six IgA mouse myeloma proteins were tested for their ability to precipitate with forty-seven different antigens. Eight precipitated with lipopolysaccharides derived from *Salmonella*, *Escherichia coli*, *Proteus*, and *Pasteurella*. Five precipitated bacterial antigens derived from the normal intestinal flora isolated from BALB/c mice. An antigen was extracted from a species of *Ascaris* which is an inhabitant of the mouse intestinal tract. This antigen reacts with the same two myeloma proteins that precipitate pneumococcus C polysaccharide.

If, in fact, antigens derived from enteric organisms are acting as immunogens and stimulating the precursors of neoplastic IgA-producing plasma cells, then such a view is subject to experimental test. It should be possible, for example, to raise BALB/c mice in the germfree state and then selectively populate the bacterial flora with a single organism with a known antigenic composition. This can conceivably lead to a predominant population of precursor immunocytes with a specificity for antigens of this single bacterium. If the hypothesis is correct and if the myelomas have their origin in these percursor cells, a large proportion of these myelomas should produce IgA with specificity for the bacterial antigen. Such experiments are under way (Potter, 1970a).

An alternative approach for directing the specificity of the plasma cell tumor has employed the use of antigens administered to the mouse along with the mineral oil. These antigens have been injected either with the oil that stimulates the tumor or by some other route. Taking a clue from the apparent total commitment by nearly all plasma cells in the rabbit to the synthesis of antibody to streptococcal antigens following intravenous administration of streptococcal vaccine, BALB/c mice have been immunized intravenously for over a period of a year and have also been injected intraperitoneally at intervals with mineral oil. High levels of precipitating antibody against the carbohydrate have been achieved, but thus far it is not certain that any of these antibodies are the products of plasma cell tumors (Eichmann, 1970). The final outcome of these and other experiments along similar lines is awaited with interest. If the experiments are successful, the means would be at hand to procure, as in conventional immunization, a large number of homogeneous immunoglobulins which have specificity for defined antigens. Because there is no obstacle to the successful transfer of a myeloma into a large number of recipient mice, there would be no limit to the potential supply of any one protein. But, most important of all, success in these experiments would be a "demonstration that the precursors of neoplastic plasma cells are active in immune responses" (Potter, 1970b).

VI. Discussion and Summation

Are the means at hand to procure at will and in a reproducible and predictable fashion, antibodies with molecular uniformity? All of the work summarized here suggests that this is so. Antibodies to certain antigens may, in fact, be much less heterogeneous than was formerly supposed. For example, rabbit antibodies to bacterial polysaccharides possess many of the features of the myeloma proteins which suggest uniformity. Most important, in occasional rabbits, these antibodies occur in concentrations between 30 and 60 mg./ml. With brisk responses such as these, sufficient antibody can be obtained for extensive structural work. Although there is as yet inadequate proof for homogeneity based on extensive amino acid sequence data, such work is progressing rapidly in several laboratories, and all preliminary data suggest that selected antibodies to microbial polysaccharides will possess a single primary amino acid sequence and, therefore, in this last respect, these antibodies will be comparable to the myeloma proteins.

At the time of the first publication which dealt with antibodies with uniform properties in rabbits immunized with streptococci, it was suggested that an examination of antisera from animals immunized with other bacteria might reveal additional antibodies with properties indicative of molecular uniformity (Osterland *et al.*, 1966). From the present advantage of hindsight, it now appears that this prediction was an understatement. For example, intravenous immunization of rabbits with pneumococci has yielded antibodies with uniform properties (Haber, 1970). Preliminary results (Gotschlich and Feizi, 1970) suggest that intravenous immunization of rabbits with living Group A meningococci yields antibodies with uniform properties to the capsular carbohydrate which is a homopolymer of *N*-acetylmannosamine phosphate (Gotschlich *et al.*, 1969). The list of possible bacteria that possess immunochemically attractive carbohydrate antigens is almost endless. Among the many choices, however, there are several which would merit attention in the future. *Streptococcus bovis*, strain 19, possesses a dextran capsule (Kane and Karakawa, 1969), and it seems very likely, in view of the large body of information available on human antibodies to dextran that an examination of the antibody response following immunization with these bacteria would be especially rewarding.

Attention should also be called to the teichoic acid antigens of the staphylococci. Torii *et al.* (1964) have identified by immunochemical means, α- and β-*N*-acetylgalactosamine teichoic acid mixtures in antigen preparations from staphylococci. Certain staphylococcal strains possess a teichoic acid which is nearly all α-linked, and others possess a teichoic

acid which is nearly all β-linked. Immunization with these staphylococci presents the interesting possibility of obtaining uniform antibodies to two specific antigens which have a nearly identical chemical structure. If an immunologist is prepared to settle for antibodies to carbohydrates and forego the advantages of antibodies to synthetic antigens, there is a potentially large number of different bacterial antigens which can be employed to generate antibodies with uniform properties.

At the moment there are three major requirements for success in achieving high levels of uniform antibodies to bacterial carbohydrates. The first is that the carbohydrate antigen must occupy the outermost layer of the bacterial surface. In the case of the streptococci, this is not a natural occurrence. Only when the protein antigens are digested away with pepsin is the underlying carbohydrate exposed. The capsular polysaccharides of the pneumococci are clearly on the periphery of the cell, and, as a result, no special treatment of the vaccine is required. Because the outermost carbohydrate antigens in some bacteria are readily washed away during the preparation of the vaccine, it may be necessary to use bacteria which are collected directly from the broth culture and which have not been processed in any way. Gotschlich *et al.* (1969), for example, found that only live meningococci, collected in the log phase of growth, still possessed the capsular carbohydrate. Late growth phase cultures which had been killed and thoroughly washed were nearly devoid of the antigen.

The second requirement for the successful stimulation of high levels of uniform antibodies is the intravenous immunization of a vaccine composed of the whole bacteria. Alternative routes of immunization are less effective. Use of the isolated carbohydrate alone is unsatisfactory. The third requirement is related to the genetic background of the rabbit. There is accumulating, although as yet only preliminary, evidence which suggests that an immune response with 20 to 50 mg./ml. of precipitating antibody is a trait which may be genetically transmitted (Braun *et al.*, 1969). Any new research program to procure rabbit antibodies with uniform properties should employ a large number of rabbits from clearly different stocks and breeds. From such a widely diverse group of rabbits, a limited number will have high responses. A portion of these high responders will have antibodies with uniform properties.

If, indeed, rabbit antibodies with molecular uniformity are at hand, to what purpose can they be employed? They should prove useful for at least three different lines of investigation. These are the structure–function relationship of antigens and antibodies and the topography of the antigen-combining site, the genetic control of the biosynthesis of the immune globulins, and the evolutionary mechanism responsible for their

diversity. Finally, use of these antibodies in specially devised idiotypic (and individual antigenic specificity) experiments should afford a means to monitor the emergence of antibody populations over prolonged periods of immunization.

It must be left for a later review to cope with what will undoubtedly be a massive accumulation of sequence data on antibodies with uniform properties such as those described here. If two antibodies are drawn from the same subclass of IgG and possess the same class of light chains and identical allotypic markers and if each is specific for a distinct but related antigenic determinant, their specific amino acid sequence should be of value to describe the unique topography of the antibody-binding site. In this connection, rabbit antibodies with restricted heterogeneity may have special application in affinity labeling experiments which have been designed to locate the antigen-binding site. Affinity labeling reagents bind specifically as hapten to the site, but, in addition, bind irreversibly to an amino acid in the site. It is then possible to identify the peptides of the heavy and light chains to which the reagent is attached. This approach to search out the antigen-binding site has been described in detail and will not be reviewed here (Singer and Doolittle, 1966). The subject is mentioned, however, because it is conceivable that the rabbit antibodies to streptococcal carbohydrates and pneumococcal polysaccharides will be useful proteins for affinity labeling studies. The technique has been employed, for example, for equine anti-β-lactoside antibodies (Wofsy *et al.*, 1967a) and for rabbit antisaccharide antibodies (Wofsy *et al.*, 1967b). The one obvious advantage of the antibodies to the bacterial polysaccharide for such affinity labeling studies is their occurrence in high concentration. This assures an ample supply of antibody for extensive investigative work.

Progress on the structure and amino acid sequence of human and mouse γ-globulins has proceeded rapidly because the occurrence of multiple myeloma in these species assured a ready source of homogeneous proteins. Now that homogeneous rabbit antibodies are available, many questions can be examined for this species as well. It has been already learned, for example, that a definite homology exists in the variable region between the human κ and rabbit light chains. When the amino acid sequences of rabbit antibody light chains (b4, and, therefore, κ) are aligned against their human κ counterparts, a definite homology is suggested between the *N*-terminus of the human and the rabbit variable regions. Such an alignment is depicted in Table IV. Since a similar homology has been noted between the common C-terminal peptides (Doolittle and Astrin, 1967; Hood *et al.*, 1967), further support is gained for the hypothesis that rabbit κ and human κ light chains descended from common ancestral genes in the variable as well as the common region. Furthermore, because of the addition of an extra N-terminal alanine in

some rabbits (i.e., R27-11) and because of the deletion of a N-terminal residue in other rabbit light chains (i.e., R24-61 slow component), it is apparent why an N-terminal analysis of pooled rabbit light chains failed to reveal significant homology with human κ chains (Doolittle and Astrin, 1967).

Although the N-terminal sequence data on the light chains of streptococcal antibodies are still fragmentary, several observations merit comment.

A comparison of the amino acid alternatives at the first three N-terminal positions of the light chains of isolated antibodies and the light chains of preimmune γ-globulin from the same rabbit indicate that immunization may select a relatively uncommon species from among the many alternatives of normal γ-globulin. For example, isoleucine accounts for less than 6% of the amino acid residues recovered from the first N-terminal position of a single rabbit's preimmune light chains. And yet, in the Group C antibody recovered from this rabbit after immunization, isoleucine was the major N-terminal amino acid of the light chains (Hood *et al.*, 1969). Furthermore, a similar result has been observed with a surprising frequency for a number of streptococcal antibodies isolated in this way (Hood *et al.*, 1970). Perhaps more striking is the fact that light chains with predominantly N-terminal isoleucine have been isolated from both Group A and Group C antibodies. This suggests that two antibodies, each with a distinct and different immunological specificity, have been drawn from the same infrequent subpopulation of γ-globulin. N-terminal sequence amino acid analysis on the light chains of 9 rabbit antibodies indicates a division into at least 3 variable region subgroups (Hood *et al.*, 1970). Furthermore, species specific residues are observed which suggest that rapid gene expansion has occurred since man and rabbit diverged.

In the earlier sections of this review, it was emphasized that at least two distinct antibody components can be isolated from a number of hyperimmune rabbit streptococcal antisera, such as example d in Fig. 17. Each of the two components in such antisera possesses unrelated individual antigenic specificity. The light chains of each component may have a distinct N-terminal amino acid sequence. This is illustrated by the data in Table IV on two antibody components isolated by preparation electrophoresis from Group C antiserum R24-61. Alanine is the N-terminal amino acid of the light chains of the slow component, whereas isoleucine is the corresponding residue of the fast component. In this rabbit, the two antibodies appear to have been drawn from different variability region subgroups.

Because rabbit antibodies which have molecular uniformity are predominantly uniform with respect to all of the allotypic loci thus far examined, they should be useful proteins for immunogenetic studies. It

should be possible, for example, to clarify the ambiguity which still surrounds the molecular determinants of certain of the allotypic specificities, and it should be possible to define in a very precise way the location of these molecular determinants on the H and L chains. Such information may eventually provide a rational basis for selecting among the various mechanisms of genetic control postulated for antibody synthesis.

It remains finally to comment on recent studies which deal with the idiotypy or individual antigenic specificity of rabbit antibodies to bacterial polysaccharides. Streptococcal antibodies produced by a rabbit during repeated courses of immunization may elicit either identical or distinct individual antigenic specificities (Eichmann *et al.*, 1970b). First and second immunization antibodies from a single rabbit which have identical individual antigenic specificities have an identical electrophoretic mobility. Furthermore, the same antiserum may possess two electrophoretically distinct antibody components, each one possessing its own distinct individual antigenic specificity. These findings are in agreement with the recent studies of Nisonoff *et al.* (1970) and by Oudin and Michel (1969a,b). Furthermore, Oudin and Michel (1969a,b) and Braun and Krause (1968) observed that a rabbit may produce antibodies with two or more idiotypic specificities when it is immunized with one or more antigens.

The recurrence during second immunization of antibodies with an individual antigenic specificity identical to that of antibodies produced during primary immunization suggests that the same cell population, present during primary immunization, has re-emerged with reimmunization and, as a consequence, synthesis of identical antibody molecules occurs in both instances. In view of the fact that an antibody-producing cell does not survive longer than a few days, recall of antibodies with specificity similar to that present during the first immunization reinforces the suggestion that cells with a memory function must be involved in the recognition of the antigen and subsequent antibody synthesis.

There is no satisfactory explanation for the occasional occurrence of a high concentration of homogeneous antibodies in rabbits following immunization with streptococcal or pneumococcal vaccines. One possibility is that a cell population with numerical superiority over all others is present before immunization and is stimulated by it. All of the individual antigenic specificity data and light-chain sequence data, however, clearly indicate that this is not the case. Instead it appears that immunization may stimulate a minor rather than a major cell population. Furthermore, the light chains of the antibody product of these cells have an N-terminal sequence which is a representation of a minor species of the normal serum γ-globulin. It would seem that the use of individual anti-

genic specificity or idiotypy and an elucidation of the amino acid sequence of specific antibody light chains will facilitate an examination of the cellular events that lead to the immune response.

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Structure and Function of γM Macroglobulins

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I. Introduction

To classify rigorously a protein one must ultimately be able to refer to the gene (or, if the protein is composed of several polypeptide chains, the set of genes) which codes for the primary structure of the protein.

Specific polynucleotide sequences may in certain instances become available (Shapiro *et al.*, 1969), but for some time to come it will be necessary to define genes in terms of amino acid sequences or genetic (antigenic) markers or both. Such data are largely unavailable for the γM immunoglobulins. These proteins can be defined, therefore, on the basis of derivative properties only. Deciding which of these properties must be present to accept a protein as a member of this class is perforce arbitrary and classification must, therefore, be considered provisional.

Historically γM immunoglobulins were first recognized in the sera of cattle and horses (Heidelberger and Pederson, 1937; Kabat, 1961). It was found that in addition to those antibodies having a molecular weight of 150,000 to 160,000 and a γ mobility on electrophoresis, a class of antibodies having a molecular weight of around 1,000,000 and a somewhat faster electrophoretic mobility than the former group, was often elicited. These macroglobulins had a higher carbohydrate content than the low molecular weight γ-globulins and were readily dissociated into subunits upon exposure to reducing reagents. Rabbit antisera were elicited which, after appropriate absorption, were specific for these proteins. Henceforth, immunoglobulins which cross-reacted strongly with such antisera and/or had physicochemical properties similar to the proteins originally described, were considered γM immunoglobulins.

Most of this later work was performed with mammalian proteins, mostly from humans, and it is only for proteins from mammalian species that the term γM immunoglobulin can be used with assurance. Although it is likely that many high molecular weight immunoglobulins from more primitively arising species are evolutionarily homologous to mammalian γM, it seems reasonable to await further sequence data before attempting a final classification. For this reason I have segregated the discussion on mammalian γM proteins from that on nonmammalian γM-like proteins. This more accurately reflects the state of our knowledge.

Many Waldenström macroglobulins have been examined with a view to understanding their structure and their relationship to γM proteins which are normally present. As with the analogous myeloma proteins, such macroglobulins are obtainable in large amounts, and, being homogeneous, are amenable to detailed chemical and genetical analysis. That some of them have well-defined combining activity (reviewed in Metzger, 1969a) makes them particularly attractive to study. It is, of course, true that, arising as they do in the course of a neoplastic process, these proteins could in certain instances be sports. Nevertheless, experience has shown that extrapolation of findings on such proteins to the structure of normally arising immunoglobulins is usually valid.

II. Isolation and Storage of Macroglobulins

A. General Methods of Isolation

The γM macroglobulins are commonly isolated by following an initial selective precipitation with fractionations based primarily on molecular size (e.g., gel filtration) or on a combination of size and charge (e.g., zone electrophoresis).

Many macroglobulins are euglobulins and are readily precipitated from serum by dilution with distilled water (Deutsch and Morton, 1958). Curves relating γM solubility to ionic strength have been published by Mandema *et al.* (1955) and Martin (1960), but, of course, variations may be expected depending on the specific protein being studied, the solvent pH, and temperature. Schultze and Heremans list a variety of solvents from which γM proteins will precipitate (see Table 42, Schultze and Heremans, 1966).

When contamination with low-density lipoproteins is a problem, raising the density of the solution and floating off the lipoproteins through centrifugation has led to effective delipidation (Chaplin *et al.*, 1965; Inman and Hazen, 1968). Lipoproteins may also be precipitated with 0.5% dextran sulfate (mol. wt. = 560,000) in the presence of 0.09 *M* $CaCl_2$ (Burstein and Samaille, 1958) as has been described by Chesebro and Svehag (1969) for the purification of rabbit γM. Precipitation at neutral pH with sodium phosphotungstate in the presence of $MnCl_2$ has also been used (Burstein, 1963).

Fractionation by size is performed by gel filtration on Sephadex G-200 (cross-linked dextran), Bio-Gel P-200 (cross-linked polyacrylamide), Sepharose (agarose beads), or related materials. For smaller quantities, ultracentrifugation is useful (Kunkel *et al.*, 1961; Stanworth, 1967). With adequately large columns, α_2 macroglobulins can be separated from the γM macroglobulins (see, e.g., Chesebro and Svehag, 1969). Similarly, if it is undesirable to have high molecular weight γM polymers present (Section III,A,*1*), they can be eliminated by successive gel filtration (F. Miller and Metzger, 1965a; Morris and Inman, 1968).

Fractionation on the basis of charge can be affected by diethylaminoethyl cellulose ion-exchange chromatography (Chaplin *et al.*, 1965; Fahey and Terry, 1967) or zone electrophoresis on starch, Pevikon (Müller-Eberhard, 1960), or agar (Onoue *et al.*, 1967). Conservative pooling of the ion-exchange effluent can also help to eliminate traces of β-lipoprotein (Chaplin *et al.*, 1965).

Excellent yields of purified rat γM macroglobulin have been reported by Fisher and Canning (1966) using sucrose density gradients

in the BIV zonal centrifuge. This method allows for large batch processing and may be particularly useful for the isolation of γM from small animal sources.

B. Specific Methods of Isolation

Advantage can be taken of a known binding activity to isolate the γM antibody. The antigen–antibody complex is washed, dissociated, and the antibody and antigen separated. The latter step is, of course, most easily accomplished if the antigen is bound to an insoluble matrix. Dissociation at acid pH's is most often used but low molarities of urea or KSCN (Dandliker *et al.*, 1967; Stone and Metzger, 1969) can also be used and may be safer. Where complex formation is promoted by low temperatures (cold agglutinins, mixed cryoglobulins), simple warming may be an effective adjunct. In general it can be stated that native macroglobulins are fairly hardy molecules (Putnam, 1959) and will withstand low pH's e.g., 0.1 *N* acetic acid (Robbins *et al.*, 1967) and elevated temperatures (<60°C.) for limited time periods (Pike, 1967; Murray *et al.*, 1965a).

C. Storage

A study on normal pooled sera conducted under the auspices of the W. H. O. International Reference Centre for Immunoglobulins (Rowe *et al.*, 1970) showed that storage of either frozen or freeze-dried serum at −20°C. was adequate. Criteria used for stability were repetitive, single, radial diffusion assays and gel filtration. In our experience with two Waldenström macroglobulins which show binding activity to human γG (Metzger, 1967) and nitrophenyl derivatives (Ashman and Metzger, 1969), respectively, storage at −20°C. has been satisfactory. Storage of isolated macroglobulins in borate–NaCl buffers (pH 8.0) at 4°C. has also not led to detectable changes in these preparations over many months.

III. Structure of Mammalian Macroglobulins

A. Physical Properties

1. Sedimentation Rate

A macroglobulin preparation isolated by one of the above-mentioned methods will usually be heterodisperse when examined by analytical ultracentrifugation. The major component—comprising about 85% of the protein—will have a corrected sedimentation rate of 18 S to 19 S, where-

as minor components of ~29 S and 38 S make up about 15 and 5% of the total protein, respectively (Deutsch and Morton, 1958; Müller-Eberhard and Kunkel, 1959).

A very wide range of sedimentation constants for each of the components has been reported in studies of large numbers of macroglobulinemic sera (as opposed to isolated macroglobulins). For example, Ratcliff *et al.* (1963) reported an average sedimentation constant of 19.9 S but a range of 14.2 S to 22.2 S for the major component. Corresponding values were 29.5 S (20.14 S–34.3 S) and 37.6 S (24.8 S–39.0 S) for the heavier constituents. A statistical analysis of forty-two sera by Filitti-Wurmser *et al.* (1964) indicated a bimodal distribution—about 60% of the major components having a mean sedimentation constant of 17.0 (range 15.6–17.9) and 40% having a mean sedimentation constant of 18.3 (range 17.3–19.3). The sedimentation constant of the major component and the intermediate one appeared related. Thus, in those sera in which the major component had a mean $s^{\circ}_{20,w}$ of 17.0 the intermediate component had a mean $s^{\circ}_{20,w}$ of 23.9; for the second group the corresponding value was 27.0. Only three of the forty-two specimens failed to show such a correlation.

It is difficult to evaluate these data. They involve several correction factors and it would be helpful to have some analyses on the purified macroglobulins from such sera to verify the adequacy of the analyses.

The concentration dependence of sedimentation appears to be highly variable even on purified preparations. In the equation $s_{obs} = s^0(1 - kC)$, where C is in grams per cent, k in three recent studies can be calculated to be 0.098 (Suzuki and Deutsch, 1966), 0.257 (F. Miller and Metzger, 1965a), and 0.648 (Morris and Inman, 1968). It seems possible that the unusual shape of the molecule (see below) may influence the concentration dependence and extrapolated sedimentation constant under relatively minor experimental variations.

Reports on the dissociability of components heavier than 19 S are contradictory. Franklin (1960) found almost complete dissociation of human 29 S components to 19 S at pH 3.5, and Lamm and Small (1966) found a halving of the molecular weight of rabbit 29 S component (1.8×10^6 to 9×10^5) in 5 *M* guanidine. Reversible dissociation of both 28 S and 32 S to 19 S components was observed at alkaline pH and elevated temperatures by Suzuki and Deutsch (1966). On the other hand, dissociation was not observed by others under conditions where cleavage of covalent bonds would not be expected (Kunkel, 1960; Ratcliff *et al.*, 1963). In the three studies mentioned above, disulfide cleavage due to trace amounts of reducing reagents or due to protein-bound sulf-

hydryls was not rigorously excluded so that this remains as a possible explanation for some of the disparate results. Nevertheless, it is clearly possible that both noncovalent and covalent polymerization of the 18 S–19 S component might occur as it does with other proteins.

The 29 S and 35 S components are evident in unprocessed sera and the 19 S component can be freed of them by gel filtration (Section II,A). Thus, although the heavy components may arise from "rough" handling of the γM monomer, they do not appear to be due to that alone nor to an equilibrium aggregation phenomenon.

Suzuki and Deutsch (1966) have investigated three macroglobulin preparations which contained an interesting 22 S component in addition to the 19 S and 28 S components. The 22 S component was converted to the 19 S species at both acid and alkaline pH and at elevated temperatures. Evidence was given that this was not a γM anti-γM antigen–antibody complex. The authors suggest that the 22 S species is a noncovalent dimer of the 19 S, but a change in the shape of the 19 S component was not excluded. Molecular weight studies on the 22 S-rich preparations could easily differentiate between these alternatives.

2. *Diffusion Coefficient*

A limited number of diffusion analyses on macroglobulins have been reported. Kabat's data (1939) on a horse antibody preparation showed a somewhat erratic concentration dependence and yielded a value of 1.8×10^{-7} cm.²/second. Values for cow and pig macroglobulins were 1.64 and 1.69×10^{-7}, respectively. The data of F. Miller and Metzger (1965a) on a Waldenström macroglobulin showed a linear negative concentration dependence, the extrapolated value being 1.75×10^{-7}. Suzuki and Deutsch (1967) reported a value of 1.71×10^{-7}, and Beale and Buttress (1969) gave a value of 1.73×10^{-7} cm.²/second for a 0.39% protein solution. A relatively high value, 2.22×10^{-7} cm.²/second was reported by Fisher and Canning (1966) for rat macroglobulin.

The diffusion and sedimentation coefficients can be used to calculate a frictional ratio (f/f_0), a number related to the hydrodynamic asymmetry of the molecule. Values close to 2 have been commonly obtained for γM (Kabat, 1961; F. Miller and Metzger, 1965a).

3. *Intrinsic Viscosity*

A broad range of intrinsic viscosity values have been reported for γM: from 0.06 deciliter/gm. (Kovacs and Daune, 1961) to 0.30 deciliter/gm. (Martin, 1960). Careful studies by Jahnke *et al.* (1958) on four γM preparations gave a range of 0.106 to 0.153 deciliter/gm. A highly purified preparation gave 0.162 deciliter/gm. in our own work (F. Miller and

Metzger, 1965a). No change was observed when the ionic strength was raised appreciably so that charge effects were excluded in the latter study.

4. *Partial Specific Volume*

Kabat (1939) measured the partial specific volume of horse antibody and obtained 0.715 cm.^{3}/gm. F. Miller and Metzger (1965a) obtained 0.723 ± 0.001 cm.^{3}/gm. for a human Waldenström macroglobulin, and a value of 0.717 cm.^{3}/gm. has been calculated (Chen *et al.*, 1969) from the amino acid and carbohydrate compositions recorded by Heimburger *et al.* (1964). A value of 0.730 cm.^{3}/gm. was similarly calculated for rat macroglobulin (Fisher and Canning, 1966).

5. *Molecular Weight*

A wide range of molecular weights has been reported for γM immunoglobulins. Several of the earlier results are listed in Kabat (1961). Most of the recent studies have yielded molecular weights between 850,000 and 1,000,000 for human Waldenström macroglobulins (F. Miller and Metzger, 1965a,b; Suzuki and Deutsch, 1967; Chen *et al.*, 1969; Beale and Buttress, 1969). A much wider range, 620,000–1,180,000, has been described for human normal and Waldenström macroglobulins by Filitti-Wurmser and Hartmann (1968a). Rat γM was calculated to have a molecular weight of 770,000 (Fisher and Canning, 1966), and rabbit γM was reported to have a molecular weight of 900,000 by Lamm and Small (1966). The "best" value (of 990,000) for the molecular weight of cow, horse, and pig macroglobulin antibody (Kabat, 1939) probably needs to be corrected downward by about 10%, since the sedimentation values on which these results were based could not at that time be adequately corrected for temperature variations occurring during the ultracentrifugal analysis (Shulman, 1953).

It is not possible to evaluate these disparate results in the absence of other data relating to the number, size, and yield of the subunits, polypeptide chains, disulfide bonds, and proteolytic fragments, as well as to the number of antigen-combining sites and the electron-microscopic pictures.

6. *Ultraviolet Absorption and Color Coefficient*

Several values for the ultraviolet absorbancy of γM proteins have been published. Those I am aware of are given in Table I along with some unpublished data. In the absence of direct data, to assume an extinction coefficient of 1.25 would seem to be safest. Data on non-mammalian proteins are given in Section IX.

TABLE I
EXTINCTION COEFFICIENTS FOR HUMAN AND RABBIT γM

Protein				
Specimen	Reference[a]	Type[b]	$\epsilon^{0.1\%}_{280\ m\mu(1\ cm.)}$	Method used[c]
γM_{Wag}	(1)	W(κ)	$1.18_5 \pm 0.002$	D.W.
γM_{Wag} (subunit)	(1)	W(κ)	1.20 ± 0.02	D.W.
γM_{Wag}	(2)	W(κ)	1.18	R.I.
γM_{Lay}	(2)	W(κ)	1.27	R.I.
γM_{Mar}	(2)	W(κ)	1.24	R.I.
γM_{Ou}[d] (γM_{Ioc})	(2)	W(κ)	1.25	R.I.
Human γM (anti-polysaccharide)	(3)	—	1.22	R.I.,K[e]
Human γM	(4)	W	1.35	D.W.
Human γM	(5)	N	1.33	?
Rabbit γM (anti-benzenearsonate)	(6)	—	1.34 (1.21)	K[f]
Rabbit γM (anti-benzenearsonate)	(7)	—	1.32 ± 0.2	K[g]

[a] References:
(1) F. Miller and Metzger (1965a).
(2) Metzger (1969b).
(3) Merler *et al.* (1968).
(4) Mihaesco (1967).
(5) Schultze and Heide, unpublished observation cited in Schultze and Heremans (1966).
(6) Onoue *et al.* (1965).
(7) Hoyer *et al.* (1968).

[b] W = Waldenström macroglobulin; N = normal pooled macroglobulins. Where light-chain type is known it is indicated.

[c] D.W. = based on dry weight; R.I. = based on refractive index increment in 0.15 *N* NaCl. Refractive index increment assumed to be 0.00188 (Kabat, 1961); K = Kjeldahl or equivalent.

[d] The protein being sequenced by Wikler *et al.* (1969); see also A. P. Kaplan and Metzger (1969).

[e] In this study the refractive index increment was assumed to be 0.0019 and the nitrogen content 15%. Agreement was said to be ~4%.

[f] The value of 1.34 was based on an assumed nitrogen of 16%. If the true nitrogen is ~14.5% (see Section III,B,*1*) then the extinction coefficient is 1.21.

[g] Nitrogen content was assumed to be 14.5%.

The good agreement between the extinction coefficient for γM_{Wag} determined on the basis of dry weight and on the basis of an assumed refractive index increment of 0.00188 (green light) (Kabat, 1961; Table I) suggests that the latter value is appropriate for macroglobulins.

An extinction coefficient at 700 mμ. for a γM macroglobulin assayed by the Folin–Ciocalteu phenol method (Kabat, 1961) has been published (F. Miller and Metzger, 1965a). Over the linear part of the curve, an absorbancy of 24.5 for a 0.1% (dry weight) solution was recorded. Values for human γG and human serum albumin were 23.2 and 15.4, respectively, in that same study.

7. *Electrophoretic Mobility and Isoelectric Point*

The isoelectric point of γM macroglobulins varies widely from protein to protein: 5.1–7.8 in one early study (Deutsch and Morton, 1958). Though of historical interest in defining levels of heterogeneity among immunoglobulins, such data do not yield useful insights into molecular structure. The variation in isoelectric points is only partially reflected in zone electrophoretic analyses, such as immunoelectrophoresis, since the hydrodynamic properties of the molecules restrict their mobility. Still, on preparative runs a relatively wide range of mobilities is observed (Kunkel, 1960).

8. *Refractive Properties*

Optically active side-chain absorption bands complicate the interpretation of optical rotatory dispersion (ORD) and circular dichroism (CD) spectra of proteins in terms of polypeptide backbone conformation. Although the ORD spectra from γG and γM molecules differ (Jirgensons, 1960; Dorrington and Tanford, 1968), there are common features which are of interest. When the data above 300 mμ. are analyzed according to the equation of Moffitt and Yang (1956), the values for a_0 are from −163 to −206 for γM and somewhat lower (−290 to −320) for γG proteins. Corresponding values for b_0 are 0–20 for both γM and γG (Callaghan and Martin, 1964; Dorrington and Tanford, 1968; Wetter *et al.*, 1966). Although these values speak against extensive helical regions, considerable β-structure may be present as in γG immunoglobulins (Ashman *et al.*, 1970). The marked changes induced by extremes of pH and high molarities of urea also suggest considerable tertiary structure in these proteins. A remarkable finding is that the latter folding appears to be divided into noninteracting domains. That is, the ORD spectrum appears to be the simple algebraic sum of the spectra for the fragments released by proteolysis of both γG and γM immunoglobulins and for the subunits released by reduction of the 19 S γM molecules (L. A. Steiner and Lowey, 1966; Dorrington and Tanford, 1968). Further details on the ORD and CD spectra of γM proteins can be found in Dorrington and Tanford (1968) and in Ashman *et al.* (1970).

9. *Fluorescence Polarization of Macroglobulin–Dye Conjugates*

The high intrinsic viscosity (Section III,A,3) and high frictional coefficient (Section III,A,2) of γM are consistent with macroglobulins being asymmetric, rigid ellipsoids. However, several other properties of these molecules are not consistent with such a picture: (*a*) the lack of interaction between the subunits once the single intersubunit disulfides are broken (Section IV,B,5); (*b*) the marked susceptibility to proteolytic

digestion leading to the formation of fragments that show no tendency for noncovalent interactions (Section IV,C,2); and (*c*) the independence of the ORD "domains" referred to above (Section III,A,8). All these latter findings suggest that the γM pentamer may have considerable flexibility and that this rather than asymmetry accounts for the hydrodynamic properties.

By studying the variation in fluorescence polarization of γM–dye conjugates with solvent viscosity, it should be possible to obtain experimental verification for such a flexible model (Weber, 1953; R. F. Steiner and Edelhoch, 1962).

Two studies that utilized this technique have been published in adequate detail to evaluate (Metzger *et al.*, 1966a; Knopp and Weber, 1969). Metzger *et al.* (1966a) studied 1-dimethylaminonapthalene 5-sulfonyl chloride (DNS) conjugates of γM, the subunits (Section IV,A) and the tryptic Fab$_\mu$ fragments (Section IV,C). The relaxation times were respectively 80 ± 6, 69, and 58 nsec. Since even a rigid sphere of appropriate mass should have given a relaxation time of about 730 nsec. for γM, they interpreted their data as indicating significant internal rotations in the nanosecond range. The relaxation times were independent of the degree of conjugation, and several experiments (Perlman and Edelhoch, 1967) failed to give evidence for rotation of the dye molecules independently of the protein.

Knopp and Weber (1969) investigated pyrene butyric acid conjugates of human γM. These conjugates have fluorescent lifetimes of 100 nsec.—about 8 times longer than the DNS conjugates—and are, therefore, more appropriate for picking up longer relaxation times. They obtained a value of 1000 ± 200 nsec. for the γM pentamer and about 200 nsec. for the reduced protein. At low values of T/η their plots showed considerable deviation from a straight line, indicating the presence of shorter relaxation times. Knopp and Weber interpret their data as indicating that the molecule is fairly symmetrical but do not comment on the implied discrepancy, therefore, with the hydrodynamic data. Both sets of investigators agree that there must be some flexibility to the molecule but the exact time constants and extent of such movements for the molecule as a whole and for the individual subunits and Fab regions remain uncertain. A recent paper by Yguerabide *et al.* (1970) reporting the use of nanosecond fluorescence polarization with γG antibodies directed to the fluorescent chromophore is promising in this respect. They were able to estimate separately the rotational correlation times of the Fab fragments and the whole γG as well as the angular range of Fab motion. Comparable studies on γM antibodies would be of obvious interest.

10. Electron Microscopic Ultrastructure

An extensive review of ultrastructural studies on γG and γM immunoglobulins was published by Green (1970) in the previous volume of this series. Briefly, the elegant studies by Svehag and his colleagues on Waldenström, normal human and rabbit macroglobulins (Svehag *et al.*,

FIG. 1. Electron micrograph of a single mouse IgM selected from a micrograph by Parkhouse *et al.* (1970). Details of preparation are given in Parkhouse *et al.* (1970).

1967a,b; Chesebro *et al.*, 1968) first gave direct visualization of the flexible circular pentamer predicted from physicochemical investigations. Study of the papain $F(c)_5\,\mu$ and peptic $F(ab')_2\,\mu$ fragments (Section IV,C) (Svehag *et al.*, 1969) added further details. It showed at least some suggestion of a dimeric structure for the $F(ab')_2\,\mu$ fragment.

Since Green's review, three new studies were completed which yielded considerably more detail than had been observed previously. Shelton and McIntire (1970) and Parkhouse *et al.* (1970) examined the macroglobulin from the Balb/c mouse myeloma, MOPC 104E, whereas Feinstein and Munn (1969) studied γM proteins from several mammalian species, as well as analogous proteins from chickens and dogfish.

The well-resolved molecule, shown in Fig. 1, is from Parkhouse *et al.* (1970). It is a pentamer made of subunits strikingly like the Y-shaped structures described for γG (Green, 1969). The average dimensions are

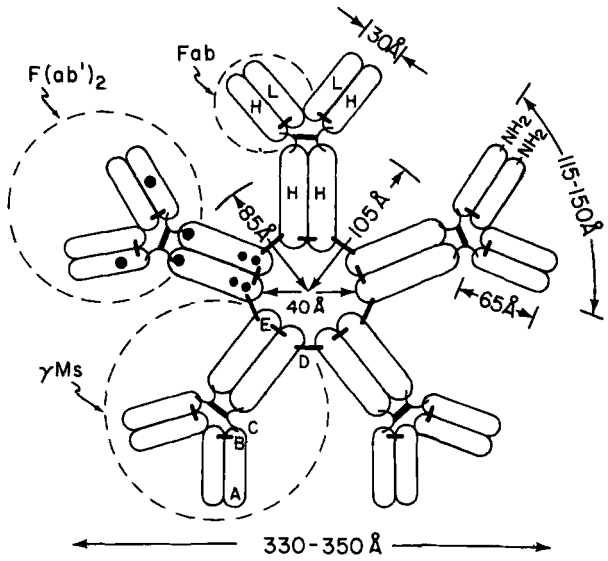


FIG. 2. Schematic representation of pentameric γM. The dimensions are the best available estimates from the studies of Svehag *et al.* (1969), Shelton and McIntire (1970), Feinstein and Munn (1969), and Parkhouse *et al.* (1970). Disulfide bonds are indicated by solid bars. The letters included in the portion of the molecule illustrating the structure of γM_s refer to the peptides described in Table III. The filled circles (for clarity shown on only one subunit) indicate the areas where attachment of polysaccharide chains has been implicated. NH_2 indicates the N-terminal ends of the polypeptide chains. The figure has been drawn with the light chains (L) on the "inside" of the heavy chains (H) on the basis of the suggestion made by Grey (1969a) and in a manner generally similar to that drawn for rabbit γG by Green (1970).

illustrated in Fig. 2. The 85 Å. diameter refers to the rim of the $F(c)_5\mu$ rings observed by Svehag *et al.;* these rings contained a central "hole" of about 40 Å. The 105 Å. diameter refers to the average distance between the center of the molecule and the "hinge" point observed by Parkhouse *et al.* (1970). The maximum span of the termini is between 115 and

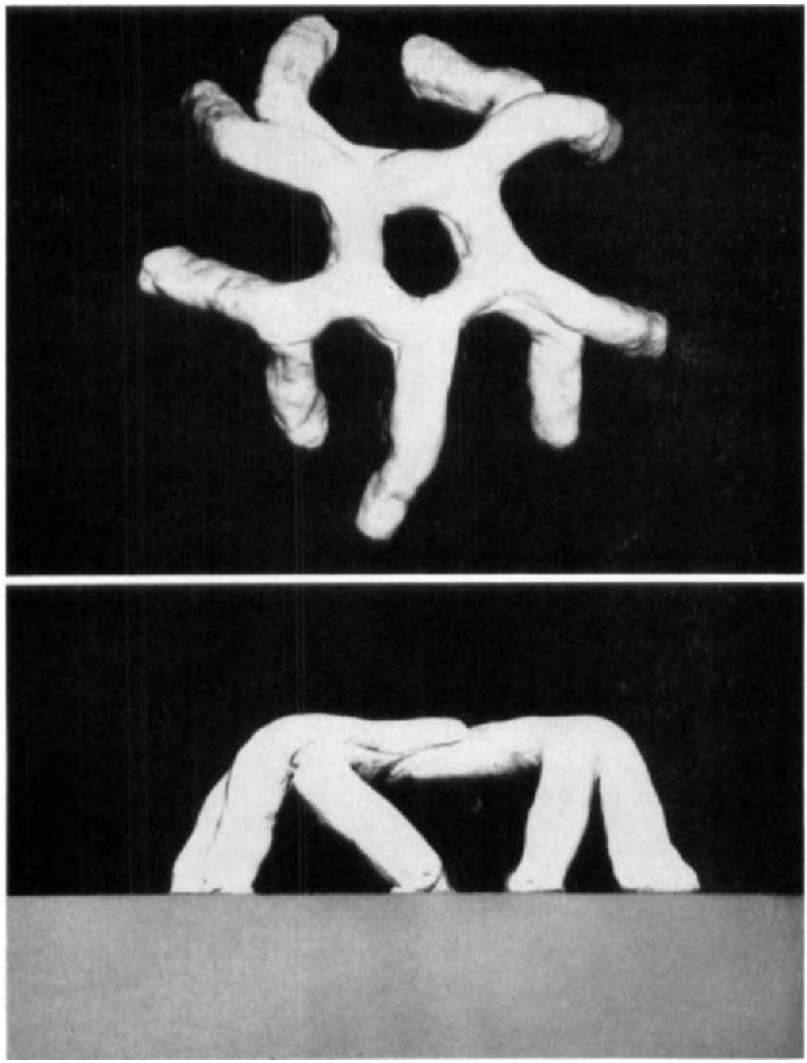


FIG. 3. (Top) Schematic model of γM with subunits opened to demonstrate the flexibility about the "hinge" region associated with the disulfide bond which connects the Fab' regions. (Bottom) Profile view of model of γM antibody bound to a particulate antigen. (From Feinstein and Munn, 1969.)

150 Å. (Shelton and McIntire, 1970). These dimensions must be considered provisional. Although the overall diameter is probably reasonably correct, the internal distances illustrated could be in error by as much as 50%, in part because the planarity of the structures is so difficult to assess.

As discussed by Green (1970) and by Feinstein and Munn (1969), the structures observed for the free-lying γM are consistent with the nominally quite different appearing pictures of γM bound to antigens (Humphrey and Dourmashkin, 1965; Feinstein and Munn, 1966; Almeida *et al.*, 1967; Svehag and Bloth, 1967; Feinstein and Munn, 1969), if one assumes that in the latter case the view is from the side. This would mean that the functional γM contains multiple hinge points resulting in a structure much more flaccid than that suggested by Figs. 1 and 2. A possible configuration is illustrated in Fig. 3.

Occasional hexameric molecules have been seen by almost all workers. That these may not be artifacts is suggested by the finding that the high molecular weight immunoglobulin of the frog *Xenopus levi* is uniformly a hexamer (Parkhouse *et al.*, 1970) (see Fig. 4). Whether such molecules ultimately result from a deviation of the normal mammalian type of biosynthesis or from a unique μ chain (see Section VII,A) is not known.

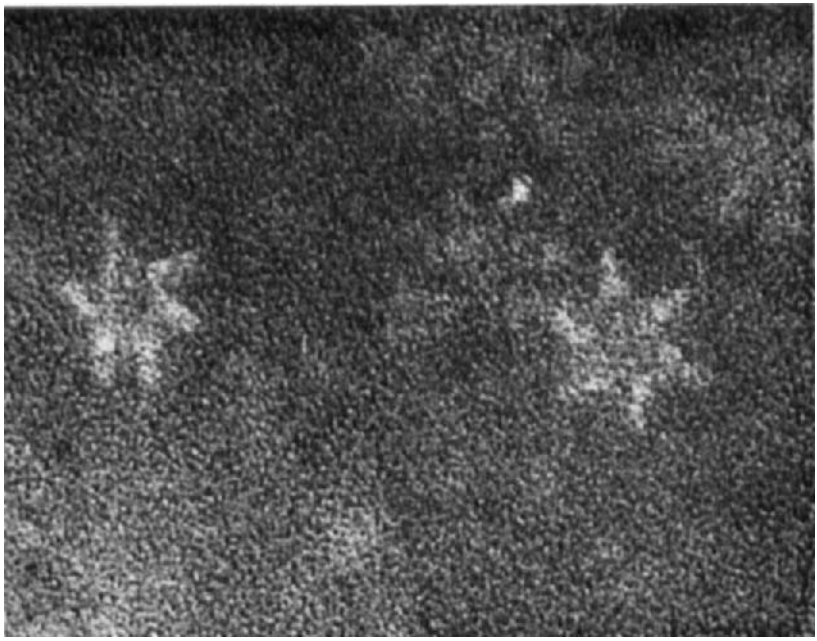


FIG. 4. Electron micrograph of hexameric immunoglobulin isolated by Dr. R. M. E. Parkhouse from *Xenopus levi*.

B. Chemical Properties

1. Amino Acid Composition and Nitrogen Content

The amino acid composition of γM immunoglobulins is unremarkable when compared to other globular proteins and especially when compared to other immunoglobulins (Metzger *et al.*, 1968) (see Section IV,B,*4,b*).

Nitrogen determinations have been published in only three instances of which I am aware. For a Waldenström macroglobulin containing 10.2% carbohydrate, 14.5% nitrogen was measured by Kjeldahl analysis (F. Miller and Metzger, 1965a). Human γG and human serum albumin each gave 16% nitrogen in good agreement with literature values. A value of 14.1% was reported for a Waldenström macroglobulin by Jirgensons (1960). The value of 13.2% reported by Fisher and Canning (1966) for rat macroglobulin seems excessively low.

2. Carbohydrate Content

It is well known (Kunkel, 1960) that γM immunoglobulins have a higher carbohydrate content than γG immunoglobulins. Innumerable carbohydrate determinations have been performed on these proteins, but the value of many of these is limited by the inadequacy of the analyses. Protein concentrations were often determined with inadequate precision and hexose determinations took no cognizance of the variation in color coefficients for different hexoses. Even more precise compositional measurements, however, by themselves lead to little insight. Only recently has clarification of the numbers, points of attachment, composition, and structure of the oligosaccharide chains been attempted.

Davie and Osterland (1968) surveyed the carbohydrate content of eight Waldenström macroglobulins. Five of the proteins had an overall carbohydrate content similar to previously determined values (9–13%) but three had somewhat less (7–8%). Concentrations for all eight γM preparations were based on a single Folin standard, and it might at first be suspected that the differences could be due to errors in the determination of protein concentrations. However, since the decreased carbohydrate content was largely accounted for by a sharp decrease in mannose and galactose (with no definite decrease in hexosamine or sialic acid, and only a small decrease in fucose content), this objection can be dismissed. No correlation with light-chain class was found.

Glycopeptides from two proteins were obtained from a Pronase digest by gel filtration and gradient elution from diethylaminoethyl cellulose. Three distinctive glycopeptides were derived from each of the proteins. Davie and Osterland's data are reproduced in Table IIA. Needless to say,

TABLE IIA
COMPOSITION OF MACROGLOBULIN CARBOHYDRATE UNITS ACCORDING TO DAVIE AND OSTERLAND[a]

	Glycopeptide[b]		
γM	I	II	III
Group I macroglobulins			
Mannose	3.0	9.0	6.0
6-Deoxygalactose	0	0	2.5
Galactose	2.0	1.0	2.5
2-Acetamido-2-deoxyglucose	1.0	2.0	5.5
N-Acetylneuraminic acid	0	0	2.0
Mol. wt. (calculated)	1,100	2,200	3,800
Units/890,000 gm.	10	10	20
Units/heavy chain	1	1	2
Group II macroglobulins			
Mannose	2.0	2.0	5.0
6-Deoxygalactose	0	1.0	1.0
Galactose	1.0	3.5	1.0
2-Acetamido-2-deoxyglucose	2.5	2.5	5.5
N-Acetylneuraminic acid	0	0	1.0
Mol. wt. (calculated)	1,100	1,700	2,800
Units/890,000 gm.	10	10	20
Units/heavy chain	1	1	2

[a] From Davie and Osterland (1968).
[b] Residues per unit to nearest one-half integer.

each of the glycopeptides listed could itself represent a mixture. Recent results by Davie and Osterland (1969) suggest that glycopeptide II may be derived from the light chains. Thus, their data indicate at least three points of carbohydrate attachment to the heavy chains. Studies on the high molecular weight proteolytic fragments of γM have also suggested at least three points of carbohydrate attachment (Section IV,C).

Spragg and Clamp (1969) examined three type-K Waldenström macroglobulins. They separated their glycopeptides from a Pronase digest by gel filtration, paper chromatography, and high-voltage paper electrophoresis. Based on the mannose content, two of their three proteins would be of the Type II proteins described by Davie and Osterland. Except for the high mannose content in the third specimen, it also looks suspiciously like a Type II protein. Although up to eleven glycopeptides were seen per protein, their data suggest two basic types, roughly similar to glycopeptides Types 2 and 3 previously described by Dawson and

Clamp (1968) for a γA myeloma protein. The compositions are consistent with Type 3, being a mixture of Davie and Osterland's Type I and II, and Type 2 similarly being a mixture of Types II and III (Table IIB). Their data suggest four to five carbohydrate chains per μ chain. Molecular weight data on tryptic glycopeptides from a Waldenström macroglobulin led Bourrillon and Razafimahaleo (1967) to a similar conclusion.

TABLE IIB
COMPOSITION OF MACROGLOBULIN CARBOHYDRATE UNITS ACCORDING TO SPRAGG AND CLAMP[a]

γM	Glycopeptide	
	3	2
Mannose	3–6	3–4
6-Deoxygalactose	0–1	1
Galactose	0–1	1–2
2-Acetamido-2-deoxyglucose	1–3	3–4
N-Acetylneuraminic acid	0	0–2
Units/890,000 gm.	20	30

[a] From Spragg and Clamp (1969).

Considerably more work will be required to delineate the site of attachment, composition, and structure of the carbohydrate moieties on γM proteins. It will be interesting to learn whether the heterogeneity of carbohydrate content is a reflection of heterogeneity in the amino acid sequence of the μ-chain common region; if so, this would indicate the presence of multiple μ-chain genetic loci (Section VII,A).

There is no glycoprotein for which the function of the carbohydrate moieties has been discovered. The γM proteins are no different. The extraordinary changes in transmembrane transport seen after minimal alterations in the carbohydrate portion of ceruloplasmin (Morell *et al.*, 1968) suggest interesting experimental approaches.

IV. Subunits, Polypeptide Chains, and Proteolytic Fragments

A. Reductive Subunits

1. Method of Isolation

Deutsch and Morton (1957) and Glenchur *et al.* (1958) first described the formation of stable subunits by reduction of γM with thiols and reaction with alkylating reagents. Structural analysis of these subunits, γM_s, is complicated by the susceptibility of intrasubunit disulfide bonds

to cleavage under the conditions commonly used to break the intersubunit bonds (e.g., 0.1 *M* 2-mercaptoethanol). Since the constituent chains are bound to each other by relatively weak noncovalent forces (Deutsch and Greenwood, 1960), partial dissociation of the subunits can occur leading to difficulties in molecular weight determinations.

The amount of dissociation may vary with the nature of the reducing reagent, alkylating reagent, and unknown factors. For example, Kishimoto *et al.* (1968) remark in a footnote that they failed to see any dissociation of μ and κ chains in their study even though their conditions were not unusual. On the other hand, Suzuki and Deutsch (1967) showed that by repetitive gel filtration the subunits could be progressively depleted of light chains.

Frank and Humphrey (1968) reported a different form of dissociation. In gradient centrifugation studies with low concentrations of iodinated anti-Forssman antibodies, they observed a major peak which sedimented at a rate consistent with half-molecules of γM_s. Similar observations are cited by Harboe *et al.* (1969).

Several investigators have prepared subunits in which the constituent polypeptide chains remained disulfide linked. F. Miller and Metzger (1965b) found that with low levels of cysteine (0.01–0.02 *M*) at pH 8.6, both inter- and intrasubunit disulfide bonds were cleaved. Re-formation of intrasubunit μ-μ and μ light-chain disulfide bonds upon prolonged incubation led to complete conversion of the γM polymer into subunits the polypeptide chains of which were not dissociable. Mihaesco and Frangione (1969), using slightly different conditions of reduction with cysteine, obtained selective cleavage of the intersubunit bonds. Mercaptoethylamine was successfully used by Morris and Inman (1968). They showed that approximately two —SH were released per mole of subunit formed (based on a subunit molecular weight of 180,000). No chain dissociation occurred under denaturing conditions. Beale and Feinstein (1969) have secured nondissociable subunits by reduction of γM at very low (0.000125 *M*) dithiothreitol concentrations, but qualitative data suggested that one of the intrasubunit disulfide bonds was cleaved in at least some of the subunits.

Subunits are most often isolated on Sephadex G-200 or Bio-Gel P-200. On diethylaminoethyl cellulose they are eluted at lower ionic strengths and higher pH's than the parent γM (Reisner and Franklin, 1961).

2. *Physical Properties*

Even where substantial chain dissociation was avoided, differences in the molecular parameters have been reported. F. Miller and Metzger (1965a) measured a molecular weight of 185,000 and calculated a

molecular weight of 178,000 from a pentameric model for γM of molecular weight 890,000. Inman and Hazen (1968) reported a value of 177,000 for their subunits, and Feinstein and Buttress (1970) have obtained 181,000. Lamm and Small (1966) obtained a molecular weight of 180,000 for the subunits of rabbit γM, although some uncertainties were encountered in those studies. Suzuki and Deutsch (1967), on the other hand, estimated the molecular weight of their subunits as 200,000. Since the latter workers obtained a molecular weight of 1,000,000 for the unreduced γM, they also concluded that the γM was a pentamer. Filitti-Wurmser and Hartmann (1968b) obtained subunit molecular weights that ranged from 1.4 to 1.9 $\times$ 10^5 daltons. Since the parent γM molecular weights varied independently of the subunits, they proposed that γM molecules may contain 4, 6, or 8 subunits alternatively.

The subunits produced by reduction fully account for the chemical composition of the whole γM and, ordinarily, reduction leads to no loss of peptide or carbohydrate material (see Section IV,C,3). The subunits have a slightly greater anodic mobility than the parent molecule (McDougall and Deutsch, 1964), and a small change in the spectrophotometric titration curve has been reported (McDougall and Deutsch, 1964). The latter might have been due to partial chain dissociation. The partial specific volume and extinction coefficient at neutral pH are unchanged (F. Miller and Metzger, 1965a). There is a marked drop in the intrinsic viscosity of the γM preparation upon reduction [0.162 to 0.080 in the study of F. Miller and Metzger (1965a)] and, in general, the subunits behave as more compact units than do the parent molecules.

Occasional antisera distinguish between subunits and parent molecules (Korngold and Van Leeuwen, 1959; Reisner and Franklin, 1961; Wollheim and Williams, 1966; Swedlund *et al.*, 1968; Gleich and Loegering, 1969), but sometimes this may simply reflect the antigenic polyvalency of the pentamer compared to the monomer, rather than unique determinants on the former (Gleich and Loegering, 1969).

3. *Specific Reassociation*

Reduced γM preparations contain subunits and polypeptide chains with free sulfhydryls, so it is not surprising that reaggregation should occur on reoxidation. Similarly, since in many assays activity is enhanced by polyvalency (Section VI,A,3), it is not surprising that many investigators were simultaneously able to recover binding activity. Of greater interest is the highly specific reaggregation of the subunits which can be obtained under appropriate conditions (Parkhouse *et al.*, 1970; Inman, 1969). That is, the subunits reassociate into pentameric γM polymers with only very low levels of intermediate-sized or heavier than 19 S

aggregates being formed. This observation complements the finding that even under conditions where only some of the polymer is reduced, little or no intermediate products are formed. Both results demonstrate the preferential stability of the pentameric polymer. It was this phenomenon that first suggested to F. Miller and myself (1965b) that the polymer was a closed (i.e., circular) rather than a linear structure.

B. Polypeptide Chains

1. Methods of Isolation

The γM proteins dissociate into heavy and light chains in much the same way as γG immunoglobulins (Cohen, 1963; Carbonara and Heremans, 1963). Under reducing conditions adequate for breaking all the interchain disulfide bonds (see F. Miller and Metzger, 1965b), the released sulfhydryls may be blocked with a variety of reagents and the chains separated by gel filtration. Sephadex G-100 or Bio-Gel P-150 equilibrated with 1 *N* acetic or propionic acid are most often used. In my own experience (using 1 *N* propionic acid and Sephadex G-100), maximal resolution is achieved at protein loads no greater than about 1 mg./10 ml. bed volume. Heavy chains separated in 1 *M* propionic acid do not release any further light chains on subsequent rechromatography in 7 *M* guanidine (Metzger, 1969b) and by several criteria are not substantially contaminated with light chains (Putnam *et al.*, 1967). For separating chains in which intra- as well as interchain disulfides have been cleaved, good results are obtained with Sephadex G-200 equilibrated with 5 to 7 *M* guanidine (Lamm and Small, 1966).

2. Yields of Chains

Accurate determination of the yields of light and heavy chains depends on the adequacy of chain separation. Thus, many values listed in the literature can only be considered approximate since the elution patterns show inadequate separation or are not described. In our separations we usually find 22% of the total recovered 280 mμ. absorbancy units under the light-chain peak and have never observed a yield higher than 27%. The extinction coefficients of the light and heavy chains in propionic acid are, in our hands, approximately equivalent ($\epsilon^{0.1\%}_{280m\mu.} \sim 1.2$) so that the absorbancy yields approximate the mass yields. Considering the molecular weights of the chains (below) the data are most consistent with a yield of one light chain for each heavy chain. The results of disulfide cleavage (below) are in support of this. Suzuki and Deutsch (1967) reported a considerably higher yield of light chains. Their separations were performed on Sephadex G-100 and in 5 *M* guanidine.

A 32% yield of absorbancy was recorded for three different proteins.[1] Lamm and Small (1966) studying rabbit γM, obtained 22–23% of the total absorbancy under the light-chain peak.

3. *Properties of Light Chains*

Early studies on the amino acid composition, molecular weight, and light-chain genetic markers consistently showed that as a group, the light chains associated with γM immunoglobulins were not distinguishable from the light chains associated with other immunoglobulins. An exception to this statement are the data on κ:λ light-chain ratios for γM relative to other immunoglobulins. Wollheim and Snigurowicz (1967) observed only twenty-five λ proteins among 125 macroglobulin "M" components, whereas among polyclonal normal macroglobulins the λ:κ ratio was more like 1:2. In an extensive series studied from November, 1966 to March, 1969 the National Cancer Institute Immunoglobulin Reference Center recorded only twenty-two λ proteins in a total of 139 Waldenström macroglobulins for which the light-chain typing was unambiguous (Woods, 1969). The percentage of λ proteins (17%) is only about half that observed with γG (36%) and γA (31%) immunoglobulins. Recently, N-terminal amino acids (Cohen and Cooper, 1968; Putnam *et al.*, 1967) and twelve partial N-terminal sequences (Niall and Edman, 1967; Edman and Cooper, 1968; A. P. Kaplan and Metzger, 1969) have been determined on light chains from γM proteins. No distinctive features were observed in these studies. Solomon and McLaughlin (1969) have described an antiserum that distinguishes between κ subgroups—i.e., κ_I and κ_{II} subgroups which end in aspartic acid and κ_{III} which ends in glutamic acid (Hood and Talmage, 1970). Nine out of ten κ chains from γM were said to be of the κ_{III} subgroup by Ouchterlony analysis, whereas only 20–30% of non-γM light chains were of this type. Neither the N-terminal data of Putnam *et al.* (1967) nor of A. P. Kaplan and Metzger (1969) support such a skewed distribution: the κ_{III} subgroup was present in only two of the ten κ chains reported on in those papers. That three of four κ light chains from γM cold agglutinins were of the κ_{III} subgroup (Cohen and Cooper, 1968) may simply represent a sampling "error"[2] or

[1] Dr. H. F. Deutsch kindly sent me the protein VI (Suzuki and Deutsch, 1967) to examine in my own laboratory. My studies indicate that our different findings are more related to differences in the experimental techniques than to any differences in the proteins used. Thus, I obtained only a 27% yield of light chains, and enumeration of the disulfide bonds gave results in agreement with our previous results (F. Miller and Metzger, 1965b).

[2] If the incidence of κ_{III} is 30%, the probability of obtaining three κ_{III} subgroup chains in a random set of four is 7.6%.

a reflection of the activity being selected for (Section VII,A). Clearly, further studies are required to clarify the apparent discrepancies.

4. *Properties of Heavy Chains*

a. Size. On the basis of molecular weight data for the γM polymer and γM_s monomer, an equivalent number of heavy and light chains, and gel filtration data, F. Miller and Metzger (1965a) suggested that the μ chains had a molecular weight of between 65,000 to 70,000. Experimental support was simultaneously obtained by Lamm and Small (1966) who studied rabbit μ chains and obtained a molecular weight of ~70,000. The majority of detailed direct determinations performed since then (Suzuki and Deutsch, 1967; Dorrington and Mihaesco, 1970) have supported these figures. The molecular weight of the chains can also be determined by amino acid composition in the following way: The cysteines contributing to the interchain bonds can be selectively cleaved (see below) and the sulfhydryls alkylated with ^{14}C-iodoacetamide of accurately known specific activity. Since the number of carboxymethyl cysteine groups per chain is known (below) a known number of moles of heavy chain can be applied to the amino acid analyzer. The summed amino acid composition should yield the molecular weight. In a recent experiment (Metzger, 1969b) the molecular weight (for the carbohydrate-free portion of the μ chain) was calculated to be ~60,000. With the carbohydrate added this would bring the total weight to between 68,000 and 70,000. A similar determination on the light chain gave 22,400.

Molecular weights ranging from 49,000 to 72,000 have been determined on μ chains from three different Waldenström macroglobulins (Filitti-Wurmser *et al.*, 1969). One would like to see additional experimental approaches applied to these materials in order to verify these results. The rather high molecular weight (75,000) obtained by Bennett (1969) rests on somewhat shaky experimental grounds, although a substantially different experimental approach by Habeeb *et al.* (1969) led to the same result. Since considerable progress is being made on the full sequence of μ chains (Wikler *et al.*, 1969), formula weights should soon be available so that belaboring this point seems unproductive.

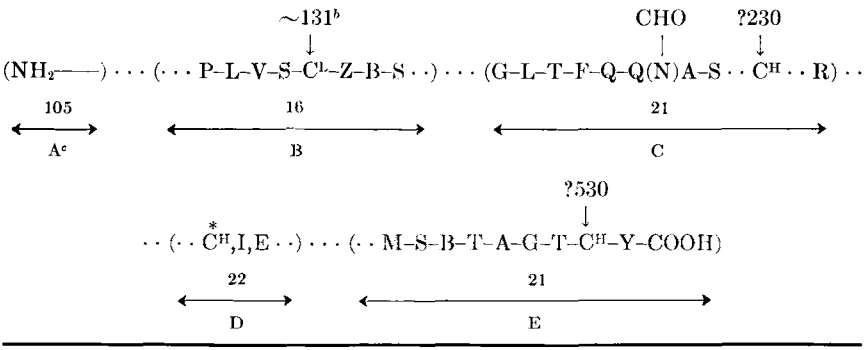
b. Amino Acid Composition. The amino acid composition of μ chains is unremarkable when compared to the composition of other immunoglobulin chains. Indeed, when examined statistically (Metzger *et al.*, 1968) heavy and light chains of several classes are remarkably similar with respect to their amino acid composition per unit weight. When calculated in this way (which obviates all assumptions), it is clear from several detailed amino acid compositions (Suzuki and Deutsch, 1967; Putnam *et al.*, 1967) that such data *cannot* be used to build molecular models—i.e., any number of light and/or heavy chains of any

assumed size would give substantially the same composition for the γM.

c. Sequence Data. The following data are available: the C-terminal nine amino acids of several μ chains (Wikler *et al.*, 1969; Grey, 1969b); a sequence of sixteen amino acids apparently homologous with

TABLE III

PRIMARY STRUCTURE OF μ CHAINS: THE INTERCHAIN DISULFIDE BRIDGES[a]



[a] The one-letter amino acid code is used (Dayhoff, 1969):

A = Ala	I = Ile	S = Ser
B = Asx	K = Lys	T = Thr
C = Cys	L = Leu	V = Val
D = Asp	M = Met	W = Trp
E = Glu	N = Asn	X = unknown
F = Phe	P = Pro	Y = Tyr
G = Gly	Q = Gln	Z = Glp or Glx
H = His	R = Arg	

[b] Numbers above the cysteine residues refer to approximate position of homologous regions in γ chains (Edelman *et al.*, 1969). The total length of ~530 residues is based on a presumed polypeptide weight of 58,000 gm. and a mean residue weight of 109 gm.

[c] Peptide isolated from μ chain: (A) N-Terminal sequence of 105 amino acids determined on protein Ou by Wikler *et al.* (1969). (B) Chymotryptic peptide sixteen residues long which contains the cysteine (C^L) which forms the μ light-chain bond. Sequence shown is by Pink and Milstein (1967). Composition of this peptide (Beale and Buttress, 1969) shows, in addition, B_1, T_1, S_2, Z_1, G_1, A_1, V_1. (C) Tryptic glycopeptide twenty-one residues long released during Fab′ μ → Fab μ conversion (Section IV,C,*2*,*b*) (Metzger *et al.*, 1966b; Beale and Buttress, 1969). The short N-terminal sequence was obtained by Edman degradation (Metzger *et al.*, 1966b). The asparaginyl-carbohydrate in position 7 is by a variety of inferential but not direct evidence. This peptide contains, in addition to the cysteine (C^H) contributing to the μ-μ bond in $F(ab')_2$, C-terminal arginine and B_2, T_1, S_1, Z_1, P_1, A_1, V_1, M_1, I_1. (D) Tryptic glycopeptide obtained from papain Fc μ fragment. It has a variable amount of carbohydrate and the cysteine (*) forming the μ-μ intersubunit bridge (Beale and Buttress, 1969). Its position is uncertain (see text); Its composition is C_1, B_3, T_2, S_3, Z_3, P_1, $G_2A_2V_1I_1F_1H_1R_1$. (E) C-Terminal tryptic glycopeptide (Beale and Buttress, 1969) which contains the C-terminal cyanogen bromide fragment sequenced by Wikler *et al.* (1969) and by Grey (1969b). The C-terminal sequence A–G–T–C–Y was also obtained from μ chains by Abel and Grey (1967). In addition to the amino acids shown the peptide contains B_1, T_1, S_2, Z_1, P_1, G_1, V_2, L_2, K_1.

an Fc peptide of rabbit γ chains (Wikler *et al.*, 1969); a composition and partial sequence of a tryptic peptide from the hinge region (Beale and Buttress, 1969; Metzger *et al.*, 1966b) (Table III); the sequence of a short region which includes the μ-chain cysteine participating in the μ light-chain disulfide bond (Table IV); a stretch of 105 amino acids from the N-terminal end of one γM (Wikler *et al.*, 1969); short sequences from the N-terminal end of nine other μ chains (Bennett, 1968; Köhler *et al.*, 1969; A. P. Kaplan *et al.*, 1970) (Table V); N-terminal amino acids from many μ chains; and tryptic fingerprint data from many proteins (Putnam *et al.*, 1967; Franklin and Frangione, 1968; Bennett, 1968).

These data permit one to arrive at the following tentative conclusions: (*1*) A substantial portion of the μ-chain sequence is common to all μ chains. Though considerably more information must be obtained, the data that we have are consistent with μ chains having an N-terminal variable region similar in length to that of γ chains (115–120 residues). (*2*) Although the data of Bennett (1968) (Table V) suggest that μ-chain-specific variable regions may exist, the data of Wikler *et al.* (1969) and A. P. Kaplan *et al.* (1970) appear to indicate quite the opposite. That is, there appears to be remarkable homology between the variable region of μ and γ chains (the variable region of all subclasses of human γ chains are so far indistinguishable). Indeed, the μ-chain sequence reported by

TABLE IV

PRIMARY STRUCTURE OF μ CHAINS: HOMOLOGIES AROUND HEAVY-LIGHT-CHAIN CYSTEINE[a,b]

	125 130 135	
μ	P–L–V–S–C^L–Z–B–S	Pink and Milstein (1967)
γ_1	G–P–S–V–F–P–L–A–P–S – S–K–S–T–S–G–G–T–A–A–L	Edelman *et al.* (1969)
γ_2	P–L–A–P–C^L–S–R	Frangione *et al.* (1969)
γ_3	P–L–A–P–C^L–S–R	Frangione and Milstein (1968)
γ^4	G–P–S–V–F–P–L–A–P–C^L–S–R–S–T–S–E(S,T)A–A–L	Pink and Milstein (1967)
γ (Rabbit)	A–P–S–V–F–P–L–A–P–C^L–C–G	O'Donnell *et al.* (1970)
γ_1	V–E–P–K–S–C^L–D–K–T–C	Edelman *et al.* (1969)
	220	

[a] As in Table III, the one-letter amino acid code is used. The numbering is based on the complete sequence of the γ_1 chain of protein Eu (Edelman *et al.*, 1969).

[b] (C^L) Cysteine implicated in heavy–light-chain disulfide bond.

TABLE V
PRIMARY STRUCTURE OF μ CHAINS: COMPARATIVE FEATURES OF N-TERMINAL REGIONS

Sub-group[a]	Protein	Heavy-Chain Class	Reference	Position 1 2 3 4 5 6 7 8 9 10
V_{HI}	Eu	γ_1	Edelman *et al.* (1969)	Z–V–Q–L–V–Q–S–G–A–E
	7 Misc. proteins	γ_{1-4}	Press and Hogg (1969) A. P. Kaplan *et al.* (1970)	——H— ——L—
	Mar	μ	A. P. Kaplan *et al.* (1970)	————
	Wag	μ	A. P. Kaplan *et al.* (1970)	Q———
	How	μ	A. P. Kaplan *et al.* (1970)	E———
	Lay	μ	A. P. Kaplan *et al.* (1970)	A———
	Koh	μ	A. P. Kaplan *et al.* (1970)	————
	Di	μ	Köhler *et al.* (1969)	————
V_{HII}	Daw	γ_1	Press and Hogg (1969)	Z–V–T–L–R–E–S–G–P–A
	Cor	γ_1	Press and Hogg (1969)	————
	He	γ_1	Cunningham *et al.* (1970)	————K——N———T
	Ou	μ	Wikler *et al.* (1969)	————T————
	(Ioc)		[A. P. Kaplan *et al.* (1970)]	
V_{HIII}	Dos	μ	Bennett (1968)	Z–S–V–A–B
	Bal	μ	Bennett (1968)	————E
	Bus	μ	Bennett (1968)	———L—
	Daw	μ	Bennett (1968)	———L—

[a] The data are grouped according to the guidelines developed at a WHO nomenclature conference held in Prague, June 1968. Subgroups HI and HII are based on the extensive sequence data for protein Eu (Edelman *et al.*, 1969) and for proteins Daw and Cor (Press and Hogg, 1969). Protein Ou for which the N-terminal sequence of 105 amino acids has been reported shows 70% homology with the first ninety-nine residues of protein Daw but only 29% homology with a comparable stretch from protein Eu. Subgroup HIII is based entirely on the pentapeptides reported by Bennett (1968).

Wikler *et al.* (1969) is actually more closely related to the γ_{Daw} sequence (Press and Hogg, 1969) than is the latter to the γ_{Eu} sequence (Edelman *et al.*, 1969). It can be tentatively concluded that μ chains may well have their variable regions coded for by the same group of genes that code for the variable regions of other heavy chains. Discussion of these data as well as their implications can be found in several recent publications (Wikler *et al.*, 1969; Edelman and Gall, 1969; A. P. Kaplan *et al.*, 1970; Metzger, 1970).

5. *Disulfide Linkage of γM Chains*

a. Methods of Analysis. Without citing "chapter and verse," a review of recent data from many laboratories convinces me that (*a*) amino acid analyses of carboxymethyl cysteine, cysteic acid, or amino

ethyl cysteine frequently give results which are off by 10% and often much more; (*b*) analyses of SH groups with 5′,5-dithiobis-(2-nitrobenzoic acid) (Ellman, 1959) can be highly accurate but are cumbersome when large numbers of samples must be assayed (e.g., from an elution pattern); (*c*) with care, reproducible and accurate results can be obtained with radioactive alkylating reagents. The latter method also permits rapid assay of large numbers of samples. The additional possibility of using double labeling (with ^{14}C and ^{3}H reagents) and radioautographic techniques allows for great flexibility. In my own laboratory we use this method exclusively when maximal accuracy is required.

b. Number of Interchain Disulfide Bonds. A detailed study of the interchain disulfides of a human γM protein has been presented (F. Miller and Metzger, 1965b), and only the conclusions will be presented here since a complete analysis is given in that paper. It was shown that there are 24–25 interchain disulfide bonds per molecule (890,000 daltons) of γM. Four-fifths of the released sulfhydryls appeared under the heavy-chain peak when the chains were separated. These data showed that the light chains were connected to the heavy chains by a single disulfide link, that there were three inter-heavy-chain bonds, and that one or two of the latter formed the intersubunit links (cf. Chaplin *et al.*, 1965). That the heavy chains contain four cysteines participating in interchain links has been shown by others. By using preparations in which only interchain bonds had been cleaved, it was shown that four unique peptides containing the participating cysteines could be isolated (Mihaesco and Mihaesco, 1968; Beale and Buttress, 1969; Beale and Feinstein, 1969, 1970). The 4:1 ratio of heavy-chain:light-chain sulfhydryls obtained after selective cleavage of interchain disulfides shows that there must be a 1:1 ratio of heavy:light chains. These data are inconsistent with the two heavy-chain:three light-chain model proposed by Suzuki and Deutsch (1967).

c. Location of Bonds. The interchain disulfide bonds can now be provisionally placed (Table III). The most N-terminal must be that connecting the light and heavy chains since it occurs in the Fab region (Section IV,C,*2,a*) (F. Miller and Metzger, 1966; Beale and Feinstein, 1969; Beale and Buttress, 1969). A short sequence around the μ-chain cysteine contributing to this bond has been reported by Pink and Milstein (1967). There is a strong hint of homology to the sequence around a comparable cysteine γ_2, γ_3, and γ_4 human heavy chains and in rabbit γ chains (Table IV). The sequence is quite unlike that around cysteine 220 which performs a similar function on γ_1 chains. Further sequences will obviously be required to substantiate this homology.

The disulfide bond C-terminal to the former one connects the Fab′ regions (F. Miller and Metzger, 1966; Metzger *et al.*, 1966b; Beale and

Feinstein, 1969; Beale and Buttress, 1969). The third relevant cysteine is found in peptide μ_3–μ_5 of Beale and Feinstein (1969) and Beale and Buttress (1969). The fourth cysteine contributing to the inter-heavy-chain bonds is a cysteine that is the penultimate amino acid residue of the μ chain (Table III). The latter residue, though contributing to an interchain bond, is not the one contributing to the intersubunit bond. The amino acid composition of the peptide thought to contribute to the intersubunit bond by Beale and Feinstein (1969) and Beale and Buttress (1969) does not contain the C-terminal tyrosine (Doolittle *et al.*, 1966, Abel and Grey, 1967; Mihaesco and Mihaesco, 1968) nor the methionine known to be nine residues from the C-terminal end (Wikler *et al.*, 1969; Grey, 1969b). Furthermore, when only the intersubunit bonds are cleaved and ^{14}C-alkylated, the C-terminal peptide contains no radioactivity (Grey, 1969b) and a tripeptide (Ile,Glu,Cys) is obtained (Mihaesco and Frangione, 1969) the composition of which is incompatible with the C-terminal sequence but is clearly compatible with the composition of peptide μ_3–μ_5 implicated by Beale and co-workers. The recently reported study by Beale and Feinstein (1970) is confirmatory.

The presence of only one intersubunit bond agrees with the conclusions of Morris and Inman (1968) (Section IV,A,*1*). In view of the composition of the C-terminal twenty-one residue tryptic peptide (peptide E in Table III) the intersubunit bond must be at least that far distant from the C-terminus of the μ chain. That the intersubunit bonds are relatively close to the C-terminal ends of the μ chains was suggested by Inman and Hazen (1968) who observed release of γM_s-sized fragments after brief incubation of γM with papain. These experiments did not, however, exclude the possibility that these fragments resulted from (*a*) papain scooping out a region of μ chain containing the intersubunit bond but lying within an intrachain disulfide loop (thereby yielding a γM_s-sized unit regardless of the points of cleavage) or (*b*) papain acting as a catalytic reducing agent and not as a protease.

6. *Chain Recombination*

Relatively little has been published on recombining the dissociated chains of macroglobulins. Gordon and Cohen (1966) recorded some observations on two γM proteins as part of a study on chain recombination by heavy chains from various classes. They observed preferential reassociation of autologous chains, similar to the phenomenon extensively documented by Grey and Mannik (1965) and Mannik (1967a) for γ chains. E. J. Miller and Terry (1968) described a unique antigenic determinant on μ–κ protenis, which could only be recovered by recombining μ chains (from either λ or κ proteins) with κ chains.

TABLE VI
PROTEOLYTIC FRAGMENTS OF γM PROTEINS

Fragment[a,b]	Mol. wt.[c] × 10^{-4}	$s^{\circ}_{20,w}$[c]	Hexose gm./100 gm. protein)	SH groups per mole after mild reduction	Antigenic determinants: Light-chain determinants	Antigenic determinants: μ-Chain determinants
I. Fab μ						
Trypsin (1–3)	4.1–4.8	3.6–3.7	0.8–1.9	2	All	Some
Chymotrypsin C (4)	4.0	3.8	N.R.[d]	N.R.	All	Some
Pepsin (5–8)	4.8–5.5	3.3–3.5	1.8–2.8	N.R.	All	Some
Papain (4, 5, 8, 9, 11)	3.7–5.5	3.5–3.8	1.6–4	2	All	Some
II. Fab′ μ						
Trypsin (1–3)	4.8–6.9	4.3–4.4	1.4–2.8	3	All	>Fab
Chymotrypsin C (4)	~6.8	N.R.	2.2	N.R.	All	>Fab
Pepsin (5–7)	6.3–7.3	4.0–4.4	5.1–5.4	N.R.	All	>Fab
Papain (5)	7.1	6.0	3.9	N.R.	All	>Fab
III. $F(ab')_2$ μ						
Trypsin (1–3)	9.5–14	6.0–6.3	1.4–2.8	6	All	=Fab′
Chymotrypsin C (4)	14	6.6	2.2	N.R.	All	=Fab′
Pepsin (5–7)	12–13	5.6–6.6	5.1–5.4	N.R.	All	=Fab′
Papain (4, 5, 9, 10)	13	7.0	3.9	N.R.	All	=Fab′
IV. Fc μ[e]						
Trypsin (3)	6.7	3.4	12	N.R.	None	Most
Papain (2, 8–12)	3.2[f]	2.9–3.2	22	2/32,000	None	Most
V. $F(c)_5$ μ						
Trypsin (3)[g]	34	11	12	N.R.	None	Most
Papain (8, 10)	32	11	22	18	None	Most

[a] Numbers in parentheses indicate the following references:

(1) F. Miller and Metzger (1966)
(2) Beale and Buttress (1969)
(3) Plaut and Tomasi (1970)
(4) Chen *et al.* (1969)
(5) Suzuki (1969)
(6) Mihaesco and Seligman (1968a)
(7) Kishimoto *et al.* (1968)
(8) Dorrington and Mihaesco (1970)
(9) Onoue *et al.* (1967)
(10) Onoue *et al.* (1968b)
(11) Mihaesco and Seligman (1968b)
(12) Mihaesco and Mihaesco (1968)

[b] For nomenclature see text.

[c] All molecular weights and sedimentation coefficients have been rounded off to two significant figures.

[d] N.R. = not reported.

[e] Obtained by reduction of $F(c)_5$ μ.

[f] Analyzed in 5 *M* guanidine–HCl.

[g] Tryptic digestion performed at high temperature. Similar results said to be obtainable using bovine α- or β-chymotrypsin (Plaut and Tomasi, 1970).

It would be of some interest to study the recombination of chains from Waldenström macroglobulins with defined binding activity.

C. Proteolytic Fragments of γM Macroglobulin

Early reports by Petermann and Pappenheimer (1941), Deutsch *et al.* (1961), and Harboe (1965) suggested that macroglobulins might be fragmented by pepsin and papain in much the same way that γG immunoglobulins can be cleaved. Only more recently, however, have precise chemical data been collected on such fragments. Table VI gives some of the results from studies with a variety of enzymes. The table is not meant to be exhaustive.

1. Nomenclature

When we performed our studies on the tryptic digestion of γM proteins (F. Miller and Metzger, 1966) the question of nomenclature arose. The 50,000-mol. wt. fragments produced from γG by papain in the presence of mild reducing agents had been called Fab fragments, whereas the dimeric 110,000-mol. wt. fragments produced by pepsin cleavage were called $F(ab')_2$ fragments. We decided to focus on the structure of such fragments rather than on the proteolytic enzyme employed and for this reason adopted the following rule: Any fragment consisting of a single heavy-chain fragment and a more-or-less complete light chain, and having other properties analogous to the papain fragment of γG, would be called Fab regardless of the enzyme used. The terms $F(ab')_2$ (and its reduction product, Fab') were similarly used even when a protease other than pepsin was employed. In conformity with the guidelines developed at a nomenclature meeting (World Health Organization, 1964) a suffix denoting the heavy-chain class of the fragment was added, e.g., Fab μ. I continue to feel that this is a reasonable approach and have organized the data in Table VI accordingly.

2. Types of Proteolytic Fragments

It is clear from Table VI that fragments closely analogous to those obtainable from γG proteins can be obtained from γM immunoglobulins. Though not indicated in the table, the assignments made on the basis of structural criteria were completely substantiated when functional macroglobulins were employed; that is, the Fab fragments contained the combining sites (Section VI,A,*2*).

a. Fab Fragment. A relatively stable fragment is obtained by proteolysis with trypsin, chymotrypsin C, and papain under the usual conditions. The susceptibility to more rapid breakdown during hydrolysis with

pepsin (Mihaesco and Seligman, 1968a; Kishimoto *et al.*, 1968) is probably related to the relatively weak heavy–light-chain interactions at the low pH's required for peptic activity. This is supported by the increased susceptibility of these fragments to papain at similarly low pH's (Mihaesco and Seligman, 1968b).

It was possible to estimate the yield of Fab fragments in several studies. From the extinction coefficients, molecular weights, and absorbancy yields, F. Miller and Metzger (1966) showed that precisely 2 moles of Fab were produced per mole γM_s (based on a molecular weight of 180,000 for the latter). Similar yields were obtained by Onoue *et al.* (1967) using papain and by Chen *et al.* (1969) using chymotrypsin C. This is evidence against a hypothetical model recently proposed by Suzuki (1969). The latter model was intended to show how the proteolytic fragmentation data might be consistent with the five-chain model of Suzuki and Deutsch (1967). In fact, all the fragmentation data along with the disulfide cleavage studies (Section IV,B,*5,b*) provide strong support for a four-chain, symmetrical subunit.

It is unlikely that the Fab μ fragments really have as wide a range of molecular weights as is indicated in Table VI. Sequence data will ultimately provide definitive values.

The Fd μ fragment (that part of the heavy chain in the Fab fragment) can be separated from the light chain by repetitive gel filtration (F. Miller and Metzger, 1966; Onoue *et al.*, 1967). It may be a useful intermediate for future sequence studies though the Fd′ μ fragment (see below) can be purified more easily.

b. Fab′ μ Fragment. This, by definition, requires both reduction of the μ–μ bond present in the $F(ab')_2$ μ region as well as scission of the μ-chain C-terminal to that bond. It is a useful fragment. It can be isolated in up to 85% yields from a 15–20 minute tryptic digest of γM_s. The Fd′ μ piece can then be isolated by gel filtration of the Fab′ on Sephadex G-100 in 1 *M* propionic acid (F. Miller and Metzger, 1966; Metzger *et al.*, 1966b). The separation of the Fd′ (which should be a useful intermediate for sequence studies) from the light chain is much superior to the separation achieved with Fd piece and light chain.

If Fab′ is redigested with trypsin, a single, nonultraviolet-absorbing, glycopeptide is released of molecular weight ~7000–8000, containing twenty-one amino acids and the cysteine contributing to the μ-μ disulfide bridge in $F(ab')_2$ (Metzger *et al.*, 1966b). Its composition is identical to that for peptide μ_2 reported by Beale and Buttress (1969) (see Table III). The presence of this hinge region is essential for an important antigenic determinant on the μ chain—an observation confirmed by all who have investigated antigenic differences between $F(ab')_2$ and Fab frag-

ments. It is of interest that this hinge peptide has only a single proline, whereas cysteines participating in inter-heavy-chain bonds in various γ chains are commonly surrounded by multiple prolyl residues (Frangione *et al.*, 1969). It is tantalizing to speculate that this may make the hinge region of γM proteins less flexible and that this, in turn, might make expression of divalency more difficult (Section VI,A,2).

c. $F(ab')_2\ \mu$ Fragment. As with Fab fragments a tremendous variation in molecular weights has been reported (Table I). Whether this is due to variability in the point of μ-chain scission, carbohydrate content, or experimental error, is uncertain.

d. Fc μ and $F(c)_5\ \mu$ Fragments. The Fc μ fragments were first isolated by Mihaesco and Seligmann (1966), Seligmann and Mihaesco (1967), and Onoue *et al.* (1967) from papain digests of γM. Mihaesco and Mihaesco (1968) confirmed the presence of the C-terminal tryosine and cysteine in such preparations. Dorrington and Mihaesco (1970) confirmed the molecular weight of 320,000 originally reported for $F(c)_5\ \mu$ by Onoue *et al.* (1968b) and showed that after reduction and dissociation in guanidine-HCl, fragments of 32,000 were released. Similarly, a 340,000-mol. wt. fragment was obtained by Plaut and Tomasi (1970) from high-temperature tryptic digests. After reduction and study in a nondissociating solvent, homogeneous fragments of 67,000 mol. wt. were found. These results confirm the presence of ten μ chains per mole γM. Adding the weights of the $F(ab')_2\ \mu$ fragments to those of the $F(c)_5\ \mu$ the data of Dorrington and Mihaesco give a molecular weight of 920,000 $[(5 \times 119{,}000) + (1 \times 320{,}000)]$, whereas the data of Plaut and Tomasi yield 815,000. Since stoichiometric yields were not obtained in either study, and variable amounts of peptides were formed, these values must be considered approximate.

The report by Plaut and Tomasi is particularly encouraging since $F(c)_5\ \mu$ was obtained in substantial yields. The extremely low yield of $F(c)_5\ \mu$ in all previous studies has greatly hampered decisive investigations of this interesting fragment.

3. *Fragmentation of Uncertain Origin*

Unusual fragmentation of γM has been described by several authors. Yakulis *et al.* (1968) noted progressive formation of Fab- and Fc-like fragments from an isolated γM cold agglutinin after reduction by sodium borohydride. Some Fc fragments were also produced in this way from rabbit γG and human γA (Yakulis *et al.*, 1969). Preincubation with 2-mercaptoethanol increased the yield of fragments. Albritton *et al.* (1970) observed a similar release of Fab- and Fc-like fragments from a Waldenström macroglobulin during incubation with 0.015 *M* mercaptoethylamine

at pH 7.2 and 37°C. The latter authors suggest that previous occult peptide cleavage led to release of fragments during reduction. Alternatively, disulfide cleavage may have increased the susceptibility of the molecule to peptic hydrolysis by enzymic contaminants. This latter mechanism would be consistent with the findings of Rimon *et al.* (1969). They found that incubation of γM proteins (particularly certain Waldenström macroglobulins) in 5 to 6 *M* urea at pH 5 to 8 led to progressive release of low molecular weight polypeptides from both μ and light chains. The reaction was not inhibited by iodoacetamide, *p*-hydroxymercuribenzoate, or ε-aminocaproate (the latter is a plasmin inhibitor) but was diminished by exposure to 60°C. They suggest that cleavage resulted from extraneous proteolytic enzymes. Klein *et al.* (1967) reported on a 7 S component which almost certainly resulted from contamination of a γM solution with *Proteus* organisms. A well-documented example of such contaminants splitting γG proteins was published by Robert and Bockman (1967).

V. Low Molecular Weight Macroglobulin-Like Proteins

Immunoglobulins antigenically indistinguishable from γM but having a considerably lower molecular weight were first clearly described in horses (Sandor, 1962; Sandor *et al.*, 1964) and then in humans (Rothfield *et al.*, 1965).

These "7 S-γM" proteins were observed in a variety of disease states: systemic lupus erythematosis (particularly in male patients) (Rothfield *et al.*, 1965; Stobo and Tomasi, 1967), dysproteinemia (Solomon and Kunkel, 1965; Gleich *et al.*, 1966), hereditary telangiectasia (Stobo and Tomasi, 1967), Waldenström's macroglobulinemia (Bush *et al.*, 1969), various other lymphoproliferative disorders (Solomon, 1967, 1969), rheumatoid arthritis (Lospalluto, 1968), and several infectious diseases (Klein *et al.*, 1967). A similar protein was found among normal infant and adult immunoglobulins and antibodies (Hunter, 1968; Perchalski *et al.*, 1968; Solomon, 1969).

Although antigenically deficient in some cases (e.g., Perchalski, 1968), by and large these low molecular weight γM-like proteins are indistinguishable from the 7 S monomers produced by reduction and alkylation of 19 S immunoglobulin. Biosynthetic studies indicate that 7 S γM is formed directly and at a different rate than 19 S γM (Solomon and McLaughlin 1970). The genetic and biosynthetic relationship of these proteins to 19 S γM remains uncertain.

Low molecular weight γM proteins can be analyzed for by gel diffusion, using gels sufficiently cross-linked to prevent diffusion of 19 S γM. Stobo and Tomasi (1967) employed 4% acrylamide gels for this purpose, whereas Solomon (1969) reported the use of 7% agarose gels. The possi-

bility that γM breakdown products are being detected (Section IV,C,3) must always be considered (Solomon, 1969).

VI. Functional Properties of Macroglobulins

A. Interaction with Antigens

1. *Comparative Aspects of γM Antigen-Combining Sites*

I have already discussed the preliminary sequence data which (with one possible exception) fail to demonstrate a subset of light-chain or heavy-chain variable regions which is unique to γM proteins. These data are so preliminary, however, that they cannot provide a definitive answer to the question of whether γM-combining sites are in any way unique.

The properties of γM and γG antibodies produced in response to immunization with the same antigen have been compared in many studies. Clearly, if we are concerned about the combining sites, only those experiments in which the polymeric nature of γM could not influence the analyses are relevant (Section VI,A,3). By and large, such studies have demonstrated the similarity of γM- and γG-combining sites. Onoue *et al.* (1935) studying rabbit anti-benzenearsonate, Jaton *et al.* (1967) studying antibodies specific for uridine, and Atsumi *et al.* (1968) studying anti-benzylpenicilloyl antibodies found that γM and γG antibodies isolated from the same sera had similar binding constants. Voss and Eisen (1968) and Mäkelä and associates (1967; Mäkelä and Kontiainen, 1969), on the other hand, obtained somewhat lower binding constants for γM and γG antibodies isolated from the same bleedings. The specificity and size of γM-combining sites have been investigated and, although in one study (M. E. Kaplan and Kabat, 1966) it was concluded that γM sites were directed to smaller determinants than γG sites were, other studies (Groff *et al.*, 1967; Haimovich *et al.*, 1969; Moreno and Kabat, 1969) failed to reveal significant differences. If we are concerned about the potentialities inherent in the γM structure, those studies that fail to demonstrate a difference seem most instructive.

Many factors influence which cells are stimulated, and the immunoglobulin end product will reflect this process as well as the potentialities of the immunoglobulin-combining sites. The subject is considered in some detail in Section X.

2. *Valence of γM*

Studies on a Waldenström macroglobulin, γM_{Lay}, which resembled "rheumatoid factor," in possessing γG-binding activity, supported the

assignments for the proteolytic fragments based on structural criteria (Metzger, 1967). Those fragments called "Fab μ" bound to the antigen, and the dimeric "F(ab')$_2$ μ" fragments not only were twice as active as the monomeric Fab, but fully accounted for all the activity in the subunits, γM_s. Subsequent studies (Stone and Metzger, 1967) showed that all ten of the γM_{Lay} Fab fragments were active. The failure of the subunits to bind more than 1 mole of antigen (γG or Fc γ) at a time (Metzger, 1967; Stone and Metzger, 1967, 1968) was attributed to steric factors. Such factors did not seem important when the ligand was small. Thus, a Waldenström macroglobulin that bound nitrophenyl derivatives with high specificity showed the expected valence of one binding site for each heavy-light-chain pair, regardless of whether proteolytic fragments, subunits, or pentamer were studied (Ashman and Metzger, 1969) (Fig. 5).

These results have been complemented by findings on non-Waldenström macroglobulins. Chavin and Franklin (1969) studied the γM component of a mixed cryoglobulin containing both κ and λ γM molecules which reacted with γG. They showed that all the Fab μ fragments were active despite the fact that only 1 mole of γG or Fc γ was bound per mole γM subunit. Merler *et al.* (1968) studied a human γM antibody separated from the serum of a normal donor who had been immunized with typhoid vaccine. A tetrasaccharide separated by paper chromatography from a partial hydrolyzate of *Salmonella typhimurium* lipopolysaccharide was employed for equilibrium dialysis. The experiments were technically difficult because it was necessary to read ligand concentrations at 210 mμ. and only the protein-free compartment of the dialysis cells could be analyzed. Nevertheless, although the data showed a moderate degree of scatter, they were consistent with ten identical combining sites per mole γM. The study by Cooper (1967) on two cold agglutinins was more indirect but his results supported the same conclusion. He used subunits from cysteine reduced γM—subunits in which the consituent polypeptide chains remain covalently linked (Section IV,A,*1*). Such subunits retained almost all the hemagglutinating capacity (and the temperature dependence) of the unreduced γM. Conventionally reduced and alkylated subunits were inactive in this study. Though it was not proven that the agglutination absolutely required bridging of cells via divalent agglutinins in this instance, it seems reasonable to assume that this was the case. If the subunits are divalent then the γM pentamer can be assumed to be decavalent. It is rather surprising that there was only a twofold difference in the hemagglutination titer for the subunits and the native γM (see next section). Schrohenloher *et al.* (1964) and Stone and Metzger (1967) also demonstrated agglutination by certain γM subunits though the activity of the latter was reduced 20–100 times compared to the unreduced γM. In these ex-

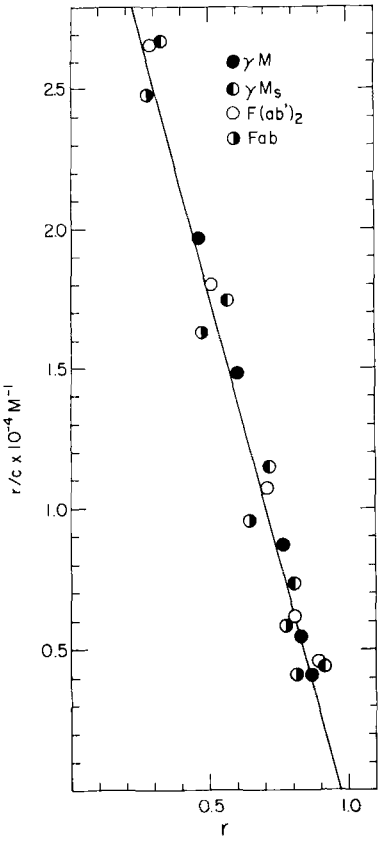


FIG. 5. Equilibrium dialysis data on a Waldenström macroglobulin, γM_{Wag}, which was fortuitously discovered to bind nitrophenyl derivatives specifically. Binding was assayed with ^{3}H-2,4-dinitrophenyl-ϵNH_2-caproic acid. The data are plotted as r (moles hapten bound per mole heavy–light-chain pair) over c (moles free hapten) vs. r. The γM, γM_s, F(ab')$_2$, and Fab were assumed to have 10, 2, 2, and 1 heavy–light-chain pairs (or equivalent), respectively. Calculations were based on molecular parameters given in F. Miller and Metzger (1965a, 1966). (Reproduced from Ashman and Metzger, 1969, by kind permission of the publishers.)

periments, however, chain dissociation could have resulted in partial inactivation consistent with Cooper's results.

Other studies on the valency of γM proteins have led to conclusions less straightforward than those described above. Results from experiments employing high molecular weight antigens (Franklin *et al.*, 1957; Lindqvist and Bauer, 1966; Schrohenloher and Barry, 1968) which showed a valence only one-half as large as expected can be interpreted as reflecting steric factors (see above) but other results, from experiments in which low molecular weight ligands were employed (Onoue *et al.*, 1965; Voss

and Eisen, 1968), are more difficult to understand. A recent paper by Onoue *et al.* (1968a) is instructive. They studied γM antibodies from rabbits immunized with azonaphthalene sulfonate groups attached to S. *typhimurium*. Their equilibrium dialysis data showed considerable heterogeneity: although the "first" few sites could be saturated at 1 to 3×10^{-6} *M* hapten, further binding was observed even at $\sim 1 \times 10^{-4}$ *M* hapten. They interpreted their data as showing equal numbers of weakly and strongly binding sites and proposed that each γM molecule had five weak and five strong binding sites. This interpretation seems unwarranted to me and results, I believe, from the tendency of the Scatchard plot to flatten out disproportionately as the weaker binding sites of a heterogeneous mixture are titrated. Their data when replotted simply as *c* (the free ligand concentration) vs. *r* (moles of bound ligand per mole of protein) fail to show either a break in the binding curve or a finite valence to which the plot can be extrapolated unambiguously. The data of Voss and Eisen (1968) apparently led to similar ambiguity. These results, therefore, do not seem to undermine seriously the conclusions described in the first part of this section. (See also Section IX.)

Frank and Humphrey (1968) approached the valency question differently. They investigated the ability of radiolabeled "subunits"[3] of purified rabbit anti-Forssman antibody to adhere to a solid antigen adsorbent. Forty to fifty per cent of the subunits failed to bind. The authors proposed that since only half of the expected amount of binding was observed, there may be only five rather than ten combining sites on each anti-Forssman molecule, although other explanations for the data were considered. Their results are provocative but too indirect to be considered definitive.

Unexpected findings were reported by Costea *et al.* (1966, 1967). They examined cold agglutinins from normals and patients with infectious mononucleosis, *Mycoplasma pneumoniae* infection, and systemic lupus erythematosis. Unlike the agglutinins from patients with idiopathic cold agglutinin disease which contained only κ-type light chains (and which were used as controls), the former agglutinins contained both κ and λ light chains. Surprisingly, the activity was completely precipitated with either anti-κ or anti-λ serum. This suggested that the vast majority of agglutinins were mixed κ,λ molecules. When such agglutinins were reduced and prior to reoxidation precipitated with anti-λ or anti-κ serum, activity was recoverable only if the anti-λ but not if the anti-κ serum was used. This result suggests that the original γM agglutinins consisted of a mixture of active κ subunits and inactive λ subunits. The results resemble those of Frank and Humphrey but, like the latter study, require further confirmation.

It is clear from a report by Coligan and Bauer (1969) that conven-

[3] These subunits appear to have been half-molecules of γM_s (Section IV,A,*1*).

tionally raised γM antibodies are not always composed of a mixture of active and inactive subunits. In their study more than 90% of the subunits derived from pooled γM anti-bovine serum albumin were bound to an antigen–cellulose adsorbent. The study by Merler *et al.* (1968) already cited makes the same point.

3. *Influence of Polyvalency*

The contribution made by the polyvalency of γM antibodies to a variety of secondary antibody–antigen interactions, remains to be assessed. It is intuitively obvious that an antibody with multiple binding sites will bind more firmly than a univalent or bivalent antibody to a multivalent antigen. Translating this intuition into precise quantitative terms has not yet been attempted and may be of little value if done entirely in the abstract. The energetics of binding will be highly dependent on the number and topology of the antigenic determinants and, correspondingly, the distribution of the combining sites in space (Section III,A,*10*; Fig. 3). There is evidence that the γM molecule has considerable flexibility (Sections III,A,9 and *10*). The different conformations need not all be energetically equivalent, however, so that the free energy of binding will be a balance between the free energy released by combining site–determinant interactions and the energy required to maximize the number of such interactions. In reactions that, in addition, involve cross-linking of translationally independent determinants (agglutination) the effect of polyvalency is even more difficult to gauge.

The situation becomes increasingly complex in reactions such as hemagglutination. Hemagglutination is not simply the cross-linking of inert particles. With antibody binding, dramatic surface changes take place which can profoundly influence local charge densities and, in turn, the relative ease with which two cells can approach (Salsbury *et al.*, 1968; Bangham and Pethica, 1960). These surface changes may be influenced by the local density of the antigen–antibody interactions and this will undoubtedly be influenced by the valence of the antibody.

An experimental analysis of this problem will require systems in which the intrinsic combining affinities and number of antibody sites, and the number and distribution of antigenic determinants, can all be determined and varied independently. Macroglobulins with homogeneous combining sites are available. Similarly, techniques for making mixed pentamers (pentamers made by reassociating subunits from active and inactive γM) have been described (Kunkel *et al.*, 1961; Jacot-Guillarmod and Isliker, 1964; Deutsch, 1969; Harboe *et al.*, 1969). Since it should be possible to conjugate inert particles with statistically defined surface densities of antigenic determinants, the problem seems experimentally approachable.

A study in which some of the many variables involved in a precipita-

tion reaction could be controlled has been reported (Stone and Metzger, 1968); it was possible thereby to rationalize many of the properties of that system.

4. *Macroglobulin Antibody–Antigen Interactions: Secondary Phenomena*

Considering the discussions in the previous three sections, it is not surprising to read in one study (Haimovich and Sela, 1968) that the relative capacity of γG and γM to inactivate phage varied 1000-fold, depending on the nature of the antigenic determinant on the phage. Similarly, a large number of factors may influence the relative efficiency of γM relative to γG antibodies in a variety of secondary phenomena, such as opsonization and bacterial clearance. Nevertheless, the value of comparisons of γM vs. γG responses is diminished only a little by our lack of a complete understanding of such secondary reactions. We are, after all, not only interested in molecular mechanisms but in a description of physiologically important events. The value of many studies (e.g., Robbins *et al.*, 1965; Chernokhvostova *et al.*, 1969) on γM antibodies in a variety of immune responses may, therefore, lie not so much in what they reveal about the capacities of γM proteins, as in what they tell us about biologically important phenomena. For example, it seems to me more important to describe what fraction of the total opsonizing capacity of an antiserum is accounted for by various immunoglobulins during the course of an immune response than to belabor the opsonizing capacity of such antibodies per microgram nitrogen in any particular case. The latter figure by itself adds surprisingly little to our understanding.

A useful review on the behavior of immunoglobulins from different classes in various assay procedures has been published (Pike, 1967). The data can be simply summarized: those subpopulations of γM not reacting with complement (see below) aside, γM antibodies participate in all types of immunological reactions other than those that involve fixation to heterologous or homologous tissues.

B. Interaction with the Complement System

Complement is a system of eleven serum proteins, an important (though not exclusive) function of which is interaction with cell membranes. The complement system is specifically activated by immunoglobulins—the antigen-combining sites of the latter determining the location where activation takes place. The initial step in complement activation is the interaction of immunoglobulin with the calcium-dependent macromolecular complex C'1 (Müller-Eberhard, 1968, 1969). Different immunoglobulins react with C'1 with different efficiencies: γ_1, γ_2, and γ_3 react well (Ishizaka *et al.*, 1967), whereas γ_4 and γ_A react

poorly if at all (Ishizaka *et al.*, 1967; Müller-Eberhard, 1968, 1969). Macroglobulins were known to activate C'1 for some time, but it was recognized only recently that, in rabbits (Hoyer *et al.*, 1968), mice (Plotz *et al.*, 1968), guinea pigs (Hyslop and Matheson, 1967; Linscott and Hansen, 1969), and humans (Mackenzie *et al.*, 1969), noncomplement-fixing γM antibodies may also be present.

The mechanisms by which γM and γG mediate complement activity is under active investigation at present. Recent studies indicate that both the initial activation of C'1 as well as later steps in the complement sequence are influenced by the class of immunoglobulin utilized.

1. Initiation of Complement Fixation

On cell surfaces a single molecule of γM suffices to fix C'1, whereas antibody "doublets" are required for fixation by γG immunoglobulins (Humphrey and Dourmashkin, 1965; Borsos and Rapp, 1965a,b). This difference may be partially explainable if activation requires saturation of two sites on a polyvalent C'1q component (Müller-Eberhard and Calcott, 1966). If there is a C'1-fixing site for each pair of immunoglobulin heavy chains, single γM molecules would be effective but two γG molecules side by side would be necessary.

Differences in C'1 activation by γM and γG also become manifest when the temperature is varied. The C'1 is efficiently activated by γG at both 4° and 37°C., whereas γM hemolysins are very inefficient at the lower temperature (Stollar and Sandberg 1966; Colten *et al.*, 1967). Using red cell–hemolysin–C'4 intermediates (EAC'4), it can also be shown that the rate and extent of activation by γM antibody is greater than when γG antibody is used (Colten *et al.*, 1969). Whereas γG antibodies fix C' optimally at equivalence ratios of antigen to antibody, γM shows maximal activity in antibody excess (Ishizaka *et al.*, 1968). At the optima the two classes of antibody were equivalently active on a molar basis in that study. Other differences between γM and γG complexes on cell surfaces and with soluble or insoluble antigens were noted by the latter workers.

2. Influence on Later Steps in the Complement Sequence

Several complement components appear to attach directly to cell membranes and not to antibody molecules (Müller-Eberhard and Biro, 1963; Müller-Eberhard *et al.*, 1966). For example, complement components remain attached even when the hemolysin is dissociated from the complex [such as by warming of cold hemolysin–cell complexes (Harboe, 1964)], and the lytic sequence may go to completion after removal of all detectable antibody (Müller-Eberhard and Lepow, 1965). Although these observations at first suggest a role for antibody only in the initia-

tion of complement fixation, current investigations suggest that the immunoglobulin class of the hemolysin may have more complex effects. Frank and Gaither (1970a) have shown that the temperature-dependent differences in lytic efficiency described above are demonstrable when several complement–red cell intermediates (EAC'1, EAC'14, EAC'4, EAC'142, and EAC'1423) are used, thus suggesting that these intermediates reflect the differences in the hemolysin used for their initiation. Later intermediates, e.g., EAC'1423567 prepared with γM hemolysin-sensitized cells lysed equally well at 37° and 4°C. In a second study, Frank and Gaither (1970b) documented substantial differences in relative titers of whole guinea pig complement and partially purified components C'1, C'2, C'3, C'4, C'5, C'6, C'8, and C'9 when assayed with γG- and γM-sensitized erythrocytes. Their results further suggest that although γM hemolysins may more effectively initiate complement fixation such sites may utilize complement components more efficiently when they are initiated by γG immunoglobulins.

It has been observed (Frank *et al.*, 1970) that cells lysed with γG antibody may have many more "holes" than those lysed with γM antibody when excess guinea pig complement is used. Human complement-induced lysis did not demonstrate such a difference. The stage at which the hemolysin acts to produce this effect remains uncertain.

The entire complement sequence is required for hemolysis as well as for other cytolytic and cytocidal effects (Inoue *et al.*, 1968; Inoue and Nelson, 1966). For other phenomena, such as precipitation of immune complexes (Paul and Benacerraf, 1965), immune adherance (Nishioka and Linscott, 1963), phagocytosis enhancement (R. A. Nelson, 1965), chemotaxis (Shin *et al.*, 1968; Ward *et al.*, 1966), anaphylatoxin generation (Cochrane and Müller-Eberhard, 1968), γM-mediated adhesion to macrophages (Huber *et al.*, 1968), and virus neutralization (Daniels *et al.*, 1969; Linscott and Levinson, 1969), the participation of only a limited number of complement component is required. The influence of the immunoglobulin class on several of the early steps in the complement sequence can be expected to have significance for these reactions also.

3. *Inactivation of γM-Initiated Complement Fixation*

Complement fixation by γM is markedly inhibited after exposing the immunoglobulin to thiol reagents. Murray *et al.* (1965b) found that with 0.03 to 0.10 *M* ethanethiol (C_2H_5SH), γM activation was differentially depressed compared to γG activation. Stollar and Sandberg (1966) achieved similar results with mercaptoethanol. Differential loss of γM complement activation can also be irreversibly affected by heating (60°C.) (Murray *et al.*, 1965a) and by exposure to 4 to 5 *M* urea at 37°C. (Cunniff *et al.*, 1968).

C. Interactions with Other Proteins

Weak associations between macroglobulins and other proteins have been described. A series of eight specimens for patients with Waldenström's macroglobulinemia showed such weak interactions with serum albumin, leading to unusual immunoelectrophoretic effects (Hartmann *et al.*, 1966). Association between γG and γM from normal individuals has also been observed (Filitti-Wurmser *et al.*, 1966), but neither reaction has been extensively characterized.

A more complete study of albumin binding to γA myeloma proteins and Waldenström macroglobulins was published by Mannik (1967b). He found complexes that would only dissociate after reaction with reducing reagents (2-mercaptoethanol was used) but not by denaturing reagents such as 5 *M* guanidine hydrochloride. References to earlier works on the association of immunoglobulins to various serum proteins can be found in Mannik's paper.

The significance of these interactions remains obscure.

D. Interactions with Cells

Specific receptor sites on macrophages and other leukocytes for the Fc region of γG immunoglobulins have been described (Uhr, 1965; Berken and Benacerraf, 1966; Rabinovitch, 1967). A separate site, sensitive to trypsin and directed toward the third component of complement can also cause binding of immunoglobulins to leukocytes by way of antigen–antibody–complement complexes ("immune adherance") (D. S. Nelson, 1963; Lay and Nussenzweig, 1968; Huber *et al.*, 1968). Macroglobulin–antibody complexes can be bound by this mechanism (Huber *et al.*, 1968; Henson, 1969) though the reaction is weaker and, curiously, may be enhanced by the presence of uncomplexed γG (Huber *et al.*, 1968).

A distinctive receptor for γM immunoglobulins on mouse macrophages which requires Ca^{++} ions and which is not destroyed by trypsin has been investigated by Lay and Nussenzweig (1969). The binding is reversed if Ca^{++} is decreased by dilution or chelation. It is unclear at present whether the receptor sites for guinea pig γM studied by Del Guercio *et al.* (1969) are similar to the latter sites. A useful discussion of the difficulties that may have led to the early failure to recognize γM receptor sites may be found in the paper by Lay and Nussenzweig (1969).

The limited data on this subject are no reflection of its importance. Phagocytosis of immune complexes is undoubtedly a major defense mechanism, and specific cell receptors for immunoglobulins can be expected to play a critical role in this process.

VII. Genetic Basis of Macroglobulin Structure

A. Evidence for Subclasses

I have already cited certain studies which suggest that human γM immunoglobulins may be divisible into subclasses. The relationship between the classes suggested by Filitti-Wurmser *et al.* (1964) on the basis of sedimentation rates (Section III,A,*1*), by Davie and Osterland (1968) on the basis of carbohydrate content (Section III,B,*2*), and by Mackenzie *et al.* (1969) on the basis of ability to fix complement (Section VI,B) is completely unknown. The possibility that low molecular weight macroglobulin-like proteins and hexameric γM molecules result from unique μ-chain loci must also be considered (Section III,A,*10* and V).

Additional evidence for subclasses comes from studies by Harboe *et al.* (1965) and by Franklin and Frangione (1967, 1968). Harboe *et al.* immunized rabbits with γM immunoglobulins isolated from patients with Waldenström's macroglobulinemia. One such antiserum when partially absorbed with normal human serum reacted with 9 out of 22 sera from patients with Waldenström's macroglobulinemia and 5 of 5 normal serums. Franklin and Frangione (1967) similarly obtained a rabbit antiserum which when absorbed with an antigenically deficient serum reacted with one-third of forty macroglobulins. The reaction was not related to the solubility properties, electrophoretic mobility, or light-chain type of the protein. Importantly, each of twenty-six normal sera reacted with the absorbed antiserum. In subsequent work, Franklin and Frangione (1968) showed that the Fab μ fragments carry the determinant in question and that tryptic peptide maps of the μ chains contained one or two distinctive spots correlating with the serological differences. In addition, these maps suggested that the seropositive and seronegative groups might each be divisible into two further subtypes.

Interpretation of these data must be provisional. An important question is whether these differences are related to variations in amino acid sequence (and, hence, to the μ-chain genes) or only to differences in carbohydrate (and, hence, of undetermined origin). Although Franklin and Frangione (1968) have obtained peptide map differences, this does not rule out carbohydrate differences which might (*a*) influence the mobility and R_f of peptides directly or (*b*) affect the site of tryptic hydrolysis. That each of twenty-six normal sera was reactive with the antiserum is strong evidence against the variations being due to allelic polymorphism. Similarly, that both noncomplement-fixing and complement-fixing γM proteins can be found in the sera of inbred mice (Plotz *et al.*, 1968) is not explainable on the basis of simple allelism.

B. Allelic Markers

Macroglobulins can potentially show allelic variability due to differences in light chains, heavy chains, or both. Among human proteins only light-chain InV variants have so far been detected. The K-type γM proteins of an individual will evidence the same InV determinant(s) as the other K-type immunoglobulins. Unlike the situation with γ_2 heavy chains —where combination of that subclass of heavy chains with κ chains may result in inefficient expression of the InV determinant (Steinberg and Rostenberg, 1969), μ chains do not appear to affect the InV marker appreciably. The light-chain allotypes of rabbits are similarly easily recognized on γM proteins.

Allelic markers referrable to μ chains have so far been found only on rabbit proteins. Here the α, μ, and γ chains appear to have a common marker: the operationally defined a locus with alleles Aa1, Aa2, Aa3 (Todd, 1963; Feinstein, 1963; Lichter, 1967; Pernis *et al.*, 1967). Quantitative precipitin data suggest that the allotype marker is identical in each of these heavy-chain classes (Pernis *et al.*, 1967) (cf. Segre *et al.*, 1969). Initial studies, furthermore, indicate that there are shared amino terminal sequences in α and γ chains which correlate with the allotype (Wilkinson, 1969a,b). Since the structural data indicate that the common regions of these heavy chains are coded for by distinctive genes, the allotypic data which indicate shared loci suggest that the heavy chains may be coded for by two separate genes. Furthermore, a genetic marker (shared or not) in the variable region would seem to place important constraints on theories of antibody variability. These matters are discussed at length in Cohn (1968), Edelman and Gall (1969), Metzger (1970), and Hood and Talmadge (1970).

Intraspecific differences in antigenic determinants, distinct from the a and b loci determinants, have been reported for rabbit γM (Kelus and Gell, 1965; Sell, 1966; Kelus, 1967). The determinants (dubbed Ms 1, Ms 2, . . .) may require the presence of certain a and b alleles in order to be expressed (Kelus, 1967). A full analysis of these markers has not yet been published.

C. Idiotypic Markers

As with other immunoglobulins, individual γM proteins may be used to prepare antisera which will be directed to structures more or less unique to the immunogen. They were first described for γM proteins by Habich (1953), and more recent references may be found in Harboe *et al.* (1969). Interestingly, some of these idiotypic determinants may be specific for proteins having a common function. For example, Williams

et al. (1968) described certain antisera that demonstrate specificity for groups of γM cold agglutinins but not for γM immunoglobulins lacking such activity. One cannot rule out the possibility that these determinants may relate to minor subclasses or alleles, but this seems unlikely (Williams *et al.*, 1968). It is interesting in this respect that thirteen of fourteen cold agglutinins reacted positively with the antiserum described by Franklin and Frangione (above) (Franklin, 1969).

VIII. Biosynthesis and Metabolism of Macroglobulins

A. Biosynthesis

1. Cellular Origins

Cells producing 19 S-γM immunoglobulin are not distinguishable as a group from cells producing other immunoglobulins, by either conventional or electron microscopy (Harris *et al.*, 1970). Both γG and γM immunoglobulins are produced by two types of cells, lymphocytic and plasmacytic, each of these types showing considerable morphological variability. In the rabbit, 80–85% of γM plaque-producing cells were of the plasmacytic form. Substantially similar results were obtained with mouse cells. The paper by Harris *et al.* (1969) describes some of the elegant techniques used to isolate and examine functionally relevant cells.

One study (Nossal *et al.*, 1964) suggests that single antibody-producing cells may switch from macroglobulin to γG synthesis, but one would like to see more extensive supportive data. Immunofluorescent data that show essentially all cells as producing a single class of heavy chain (Cebra *et al.*, 1966) do not necessarily conflict with Nossal *et al.*'s results if the switchover time is short. More extensive data on cells in tissue culture indicate that single cells can produce both γM and other immunoglobulins (Fahey and Feingold, 1967; Takahashi *et al.*, 1968) but one must be cautious about extrapolating from these data to physiological situations. As discussed elsewhere (Metzger, 1970), the data on the homogeneity of immunoglobulin receptors on individual presumptive antibody precursor cells are conflicting.

2. Assembly

The first study on the cellular assembly of γM molecules has just appeared. Parkhouse and Askonas (1969) studied the incorporation of tritiated leucine into γM proteins by cell suspensions of the Balb/c mouse tumor, MOPC 104E. The cells secreted γM and light chains in a

1:2 weight ratio with a lag period of 20 to 30 minutes. Within the cells, a 7 S component (presumably γM_s) accumulated and even after 3 hours of incubation only traces of completed 19 S molecules were detected in the cell lysates. On the other hand, essentially all of the secreted macroglobulin was of the 19 S variety. The authors conclude that "The processes of polymerization and secretion are . . . intimately related, affording a mechanism for the selective secretion of the large molcules."

As indicated in Section VI,A,2, the light chains of individual γM molecules have been said to be mixed κ and λ (Costea *et al.*, 1966, 1967), suggesting postsecretion assembly of subunits or, alternatively, homogeneous with respect to light-chain allelic markers (Schmale *et al.*, 1969), suggesting assembly prior to secretion. Data on allelic markers on the μ-chains support the latter result (Pernis *et al.*, 1967).

B. Distribution

Macroglobulins are distributed predominantly in the intravascular pool both in rabbits (Taliaferro and Talmadge, 1956) and humans (Cohen and Freeman, 1960; Barth *et al.*, 1964). Unlike γG antibodies, no extensive maternal–fetal transport of γM immunoglobulins takes place. In a study with iodinated proteins in humans, Gitlin *et al.* (1964) demonstrated some transfer of γM—the fetal blood level reaching ~10% of the maternal concentration, but most fetal γM (mean concentration 0.10 mg./ml.) appears to be accounted for by local synthesis (Van Furth *et al.*, 1965). There is no correlation between γM (or γA) levels in the maternal and fetal sera (Stiehm and Fudenberg, 1966; Johansson and Berg, 1967).

C. Rates of Synthesis and Catabolism

An extensive review of immunoglobulin metabolism has appeared recently (Waldmann and Strober, 1969) and I will, therefore, summarize the data on γM only briefly.

In humans, synthesis of γM generally reaches substantial levels around the twentieth week of gestation (Van Furth *et al.*, 1965; Toivanen *et al.*, 1969). In humans, 4.5–6.9 mg. of γM are synthesized per kilogram per day—equivalent to 2×10^{17} molecules for the "standard" 70-kg. individual. This is only about one-twentieth the number of γG molecules produced per day. On the other hand, the fractional catabolic rate (see Waldmann and Strober, 1969) is 2–3 times that of γG, and this rate is unaffected by the serum concentration over a greater than 1000-fold range (Barth *et al.*, 1964). As with other immunoglobulins, the sites of γM catabolism are uncertain. The lower synthetic rate and higher cata-

bolic rate account for the relatively low level of γM in normal human serums: 0.8–0.9 mg./ml.

Many other details regarding the metabolism of γM and other immunoglobulins in both normal individuals and in patients with a variety of disease states will be found in Waldmann and Strober's review.

IX. Macroglobulin-Like Proteins from Nonmammalian Species[4]

Cyclostomes and higher forms synthesize high molecular weight antibodies which resemble mammalian immunoglobulins. Some properties of these proteins are listed in Table VII. Not shown in the table are the recent electron-microscopic results of Feinstein and Munn (1969) which indicate that dogfish and chicken high molecular weight antibodies have a configuration very similar to γM from several mammalian species. At the risk of being overconservative, however, it seems prudent to reserve judgement on the extent to which these nonmammalian high molecular weight antibodies are homologous to mammalian γM (i.e., are directly derived from a common ancestral heavy-chain gene). For example, it is impossible to exclude rigorously that they may be more closely related to mammalian γA.

Where measurements have been made, the heavy chains of these proteins appear to have a molecular weight of ~70,000, similar to the most frequently observed value for mammalian μ chains. The molecular weight data for the whole immunoglobulins as well as the electron-microscopic data cited above indicate that many of these proteins are likely to have a pentameric structure. Recent evidence for a hexameric "γM" from the frog *Xenopus levis* (Parkhouse *et al.*, 1970) (Fig. 4) and micrographs of the high moleclar weight antibodies of carp which appear tetrameric (Shelton, 1970) (Fig. 6) show, on the other hand, that each system deserves independent exploration.

Many nonmammalian sera show a prominent 7 S immunoglobulin component. In the elasmobranchs this 7 S protein is difficult to distinguish from the 7 S subunits derived by reduction from the 18 S component (Marchalonis and Edelman, 1965; Clem and Small, 1967). The proteins have similar antigenic properties, and their constituent polypeptide chains have similar disc electrophoretic mobilities, molecular weights, amino acid compositions, and tryptic peptide maps. One distinguishing feature observed by Clem and Small (1967) remains unexplained. They found that, whereas the 18 S antibodies lost their agglutinating capacity upon reduction, the 7 S antibodies did not. Possibly,

[4] Two useful reviews appeared too late to be discussed in this article: Clem and Leslie, 1970; Grey, 1969c.

TABLE VII

NONMAMMALIAN MACROGLOBULIN-LIKE PROTEINS[a,b]

Properties	Cyclostomes				Elasmobranchs (3)		Teleosts (4)		Amphibia (5)		Avian (6)	
	Pacific hagfish (1)[c]		Lamprey (2)									
	High	Low	High	Low	High	Low	High	Low	High	Low	High	Low
I. Structural												
Sedimentation rate	>28 S	—	14 S	6.6 S	~19 S	~7 S	16 S	6.4 S	18 S	6.7 S	15–16 S	7.1 S–7.4 S
Mol. wt. × 10^{-5}	N.R.	—	N.R.	1.0	8.7 ± 0.6	1.6 ± 0.09	9.0	1.2	N.R.	N.R.	8.9	1.7–1.8
$\epsilon^{0.1\%}_{280m\mu}$	1.45	—	N.R.	N.R.	1.34	1.38	1.38	1.66	N.R.	N.R.	1.27	1.3–1.5
Carbohydrate(%)	3.4 (hexose)	—	N.R.	N.R.	3.7 (hexose)	3.5 (hexose)	"High"	"Low"	10.8 (total)	2.1 (total)	2.6 (hexose)	2.2 (hexose)
Heavy-chain mol. wt. × 10^{-4}	N.R.	—	N.R.	7.0	7.1	7.1	7.0	4.0	7.2 ± .2	5.4 ± .2	7.0	6.7
Light-chain mol. wt. × 10^{-4}	N.R.	—	N.R.	2.5	2.2	2.2	2.2	2.2	2.0 ± .1	2.2 ● .1	2.2	2.2
II. Functional												
Valence	N.R.	—	N.R.	N.R.	5–10	1–2	5–10	1–2	N.R.		N.R.	2

[a] Numbers in parentheses indicate the following references:

(1) Thoenes and Hildemann (1970).

(2) Marchalonis and Edelman (1968).

(3) Marchalonis and Edelman (1965); Clem and Small (1967); Voss *et al.* (1969); Klapper *et al.* (1970); Clem *et al.* (1967); Suran *et al.* (1967).

(4) Clem and Small (1970).

(5) Marchalonis and Edelman (1966).

(6) Leslie and Clem (1969); Gallagher and Voss (1969); Orlans *et al.* (1961).

[b] "High" and "Low" indicate high and low molecular weights, respectively. N.R. = not reported.

[c] A low molecular weight immunoglobulin has not been reported for this species.

the 18 S antibody sites had a rather low intrinsic binding affinity which in the polymer was amplified because of polyvalency.

The relationship between the 7 S and the 18 S immunoglobulins is still uncertain as is true for the 7 S-γM-like proteins in mammals (Section V). During an immune response the ratio of 7 S to 18 S activity may increase (Clem and Small, 1967), suggesting the possibility that the 7 S and 18 S may be more distinctive immunoglobulins than present data would suggest.

Although the situation in teleosts remains unclear (Clem and Small, 1970), in higher forms (amphibia, etc.), 7 S components distinctively different from γM proteins are discernible (Marchalonis and Edelman, 1966).

Data on the valence of nonmammalian immunoglobulins are in a confusing state of affairs at this writing—another property which they apparently share with mammalian γM! The recently published study of Voss *et al.* (1969) on lemon shark anti-dinitrophenyl antibodies concludes that there is one effective site per 7 S unit and five per pentamer, but close examination of their data suggests that their extrapolations may be somewhat arbitrary. Clem and Small (1968, 1970) studied the valence of immunoglobulins of the giant grouper (a marine teleost), but a definite value cannot yet be assigned. The extreme heterogeneity (and rather low average binding constants) evidenced in the published binding data suggests that a useful approach may be to isolate a more limited, tightly binding set of molecules——for example, by fractional precipitation. The 7 S components have sometimes been reported to have bivalent properties (Suran *et al.*, 1967; Clem *et al.*, 1967; Clem and Small, 1967) and other times not to have (Voss *et al.*, 1969). Larger amounts of material and study of Fab and $F(ab')_2$ fragments (Klapper *et al.*, 1970) may help to resolve the issue.

X. Role of Macroglobulins in the Immune Response

So far, I have concentrated on the structure of γM macroglobulins, on their interaction with antigen, the complement system, and with certain cells. Ultimately, it is desirable to incorporate these structural–functional relationships into a coherent scheme for the role—particularly for any special role—that γM antibodies perform in the immune response.

Antibodies appear to function in two important ways in the immune response. First, cell-bound antibodies serve as antigen receptors on those cells that are the immediate precursors of antibody-producing cells, those cells that mediate cellular immune reactions, and those antigen-sensitive "helper" cells that appear to facilitate the productive interaction between antigen and antibody precursor cells (Metzger,

1970). Second, secreted antibody participates in the control of further specific antibody production apparently by influencing the availability of antigenic determinants to the cell-bound receptors (Uhr and Möller, 1968).

In this section I shall consider some of the data which suggest that γM antibodies may subserve these functions in unique ways.

A. Macroglobulins As Antigen Receptors

There is little information with respect to the nature of the antigen receptor on helper cells or on those cells that mediate cellular immunity. The weight of evidence is in favor of their being immunoglobulins (Metzger, 1970). One study implicates conventional light chains on these receptors (Greaves *et al.*, 1969), but there is no information as to the type (if any) of heavy chain involved. On the other hand, there is substantial evidence that immunoglobulins of all classes are involved on those cells which will produce antibodies or daughter cells which will. Macroglobulins can be envisioned as playing a special role as receptors on such cells for one or more of the following reasons: (*1*) the combining sites of γM antibodies may exhibit certain specificities not present among immunoglobulins belonging to other classes; (*2*) the polyvalency of γM receptors may influence the ease with which the cells bearing such receptors are triggered; and (*3*) γM-bearing cells may have unique functions.

The first point has already been discussed (Section VI,A,*1*). There are no substantial structural or functional data which suggest that γM antibodies have a unique set of specificities.

Data with respect to the second point have been presented by Mäkelä and associates (1967; Mäkelä and Kontiainen, 1969). Reasoning that a multivalent receptor would react more strongly than a bivalent receptor with multivalent antigens, they studied the influence of antigenic valency on the class of antibody produced. Their initial results suggest that, indeed, a multivalent antigen markedly enhances the γM response compared to that observed with a paucivalent antigen. If the γM-bearing cells were partly being triggered on the basis of the receptor valency rather than simply on the basis of the intrinsic binding affinity of receptor sites, then the γM antibody sites might have a lower intrinsic binding constant than the simultaneously produced γG antibodies. Some experimental confirmation of this prediction has been obtained (Mäkelä *et al.*, 1967; Mäkelä and Kontiainen, 1969). The data suggest that early in an immune response γM receptor-bearing cells might be stimulated prior to significant cell selection by antigen (see Siskind and Benacerraf, 1969). Whether this accounts for the frequently

observed fact that γM antibodies tend to be preferentially synthesized in the early stages of the immune response is uncertain but it is certainly likely that it contributes to the phenomenon.

Several groups (Naor and Sulizeanu, 1967, 1969; Humphrey and Keller, 1970; Ada and Byrt, 1969; Ada *et al.*, 1969; Byrt and Ada, 1969) have recently reported on the distribution and effect of highly radioactive antigens added to unprimed lymphoid cells. Both Humphrey and Keller and Ada and associates found that with such "hot" antigens the immune response could be inhibited—presumably by killing the cells to which the radioactive antigen had bound. Significant here is that such uptake has so far been inhibited only by anti-light-chain and anti-μ-chain sera (Ada *et al.*, 1969). It is not known whether helper cells or antibody precursor cells or both are being affected in this study, but it suggests that there are certain lymphoid cells bearing γM receptors on their surface that may be critical to the production of serum antibodies. There is evidence that γM antibodies were the first to evolve (Section IX) and they are usually the first to appear in ontogeny (Good and Papermaster, 1964; Sterzl and Silverstein, 1967). It would not be surprising if the mature immune response continued to reflect the special evolutionary position of γM immunoglobulins.

B. Macroglobulins in the Control of Antibody Synthesis

The specific role of secreted γM antibodies in the control of antibody production is complicated and there is much conflicting information (Uhr and Möller, 1968). A recent study by Henry and Jerne (1968) gave some clear-cut and provocative results. They administered purified γM and γG antibodies before or shortly after immunization with a homologous antigen. The γG antibodies suppressed the subsequent immune response, whereas the γM antibodies markedly enhanced it. Mixtures of γG and γM antibodies gave the expected algebraic sum of the individual effects. Further studies are required to know just how general these effects are and the mechanism of action. Henry and Jerne suggest that 7 S antibodies may cover immunogenic determinants, whereas cytophillic γM antibodies, by binding antigen to macrophages, may promote contact between antigenic determinants and relevant receptors on lymphocytes.

XI. Prospects

The weight of evidence now favors a molecular model of γM consisting of a circular (usually pentameric) array of equivalent symmetrical, four-chained subunits, each containing two equivalent (though not

always simultaneously available) antigen combining sites. The evidence against such a model has been cited but, in my view, it is unconvincing. Those proposing more exotic models have yet to prove their case.

Those pursuing the primary sequence of the μ chain will settle the remaining ambiguities about the size of these proteins, and further molecular weight studies on garden-variety macroglobulins seem to me a bad investment of effort at this juncture. Though occasional crystalline γM proteins have been described, I am not aware of any promising

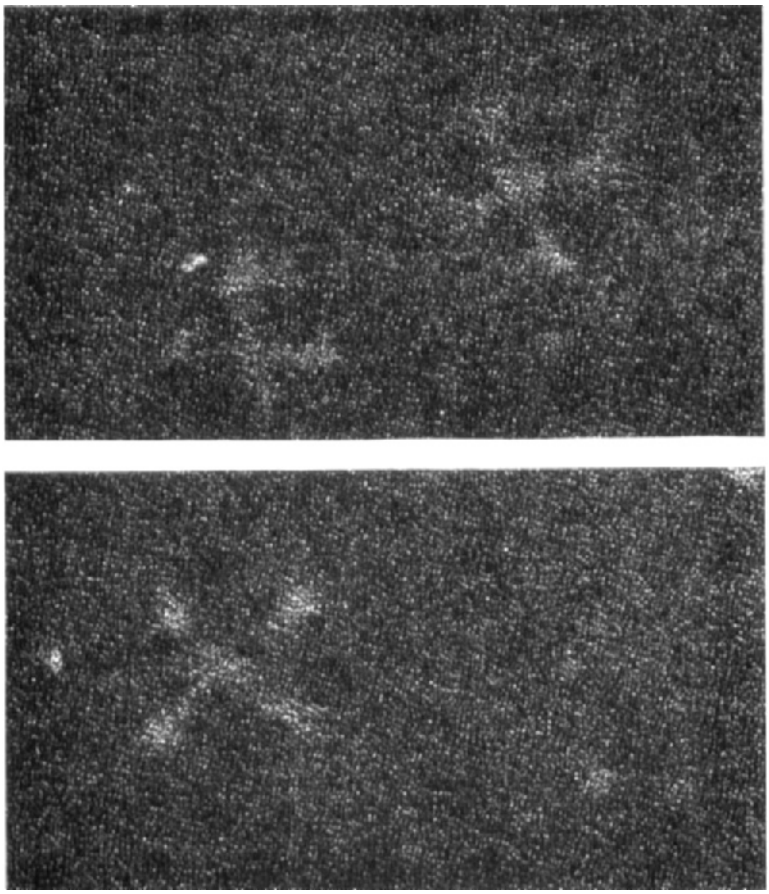


FIG. 6. Electron micrograph of tetrameric immunoglobulin isolated by Drs. M. Smith and E. Shelton from carp. (The micrograph was kindly provided by Dr. E. Shelton.)

diffraction studies and we may, for some time to come, have to satisfy ourselves with extrapolations from X-ray patterns of γG immunoglobulins and with the more indirect, but increasingly sophisticated, optical methods of conformational analysis.

The most interesting problems remain: How do these molecules function—both as cell receptors and as effectors for antigen disposal? If they were the most primitively arising immunoglobulins, what came before them? Does their present role still reflect their putative unique place in the origins of the immune response? Providing the chemical answers to these questions will require the combined talents of protein chemists and cellular biologists.

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Transplantation Antigens

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I. Introduction

The early recognition that genetic constitution plays a key role in the fate of tissue grafts (histocompatibility) (Little and Johnson, 1922; Loeb, 1930; Snell, 1948) led to many investigations which showed that in each species studied thus far there is a single strong histocompatibility locus: the H-2 locus of mice (Snell, 1948; Shreffler, 1967), the HL-A locus of man (van Rood and Ernisse, 1968; Dausset *et al.*, 1965; Cepellini *et al.*, 1968), the Ag-B (H-1) locus of rats (Palm, 1964; Stark *et al.*, 1968), and the B locus of chickens (Jaffe and Payne, 1943; Crittenden *et al.*, 1964). Presumably it is the antigenic products of the strong histocompatibility locus or of multiple weak loci operating in concert (Graff *et al.*, 1966) which play a dominant part in the rejection phenomenon and are, thus, of great biological interest. Serological studies suggested that the gene product(s) possesses several antigenic specificities, and from genetic studies it appears that these are determined by a single chromosomal region (Snell *et al.*, 1964). The gene product(s) of this chromosomal region appears to be essential for cell function since determinants of histocompatibility antigens can be demonstrated on all cells and can be detected on cells perpet-

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uated in tissue culture (Spencer *et al.*, 1964; Gangal *et al.*, 1966). Since allografting is not a natural occurrence, one would expect that survival pressure would have long discarded these polymorphic antigenic components unless they played an important role in cell structure or function. It has been proposed that histocompatibility antigens mediate transport (Sanderson and Davies, 1963; Pardee, 1968) or, more probably, cell contact and recognition phenomena (Moscona, 1963; Möller and Möller, 1966; Amos, 1966; Humphreys, 1967; Crandall and Brock, 1968). Burnet (1970) postulated that the generation within a species of a large polymorphism of histocompatibility antigens and of a diversity of immune patterns for cell receptors developed in early vertebrate evolution mainly as a protection against somatic cells which became neoplastic and present a new antigenic profile. Jerne (1970) proposes that self-tolerance and antibody diversity are achieved by the same process by postulating that germ-line antibody genes are structural genes for antibodies directed against histocompatibility antigens and that the suppression of "forbidden" clones of cells permits a diversity of mutant cells to emerge. Histocompatibility antigens as products of fell-defined genetic systems are, thus, not only important in elucidating the nature of the rejection phenomenon but may also provide a key to the understanding of basic immunological processes.

Considerable efforts have been made during the last decade to solubilize transplantation antigens from their site on the surface of cell membranes, owing largely to the discovery by Medawar (1963) that nonparticulate transplantation antigens administered intravenously tend to induce prolonged graft survival. A whole array of procedures has been employed to solubilize these leukocyte antigens using diverse methods such as detergent and organic solvent extraction, digestion with numerous proteolytic enzymes, and application of high- and of low-frequency sonic energy in an attempt to obtain large enough amounts of these antigens sufficiently purified to evaluate their immunogenic and tolerogenic capacities.

The extraction of spleens and of tumor cells with detergents produced immunogenic murine transplantation antigens (Kandutsch, 1960; Manson and Palm, 1968) and human transplantation antigens (Metzgar *et al.*, 1968; Bruning *et al.*, 1968) which were, however, not readily water-soluble and proved difficult to characterize by chemical means. Organic solvent extraction of murine spleen and tumor cells also produced immunogenic transplantation antigens which were composed largely of such highly complex lipoproteins that they could neither be extensively purified nor characterized (Kandutsch, 1960; Manson and Palm, 1968).

Murine transplantation antigens were obtained in solubilized form following digestion of spleen and ascites tumor cells with proteolytic

enzymes (Davies, 1966b, 1967; Nathenson and Davies, 1966; Summerell and Davies, 1969; Shimada and Nathenson, 1969). Similarly, proteolytic digestion of human spleen cells (Sanderson and Batchelor, 1968; Sanderson, 1968) and of cultured leukocytes derived from donors with lymphoid malignancies (D. L. Mann *et al.*, 1968, 1969a,b) resulted in the extraction of solubilized alloantigens.

The longtime and widespread use of sonic energy to liberate water-soluble substances from intracellular, intraorganelle, and membranous locations on bacterial and animal cells (Grabar, 1953) led to the early application of this method in an attempt to solubilize transplantation antigens. Billingham *et al.* (1956b) applied probe-mediated, high-frequency, high-intensity ultrasound (20 kc./sec., 60 W.) and detected only small amounts of soluble, immunogenic mouse transplantation antigen, whereas Haughton (1964) found that antigen was released and then rapidly inactivated after exposure to ultrasound. On the other hand, application of low-frequency, diaphragm-mediated sonic energy (9–10 kc./sec.; 15 W.) made it possible to obtain reasonable amounts of water-soluble transplantation antigens from mouse spleen cells (Kahan, 1964 1965), from guinea pig spleen (Kahan and Reisfeld, 1967) and sarcoma cells (Kahan *et al.*, 1969), from dog spleen cells (Dagher *et al.*, 1967), from human spleen cells (Kahan *et al.*, 1968b), and from cultured leukocytes derived from normal donors (Reisfeld *et al.*, 1970a).

II. Extraction and Solubilization of Transplantation Antigens

The whole concept of solubilization of histocompatibility antigens from their site on the cell membrane surface is based upon the assumptions that (*1*) antigen molecules can be isolated independent of the membrane structure, (*2*) the solubilized product represents the antigen molecule with its antigenic determinants more or less in the same form as on the cell membrane, and (*3*) there are no immunologically significant intermediate linkages between solubilized antigens and other constituents of the membrane.

Definitions vary as to what constitutes a solubilized antigen. Many investigators consider an antigen to be in a soluble form if it does not sediment when subjected to centrifugation at 100,000*g*, i.e., is free of any visible cell membrane fragments. This concept is questionable since Rapaport *et al.* (1965) found that antigen which did not sediment at 100,000*g* did contain membrane fragments, as determined by ultrastructural studies of sediments obtained from these "soluble" antigens following centrifugation at 200,000*g*. Some of the definitions as to what constitutes a "soluble" and "stabilized" antigen preparation are not too meaningful. Davies (1968) has distinguished "solubilized" from "stabi-

lized" antigens on the basis that the latter are chemically complex polymolecular lipoprotein complexes which do not sediment in the presence of detergents during short-term high-speed centrifugation and which are immunogenic but not separable molecular entities. Soluble antigens as defined by Davies (1968) can be fractionated into molecular entities carrying different antigenic determinants and possessing binding capacity for cytotoxic antibodies. Blandamer *et al.* (1969) defined "as an acceptable demonstration of solubility that the antigenic material should be able to pass through a gel filtration column and not be eluted with the void volume." To use the behavior of antigenic material on Sephadex columns is not entirely acceptable since it is known that these materials can easily aggregate and thus be excluded. Most properly, the solubility of a substance should be defined by its characteristic solubility in any given solvent that does not denature it. Since most antigenic preparations isolated thus far are complex mixtures of proteins and conjugated proteins subjected to intricate protein–protein interactions, classic solubility characteristics, such as those used to define protein purity, are hardly applicable. Thus, at present, it is most useful to define solubility in practical terms, by the absence of membrane fragments detectable by ultrastructural methods and by the applicability of methods, such as ion-exchange chromatography and gel electrophoresis, which resolve antigen preparations *without* serious loss of biological activity due to insolubility at varying protein concentrations in a number of aqueous solvents.

The question of solubility may be of critical importance during the evaluation of purification especially in the assessment of yield. It is conceivable that the loss of antigenic units may reflect the precipitation of insoluble antigenic materials contaminating the "soluble" preparations. A useful and practical criterian of solubility consists of prolonged dialysis of the antigenic preparations against distilled water followed by extensive ultracentrifugation at 130,000*g* without serious loss of antigenic activity of the supernatant.

Numerous attempts have been made to solubilize transplantation antigens from cell membranes with detergent and organic solvent treatments, with proteolytic digestion, and by application of sonic energy.

A. Detergent Extraction

1. Triton and Potassium Cholate

Kandutsch (1960) found that 5% Triton X-100 was able to extract an antigenic material from particulate fractions of the A strain mouse ascites tumor Sarcoma I. This material was almost completely insoluble in water in the absence of detergent and at pH values in the region of

neutrality. A molecular weight of less than 200,000 was stipulated since the sedimentation coefficient ($s_{20,w}$) in the presence of Triton was 2.65 (Kandutsch, 1963). The yield of the whole Triton extract comprised from 30 to 40% of the weight of the freeze-dried cell particulate fraction. Treatment of this extract with 0.1 *M* sodium phosphate buffer, pH 6.9 and with 0.14 *M* saline resulted in a preparation (5–8% by weight of the lyophilized particulate fraction) which did not sediment when centrifuged at 100,000*g* for 30 minutes. This extract, which was soluble in dilute salt solution when treated with snake venom, of which the active component was thought to be phospholyase A, could be readily dissolved into a slightly opalescent solution in 0.1 *M* tris buffer, pH 7.9. However, when centrifuged at 100,000*g* for 2 hours, approximately 50% of this material was sedimented. The snake venom-treated preparation showed only one major component by moving boundary electrophoresis (pH range 6.6–9.0), but ultracentrifugal analysis revealed this material to be quite polydisperse suggesting that the antigen was either a collection of polymeric units or still contained small cell membrane fragments. It is quite possible that actual electrophoretic heterogeneity of this complex substance was masked by the alteration of its overall charge due to the presence of lysophosphatide groups following enzymatic treatment. A similar problem arises when other detergents, e.g., sodium dodecyl sulfate, are applied in electrophoretic characterization of antigen preparation. It is also feasible that the polydispersity observed was due to extensive chemical complexity rather than to polymeric forms of a single substance. The enzyme-treated material seemed to be composed of lipo- and glycoproteins since analyses showed the following: nitrogen, 8.91%; hexose, 2.38%; phosphorus, 0.97%; lipid, 33.7%. In an attempt to elucidate the chemical nature of the antigenic determinant, Kandutsch (1963) extracted the antigen with chloroform–methanol and found that most of the antigenic activity was destroyed as was the case when more purified preparations were treated with 0.004 *M* sodium–*m*-periodate. Kandutsch and Stimpfling (1966) also attempted enzyme treatments of their antigenic preparations. Thus trypsin [enzyme/substrate ratio (E/S) = 1:5; 2 hours at room temperature] did not markedly affect antigenic activity and resulted in little fragmentation since most of the digest was excluded by Sephadex G-200. Pronase (E/S = 1:10; 17 hours at room temperature) caused 75% of the material to be included in Sephadex G-200. However, this included material had little or no antigenic activity.

In a recent paper, Hilgert *et al.* (1969) evaluated the effectiveness of three detergents—Triton X-100, Triton X-114, and potassium cholate—to solubilize H-2 antigen from a particulate fraction of Sarcoma I. The ^{51}Cr-cytotoxicity assay was used to measure antigenic activities. Triton

X-100 was found to be least suitable since, although the detergent extract contained 50% of the initial activity, about two-thirds of it was lost when acetone and ether had to be used to remove the detergent. Triton X-114 was more advantageous to use than X-100 since it could be used for a shorter period of time (2 hours compared to 10 days) and at lower concentrations (3 μl. of 20% Triton X-114 as compared to 10 μl. of 20% Triton X-100 per milligram of protein). Most of the Triton X-114 could be removed with ether without any detectable loss of antigenic activity after saturation of the water phase with ammonium sulfate. However, this preparation was insoluble in the absence of a detergent in dilute salt solutions at pH 7. Gel filtration on either agarose or Sephadex G-200 in the presence of 1% Triton X-100 did not result in any significant concentration of antigenic activity.

Attempts to solubilize the antigen with potassium cholate (varying KCl concentration from 0.14 to 3 *M* while cholate was varied from 0.1 to 1%) resulted in approximately the same yield (20–50% of the initial antigen activity) with the same increase in specific activity (two- to four-fold) as did solubilization with Triton X-114. The advantage claimed was that exposure to organic solvents was not necessary to remove the detergent. Fractional ammonium sulfate precipitation did not result in any significant separation of five H-2 specificities; however, much of the protein was not retained on Sephadex G-200 and was either aggregated or had a relatively high molecular weight. The specific activity of the retained fraction was not significantly higher than that of the excluded fraction.

2. *Deoxycholate*

Metzgar *et al.* (1968) employed (0.5%)deoxycholate to disrupt human tissue culture cells (KB and W2-38 cell lines or chimpanzee lymph node cells) and were able to solubilize the 4^a and 4^b antigenic determinants of the human HL-A system. After the addition of deoxycholate, deoxyribonuclease (2.2 mg./10^9 cells) was added and the mixture incubated for 15 minutes at 37°C.; $MgCl_2$ (0.08 *M*) was then added and the mixture incubated for an additional 15 minutes. The suspension was adjusted to 0.4 *M* $MgCl_2$ concentration to precipitate deoxycholate and centrifuged at 100,000*g* for 1 hour; the supernatant was dialyzed against two changes of phosphate-buffered saline overnight, recentrifuged at 100,000*g* for 30 minutes, and stored at —75°C. This antigenic preparation specifically inhibited agglutination of leukocytes, mixed agglutinations, and cytotoxicity reactions and induced the accelerated rejection of donor skin grafts. Metzgar *et al.* (1968) also extracted lymph nodes from chimpanzee

donors by the same procedure and employed these extracts to test for inhibition of agglutination by human isoantibodies. The chimpanzee lymph node extract inhibited the reactions of one human antiserum with a panel of cells from four human donors. It was concluded that, although the amount of isoantigen recovered could not be accurately measured, from 20 to 30% of the activity of the intact cells was recovered by this extraction procedure. Although other investigators (Brent *et al.*, 1962b) failed to obtain antigenic activity after treatment of mouse tissues with various detergents, Metzgar *et al.* (1968) contend that their short-term (30-minute) treatment with deoxycholate followed by its quick removal contributed to their success. Apparently, long-term (24-hour) detergent treatment also failed in their hands to yield active antigen preparations from mouse tissues.

Bruning *et al.* (1964, 1968) were able to use deoxycholate to extract HC-B antigens, the products of a less important genetic locus than the major HL-A locus, from the cell membrane sediments of placental tissue. This procedure involved the treatment of placental tissue particulate fraction with 0.5% deoxycholate for an unspecified period of time and then centrifugation of the extract at 100,000*g* for 2 hours followed by dialysis of the supernatant against distilled water (3×24 hours) and lyophilization. From 150 to 200 gm. of placental tissue was obtained 200–500 mg. of material which contained 5^a and 5^b antigens as determined by the neutralizing activity of these antigens in agglutination inhibition assays.

Gel filtration of the extract on Sephadex G-200 showed that antigenic activity could only be recovered from a fraction that eluted in the void volume even when the extract was previously treated with enzymes such as chymotrypsin, lysozyme, or phospholipase A. It is, of course, possible that residual deoxycholate may have prevented enzymatic action.

3. *Decyl and Dodecyl Sulfate Extraction*

Manson and Palm (1968) obtained a solubilized material by extraction of murine microsomal lipoproteins with sodium decyl and dodecyl sulfate. This material specifically inhibited alloantibody and remained dispersed after removal of the detergent. Sedimentation constants ascertained ranged from 15 to 55 and permit only speculation concerning the actual molecular weight of these preparations which apparently were composed of highly complex lipoproteins.

Manson *et al.* (1963) also obtained microsomal lipoprotein particulates by exposing mouse tumor cells to 1500 p.s.i. for 15 minutes followed by decompression in a nitrogen bomb and ultracentrifugation of the sus-

pension. This particulate lipoprotein material was shown to inhibit hemagglutinating antibody and to contain homograft-sensitizing activity at dose levels (intraperitoneal) from 25 to 50 μg.

B. Extraction with Organic Solvents

Another extraction method to obtain solubilized histocompatibility antigens involved the use of organic solvents. Morton (1950) demonstrated that intracellular enzymes could be both released and solubilized by extraction of various cell particulate fractions. Butanol was found to be a most effective solubilizing agent for enzymes including phosphatases, peptidases, dehydrogenases, esterases, and transaminases. In contrast, some of the intercellular enzymes studied were destroyed by extraction with papain, autolysis, lipases, or trypsin. Because of its relatively low solubility, *n*-butanol saturates aqueous solutions without causing any serious loss of activity when extractions are carried out at 0° to —2°C. It is postulated that butanol competes most effectively for the polar side chains of proteins with the alcohol displacing the lipids and, thus, causing dissociation of lipoproteins and protein–protein complexes. Morton (1950) also obtained some evidence that phosphate esters are preferentially extracted by butanol thus destroying the structural integrity of cell membrane-bound lipids resulting in the solubilization of membrane proteins.

Kandutsch (1960) used butanol to solubilize the antigenic activity of a membranous fraction obtained from water-lysed Sarcoma I mouse ascites cells. The butanol extract of the cell particulate fraction was centrifuged at 105,000*g* (1 hour) at room temperature, the supernatant poured into 10 volumes of cold acetone, and the resultant precipitate washed with ether and dried *in vacuo*. Saline suspensions of this material enhanced the survival of grafts. Manson and Palm (1968) liberated from 40 to 75% of the H-2 and non-H-2 antigenic activity of microsomal lipoproteins with butanol. The lipid content of the solubilized material was reduced from 42 to 28% but still existed in a highly aggregated form with a particle weight estimated from an *s* value of 56 to be as high as 6×10^6. This material was ineffective in inducing accelerated graft rejection when injected intraperitoneally but caused an accelerated allograft response when injected subcutaneously in Freund's adjuvant. The butanol-solubilized material also elicited formation and anamnestic rise of H-2 antibody and inhibited donor-specific hemagglutinins and cytotoxins. Harris *et al.* (1968) used Triton X-100 treatment and butanol extraction to solubilize antigen from cell membrane fragments of rabbit lymph nodes and spleens. Analyses of these extracts by sucrose density gradient centrif-

ugation and diethylaminoethyl (DEAE)-cellulose chromatography indicated them to be of highly complex chemical nature.

A number of investigators have tried to extract soluble histocompatibility antigens with detergents and organic solvents from a variety of animal and human tissues. However, the overall results have, in retrospect, not been encouraging. Although many of the extracted materials showed good antigenic activity and specificity and often immunogenicity, they were generally found to be such extremely complex mixtures that there was little or no effort made to purify them further and to characterize them by physicochemical methods. Furthermore, there is well-founded and reasonable doubt that these materials instead of being really truly water-soluble were often composed of small membrane fragments in a colloidal suspension. Although it may yet be feasible to obtain truly soluble antigens with new detergents, at present it appears that this approach has not been too rewarding.

C. Antigen Extraction by Proteolysis

1. Autolysis

Digestion of membrane fragments of mouse spleen and tumor cells and human lymphoid cells with proteolytic enzymes has been used by several investigators to solubilize histocompatibility antigens.

In order to solubilize murine histocompatibility antigens, Nathenson and Davies (1966) washed mesenteric lymphoid, thymus, and spleen cells for 20 minutes with 0.8 and 0.7% saline. The extract was centrifuged at 600*g* for 15 minutes and the combined supernatants centrifuged at 105,000*g* for 90 minutes. The sediment contained at best 70% of the activity of the original cells. This crude insoluble lipoprotein fraction was suspended in tris–HCl buffer, pH 7.4, and decreased the antigenic activity. The autolysate was centrifuged at 105,000*g* for 1 hour, and the supernatant was tested for antigenicity by its capacity to inhibit cytotoxic antibody. The absolute amount of solubilized activity thus obtained did not exceed 20% of that in the insoluble starting material, i.e., 14% of the activity of the original cells. Although antigens could be solubilized from normal C3H mouse lymphoid tissue by autolysis, this method failed when applied to BP 8 ascites tumor cells. In this case, the crude membrane fraction was incubated with ficin for 1 hour at 37°C. at an E/S ratio of 1:16. Ficin digestion for more than 1 hour destroyed the antigenic activity of the membrane fraction.

The crude ficin containing at least eighteen proteolytic enzymes was partially purified by gel filtration on Sephadex G-75. Only the eluate of the included volume was used for digestion of the crude membrane frac-

tion. The enzymatic digest was centrifuged 105,000g for 1 hour and passed over Sephadex G-75. Antigenic activity was found only in the included volume in accord with previous knowledge of the behavior of solubilized murine alloantigens upon gel filtration (Kahan, 1965).

Autolytically solubilized antigen was passed over Sephadex G-75 and all antigenic activity was found in the excluded fraction in the void volume. Gel filtration of this material on Sephadex G-200 resulted in removal of some inactive protein and in a fourfold increase of activity. Stepwise elution chromatography of these antigen preparations on DEAE–Sephadex with increasing sodium chloride concentration (0.1–0.15 *M*) resolved three fractions, two of which showed a twenty-fold increase in activity. Antigens thus prepared (*1*) could be lyophilized, (*2*) were soluble in aqueous solvents at pH 6.5 at a concentration of 1 mg./ml., (*3*) were heat-labile (2 minutes at 60°C.), and (*4*) were stable at 37°C. only in the pH range 6–9. Ultrasonication (30 seconds; 60 W.; 20 kc.) resulted in a 20% loss in activity, and exposure to urea (6 *M*) at 37°C. irreversibly inactivated the antigen within 20 minutes. Analyses of this antigenic material showed protein 60–64% by weight, amino sugar 8.5%, and hexose 7%. Phosphate content was less than 0.2% and lipid content was not determined. All three DEAE-Sephadex peaks contained the same antigenic specificities, i.e., there was no apparent separation of H-2 specificities. From 600 mice, approximately 1 gm. crude membrane fraction was obtained, 90 mg. of which was eluted from Sephadex G-75 (one- to twofold increase on specific activity), and the two most active Sephadex fractions contained 5 mg. with a twenty-fold increase in activity. From the Sephadex elution patterns (G-75 and G-200) the molecular weight was estimated to be between 75,000 and 200,000.

Davies (1967) passed an autolysate of murine spleen membrane fragments over a column of Bio-Gel P-300 and tested various fractions differentiated from a rather diffuse elution pattern for inhibition of immune cytolysis. The portion of the eluate that was partially in the included volume of the column and comprised a relatively large portion of the total protein effluent was further purified on DEAE-Sephadex using a straight-line salt gradient (0.05 *M* tris, pH 7.4, plus NaCl to get a molarity from 0.1 to 0.35). Biological activity was localized in the middle of a broad protein (O.D. 280) distribution pattern. Electrophoresis of this material on a sheet of acrylamide gel (pH 7.2; 300 mA.; 15 V./cm.; 15.5 hours) revealed a relatively diffuse electrophoretic zone situated between the α- and β-globulins of an adjacent reference pattern of normal mouse serum. Another adjacent strip containing 20 μg. of antigen was cut into sections and eluted with water and activity was

found on one of three occasions in the electrophoretic region that showed the broad protein zone. Specific inhibition of cytotoxicity could be obtained, depending on the H-2 specificity tested, with 0.024 μg. to 1.3 μg./ml. Antigen potency was measured as the amount (weight per milliliter) required to give 50% inhibition of a 75% lytic dose of antibody, i.e., a 37.5% inhibition of lysis was considered significant.

Davies maintained that, judging from his DEAE–Sephadex elution profile, there were only three major protein components. This is not a convincing argument since it is well known that DEAE elution peaks resolved from chemically complex material always contain many families of protein components. Davies further contended that "it still seems most likely that for H-2 some moiety attached to the protein is responsible for the isoantigenic specificity." He felt that "the possible low density suggests carbohydrate is certainly present." In this study the different antigenic specificities determined by a particular H-2 allele could not readily be separated. The active peak on DEAE-Sephadex showed coincidence of several if not all the detectable specificities of H-2^k. Also, crude, autolysed antigen from an F_1 mouse donor was reacted with an alloantiserum specifically directed against one of the parental strains and then the antigen–antibody complex was isolated by gel filtration. This complex was found to contain the antigenic specificities of the other parent, indicating to Davies (1967) that both phenotypes were present on a single molecular species.

Davies (1968) points out that the same solubilized antigenic product could be obtained from mouse lymphoid tissue by autolysis and by digestion with either ficin, bromelain, or papain. This argument is not convincing since it is solely based on the similarity of respective Sephadex elution patterns. When solubilized antigen was passed over Sephadex G-25, most of the activity appeared in the excluded volume although some activity was in the included volume. This antigenic material appeared, however, to have nonspecific activity and only thorough serological studies could determine whether there was, indeed, a very small fragment with specific antigenic activity.

Davies (1967) applied the same autolysis procedure used to solubilize murine H-2 antigens to obtain solubilization of human isoantigens from spleen cells. From an average spleen (2.5 gm. dry weight of crude membrane fraction) he obtained about 300 mg. of solubilized material. After passage over Bio-Gel P-300, a fraction comprising 45 mg. of material was obtained which by inhibition of platelet complement fixation tests was shown to contain specific antigenic activity.

Halle-Panenko *et al.* (1968) employed autolysis for 3 hours at 37°C. (pH 7.4; protein concentration 0.6%) to solubilize alloantigens from both

normal murine (A_N) cells and from BP 8 tumor cells. Soluble extracts of the latter were found to induce consistently the formation of hemagglutinating antibody and, in two cases, the induction of cytotoxic antibody as well as the accelerated rejection of allogeneic skin grafts. Ion-exchange chromatography of soluble antigen extracts on DEAE-cellulose and subsequent analyses of column eluates by acrylamide gel electrophoresis revealed highly complex patterns especially in fractions with antigenic activity. No attempts were made to purify or characterize these antigen fractions.

2. *Antigen Extraction by Papain Digestion*

Human isoantigens were solubilized from spleens by autolysis and by papain treatment of crude membrane fractions obtained by hypotonic salt extraction (Davies *et al.*, 1968a,b). Isoantigens were evaluated by their inhibitory capabilities for cytotoxic antisera and for platelet complement fixation. Crude salt-extracted membrane fractions were allowed to stand 2.5 hours at 37°C., centrifuged at 120,000*g* for 90 minutes, and the supernatants were found to contain the autolysed alloantigens. The sediment, resuspended in 0.05 *M* tris buffer, pH 8, was incubated with papain (E/S = 1:150) in the presence of 0.35 m*M* cysteine for 45, 90, and 180 minutes. Enzyme digestion was most effective at 180 minutes and was stopped by the addition of iodoacetate. Papain was removed by passage of the material over Sephadex G-75 columns. These antigens were fractionated either by autolysis, papain digestion, or both procedures combined, by gel filtration on Bio-Gel P-300, and by ion exchange chromatography on DEAE-cellulose or DEAE–Sephadex columns. The point was made that there are "good" and "bad" spleens, and only the former could be efficiently extracted by papain digestion. In the mouse, it was found that the efficiency of solubilization of isoantigens varies from strain to strain. Much was made of the observation that these relatively crude solubilized extracts containing either H-2 or HL-A alloantigens behaved similarly on DEAE–cellulose. However, in both cases, relatively poor resolution was obtained. The antigens also showed similar profiles upon gel filtration on Sephadex G-200. From this and from the fact that the same methods were able to extract HL-A and H-2 isoantigens from lymphocyte membranes, Davies *et al.* (1967) deduced that mouse and human histocompatibility antigens were homologous, i.e., molecules with closely similar composition.

Although this is certainly a possibility the antigens isolated thus far are chemically highly complex and heterogeneous and arguments based solely on these data are not too convincing. Whether or not histocompatibility antigens of different species are homologous in a manner

analogous to immunoglobulins will become evident only after thorough chemical analyses are performed on highly purified antigen preparations.

Boyle (1970), analyzing homogenates obtained from human cadaver spleens, found the major HL-A activity associated with a microsomal sediment which could be further fractionated by sucrose density gradient centrifugation, the activity being associated with two of three major components. Antigens were assayed by either inhibition of leukagglutination or lymphocytotoxicity. The sediment could be partially solubilized at pH 10 or in 6 *M* urea which resulted, however, in a 90% loss of antigenic activity. Phospholipase A from *Crotulus adamantus* did not solubilize antigenic activity to any significant extent. Digestion of the sediments with papain (E/S = 1:100) for 1 hour at 37°C. in the presence of 0.005 *M* cysteine at pH 7.2 solubilized HL-A antigenic activity. However, the sole criteria of solubility applied are the absence of either visible sediment after ultracentrifugation at 105,000*g* for 30 minutes or the lack of protein stainable material at the origin of a cellulose acetate strip following electrophoresis. By this method, one cathodic and four anodic components could be detected. These conditions of papain digestion yield solubilization of 20 to 50% of HL-A alloantigens containing 30–40% of the protein present in the cell membrane sediment. Additional purification was found by gel filtration on Sephadex G-150 where several antigenic specificities were detected in a relatively broad zone located between the excluded and included volumes. High specificity was claimed based solely on the observations that a single specificity not detectable on the donors' spleen cells was also absent from the Sephadex eluate which was found to contain the four antigenic specificities detected on the donor cells. Specificity ratios, antigen concentration, and cytotoxicity units at which tests were carried out are not supplied making it difficult to evaluate this study.

Sanderson and Batchelor (1968) used insoluable papain since they found considerable nonspecific inhibition of cytotoxicity even when the enzyme had been inactivated by iodoacetate. Antigens were evaluated by their capacity to inhibit specific cytotoxic antibodies. Sanderson (1968) emphasized the importance of the specificity of this inhibition since a variety of nonspecific substances could cause inhibition of cytotoxic antibodies. The specificity was evaluated by the specificity ratio. This ratio was expressed by the amount of antigen needed to inhibit donor-positive antiserum vs. the amount that can be used without getting any inhibition of donor-negative antiserum. The higher the ratio the greater the specificity which is probably the best index of the degree of alloantigen purification attained.

Autolysis-solubilized material gave a low specificity ratio (SR) (1–5)

but contained all the specificities possessed by the spleen donor. Papain-solubilized material gave a higher SR (as high as 120) but did not possess all the specificities present on the donor's peripheral lymphocytes. Sanderson (1968) pointed out that, in the case of antigens solubilized by autolysis, serologically active substances were bound together with non-specific inhibitors, and, hence, no real purification of antigenic specificities was achieved. Estimates of the molecular weight of papain-solubilized HL-A antigens were approximately 45,000 based on elution patterns of calibrated Sephadex G-200 columns. On the other hand, human alloantigens solubilized by autolysis were claimed to have molecular weights as high as 200,000.

Shimada and Nathenson (1969) recently described some chemical properties of solubilized H-2 alloantigens with H-2^b and H-2^d genotypes. The authors started with 4000 spleens of either C57Bl/6 or DBA/2 mice. The crude particulate fraction was found to contain 35% of the protein of the original homogenate but 90% of the original alloantigenical activity, thus providing a 2.5-fold purification. Alloantigens were solubilized from this crude particulate fraction by autolysis (1 hour at 37°C.) followed by papain digestion (E/S = 1:38) for 1 hour at 37°C. The enzyme digest was centrifuged at an average of 78,000*g* for 2 hours—a speed insufficient to sediment membrane particles—and the supernatant was subsequently fractionated by gel filtration, ion-exchange chromatography, and acrylamide gel electrophoresis.

The papain procedure was found better in this study than the autolysis method, both will respect to yield and reproducibility. Yields of alloantigens obtained by autolysis of cell membrane fragments at 37°C. were maximal at 1 hour and did not change during an additional 8-hour incubation. Antigen yields obtained by autolysis were found to be only 5–10% of that obtained by papain digestion—a claim which differs from previous observations both by Davies (1967) and by Nathenson and Davies (1966) who noted that considerably higher antigen yields were obtained by autolysis. Apparently, autolysis solubilizes murine alloantigens to a much lesser extent than papain; however, essentially all the antigenic specificities of the cell donor are present in the autolysate. It is, indeed, possible that autolysis, at 37°C., although found less efficient by Shimada and Nathenson (1969), solubilizes mainly specificities located in areas easily accessible to enzymes (cathepsins) present in finite concentration. On the other hand, papain added in excess, is more efficient and probably attacks areas on the membrane not accessible to cathepsins and, in the process, inactivates some of the specificities which it solubilizes as well as some antigen which it cannot remove from the membrane.

Experiments designed to determine E/S ratios and incubation times showed that incubation periods in excess of 1 hour resulted in loss of antigenic activity as did E/S ratios lower than 1:30. An E/S ratio of 1:11 was shown to cause approximately a 33% loss in specific activity (Shimada and Nathenson, 1969).

Fractional ammonium sulfate precipitation, gel filtration, and ion-exchange chromatography were applied in an effort to purify papain-solubilized H-2^b and H-2^d alloantigens. Partially purified antigens of each genotype resolved into three components each following acrylamide gel electrophoresis at pH 9.4. Three electrophoretic components within each genotype were claimed to be separated by the same electrophoretic procedure apparently containing the same antigenic profile and amino acid composition. Only small differences in arginine and glutamic acid were observed between H-2^b and H-2^d alloantigens. However, the significance of these amino acid compositions is difficult to evaluate since only a single analysis was performed and, thus, it is impossible to determine the standard error and the significance of other apparent amino acid differences (Shimada and Nathenson, 1969).

The molecular weights of murine H-2^b and H-2^d alloantigens were estimated by gel filtration and sucrose gradient centrifugation. It was claimed that antigens from both genotypes could exist either as 65,000–75,000 or as 40,000 molecular weight fragments. Should these estimations prove correct, then one can either attribute this variation in size to variable fragmentation by papain or to selective aggregation following papain fragmentation.

Yields of crude, papain-solubilized alloantigen varied with antigenic specificities and ranged from 15.9 (H-2.5) to 2.6% (H-2.31), whereas yields of autolytically solubilized antigen ranged from 1.77 to 0.93%. In the best case (H-2.5), there was usually from one purification step to the next, a two- to eightfold increase in specific activity and about a 700-fold increase in activity concomitant with an overall loss during purification of approximately 85% of the alloantigenic activity originally present in the crude cell membrane extract.

D. L. Mann *et al.* (1968) described the isolation of alloantigens from continuous cultures derived from the lymphoid tissue of human donors with lymphoid malignancies. Crude papain (E/S = 2:1) was used for 1 hour at 37°C. to solubilize alloantigens from frozen cells washed with isotonic and hypotonic salt solutions. It is of interest that with this extremely high E/S ratio of 2:1 (even though crude papain was used) from 40 to 50% of the total isoantigenic activity was recovered. However, in a subsequent paper D. L. Mann *et al.* (1969b) showed that with crystalline papain (E/S = 0.5 unit mg. protein) only 20% of the mem-

brane activity could be recovered. Ammonium sulfate-precipitated (0.75 saturation) protein was centrifuged at 40,000*g* for 80 minutes and the dialyzed supernatant further fractionated on Sephadex G-150. The majority of the HL-A-3 alloantigenic activity of the cell line RAJI was found in the included fraction, whereas in a subsequent study using a different cell line (R-4265) the only activity tested with a monospecific antiserum (HL-A-2) was found mainly in the excluded volume (D. L. Mann *et al.*, 1969b). Human and mouse cell membrane extracts obtained by papain digestion were admixed and placed on a Sephadex G-150 column. From the relatively broad but superimposable activity curves obtained, it was concluded that papain-solubilized fragments may have similar molecular weights (50,000–70,000) and similar structures in both species. Since these are rather complex and, possibly, in part aggregated materials, this statement seems to be an oversimplification, especially since it is well established that substances which appear to have identical K_D values on Sephadex have more often than not decided chemical differences.

In their most recent study of cell lines RAJI and R-4265, D. L. Mann *et al.* (1969b) supply figures for the recovery of HL-A-3 alloantigenic specificity (RAJI) indicating that from 3.3 gm. of cells (3.8×10^9 cells) they obtain 0.09 mg. protein with a twenty-fivefold increase in activity per milligram, representing approximately 3.5% of the activity of the initial membrane extracts. Since amounts of protein and total units of activity recovered for LA-2, 4d and 6b specificities (R-4265) are not supplied (only units of activity per milligram protein are given), it is difficult to compare the antigen yields from these two cell lines.

Antigenic activity was determined by the capacity of alloantigens to inhibit cytotoxic effects of specific alloantisera and complement against ^{51}Cr-labeled target cells. An antiserum dilution which would cause lysis resulting in 60–70% release of radiolabel from these target cells was designated as the lytic end point, i.e., 100% lysis. The reciprocal of the dilution of the alloantigen which caused 50% inhibition of lysis, i.e., 30–35% ^{51}Cr release, was used to express units of alloantigenic activity. Thus in the most purified antigen preparation, approximately 0.05 μg. antigen could reduce the lysis of 100,000 target cells from 70 to 35%.

a. Lability of Antigenic Determinants. Papain which can readily solubilize some of the alloantigenic specificities of lymphocyte membranes can apparently also selectively destroy antigenic specificities. Sanderson (1968) found some antigenic specificities were not stable to papain and were, in fact, even destroyed in the insoluble membrane fractions remaining after papain digestion. Shimada and Nathenson (1969) found that recovery of antigenic activity varied among alloantigens with different genotype and among antigenic specificities within each of the

genotypes. Thus only about half the recovery was obtained with H-2^d as compared to H-2^b alloantigens. From H-2^b antigens the H-2.5 activity was recovered in yields almost two magnitudes higher than the H-2.2 activity, and from H-2^d antigens the recovery of H-2.4,10,13 activity was more than a magnitude greater than that of H-2.31 activity. It is unclear whether these differences were properties of the antigen or of the antibody-detection system.

Shimada and Nathenson (1969) also found that only about 18% of H-2.5 activity present in the crude particulate fraction was solubilized (60% of activity remaining on the membranes), whereas H-2.2 was only solubilized to the extent of 5.5% with 67% activity remaining in the crude particulate fraction. It seems that in each case about 25% activity was either lost or destroyed. Moreover, H-2.2 which was solubilized to a much lesser extent was not found associated with other specificities such as H-2.33 and H-2.28. Some specificities, i.e., H-2.22, were destroyed on the membranes and not solubilized at all. So far as the H-2^d alloantigens were concerned, H-2.4,10,13 were solubilized to the extent of 16% with only 22% activity remaining in the crude particulate fraction. In this case it seems that about two-thirds of activity was not accounted for, i.e., was destroyed by papain even while remaining in the particulate fraction. Only 3.6% of the H-2.31 activity was solubilized, but in this case 74% of activity remained on the membrane fragments. It is of considerable interest that the particulate fraction once digested with papain could be redigested and additional alloantigens (from 2 to 10%) could be solubilized. However, no activity remained in the particulate fraction following this treatment. This antigenic activity which amounted often to as much as 70–75% of the initial activity was completely destroyed by papain, i.e., this enzyme could not remove the vast majority of antigens on the cell membrane fragments, but apparently it could readily destroy their serological activity once the easily solubilized antigens (15% of the total) were removed. In this regard, D. L. Mann *et al.* (1969b) also reported poor recoveries of HL-A alloantigenic specificities 4a, 4d, 6b following papain digestion of cell membranes derived from human lymphoid cells in culture.

3. *Antigen Extraction with Trypsin*

Edidin (1967) prepared a stable insoluble stroma by extracting pools of mouse embryo lymph nodes, spleen, liver, and thymus with hypertonic salt solutions containing exhylenediaminetetraacetate (EDTA) (1.14 *M* NaCl, 0.02% EDTA). This insoluble material was treated with trypsin (pH 7.4) in the presence of 5 *M* urea, followed by extraction with 0.14 *M* phosphate-buffered saline containing EDTA, centrifugation at 8000*g*, and finally phenol (88%) extraction of the resultant supernate which was

lyophilized. Gel filtration of this material on Bio-Gel P-2 yielded soluble antigens which specifically inhibited anti-H-2 cytotoxic alloantisera. These antigenic materials could be further purified by isolation from specific antigen–antibody complexes dissociated by dilute acid treatment. Antigens thus prepared seem to be peptides or glycopeptides which were retarded on Sephadex G-25 and G-10 and were readily dialyzable and soluble in 5% trichloroacetic acid (TCA). The isolated antigen preparations were chemically complex and seemed to consist of many antigenic determinants linked on a single molecule.

The use of a highly specific enzyme such as trypsin has as yet not been fully investigated and may possibly prove to be a useful method to solubilize alloantigens from cell membrane surfaces or from insoluble fragments bearing antigenic determinants.

4. Limitations of Enzymatic Solubilization Methods

The autolytic method first described by Nathenson and Davies (1966) has now been found, generally less efficient and less reproducible than the "papain method" (Shimada and Nathenson, 1969; Sanderson, 1968). Autolysis, generally ascribed to the action of cathepsins seems to attack the membranes more slowly than papain and "solubilize" large molecular weight (10^6) fragments containing all the antigenic specificities present on the peripheral lymphocytes of the cell donor. In most cases, digestion periods can safely be extended from 1 to 8 hours without either decreasing the yield. However, the yields of antigen liberated by autolysis from the cell membranes vary considerably. Nathenson and Davies (1966) obtained a cell extract which contained 70% of the activity of that of the original cells, 20% of which, i.e., 14% of the activity of the original cells, was solubilized. Recently, however, Shimada and Nathenson (1969) found that autolysis liberates only from 1 to 2% of the activity present in the crude cell extract. It seems logical that extended periods of autolytic digestions can safely be used since no enzyme is added, and the native cathepsins, which are present in finite amounts, are simply allowed to act on the cell membranes. However, human alloantigens solubilized by autolysis have much less antigenic specificity (SR = $\sim$1) than papain-solubilized antigens (SR = $\sim$120) (Sanderson, 1968). The large molecular weight of the solubilized material and the finding that it almost always elutes in the void volume of Sephadex G-200 (Shimada and Nathenson, 1967; Sanderson, 1968) while containing all detectable antigenic specificities of the donor cells leads one to suspect that autolysis may possibly "solubilize" small membrane fragments. This assumption gains further support if one realizes that all of the autolytically "solubilized" preparations were always subjected to short-term centrifugations never exceeding

120,000g. It would be of interest, in view of the findings of Rapaport *et al.* (1965), to examine the ultrastructure of these preparations or to expose them to prolonged centrifugation periods at 200,000g and above.

Papain has been used in a variety of ways in an attempt to solubilize with the highest possible yields of the alloantigens present on the cell membrane. Various enzyme substrate ratios have been used, varying from E/S ratios of 2:1 (crude enzyme used) to 1:150. However, there is general agreement that incubation in excess of 1 hour results in inactivation of both human and murine alloantigens and that certain alloantigens either cannot be solubilized by papain or are obtained in very poor yield. In fact, some alloantigens are not solubilized at all but are destroyed on the membrane. It is of interest that, although a second treatment with papain solubilizes additional small amounts of antigens, it destroys all the remaining antigenic activity which is often as much as 60–70% of that present on the original cell extract.

Up to 700-fold purifications have been achieved for murine antigens (Shimada and Nathenson, 1969), unfortunately without any serious attempt to evaluate specificity ratios of these purified materials. Critical evaluations of purification schemes of human alloantigens can claim maximal specificity ratios of ~120 (Sanderson, 1968). The problems of papain solubilization of histocompatibility antigens can be illustrated by a close look at the data of Shimada and Nathenson (1969). From 4000 mouse spleens, which these investigators calculated to contain ~40 mg. antigen, papain solubilizes about 130-fold as much protein as antigen present (5310 mg.) and subsequent purification at best resulted in three electrophoretic fractions containing a combined total of 1.43 mg. The activity units per milligram protein were increased 700-fold but at the same time 98% of the activity units of the crude extracts were lost during the process of purification.

D. L. Mann *et al.* (1969b) have shown that papain causes from 15 to 20% nonspecific inhibition of cytotoxic activity. These authors, furthermore, claimed that the nonspecific effects were eliminated by a 1:2 dilution of their test antigen. Sanderson (1968), however, was sufficiently worried about the nonspecific cytotoxicity of papain to consider mandatory the removal of the enzyme by chromatography following digestion or to use insoluble papain which could be easily removed by centrifugation.

D. Solubilization by Sonication

Exposure of mouse and human tissues to sound and ultrasound has been widely used in an attempt to liberate soluble histocompatibility antigens. The activity of the liberated antigens depends upon the condi-

tions of sonication. Substantial quantities of potent antigens are released from cells and their membranes by exposure to low-frequency, low-intensity sound; however, only small quantities of less active material are liberated by high-frequency ultrasound. Thus, Billingham *et al.* (1956b) could detect only small amounts of immunogenic mouse transplantation antigen in the soluble fraction and, similarly, Haughton (1964) found that antigen was released but rapidly inactivated after exposure to ultrasound generated at 20 kc./second with a 60-W. probe.

On the other hand, in accord with a large body of evidence (Chambers and Florsdorf, 1936; Haas, 1943; Stumpf *et al.*, 1946; Pappenheimer and Hendree, 1949; Hogebroom and Schneider, 1950), sonic energy mediated by a diaphragm of 9 to 10 kc./second liberates active, water-soluble components from intracellular, intraorganelle, or membranous locations. The disparity between the ability of these two forms of sound to liberate active histocompatibility antigens is probably related to the more pronounced oxidative, bond-breaking, and depolymerizing effects of the probe-generated 20-kc./second ultrasound with its propensity toward the development of local heating and the generation of eddy currents. There is apparently a relatively narrow region of intensity in which sonic energy causes solubilization without inactivation of the histocompatibility antigens. The effects of sonic ($<$16,000 cycles/second) and of ultrasonic ($>$16,000 cycles/second) energy have been critically reviewed (Grabar, 1953; Hughes and Nyborg, 1962). Exposure to sound breaks up animal and bacterial cells as well as molecules in solution by the generation of heat, by oxidative effects, by mechanical effects including an agitation effect analogous to foaming, and by a frictional effect. The most important effect, however, is gaseous cavitation with rapid expansion and violent collapse of the dissolved air within the fluid. The extent of these effects depends upon the intensity and frequency of the applied sound and upon the physical state of the exposed material.

Exposure to low-intensity sound (9–10 kc./second) liberated water-soluble histocompatibility antigens from mouse spleen, lung, kidney, and liver cells (Kahan, 1964a,b, 1965; Zajtchuk *et al.*, 1966) and their cell membranes (Haene-Severns *et al.*, 1968); from guinea pig spleen, lung, kidney, liver (Kahan, 1967; Kahan and Reisfeld, 1967; Kahan *et al.*, 1968a) and sarcoma cells (Kahan *et al.*, 1969); from dog spleen cells (Dagher *et al.*, 1967); from human spleen cells (Kahan *et al.*, 1968b); from human lymphoid cells grown in long-term continuous culture (Reisfeld *et al.*, 1970a); and from lung, liver, spleen, and kidney of 3, 4, and 5½-month-old human fetuses (Pellegrino and Kahan, 1970).

A brief exposure (3–5 minutes) of murine and guinea pig cell suspensions to low-intensity, diaphragm-mediated sound (9–10 kc./second;

15.5 W./cm.^{2}; 4°C., Raytheon Model DF 101 magnetostrictive oscillator) liberates 12–15% of the total immunogenic activity of the treated suspensions in soluble form. Titrations of the duration of sonication have shown that antigen release occurs after the cell surface membrane is disrupted but prior to complete cytoplasmic and nuclear disruption. This point correlates with a 90% decrease in cell count on a Coulter counter in a suspension containing 40–50 $\times$ 10^6 cells/ml. The debris and cellular membranes could be removed by ultracentrifugation at 130,000*g*. It should be noted that even prolonged centrifugation at 200,000g failed to yield any detectable membrane sediment following ultrastructural analysis. The antigenic principle from guinea pigs purified by gel filtration of the supernate on Sephadex G-200 (0.2 *M* tris, 0.5 *M* glycine, 0.5% mannitol, pH 8.0) eluted at the front of the inner volume (K_d 0.92).

The active fraction (Sephadex Fraction I) from guinea pigs (*1*) induced the specific accelerated rejection of test allografts (Kahan and Reisfeld, 1967), (*2*) elicited specific delayed-type hypersensitivity reactions upon intradermal challenge of allogeneic hosts that had been presensitized with donor-type grafts (Kahan, 1967), (*3*) participated with sensitized cells in third-party local passive transfer reactions in syngeneic hosts (Kahan, 1967) or (*4*) in irradiated hamsters (Kahan *et al.*, 1968a), and (*5*) stimulated blast transformation of lymphocytes *in vitro* (Kahan *et al.*, 1968a) (see below).

This Sephadex Fraction I from guinea pig spleen is chemically complex and contains at least seventeen components which can be resolved by discontinuous acrylamide gel electrophoresis at pH 9.4 (Kahan and Reisfeld, 1967) (Fig. 1). Of these seventeen components, only a single component (component 15; R_f 0.73–0.74) possesses transplantation antigenic activity. Component 15 is immunogenic—intradermal administration of 1 to 3 μg. of strain 2 component 15 in polyacrylamide gel, which is known to be a good adjuvant (Raymond and Weintraub, 1963), to allogeneic strain 13 hosts accelerated the destruction of donor-type strain 2 grafts but not of strain 13 isografts (Kahan and Reisfeld, 1969a). The antigenic activity of component 15 was also demonstrated by elicitation of a specific delayed-type hypersensitivity response by intradermal challenge of presensitized allogeneic hosts with 0.1 μg. of antigen (Kahan and Reisfeld, 1967). The purified antigen could thus not only induce a state of specific transplantation immunity but also could elicit expressions of delayed-type hypersensitivity following the induction of immunity by skin or tumor grafts. Observations such as these strongly suggest that this component contains a determinant against which hosts develop sensitivity following allografting.

Component 15 has been shown to be electrophoretically homogeneous

FIG. 1. Disc electrophoresis pattern of Sephadex Fraction I (pH 9.4, 7.5% acrylamide gel) applied 350 μg. of protein.

as shown by the appearance of a single electrophoretic band following re-electrophoresis of ^{125}I-labeled component 15 (Fig. 2) with and without 8 *M* urea while varying the porosity of the gel (5, 10, and 15%) (Kahan and Reisfeld, 1967, 1968a, 1969b).

The molecular weight for electrophoretically homogeneous strain 2 and strain 13 guinea pig transplantation antigen was found to be 15,000, assuming a partial specific volume of 0.74. The three techniques used to determine molecular weights were (*1*) ultracentrifugation employing the Yphantis sedimentation equilibrium method with interference optics, (*2*) gel filtration in the presence and in the absence of 5 *M* guanidine hydrochloride (Fig. 3), and (*3*) calculation from the amino acid composition.

The amino acid compositions of antigens isolated from strain 2 and strain 13 guinea pigs were quite characteristic and reproducible. Of interest were the distinct amino acid differences observed between the electrophoretically homogeneous components 15 prepared from the two histoincompatible lines of guinea pigs (Table I). Hexosamine and half-cystine were not detectable and methionine was only present in trace amounts. There were significant differences at the $P < 0.01$ level in the content of serine, alanine, valine, isoleucine, leucine, and possibly tyrosine and phenylalanine. The differences ranged from 1 to 7.5 mole % (Kahan and Reisfeld, 1968b). In connection with the recent advances in the genetic code, it was considered of interest that a single base substitu-

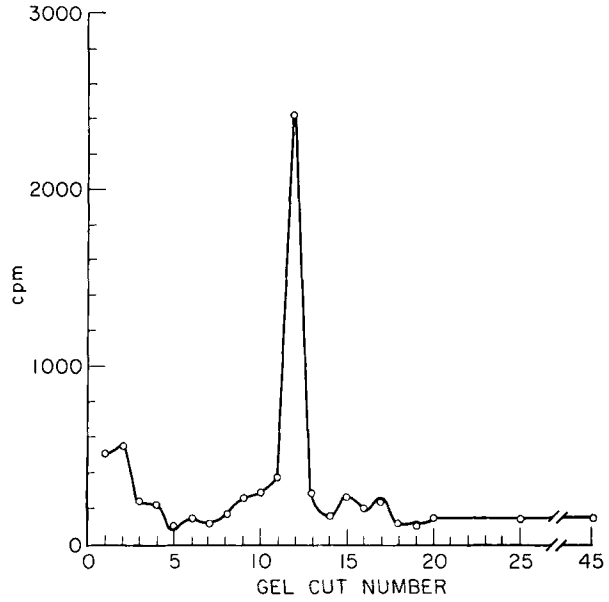

FIG. 2. Re-electrophoresis of ^{125}I-labeled strain 2 guinea pig transplantation antigen (7.5% acrylamide gel, pH 9.4). The gel was cut into forty slices and monitored for radioactivity.

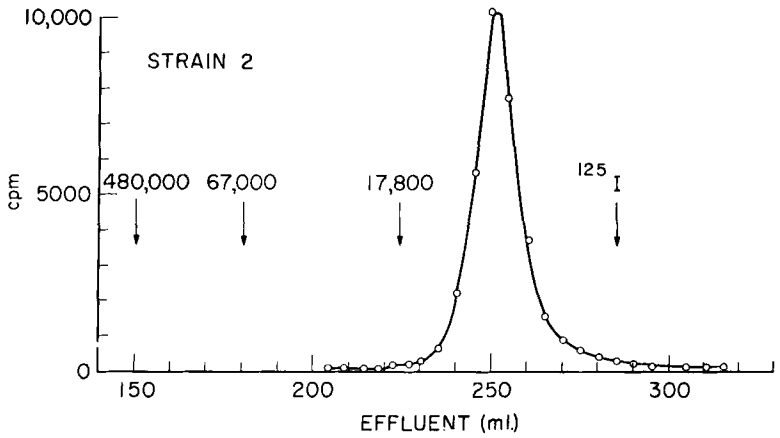

FIG. 3. Gel filtration pattern of ^{125}I-labeled strain 2 guinea pig transplantation antigen. The Sephadex G-200 column equilibrated with 0.1 *M* ammonium bicarbonate was calibrated with compounds of known molecular weights as indicated.

TABLE I
DIFFERENCES IN AMINO ACID COMPOSITION BETWEEN STRAIN 2 AND STRAIN 13 GUINEA PIG TRANSPLATION ANTIGENS

Amino acid	Strain 2 (mole %)	Strain 13 (mole %)	Difference, strain 2–strain 13 (mole %)	Difference in No. of residues, strain 2–strain 13 (No./mole)[a]
Serine	10.95	18.87	−7.92	−11.1
Alanine	11.40	7.96	+3.44	+4.8
Valine	6.46	5.48	+0.98	+1.4
Isoleucine	4.46	3.32	+1.14	+1.6
Leucine	8.58	7.19	+7.19	+1.9

[a] Based on the assumption that there are 140 residues per mole of antigen.

tion in serine (UCU, UCC, UCA, AGU, AGC) yields isoleucine (AUU, AUC), leucine (UUA, UUG), and alanine (GCU, GCC, GCG) and that a two-base change yields valine (GUU, GUC, GUA, GUG) which had the smallest difference considered significant. The remaining amino acids were present in strikingly similar amounts in the antigens isolated from the two strains of guinea pigs. These data suggest that guinea pig transplantation antigens possess allotypic specificities related to protein structure in analogy to the polymorphic genetically segregating antigenic determinants found on the serum proteins of numerous species. There seems to be a correlation between the allotypic specificity and the amino acid composition of the polymorphic substances similar to those observed with rabbit, immunoglobin G, light and heavy polypeptide chains (Reisfeld *et al.*, 1965; Koshland *et al.*, 1968). It is reasonable to assume that, although not all of the observed amino acid differences may be related to the antigenic specificity, at least some of the amino acids are involved in determining the characteristic immunological properties of these molecules.

The significance of this protein polymorphism in relation to the chemical nature of the antigenic determinant becomes even more apparent since there is no detectable lipid or carbohydrate at levels greater than 1%. Following lipid extraction of component 15 with chloroform–methanol (2:1 v/v) and Folch partition, thin-layer chromatography was performed on silica gel G, staining selectively for lipids, glycolipids, and cholesterol esters (Kolodny, 1968a,b). Hexose and pentose content was determined by the cysteine–sulfuric acid method (Dische, 1949).

1. Human Spleenic Antigens

To prepare soluble HL-A alloantigens, spleens were obtained from five donors, cell suspensions were prepared, and subjected to brief treat-

ment with sonic energy, much in the same manner as that described for the solubilization of guinea pig transplantation antigens. The purification procedure involved ultracentrifugation of the sonicate at 130,000*g*, gel filtration of the concentrated supernate on Sephadex G-200, purification of the active fraction (Sephadex Fraction I) by preparative acrylamide gel electrophoresis resulting finally in the isolation of an electrophoretically homogeneous (R_f 0.80) HL-A alloantigen (Fig. 4). Molecular weight determinations of this HL-A alloantigen revealed a component which was 94% monodisperse with a molecular weight of 34,600 and a 6% aggregated moiety with a molecular weight of 150,000. Calculation of the molecular weight from the amino acid composition of the HL-A alloantigen yielded a value of 33,000, in good agreement with the results obtained from ultracentrifugal analysis (Kahan and Reisfeld, 1969b). The amino acid composition obtained from a single analysis of 47 μg. of electrophoretically homogeneous alloantigen (HL-A-3, HL-A-5) showed general characteristics similar to those of guinea pig transplantation antigens, i.e., no detectable amounts of methionine, half-cystine, and hexosamine. In parallel with chemical analyses of guinea pig transplantation antigens, employing the same sensitive methods, no carbohydrate or lipid could be detected within the limitation (1%) of the methods employed (Kahan and Reisfeld, 1969b).

It is of some interest that the amino acid compositions of human and guinea pig alloantigens solubilized by sonication are relatively similar. Although these data are certainly limited, they suggest that there might, indeed, be some structural homology between some histocompatibility

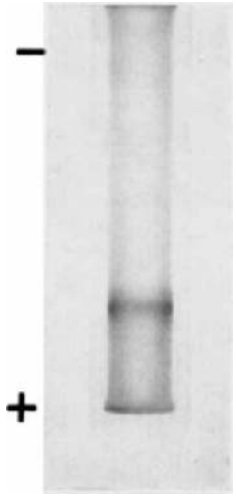

FIG. 4. Disc electrophoresis pattern of purified HL-A alloantigen isolated from cell line RPMI 1788 (pH 9.4, 7.5% acrylamide gel) applied 100 μg. of protein.

antigens of different species, possibly analogous to the well-documented structural homology of immunoglobulin classes and subclasses of certain species. Further support for this hypothesis will have to come from detailed structural analyses of highly purified histocompatibility antigens.

Both Sephadex Fraction I and electrophoretically purified HL-A alloantigens elicited specific Arthus-type hypersensitivity reactions only in individuals who were producing cytotoxic antibodies directed against antigenic specificities present in the spleen donor. The purified HL-A alloantigens specifically inhibited a series of eleven cytotoxic alloantigenic antisera (at 2 and 4 cytotoxicity units), nine of which were monospecific, in a pattern consistent with both the direct and absorption HL-A typing. To employ meaningful blocking studies with antigen, more than 1400 cytotoxicity tests were required to ascertain the antigenic specificity of each preparation, and for cross-check five preparations were investigated. Each of the antigens prepared contained different mosaics of the HL-A antigenic determinants 1, 2, 3, 5, 6, 8; Torino-11 and Torino-12. Soluble HL-A antigens were also extracted by the same procedures from a series of cadaver spleens, thymus, kidney, and lung (Pellegrino and Kahan, 1970).

2. *Antigen Extraction from Human Lymphocyte Cultures*

The limited source material from the spleen of a single donor proved to be a major stricture in this work. To overcome this difficulty, soluble HL-A antigens were obtained by exposure of 20×10^9 lymphocytes of a continuous cell line derived from a normal donor to a brief exposure (2 minutes) of low-intensity sound (Reisfeld *et al.*, 1970a). Following ultracentrifugation of the sonicate at 130,000*g*, the supernatant was concentrated and subjected to gel filtration on Sephadex G-200. Sephadex Fraction I (K_d 0.92) was concentrated, dialyzed against Hank's solution, and tested for its ability to inhibit the cytotoxic reactions of monospecific alloantisera. The antigen inhibited the action of these antisera in a pattern identical to that found upon direct and absorption typing of the donor's peripheral lymphocytes. The phenotype of the peripheral lymphocyte (HL-A1—, 2+, 3—, 4+, 5—, 6+, 7+, 8—) determined with twenty-one specific alloantisera reflecting sixteen distinguishable HL-A antigenic determinants was identical to that previously reported for the cultured lymphocytes (Papermaster *et al.*, 1969). The inhibition curves (Fig. 5) showed that, in quantitative terms, antigen prepared from 8×10^3 cells was sufficient to inhibit significantly anti-HL-A2 antiserum at 2 cytotoxic units (see below). However, antigen prepared from as many as 2.5×10^7 cells was unable to effect the cytotoxic reactions of antisera directed against HL-A1,3,8, and Torino-11 determinants, all of which the donor

lacked. The specificity ratio, i.e., the reciprocal concentration required to inhibit an alloantiserum directed against a determinant present on the donor's cells vs. that required to inhibit an indifferent alloantiserum was 300 $[(2.5 \times 10^6)/(8 \times 10^3)]$. The specificity ratio of this relatively crude alloantigen solubilized by sonication was thus 2–3 times greater than that reported for papain-solubilized human alloantigen purified by ion-exchange chromatography (Sanderson, 1968). Antigens prepared from cultured cell lines showed only a slight deterioration in activity following storage at 4°C. for over 2 months and at —70°C. for over 6 months, in spite of repeated freezing and thawing, which is in contrast to the lability of antigen prepared from human spleen at this stage of purification.

The cell line from a normal donor has certain advantages over a line derived from a donor bearing a lymphoid malignancy, since the application of the latter is not completely satisfactory in view of (*1*) the possibility of transmission of oncogenic agents to normal patients during biological studies; (*2*) the possible interrelation between tumor antigenic determinants and histocompatibility antigenic determinants (G. Klein,

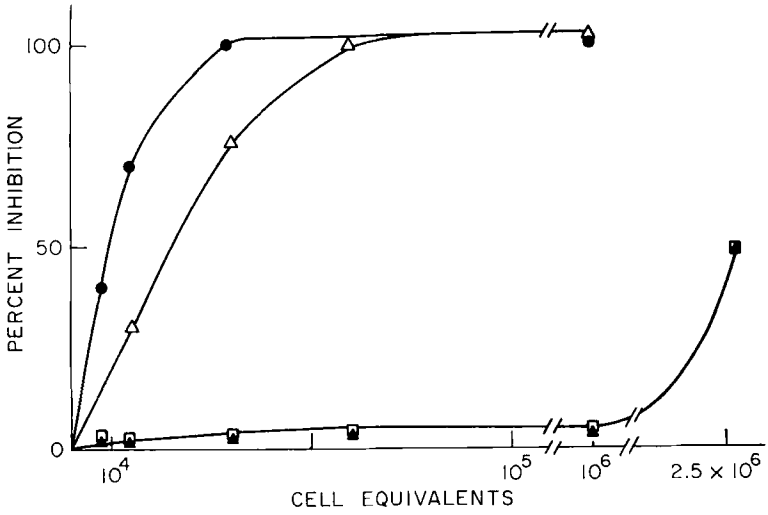

FIG. 5. Inhibition of cytotoxic reactions of typing antisera employed at 2 cytotoxic units by Sephadex Fraction I (RPMI 1788). (1) ●—● Torino 11.03 (HL-A2); (2) △—△ Cutten (HL-A7); (3) ▲—▲ Torino-32.19 (HL-A1); (4) □—□ Storm (HL-A3).

$$\% \text{ inhibition} = 100 - \frac{\% \text{ cells killed in presence of inhibitor}}{\% \text{ cells killed in absence of inhibitor}} \times 100$$

Cell equivalents are the number of cells from which the antigen was extracted.

1966); (3) the observations that extracts from a malignant source were chemically more complex than those from normal tissue (Kahan *et al.*, 1969); and (*4*) the need to have a living donor for long-term continuous comparisons between his peripheral lymphocytes, his cultured cells, and the antigen extracted from them. Thus, although the malignant cell lines offered a temporary solution to the problem of a uniform and abundant source for the extraction of transplantation antigens, the application of normal cell lines offers a new dimension to the field.

3. *Limitations of the Sonication Procedure*

The use of low-intensity sound to liberate water-soluble alloantigens has the same major limitations inherent in other solubilization methods. Thus, the sonication method also solubilizes relatively large amounts of nonspecific cellular materials together with relatively small quantities of alloantigens. Sonication does not seem to yield significantly larger amounts of soluble antigen than do other methods. However, it is difficult to compare yields reported by various investigators employing different methods since both procedures and criteria used to evaluate antigenic activity and specificity differ considerably.

However, the application of low-intensity sound offers some advantages in that the materials obtained are truly water-soluble as well as immunogenic and can be obtained as an electrophoretically homogeneous moiety which is essentially protein in nature. Furthermore, all the HL-A alloantigenic determinants detectable on the donor's peripheral lymphocytes can be obtained in the highly purified antigen preparation. The method can, thus, be employed to isolate a chemically well-characterized homogeneous antigen which can subsequently be cleaved by both chemical and enzymatic methods to ascertain the molecular and chemical nature of the alloantigenic determinants.

E. Other Methods for Antigen Extraction

Hypertonic salt solutions have been successfully applied to extract soluble HL-A alloantigens from human lymphocytes in long-term tissue culture. Cells were washed with Hank's balanced salt solution and then suspended in Hank's solution containing 3 *M* KCl (10 ml/10^9 cells) and stirred gently for 16 hours at 4°C. The extract was then centrifuged at 130,000*g* for 2 hours and the clear supernate once dialyzed against Hank's solution contained specific alloantigenic activity as measured by its capacity to inhibit the cytotoxic action of operationally monospecific alloantisera. The specificity ratios of such crude preparations range from 50 to 100. By this method and with the same salt concentration, from 10–15% of the antigenic activity of the crude cell particulate fraction,

containing about 15–20% of the total cell protein, was solubilized (Reisfeld and Kahan, 1970).

A cationic detergent, sodium lauroyl sarcostinate, has also been used successfully to solublize HL-A alloantigens from human lymphocytes in tissue culture. Cells (10^7/ml.) were homogenized with a Teflon homogenizer (4°C.) in Hank's balanced salt solution containing 0.7% (w/v) detergent. The homogenate was centrifuged at 130,000*g* for 12 hours (0°C.) and the supernatant placed at 0°C., a temperature at which much of the detergent crystallized. The supernatant thoroughly dialized against Hank's solution specifically inhibited the cytotoxic activity of operationally monospecific alloantisera. This procedure solubilized from 15 to 20% of the antigenic activity of the cell particulate, but contained only approximately from 3 to 5% of the total cell protein (Reisfeld *et al.*, 1970a,b).

III. Physical and Chemical Nature of Transplantation Antigens

A. Chemical Nature of Alloantigenic Determinants

Many investigators have attempted to determine the chemical nature of histocompatibility antigens. Progress in this was hampered by the extreme chemical complexity of most antigen preparations studied, and, consequently, histocompatibility antigens were considered to be almost every chemical entity known to man. The antigenic determinants were thus at various times considered to be either deoxyribonucleic acid (DNA), lipid, carbohydrate, or protein. Many studies attempted to evaluate the effect of a series of conditions and reagents on the activity of highly complex alloantigen preparations. Although such studies were generally quite useful as a guideline for isolation procedures, the vast chemical complexity of most of the antigen preparations studied seriously limited their value in obtaining conclusive information with regard to the physicochemical nature of alloantigenic determinants.

1. Deoxyribonucleic Acid

The pioneering experiments of Billingham *et al.* (1956b) indicated that a nuclear subcellular fraction could immunize recipients against subsequent grafts. Since these authors found that deoxyribonuclease but not ribonuclease or trypsin inactivated the preparation, they suggested that DNA determined transplantation specificity. However, neither Haskova and Hrubeskova (1958) nor Medawar (1958) were able to elicit accelerated rejection with purified DNA. Even more conclusively, Castermans and Oth (1956) proved that a component other than DNA carries the antigenic specificity when they found that extraction of a

nuclear homogenate with sodium chloride yielded an active supernatant that lacked DNA and an inactive sediment that contained DNA.

2. *Lipid*

Intensive efforts by several investigators (Herzenberg and Herzenberg; 1961; Lejeune *et al.*, 1962; Davies, 1962; Manson *et al.*, 1963) suggested that water-insoluble materials containing approximately equal proportion of lipid and protein and with a low carbohydrate content mediated transplantation immunity. These lipoproteins induced accelerated graft rejection, elicited the formation of specific alloantiserums, and inhibited the reactions of these serums *in vitro*. Davies (1966a) found that protein precipitated after organic solvent extraction was inactive and that there was an increasing porportion of lipid with increasing degrees of antigen purification. However, more recently, purified, soluble murine alloantigens were found to contain no detectable lipids or phospholipids (Shimada and Nathenson, 1969). It has become quite apparent that more purified materials containing little or no detectable lipid possess all of the attributes of transplantation antigen (Kahan and Reisfeld, 1967, 1969a, 1969b; Kahan *et al.*, 1968b) and that lipoidal fractions by chloroform–methanol or ethanol extractions have no alloantigenic activity (Graff and Kandutsch, 1966).

3. *Carbohydrate*

Billingham *et al.* (1958) showed in later work antigenic activity in the sediment of cells that had been exposed to ultrasound and centrifuged at 27,000*g*. The antigenic determinant in this preparation was proposed to be a mucoid since its biological activity was drastically reduced by two reagents: (*1*) receptor-destroying enzyme, a complex mixture from *Trichomonas foetus*, and (2) periodate (0.005–0.01 *M*). Several other investigators (Kandutsch and Reinert-Wenk, 1957; Kandutsch and Stimpfling, 1966) felt that carbohydrate moieties could be part of the antigenic determinants of transplantation antigens since they found inactivation following periodate treatment. However, none of these studies clearly demonstrated specific effects on the carbohydrate moiety per se, e.g., specific oxidation products, and at the same time they documented that periodate did not adversely affect amino acids and, thus, protein configuration. Kandutsch and Stimpfling (1966) have shown that when mouse transplantation antigens are exposed to periodate (0.001 *M*), there are marked changes in the content of several amino acids including tyrosine, isoleucine, and leucine. It is, indeed, difficult to ascribe the nature of the antigenic determinant to carbohydrate on the basis of the reaction of highly complex mixtures with relatively nonspecific oxidizing agents such as periodate.

In further attempts to implicate carbohydrate as the antigenic determinant of histocompatibility antigens, Brent *et al.* (1961) found that some polysaccharides with Forssman affinities—blood group substance A (but not B, H, or Lea), Type XIV pneumococcal polysaccharide (but not Types I, II, or V), and *Shigella shigae* polysaccharide—inhibited the agglutination of erythrocytes by alloantiserum in a fashion analogous to the hapten inhibition observed with blood group isoantiserums. Davies (1966a) found that D-galactopyranose-β-(1 $\rightarrow$ 4)-D-glucosaminoyl residues partially inhibited alloantiserum against specificity H-2 18 (R) and that *N*-glycolyl neuraminic acid specifically inhibited some mouse alloantiserums. However, these studies have not been continued and have at least thus far failed to yield much insight into the chemical nature of the determinants, since the observed effects were generally very weak.

Shimada and Nathenson (1969) found that papain-solubilized alloantigens from spleen cells of mice with H-2^b and H-2^d genotype are are glycoprotein with an 80–90% protein moiety. They base their claim on total neutral carbohydrate estimations by the orcinol method and by determinations of sialic acid and glucosamine. These determinations were related to dry weight. However, the amount of purified material was so small that dry weight could not be determined, and a 90% value for the protein content of the dry weight had to be assumed. On the basis of these assumptions, neutral carbohydrate was calculated to range from 3 to 5%, hexosamine from 3 to 4.4%, and sialic acid from 0.9 to 1.3%. Whether or not these carbohydrate moieties are part of the antigenic determinant per se has not been determined thus far.

D. L. Mann *et al.* (1969b) used papain to solubilize human alloantigens from two cell lines bearing different alloantigenic specificities. These alloantigens were found to contain from 5 to 8% orcinol-reactive carbohydrate. No hexosamine could be detected, which is curious since, with the exception of collagen, there are no known mammalian glycoproteins that lack hexosamine. In fact, recent studies have shown that hexosamine is present in human platelet membrane glycoproteins (Pepper and Jamison, 1969). As pointed out by D. L. Mann *et al.* (1969b), there is as yet no decisive information, which can be drawn from their data, indicating that the carbohydrate portion is responsible for alloantigenic specificity of HL-A antigens.

4. Protein

There is a considerable body of evidence which suggests that polypeptide is essential for antigenic activity. Thus, Kandutsch and Reinert-Wenk (1957) observed irreversible destruction of antigenic activity after exposure to protein denaturants, e.g., 50% urea, 90% phenol, aqueous alcohol, heat, and pH values less than 4 and greater than 9. Kandutsch

and Stimpfling (1966) have also shown that pronase degrades antigenic material to an inactive lower molecular weight substance and have suggested that the previously observed resistance of crude fractions to other proteolytic enzymes may have been due to an inaccessibility of the enzyme to the active region of the antigens. Furthermore, Graff and Kandutsch (1966) found that deoxycholate treatment and succinylation, both of which affect protein configuration, partially destroyed the immunogenic properties of detergent-solubilized antigenic extracts.

Bruning *et al.* (1964, 1968) extracted HL-B antigens with deoxycholate from cell membrane sediments of placental tissue. The solubilized antigens were treated with a variety of agents capable of denaturing or extracting either protein, lipid, or carbohydrate. The serological activity of antigens thus treated convinced these investigators that protein structure and not carbohydrate or lipid moieties determined the activity of these antigens.

Davies (1967) found that some H-2 alloantigens were more readily heat-denatured than others. Thus H-2, EK(5.11) was labile when exposed to 37°C. for 7 minutes at pH 4.5, H-2,C(3) was not affected by this treatment.

D. L. Mann *et al.* (1969b) investigated the stability of HL-A alloantigen which had been partially purified by gel filtration but remained chemically quite complex as judged by acrylamide electrophoresis patterns. They found marked and irreversible loss in activity when antigen preparations were exposed for 24 hours (4°C.) to NaCl concentrations less than 0.15 *M* or to pH values less than 6 or greater than 10. Temperatures greater than 4°C. caused progressive inactivation, whereas exposure to 56°C. resulted in almost complete loss of antigenic activity. Whether these treatments had varying effects on different antigenic determinants cannot be ascertained from these studies since antigenic activity was only measured by cytotoxic inhibition of a highly polyspecific antiserum.

Additional evidence which suggested that polypeptide was essential for the antigenic specificity of alloantigens came from all the investigators who used the proteolytic enzyme papain for the solubilization of alloantigens from lymphocyte membranes. This enzyme could only be allowed to react with cell membranes for a limited period of time (approximately 1 hour) since longer digestion periods usually caused rapid loss of antigenic activity (Sanderson and Batchelor, 1968; D. L. Mann *et al.*, 1969b; Shimada and Nathenson, 1969). It is, indeed, striking that redigestion of cell membranes with papain rapidly and completely destroyed all the antigens that were not extracted during the initial digestion (Shimada and Nathenson, 1969).

In view of these observations, it is difficult to imagine that moieties

other than proteins make up the vast majority of antigenic determinants of human and mouse alloantigens. One can, of course, argue that (*1*) polypeptide configuration is crucial to express alloantigenic determinants composed of carbohydrates or (*2*) carbohydrates are instrumental in bringing about a given polypeptide configuration which expresses a certain alloantigenic specificity. Neither of these arguments seems particularly compelling in view of the observation that electrophoretically homogeneous guinea pig transplantation antigens and human alloantigens do not contain either carbohydrate or lipid at the 1% limit of the analytical method (Kahan and Reisfeld, 1968b, 1969b). Thus, in the case of guinea pig antigen (molecular weight 15,000) there is at most one residue, and, in the case of human antigen (molecular weight 34,600), there are at most two residues of carbohydrate per molecule. These hypothetical values are advanced only because it is simply impossible with available analytical methods to rule out absolutely the presence of a very small carbohydrate moiety in a relatively large molecular weight protein molecule. On the other hand, amino acid analyses of electrophoretically homogeneous transplantation antigens from two histoincompatible inbred strains of guinea pigs strongly suggest that their antigenic determinants depend upon protein structure because they show marked and reproducible differences in their amino acid composition (Kahan and Reisfeld, 1968b). Although it is not presently known whether the antigenic products of the two histoincompatible, inbred strains examined are the result of single or multiple gene differences, the data suggest that these transplantation antigens possess genetically segregating (allotypic) specificities related to protein structure possibly in analogy to the polymorphic antigenic determinants found on the serum proteins of numerous species. Finally, the mediation of transplant rejection by a cell-bound immune response implicates polypeptide specificities since, as Holborow and Loewi (1967) have summarized, "there is practically no evidence that man or animals develop delayed type hypersensitivity toward polysaccharides, and in that respect they differ sharply from proteins."

Furthermore, the distinct and reproducible amino acid differences observed between transplantation antigens prepared from two histoincompatible lines of guinea pigs suggest that these antigens possess allotypic specificities related to protein structure (Kahan and Reisfeld, 1968b). The polymorphism of the primary protein structure of these antigens is strikingly similar to that observed in rabbit immunoglobulin light chains with different allotypic specificities (Reisfeld *et al.*, 1965). Since histocompatibility antigens are readily distinguishable products of well-defined alleles which are distributed among some but not all mem-

bers of the same species, these polymorphic forms of genetically segregating antigenic determinants can be considered to form an allotypic system analogous to that described for serum immunoglobulins (Oudin, 1966). In both cases there is a correlation between allotypic specificity and the amino acid composition of these polymorphic substances.

It is impossible at this point to rule out that either lipid or carbohydrate moieties express a specific alloantigenic specificity *per se* or that they confer a unique protein configuration that determines such a specificity. There is little doubt that carbohydrate moieties present on cell membranes and on some alloantigens may have an important physiological role which will probably become apparent from future research efforts. However, at present, it seems that most, if not all, antigenic determinants of alloantigens are polypeptide in nature in view of the data summarized here and since the large number of amino acid sequence analyses and immunogenetic studies of human, mouse, and rabbit immunoglobulins thus far show that only polypeptides express genetically segregating antigenic (allotypic) determinants.

B. Physical Nature of Alloantigens

1. Molecular Heterogeneity of Alloantigens

The notion that alloantigenic determinants might be associated with different molecular species of the cell membrane received some of its stimulus from the proposal that two closely linked chromosomal regions may determine the genetic control of HL-A alloantigens (Ceppellini *et al.*, 1968; Kissmeyer-Nielsen *et al.*, 1968; Dausset *et al.*, 1969) and from the work of Boyse *et al.* (1968) indicating that H-2^d and H-2^k alloantigens were distant from each other on the membrane of mouse thymocytes.

Several investigators observed that following solubilization by papain, both human and murine alloantigens could be fragmented into entities that lacked some of the specificities of the donor and, in some cases, contained only a single detectable antigenic specificity as determined by their ability to inhibit the cytotoxicity of a very limited number of operationally monspecific alloantisera (Sanderson, 1968; Davies, 1969; D. L. Mann *et al.*, 1969a,b).

Davies (1969) used either autolytically or papain-solubilized antigen extracts to show that by chromatography on DEAE–Sephadex columns he could differentiate, from very complex protein effluents patterns, various molecular species carrying only one or, in some instances, several H-2 specificities. Some H-2 antigens of a single phenotype are obtained in this manner. Thus, from H-2^b mouse antigen, H-2.5 specificity was obtained essentially free from H-2.8 and, in turn, H-2.8 was obtained

free from H-2.5. These antigenic specificities were separated by DEAE-Sephadex chromatography, applying a simple straight-line salt gradient. Sanderson (1968) initially failed to separate HL-A alloantigenic specificities by gel filtration on Sephadex G-200. Instead, alloantigenic specificities which had a relatively high specificity ratio (SR $\sim$ 100) emerged together in a region where calibration curves indicate a molecular weight of 45,000. A smaller peak of aggregated material was found in the excluded volume containing the same antigenic specificities but with a very low specificity ratio (SR $\sim$ 1). This observation is of some interest in view of the data of D. L. Mann *et al.* (1968, 1969a,b) who always found antigenic activity (in some cases the majority) in the excluded volume of a Sephadex G-150 column; however, these investigators have not reported any specificity ratios for their separated alloantigens.

D. L. Mann *et al.* (1969a) claimed molecular heterogeneity of human alloantigens based on their ability to separate alloantigenic determinants from both subloci HL-A2, HL-A4, and HL-A7, respectively. It is interesting that all alloantigenic specificities analyzed were found both in the excluded and included volumes. However, most of the HL-A2 activity was in the excluded volume whereas most of the HL-A4 and HL-A7 activities were found in the included volume of the Sephadex G-150 eluate. Moreover, although somewhat overlapping, the peak values of HL-A4 and HL-A7 activities appeared in slightly different elution positions (no elution volumes or K_d values were given) with a smaller peak of HL-A4 activity appearing in yet another elution position together with small amounts of HL-A2 and HL-A7 activities.

In a more recent study of the same lymphoid cell line (R-4265), D. L. Mann *et al.* (1969b) showed a Sephadex G-150 elution profile which depicts only one alloantigenic specificity (HL-A2) determined by an operationally monospecific antiserum. The majority of this specificity was again found in the excluded volume. The area of the effluent pattern in which previously (D. L. Mann *et al.*, 1969a) several alloantigenic specificities were shown to be resolved was now covered by a large peak indicating specificities by a highly polyspecific antiserum (Iochum) which reacts with lymphocytes from 96% of a normal population. Within this activity region of the included volume, also a small peak of HL-A2 specificity appeared. It is surprising that apparently separated components with different alloantigenic specificities could not be separated at all on acrylamide gel electrophoresis but appeared within one electrophoretic zone. Shimada and Nathenson (1967) found that, by Sephadex gel filtration, murine alloantigens could be separated into entities with different H-2 specificities. In a more recent study (Shimada and Nathenson, 1969), it was observed that, although this was not possible for H-2^4

alloantigens, there was some separation of H-2^b alloantigenic specificities H-2.5 and H-2.2, both of which were in the included volume of a Sephadex G-150 column. However, it was not possible to separate these alloantigenic specificities by acrylamide gel electrophoresis. Both of the purified preparations (H-2^b and H-2^d) resolved into three electrophoretic components each of which carried essentially the same combination of antigenic specificities. In order to reconcile these electrophoretic analyses with the gel filtration data, one has to propose that electrophoresis resolved components with different overall charge properties from each of the two antigen preparations. It is probable that, since only a 7.5% acrylamide gel was used, most separation occurred on the basis of charge rather than size. Since the electrophoretic components have basically the same antigenic profile and also relatively similar amino acid compositions, it seems, indeed, feasible that they represent a series of fragments produced by papain. It is possible that antigen separation on Sephadex occurs due to the association of antigen fragments with other proteins and that these protein–protein interactions are minimized by the conditions of acrylamide gel electrophoresis.

From the proposal that at least two closely linked chromosomal regions determine the genetic control of HL-A alloantigens (Ceppellini *et al.*, 1968; Kissmeyer-Nielsen *et al.*, 1968; Dausset *et al.*, 1969), one can readily deduce that either (*1*) two structural cistrons code for separate molecules with either one or the other series of determinants or (*2*) one structural cistron with multiple mutational sites is able to do the same. Although such hypotheses (D. L. Mann *et al.*, 1969a) may eventually be useful to understand the genetic mechanism that controls the expression of histocompatibility antigens, it seems that the lack of chemically well-defined alloantigen molecules makes it difficult at present to test such hypotheses at the molecular level.

Several hypotheses can be advanced in an effort to explain the finding of alloantigenic specificities on components separable by either gel filtration (D. L. Mann *et al.*, 1968, 1969a,b; Shimada and Nathenson, 1967, 1969) or by cellulose or Sephadex ion-exchange chromatography (Sanderson, 1968; Davies, 1969). Since a nonspecific proteolytic enzyme such as papain is allowed to react in large excess with a highly complex mixture of cellular proteins, it is conceivable that the enzyme cleaves in a random fashion, not only materials containing antigenic determinants but also other proteins. Protein–protein interactions and aggregation could account for some of the separations observed on gel filtration and ion-exchange chromatography. Fragments containing alloantigenic determinants could certainly associate with proteins bearing no alloantigen determinants which may or may not have been cleaved by papain. When

proteins are cleaved into different fragments, their binding capacity for other substances also may change, further complicating the picture. There is little doubt that, following proteolysis, cell membrane fractions, whether separated by gel filtration or ion-exchange chromatography, are highly complex mixtures (Halle-Panenko *et al.*, 1968; D. L. Mann *et al.*, 1969b; Shimada and Nathenson, 1969), and it is no surprise that the same antigenic determinants often appear in different portions of a chromatogram. Furthermore, since papain can apparently easily destroy alloantigenic activity (Sanderson, 1968; D. L. Mann *et al.*, 1969b; Shimada and Nathenson, 1969), it seems quite feasible that this nonspecific proteolytic enzyme may produce a number of fragments that lack certain antigenic specificities, and that then can be resolved by either gel filtration or ion-exchange chromatography. In fact, it does not seem unreasonable to assume that papain, which can easily destroy membrane-bound specificities (Shimada and Nathenson, 1969), can also "silence," i.e., selectively inactive, certain alloantigenic determinants made more susceptible once a given antigenic fragment is removed from the membrane and is then exposed to the large excess of papain present during the 1-hour digestion period.

Although the molecular heterogeneity of alloantigens certainly poses a challenging and intriguing problem, it is difficult to draw any meaningful conclusions on the basis of presently available experimental evidence. It would probably be profitable to solubilize alloantigens without the aid of nonspecific proteolytic enzymes and then apply chemical and enzymatic methods only to thoroughly purified and characterized entities in an attempt to separate and characterize fragments bearing different alloantigenic determinants.

2. *The Homogeneity Problem*

The selection of criteria for homogeneity of polymorphic proteins and glycoproteins solubilized from cell membranes poses some problems. As pointed out previously, all the methods presently in use suffer from a lack of selectivity, i.e., they tend to solubilize large amounts of complex materials from the cell membrane containing relatively small quantities of alloantigens. This leaves one with the "needle in the haystack" problem, the end result of which is in the best case, the isolation of an extremely small quantity of antigenic material. This places a severe limitation on rigorous determinations to establish homogeneity of this material. Consequently, the only techniques used are those that require little material, i.e., acrylamide gel electrophoresis and analytical ultracentrifugation.

Discontinuous acrylamide gel electrophoresis is, when properly exe-

cuted, a sensitive technique with excellent resolving power. The method is most discriminating and is at its best when used with varying acrylamide pore sizes, urea, and radiolabeled samples; it is a poor criterion of electrophoretic homogeneity when these parameters are not applied. Then "bandsmanship" takes over, i.e., any "single band" is interpreted as a sign of homogeneity, no matter whether it is barely visible or whether there is an underlying or tailing smear of stained but unresolved proteins.

Shimada and Nathenson (1969) found that murine alloantigen preparations, which had been purified by gel electrophoresis and ion-exchange chromatography, when applied to acrylamide electrophoresis showed three components (R_f 0.36, 0.38, and 0.40). These components were poorly resolved and contained much underlying and tailing material. Re-electrophoresis of one of these H-2^b components (R_f 0.38) in the presence of sodium dodecyl sulfate (SDS) resulted in the appearance of a single 2–3-mm. wide zone which in itself was considered an indication of electrophoretic homogeneity. It was pointed out that H-2^d electrophoretic components showed, under the same condition, an additional component, estimated as 10% of the total, based solely on its dye intensity. Estimate of so-called "minor components" based simply on dye intensity have often proven to be erroneous especially when the band appears diffuse. Estimates of components based on scanning of gels containing radiolabeled materials has proven to be a much more reliable method. The choice of SDS gel to indicate electrophoretic homogeneity is rather unfortunate since the strong negative charges present on the detergent overwhelm all charge differences, so that any small charge differences between protein components are hidden and only large size differences can resolve proteins in this system. Incidentally, this electrophoresis system also lacks the discontinuous voltage gradient which is the key to the sharp resolution of disc electrophoresis. Furthermore, SDS gels, to be most informative, should be run at a number of different acrylamide concentrations to make full use of the size resolving power which is the main attribute of these gels. As shown previously (Kahan and Reisfeld, 1969b), to make the most efficient use of the high resolving power of the discontinuous acrylamide system, it is useful to radiolabel the material to be characterized and then to carry out electrophoresis in urea, followed by the screening of narrow gel slices. Only a sharp, single peak resolving within one gel slice is indicative of electrophoretic homogeneity. Moreover, since the radiolabel has a sensitivity of several magnitudes greater than the Coumassie dye, one can evaluate much more accurately the existence and quantitative extent of an underlying or tailing unresolved protein zone. These criteria previously applied by others (Kahan and Reisfeld, 1969b) to test the degree of electrophoretic

homogenity of an alloantigen preparation have not been used by Shimada and Nathenson (1969), and the homogeneity of their antigen preparation is thus difficult to assess.

D. L. Mann *et al.* (1969b) showed that pooled Sephadex eluates with alloantigenic activity appeared as a complex pattern following acrylamide gel electrophoresis and that a 2–3 mm. wide, poorly resolved, zone containing all the antigenic activity when eluted and re-electrophoresed, appeared again as a broad, 2–3-mm. wide band. However, this electrophoretic zone was not readily visible to the eye, and had to be indicated by superimposed lines. From these data, it is difficult to determine whether this preparation is electrophoretically homogenous.

Only one serious effort was made thus far to use analytical ultracentrifugation data in an effort to determine the size homogeneity of an alloantigen preparation. Thus, electrophoretically homogeneous guinea pig transplantation antigen was subjected to ultracentrifugation, employing the Yphantis sedimentation equilibrium techniques, and was found to be 91% monodisperse with a molecular weight of 15,000 and a 9% aggregated moiety with a molecular weight of 120,000. Human alloantigen was found to be 94% monodisperse with a molecular weight of 34,600 and a 6% aggregated moiety with a molecular weight of 150,000 (Kahan and Reisfeld, 1969b).

3. *Cell Surface Location of Alloantigens*

The structure of cellular membranes, according to the traditional model of Danielli and Davson (1935), is thought to consist of phosphatide bilayers, the surface of which are coated with membrane proteins linked to the lipid head groups by ionic and/or hydrogen bonds. Wallach and Gordon (1969) believe that this traditional view of the membrane structure is not compatible with the data obtained from recent studies utilizing infrared and nuclear magnetic reasonance spectroscopy as well as measurement of ultraviolet optical rotatory dispersion and circular dichroism. They feel that membranes respresent a complex mosiac of organized assemblies of lipoproteins with certain architectural organizations. According to their model, membrane proteins are located on both of the membrane surfaces and also within the apolar core of the membrane. Surface-located proteins are considered to be coiled irregularly, whereas penetrating protein segments are thought to be helical rods with a hydrophobic face. These investigators also propose that the intact cell surface is not uniform, but that it consists of discrete patches with surface areas of 1.2×10^6 sq. Å. and that these patches fall into a number of groups with distinct protein compositions. Present evidence, based upon observations with (*1*) fluorescent alloantibody

(Möller, 1961; Cerottini and Brunner, 1967; Gervais, 1968), (2) agglutinating alloantibody (Gorer and Mikulska, 1954; Amos, 1953), (3) antibody absorption before and after cell rupture (Haughton, 1964) and (4) purified fractions (Ozer and Hoelzl-Wallach, 1967; Popp *et al.*, 1968), indicates that the majority of the strong alloantigenic determinants are intimately associated with the cell surface membrane.

To get an idea of the cell surface location of alloantigens, Boyse *et al.* (1968) have attempted to determine the position on the surface of murine thymocytes of five alloantigenic systems (H-2, θ, Ly-A, Ly-B, and TL) which exhibit both genotypic and phenotypic variability. The principle used to plot these antigen positions is that when two antigens are situated close together, the absorption of antibody by one of them impedes the absorption of antibody by the other. This "blocking test" provides then a measure of the proximity of a pair of antigens on the cell surface. This test does, of course, bear no relation as to whether two or more determinants are on the same molecule of a soluble antigen preparation. Thus H-2^d and H-2^k murine alloantigens have been found distant from each other on the membrane but appeared on soluble antigen in the same chromatographic fraction (Davies *et al.*, 1967). It is believed that the alloantigens of the five systems examined comprise a single cluster, i.e., a basic unit which is a repetitive feature of the membrane surface. Studies of H-2 alloantigens on lymphocyte membranes are more difficult since these antigens are present in such a high concentration that they form a confluent pattern. Davis and Silverman (1968) employing the ferritin-labeled antibody technique could, however, detect clusters of labeled H-2^d and H-2^b alloantibodies on lymphocyte membranes.

The susceptibility of murine and human alloantigens to a variety of protein denaturants and the lack of detectable carbohydrate and lipid on preparations of electrophoretically homogeneous guinea pig and HL-A antigens suggest a protein nature for the "active site" of these antigenic substances. The various physical, chemical, and enzymatic methods employed to remove these materials from membrane surfaces, e.g., to solubilize them, most likely change the native configuration of these alloantigens. In this regard, it is interesting that Sanderson (1968) feels that a certain "macromolecular integrity" is needed for these alloantigens to be expressed. Should this be the case, then it is difficult to see how a nonspecific proteolytic enzyme such as papain can "solubilize" some antigens with enough "macromolecular integrity" to express specific antigenic determinants, whereas a large amount of residual antigens expressing these same determinants remains in or on the cell membrane where it can even be destroyed by papain without ever being solubilized.

These observations, together with those indicating that only a minor portion (15–25%) of the alloantigens can be solubilized by existing methods, make it necessary to postulate that there are some antigens that can be solubilized relatively easily, whereas others resist solubilization by presently available methods. In the light of the foregoing discussion concerning the nature of the cell membrane, it seems feasible that antigens associated with macromolecular entities on the membrane by means of ionic bonds or hydrogen bonds may be solubilized by either proteolysis, detergents, sound, or hypertonic salt extraction. On the other hand, many antigens cannot be solubilized by any of these treatments and could, thus, be located within the apolar core of the membrane, highly interacted with hydrophobic lipid moieties. It is obvious that at present no real conclusions can be drawn and that considerable research will have to be done to understand the physicochemical nature of the attachment of histocompatibility antigens to lymphocyte cell membranes.

IV. Biological Activity of Extracted Transplantation Antigens

The ability of tissue extracts to accelerate the destruction of donor-specific grafts (Billingham *et al.*, 1956a,b), the second-set phenomenon (Medawar, 1944), demonstrates the activity of these extracts as transplantation antigens. Although this system most meaningfully reflects the role of the substances in histocompatibility, it is a cumbersome, insensitive, only crudely quantitative, and time-consuming technique for the screening of putative antigens. Therefore, more rapid, flexible systems dependent upon delayed-type hypersensitivity or upon humoral antibody have been routinely employed for antigenic detection.

A. Transplantation Compatibility Assays

1. Accelerated Rejection of Allografts—Skin Transplants

Loeb (1930) suggested that if transplantation resistance depended upon an immune mechanism, a second graft transplanted onto a host when his reaction against the first graft from that donor was at its height, would be attacked with equal intensity at once. Medawar (1944) succeeded in demonstrating that challenge grafts obtained from the donors of the sensitizing transplant were destroyed more rapidly than transplants from third-party donors and that the resistance induced by allografting was systemic. Billingham *et al.* (1956a) showed that pretreatment with subcellular extracts immunized recipients specifically against donor-strain skin grafts and, thus, established that transplantation antigenic activity did not depend upon intact cells.

In routine practice, an antigenic preparation is administered to an

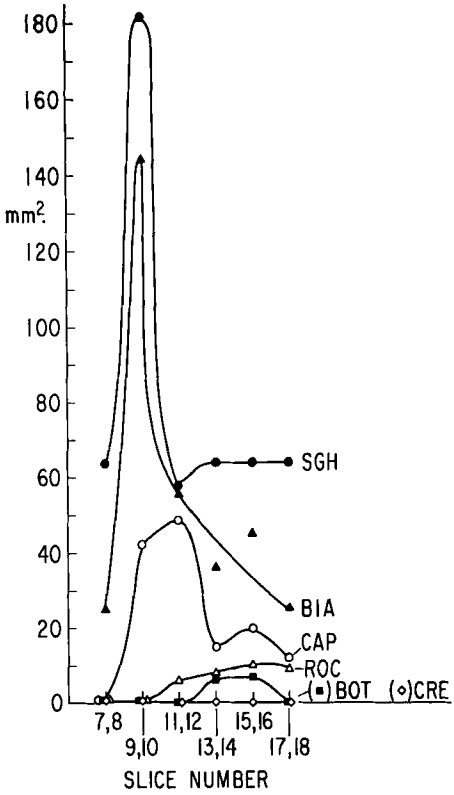

FIG. 6. Cutaneous reactions of preimmunized individuals SGH, BIA, CAP and of normal individuals BOT and ROC, to acrylamide gel cuts after electrophoresis of NAV Sephadex Fraction I.

allogeneic host and, after an interval of a few days, the recipient is challenged with grafts derived from the donor strain and from a third-party strain, i.e., a strain different than that of the donor or the recipient. In Fig. 6, the host has received soluble antigen extracted from the spleens of the strain of the left (upper) graft and later was challenged with skin transplants from the donor strain and from a third-party strain (the right lower graft). The specific accelerated rejection of the upper graft in this figure revealed the presence of transplantation antigenic substances in the original extract. There are several significant factors in the performance of this assay.

a. Route of Administration of Antigen. Medawar (1957) demonstrated that in rabbits administration of leukocytes by the intravenous route was less efficacious than by the intradermal route in the induc-

tion of allograft immunity. In the later work of Billingham and Sparrow, intravenous injection of trypsin-dissociated epidermal cells resulted in prolonged skin graft survival, whereas intradermal administration of these cells resulted in accelerated rejection of the transplants. The phenomenon was confirmed in the case of pretreatment of rabbits with spleen cells (Billingham *et al.*, 1957). Indeed, Merrill and colleagues (1961) failed to detect sensitization of human beings preimmunized with intravenous leukocyte injections. This difference is not as pronounced in the mouse (Billingham *et al.*, 1957): administration of relatively high doses of spleen cells (250,000 and 5×10^6 cells) were equally effective by the intravenous subcutaneous, intramuscular, intradermal, or intraperitoneal routes. By the intraperitoneal route 2000 cells provoked just perceptible immunity, and in the range of 2000 to 5×10^6 cells the dose–response curve appeared quite flat. In the guinea pig the intravenous and intraperitoneal routes were equivalent at the 50×10^6 cell dose (Billingham *et al.*, 1957).

A. P. Monaco *et al.* (1965) found no significant difference in either the degree of allograft immunity induced or in the time course of its development following intravenous or intraperitoneal administration of a membranous transplantation antigenic fraction prepared by homogenization. In contradistinction to these particulate materials, the result obtained after administration of "semisoluble" or soluble antigenic fractions depended upon the route of administration. The weight of evidence with transplantation antigens, just as with a variety of other immunologically potent materials, indicates that solubilized materials tend to be tolerogenic when administered by the intravenous route and immunogenic when administered by the subcutaneous route. The superiority of the subcutaneous route for the administration of transplantation antigens was established in murine systems with sonicated (Kahan, 1965) and detergent-solubilized materials (Graff and Kandutsch, 1966). Furthermore, Brent *et al.* (1962b) demonstrated that several soluble preparations did not sensitize when administered intravenously—a situation that parallels the dependence of the Sulzberger-Chase phenomenon on the portal vascular system. Further studies on tolergenicity of antigens (as discussed below) have employed the intravenous route. Immunogenicity studies, on the other hand, generally have utilized the subcutaneous or intraperitoneal routes of administration.

b. Length of Interval between Antigen Administration and Challenge Skin Grafting. The kinetics of the induction of homograft immunity are poorly understood. There is little doubt that the sensitizing activity and the permanence of the immunity so induced depend upon the form of the transplantation antigen employed for immunization. Thus, sensi-

tization with skin grafts results in a durable state of immunity which is definitely in force at 120 days (Billingham *et al.*, 1954a,b), although there has been decline between 15 and 120 days. The most violent immune reaction, the white graft reaction (Rapaport *et al.*, 1958), is detected in challenge grafts applied within 7 days after the rejection of a first-set skin graft. This powerful immunity persists only a short time; challenge grafts applied at longer intervals after immunization show less violent destruction which decreases in severity as the interval between sensitization and challenge increases.

The micrograft experiments of Billingham *et al.* (1959) revealed that a graft of but 1.5 mm.^{2} which was estimated to contain but 50,000 cells provoked a state of immunity which was still maximal after 30 days, i.e., just as intense as stimulation from a larger graft, and much more potent than an equal number of leukocytes administered intraperitoneally. Indeed, the difference between the sensitizing ability of the skin graft and dissociated cells may be due to local granuloma formation in the skin at the site of the graft, to the ability of the intact graft to attract cells and imprint more successfully on their immunological memory, to the nature of the epidermal antigen, or to the nature of the antigen associated with the blood vessels present in the graft.

Kinetic studies on the induction of immunity following skin graft sensitization have shown that 3–4 days of contact of the graft with the bed are essential for a second graft to be destroyed in accelerated fashion. McKhann and Berrian (1959) documented that grafts applied on the same day as the challenge graft was removed, i.e., at 4 days, had little breakdown but that grafts applied after a 2-day interval (i.e., on the sixth day) were entirely destroyed, having failed to establish themselves at all, i.e., white grafts. These experiments suggest that although 4 days was a sufficient interval of contact for immunity to be induced, it was not a sufficient length of time to reach a level of immunity sufficient to destroy a fresh graft; an extra 2 days of maturation of the response was necessary. As the time was increased beyond the 2-day interval, there was attenuation of the immunity such that some initial healing did occur. It was of interest that continuous contact was not essential; for grafts placed on new beds, or new grafts applied to the same bed or to new beds, also inducted sensitization and subsequent accelerated rejection. These observations thus demonstrated that there were no special properties endowed in the graft or its bed.

Administration of subcellular transplantation antigen produce a more induction of transplantation immunity elicits a different kinetic picture. McKhann and Berrian (1959) injected cells intraperitoneally on the −3, −2, or −1 day prior to grafting, eliciting complete rejection of

the challenge grafts. When cells were administered on the day of, or 1 day after, grafting there was accelerated rejection with less but still a perceptible effect when administered on the third day after grafting.

Administration of subcellular transplantation antigen produce a more attenuated immunity. Although A. P. Monaco *et al.* (1965) found that the sensitizing capacity of a 105,000-G. sediment of homogenized cells was just as great as that of an equivalent number of cells, most investigators found the extracted antigens to be much less potent than intact cells (Brent, 1958; Celada and Makinodan, 1961; Kahan, 1965). Quantitative studies on murine sonicated antigen revealed that although 280,000 whole spleen cells were required to induce immunity against allografts, 10.7×10^6 sonicated cell equivalents, about thirty-five-fold greater, were required to induce the same degree of sensitization (Kahan, 1964a). The difference in potency between whole and disrupted cells has been attributed to (*1*) the destruction of labile antigens or their carriers (Brent, 1958), (*2*) the increase in antigen dosage due to cell division of viable cells, (*3*) a longer persistence of viable cells in the host resulting in a more prolonged antigenic stimulus (Celada and Makinodan, 1961), (*4*) the migratory capacities and homing tendencies of intact lymphocytes (Fichtelius, 1958; Murray, 1964; J. Bainbridge *et al.*, 1966), and (*5*) the possible greater potency of transplantation antigen located on intact cell surfaces to interact with cell-bound recognition systems.

Indeed, there is a further decline in immunogenic activity as the antigenic principle is purified from the disrupted cell fractions. Partially purified antigenic fractions show not only a rapid induction of immunity, similar to that seen with the administration of dissociated cells, but also an abrupt decline in sensitivity. The rate of decline in sensitivity is inversely proportional to the dose of antigen administered, but in all cases reported, there is less than 10% of the original activity at 14 days after antigen administration (Kahan, 1964a,b). Furthermore, of the immunogenic activity of the total nonviable cell, 22% of the activity can be detected in the 105,000g supernate of sonicated cells (in a soluble form) i.e., 48×10^6 cell equivalents of soluble antigen are required to equal the immunogenic effect of 10.7×10^6 sonicated cell equivalents (Kahan, 1964a). Although it is possible that in the course of purification a substantial proportion of the activity is lost due to denaturation of the antigenic material, it is also possible that contaminant materials without immunological specificity appreciably contribute to the immunogenic effect of the active principle. In this regard, Messina and Rosenberg (1962) found the delayed-type hypersensitivity response to egg albumen

administered intramuscularly was increased by inoculation of intact or disrupted spleen cells. Braun and Nakano (1965) and Merritt and Johnson (1965) found that administration of DNA increased the early antibody response to antigens.

Work on sonicated murine antigens has revealed that it is not merely the presence of contaminant membranous and other subcellular components which is responsible for the greater immunogenic effect of crude fractions. The activity of solubilized 105,000*g* supernates as transplantation antigens was not appreciably increased by preincubation admixed with membranous fractions derived from the same strain as the recipient who received the immunizing antigen injection (Kahan, 1964a). These data suggested that the state of the antigen itself, rather than the presence of accessory membranous material, was the major factor in determining its immunogenic capacity. It has been frequently observed that antigenic sediments are more potent than their mother liquors (Brent *et al.*, 1962b; Kahan, 1964a; A. P. Monaco *et al.*, 1965). In work on sonicated murine transplantation antigens, the 20,000-G. microsomal-lysosomal membrane fragment fraction was 4 times more potent than its original 2000*g* post nuclear mother liquor (Kahan, 1964a). These observations are consistent with those of Dresser (1962) who found that the sediment of bovine γ-globulin was more immunogenic than the original preparation and proposed that aggregated materials possess higher inherent adjuvanticity. He has suggested that the immunogenic activity of commercial bovine γ-globulin was due to the relatively small fraction of aggregated, possibly partially denatured material present. Interference optics ultracentrifugal analyses of purified guinea pig and human sonicated antigens (Kahan and Reisfeld, 1969b) have revealed that a small fraction of the antigen exists in a highly aggregated form of over the 100,000–200,000 molecular weight range. This fraction may be the potent antigen in the induction of immunity.

c. Scoring of Challenge Skin Grafts. When donor and recipient differ at a strong histocompatibility locus, the nature of the challenge graft used to demonstrate an immune state does not appear to be important. In work on sonicated murine antigens, destruction of the DBA-2 Cloudman tumor provided just as reliable an index of immunity as did accelerated rejection of skin allografts (Kahan, 1965). However, when dealing with graft survivals exceeding 15 days, i.e., weaker histocompatibility loci, skin grafts were preferable to tumor transplants as an index of immunity. Neoplastic tissue is frequently able to override minor histocompatibility barriers (Gorer *et al.*, 1948; Snell *et al.*, 1946) and may also demonstrate antigenic simplification after repeated transplantation (Little and Gorer, 1943).

Although some investigators have relied upon gross observation of graft survival (Billingham *et al.*, 1956a; Manson *et al.*, 1963; A. P. Monaco *et al.*, 1965) as an appropriate measure of immunity, histological scoring is generally felt to be a preferable method (Billingham *et al.*, 1958; Brent *et al.*, 1962a; Kahan, 1965; Al-Askari *et al.*, 1966). The survival end point of skin allografts can only be determined with acceptable accuracy by naked-eye inspection if the latent period before tissue breakdown lasts at least 10 days, so that the graft can cast off its cuticle and original pelt of hair and expose its epithelial surface to direct inspection. In the case of rapid and violent rejections across strong histocompatibility differences, the prerequisites of naked-eye inspection are not fulfilled, and histological examination of the epithelial survival is essential (Billingham and Medawar, 1951).

Several methods of histologically assessing and classifying graft survival have been described. Billingham *et al.* (1954a) provided an accurate method to compute survival times based upon histological analysis of serial sections of the challenge graft to determine the overall epithelial survival. By application and statistical methods, they obtained a mean survival time (MST) which was analogous to the LD_{50} method of drug toxicity studies.

The histopathology of the allograft reaction is not well understood. Histological examination of first-set grafts reveals that the immunological attack against them begins asynchronously throughout the transplant. Inflammatory changes and infiltration of host leukocytes are maximal at about 6 days. At 1 to 3 days later, there is the beginning of overt tissue breakdown and, after an additional 3–4 days, the process is completed, showing dilatory progress of the phenomenon after the reaction has been initiated (Billingham *et al.*, 1954b).

A point of interest and a source of considerable confusion was the observation that the histopathology of second-set reactions could differ quite remarkably. Billingham *et al.* (1954b) showed that the homograft on a normal, nonimmune mouse had an inflammatory and infiltrative reaction, i.e., an internal phenomenon, whereas the homograft reaction on an immunized mouse occurred prior to revascularization at the graft–host interface. Animals who had not been rendered immune by administrative of a putative antigen, underwent transient proliferation of the graft epithelial layer without evidence of cell death at the sixth postgraft day. In contradistinction, other investigators described the histological pattern of second-set reactions as an acceleration of the processes of firstset rejection, viz., infiltration of the graft with lymphocytes and temporary hyperplasia of the graft (Eichwald and Lustgraaf, 1961; Andre *et al.*, 1962).

Indeed, these two conditions were demonstrated by Eichwald *et al.* (1966) to be polar extremes of a single continuum. They found that tail skin grafts applied 10 days following sensitization with either skin grafts or with 20×10^6 spleen cells showed one of three typical histological appearances: (*1*) white graft—a particularly intense reaction in which there is no vascularization of the skin graft; (2) red graft (hemorrhagic necrotic) revealing engorgement, hemorrhage, epithelial necrosis, and lack of dermal infiltration; and (3) blue (infiltrative-hyperplastic) grafts without much change in the epithelium but with a premature subdermal activity when examined at 6 days after grafting. Stetson (1959) initially felt that white graft immunity differed qualitatively from accelerated rejection responses in that the former was due to circulating antibody which could be transferred with serum, whereas the latter was an anamnestic response mediated by cell-bound antibody. However, some investigators (*1*) have failed to transfer white graft immunity with serum (Brent *et al.*, 1959; Eichwald *et al.*, 1966), (2) have been able to transfer white graft immunity with cells (Eichwald *et al.*, 1966), and (3) have noted the incidence of white grafts to be high even in donor–host combinations in which antibodies have not been demonstrated after grafting. Eichwald *et al.* (1966) demonstrated that from a genetic viewpoint, red and white grafts did not differ but reflected quantitative differences. Not only could H-2, but also non-H-2 antigenic differences yield white grafts, e.g., 5/8 DBA-2 grafts on Balb-c mice were white, and data on F_2 hybrids suggested that this effect was due to the cumulative action of multiple weak loci. Thus the incidence of white grafts depends upon the number and type of antigenic differences between donors and recipients. Blue grafts were seen in congenic lines differing at the H-1 and H-4 loci. Interestingly, the result across the Y male chromosomal antigen varied with the technique of sensitization: intraperitoneal injections of splenic cells produced blue grafts; subcutaneous injections with challenge at 8 to 15 days produced red grafts; and subcutaneous sensitization with longer intervals before challenge yielded blue grafts.

In addition to the histological method of assessment of graft survival, there are two other acceptable techniques. In the stereoscopic method the blood flow through the subdermal vessels is assessed in order to determine the time of circulatory standstill. This survival time is approximately 2 days less than the histological mean survival time (Taylor and Lehrfield, 1953a,b). The third method of assessment, also of an all-or-none character, is the survival test of Medawar (1944). The challenge graft which has resided on the sensitized animal is transferred back onto a member of the donor strain and observed for its survival in an

isogenic environment. A graft that has been completely destroyed can thus be distinguished from a graft with some surviving epithelium.

It is of no small consequence that appreciable variations occur in the responses of the recipient animals. Obviously, the stronger the antigenic stimulus, the less the degree of variation. Billingham *et al.* (1954b) assessed some of the factors affecting variability in the second-set assay system. Although residual heterozygosity within inbred lines is possible, it has been found to be minimal in strictly inbred lines evaluated at a single point in time. However, it is known that there can be differences between sublines and even secular drift in the antigenic constitution of a single subline examined over 10 to 20 generations (Gruneberg, 1954; Hildemann *et al.*, 1959). It appears that differences in graft dosage are minor effects (Medawar, 1945), except when specifically examined over an exceedingly wide range of graft sizes. There are differences in the physiological state of the graft, e.g., different stages in the hair activity cycle, and in the physiological state of the individual, e.g., as related to stress status since corticosteroids are known to affect survival (Sparrow, 1954). However, the primary source of variation is in the surgical technique affecting healing and vascular penetration of grafts.

d. Dose–Response Relationships. Billingham *et al.* (1957) found a rough correlation between the number of spleen cells administered and the severity of the second-set reaction. L. T. Mann *et al.* (1959) and Corson *et al.* (1967) found a direct relation between the histological extent of destruction, employing six distinguishable classes of epithelial survival, and the logarithm of the number of cells administered. They appeared to be able to measure a differential response over a twenty-fold range from 50,000 to 1×10^6 cells. These findings were confirmed in the rat by Steinmuller and Weiner (1963). On the other hand, employing gross inspection to determine the extent of epithelial survival, A. P. Monaco *et al.* (1965) found that large differences in the dosage of membranous antigenic fraction reflected only small differences in graft survival. Quantitative studies on sonicated murine antigens revealed linear relationships between histological classes and the logarithm of the cell dosage, facilitating assessment of the extent of purification, the kinetics of sensitization, and the reproducibility of the response to solubilized antigens (Kahan, 1965).

In the low dose range, the immunological response to sensitization appears to be graded in linear fashion. The administration of allogenic cells in divided doses rather than in single doses produces a distinct dose–response relation. Hildemann *et al.* (1959) administered 300 whole

cells at 4-day intervals, yielding a range of 3000 to 27,000 cells and inspected the grafts grossly for their degree of epithelial destruction. Over this range the median survival time decreased stepwise from 9.8 ± 0.5 days to 7.9 ± 0.8 days in a curvilinear fashion when plotted as the median survival time versus the logarithm of the cell dose. The authors postulated that the curvilinear response to multiple injections as opposed to the rectilinear response with single injections might reflect a difference in the nature of the immune response to cumulative injections. A level of maximal immunity was predicted to be at 50,000 intact cells (in divided doses) which is twenty-fold less than the 1×10^6 cells required in their system when a single dose of cells was used for sensitization.

e. Minimal Doses for Induction of Immunity. Because of the difficulties of execution and evaluation of transplantation assays, quantitative data are exceedingly difficult to accumulate and comparative data between antigenic fractions prepared by different methods are unavailable. Manson *et al.* (1963) found that 25-50 μg. of membranous microsomal lipoprotein prepared by differential centrifugation and flotation on 1.75 *M* sucrose, administered intraperitoneally 4 days prior to skin graft challenge caused just-significant skin breakdown. The macroscopic mean survival time was decreased from 9.2 to 7.2 days by inoculation of 100 μg. of antigen. The minimum dose of 50 μg. represented the sensitizing activity of 10^7 tumor cells, whereas the 25-μg. dose represented 5×10^6 spleen cells.

The water-soluble postmicrosomal 105,000*g* (or 198,000*g*) murine antigenic fraction was barely detectable at 48×10^6 cell equivalents, and 4 times this amount of antigen was required to obtain maximal sensitization (E. R. Owen *et al.*, 1968). In the guinea pig system, 1–3 μg. of acrylamide-purified antigenic component were sufficient to elicit maximal rejection in all recipients sensitized with the preparation (Kahan and Reisfeld, 1969a). This dose in equivalent to 70 pmoles per host or 0.28 pmoles per gram body weight which is about 0.001 of the minimum circulating antigen concentration necessary to induce tolerance in most systems (see below). The transplantation antigenic activity of materials solubilized by enzymatic methods is not yet available but, certainly, from the experience with sonicated antigens, there is no reason to assume *a priori* that these materials when solubilized from the cell surface have lost their capacity to sensitize if they are administered by the appropriate route and in the appropriate form. Although the inherent adjuvanticity of acrylamide may have contributed to the potency of the sonicated purified antigen, the active principal is known by physiochemical methods to exist in appreciable aggregated forms ranging in size to almost 20 times the monomer molecular weight. Thus, possibly antigen adjusted

to its polymer state could be used as a powerful immunogen, and antigen prepared in the monomer state would function as an effective tolerogen.

2. *Accelerated Rejection of Allografts—Lymphoid Tissue Transplants*

Deutsch (1899) showed that splenic grafts from guinea pigs actively immunized against *Bacillus typhosus* caused the transient appearance of agglutinins in low titer in the normal hosts into which they had been implanted. In the murine system, Mitchison (1957) was able to distinguish individuals preimmunized against donor-strain tissue from those individuals who were nonimmune by the rapid decline in adoptively acquired antibody production in the former group. This assay was used by Celada and Makinodan (1961) who transferred 24×10^6 spleen cells from donor mice primed with sheep red blood cells into heavily X-irradiated allogeneic mice primed with antigenic extracts. If the extract immunized the host, then the transferred cells were rejected, and no sheep hemagglutinins could be detected at the sixth day following transfer and with simultaneous stimulation of the recipient with sheep erythrocytes. Maximal stimulation of hosts occurred with a 4-day interval between intraperitoneal cell administration and challenge with transferred cells. In this assay system, 600 viable bone marrow cells was the 50% rejection dose (RD_{50}) or 4000 cells killed by irradiation or freezing and thawing, i.e., about a 100-fold difference. The RD_{50} of spleen cells was 10^5, i.e., about the same as the activity of spleen cells in eliciting accelerated rejection. After extensive work with a similar assay in rabbits, Harris *et al.* (1968) applied their techniques to the assay of detergent-extracted antigens.

A similar system is the transplantation chimera system of Simonsen and Jensen (1959). Transplantation of adult cells into immature hosts unable to resist them allows these cells to perpetuate an immune response against the alloantigens present in the recipient. On characteristic of this response is hepatosplenomegaly, which can be quantitated as the index of enlargement, viz., the spleen weight of organs from injected vs. control animals. The conditions of this assay system can be adjusted, e.g., by titration of the number of transferred cells, so that cells derived from a normal donor would not cause splenomegaly. Preimmunization could thus be measured as the splenic index. The opposite possibility, of measuring immunotolerance by demonstrating the inability of the transferred cells to respond to the host's transplantation antigen, has been attempted with crude alloantigens without significant success.

3. *Prolonged Survival of Allografts*

a. Enhancement. Flexner and Jobling (1907) described the promotion of a transplantable rat sarcoma following pretreatment with emulsified, heat-killed tumor cells—the phenomenon of immunological enhancement. Kaliss (1958) showed that humoral antibody mediated immunological enhancement: sera from animals which had been pretreated with certain antigenic preparations were capable of passively transferring a state of altered reactivity to normal recipients (Kaliss and Malomut, 1952). The humoral antibody appeared to function by a central action or immunocompetent cells rather than by a peripheral combination with antigen (Snell *et al.*, 1960; Uhr and Baumann, 1961).

The administration of antibody to normal hosts can produce two disparate effects on subsequent grafts—sensitization and enhancement. Which one of these two responses predominates depends on several factors. One factor is the nature of the immunizing stimulus. If the stimulus is feeble and fleeting as with killed cells, there is a greater chance of achieving enhancement than with normal tissue grafts (Kaliss, 1966). Second, Gorer and Kaliss (1959) suggested that the difference between immunity and enhancement depended upon the amount of transferred antibody: 0.5 ml. yielded resistance against a chemically induced sarcoma, whereas 0.1 ml yielded enhancement. Not only could the administration of antibody to hosts accelerate the rejection of normal tissue grafts (Stetson and Demopoulos, 1958; Siskind and Thomas, 1959; Chutna and Pokovna, 1961) but also enhancement could be detected in immune hosts once antibody synthesis had reached its height (Kaliss, 1952), reflecting the bimodal biphasic immune response of the host (Kaliss, 1966). The third factor is the timing of the graft challenge. The host immune response became less efficient as the expression of the delayed-type hypersensitivity component declined (Batchelor, 1963), in part due to the interference of excess humoral antibody with the cellular expression of delayed-type hypersensitivity (Voisin and Krinsky, 1961; Batchelor and Silverman, 1962). Conversely, delayed-type hypersensitivity seemed to predominate during periods of modest antibody production, e.g., following X-irradiation (Salvin and Smith, 1959) or after immunization with relatively small doses of antigen delivered as either antigen–antibody complexes (Uhr *et al.*, 1957) or into the isolated intradermal site.

In several instances the prolonged survival of normal tissue grafts has been ascribed to immunological enhancement. The prolonged survival of ovarian tissue described by Parkes (1958) was probably related to enhancement or to the nature of the endocrine tissue. Similarily, it

may be that some instances of prolonged survival of kidney allografts were due to the ability of this tissue to respond to enhancement. The prolonged survivals observed in the Syrian hamster by Hildemann and Walford (1960) may also have been related to enhancing phenomena.

Several investigators have succeeded in prolonging the survival of normal tissue grafts by antigen pretreatment. Billingham *et al.* (1956c) prolonged skin graft survival after pretreatment with lyophilized donor-specific tissue antigens. Nelson (1962) reported significant prolongation of guinea pig skin grafts following pretreatment with donor spleen cell homogenates. Heslop (1966) obtained prolonged survival after treatment with intact, alcohol- or heat-killed spleen cells.

Brent and Medawar (1959, 1961) showed that injection of 1.0 ml. of specific hyperimmune antibody administered on the day of or as long as 3 days after grafting lowered the sensitivity that would have otherwise resulted from the intraperitoneal injection of cell extracts. Although, when acting by itself, 1.0 ml. of hyperimmune serum had no more than a barely discernible power to prolong the survival of A-strain skin grafts, when the serum was combined with 2.5 mg. of semisoluble antigen survival was significantly prolonged. In elegant experiments, Voisin and Krinsky (1961) pretreated neonatal hosts with donor-antihost alloantiserum and decreased the incidence of runt disease following the transfer of immunologically competent cells.

The antigen responsible for graft enhancement are presumably in part related, if not totally identical, to those affecting graft rejection. Kandutsch and Stimpfling (1962) felt that enhancement is a more sensitive assay for antigenic activity than sensitization, since the former phenomenon depends upon humoral antibody production, which, in their hands, was easier to elicit than graft immunity.

An assay system dependent upon immunological enhancement was developed by Kandutsch and his colleagues. After administration of antigenic preparations distributed over three intraperitoneal injections, 4 days apart, with a 10-day interval after the final injection bits of tumor allografts were transplanted subcutaneously into the host. The substance possessed biological activity if the tumor grew progressively until the death of the host. In the experiments of Kandutsch and Reinert-Wenck (1957), 1.5 mg. of lyophilized Sarcoma I (A strain) administered to B10D2 mice resulted in 50% deaths. Their initial work in fractionating the antigenic preparation revealed the enhancing activity to be distributed among various membranous fractions. Kandutsch (1960) found that (*1*) extraction with sodium dodecyl sulfate inactivated the cells and eluate, (*2*) distilled water, 0.1 *M* potassium phosphate, freezing and thawing, and deoxycholate vielded negligible activity, and (*3*) Triton was most

efficient at releasing the activity from the membranes. About 5 mg. of the Triton-solubilized material sufficed to kill 50% of the animals, whereas the residue was essentially inactive. Truly water-soluble material was obtained by digestion of the Triton-soluble water-insoluble fraction with *Crotaleus adamanteus* snake venom (E/S = 1:40). The detergent-solubilized fractions possessed not only enhancing activity but also homograft-sensitizing potency, confirming the hypothesis that the antigenic determinants mediating these phenomena were closely related (Graff and Kandutsch, 1966).

Zimmerman *et al.* (1968) administered subcellular fractions to allogeneic hosts in an attempt to prolong canine renal allograft survival. These transplants had a mean survival time of 10 days. Following pretreatment with three intravenous injections of crude nuclear fraction over a period of 3 weeks (25 mg. antigen total), renal allograft survival was extended to 16 days. Hosts treated with more than 25 mg. showed accelerated rejection. Application of the sonic method of preparation of transplantation antigens yielded more active fractions. Administration of 1 to 5.7 mg./kg. of 105,000*g* cell sap supernate fraction failed to improve survivals after allografting. However, when 1 mg./kg. of methylprednisolone and 2 mg./kg. of azothiaprine were administered daily after transplantation into a host that had received 1–2 mg./kg. of antigen in six doses over a 2-week period, the survival time was dramatically prolonged to a mean of 144 days and a maximum survival of 369 days (Wilson *et al.*, 1969a,b). A significant but far less impressive prolongation was obtained by the combination of antigen and antilymphocytic serum. Wilson (1970) feels that these results are best interpreted as representing immunological enhancement, because (*1*) the enhancement is more obvious after a period of 6-week pretreatment which yields demonstrable cytotoxic antibody, (2) discontinuation of the immunosuppressive regimen results in prompt rejection suggesting that true tolerance was not established, (3) cell-bound complement-fixing antibodies can be detected in recipient splenic microsomes at the time of transplantation, suggesting that the host was in the process of an immune response (Wilson and Wasson, 1965), and (*4*) there is a statistical correlation between serum cytotoxic antibody titers in the recipient and the allograft survival time, and the antibody titer appears to be related to the antigen dosage and the time course of administration.

b. Tolerance. The hypothesis of Burnet and Fenner (1949) that permanent tolerance to allogeneic tissue could be established by inoculation of foreign cells into immature hosts was confirmed by R. D. Owen (1945) and by Billingham *et al.* (1956a). Immunological tolerance has been defined as a central failure of responsiveness in which immuno-

logically competent cells become unable to initiate synthesis of a restricted range of antibodies following exposure to antigen (Dresser and Mitchison, 1968). Dresser and Mitchison (1968) distinguish three types of antigens: nonimmunogenic, weakly immunogenic, and strongly immunogenic materials. Nonimmunogenic antigens paralyze but do not immunize unless administered in conjunction with adjuvant, e.g., ultracentrifugally purified γ-globulin (Dresser, 1961) and poly-D-amino acids (Janeway and Sela, 1967). The weakly immunogenic antigens immunize at a dosage greater than that required for paralysis and, thus, show two discrete zones of immunological paralysis (low and high dosage) separated by a factor of 10^4 (Mitchison, 1964). Although a weakly immunogenic system had been constructed by testing the response of mice to particular F_1 hybrid cells (D. R. Bainbridge and Gowland, 1966) or by employing donor–recipient combinations differing at minor histocompatibility loci (McKhann, 1962), the antigens measured in transplantation are usually of the strongly immunogenic type, i.e., they immunize at a dose below the tolerogenic level and paralyze at high dosages. The phenomenon of paralysis with strongly immunogenic antigens was discovered by Glenny and Hopkins (1924), redefined by Dixon and Maurer (1955), and reviewed by Smith (1961).

Gowland (1965) has reviewed the problem of induction of transplantation tolerance in adult animals. He has pointed out that the genetic relation between the donor and recipient is a very important factor in determining the facility of induction of tolerance. It is approximately 20 times more difficult to overcome a strong H-2 barrier than it is to overcome a relatively weak H-1, H-3 genetic difference. One might predict that the application of congenic lines differing at a relatively restricted number of strong specificities would simplify the induction of transplantation tolerance based upon the findings of J. Klein (1966), viz., when the spectrum of H-2 antigen representation was limited, graft survival time was prolonged. However, Hilgert (1967) has observed that, although such an H-2 system with prolonged first-set graft survival is barely distinguishable from a weak genetic system on the basis of the MST, it does show distinct immunological properties. Although the administration of 5×10^8 cells yielded permanent tolerance in some animals differing at weak genetic loci, pretreatment of animals differing at the H-2 locus with the same number of cells uniformly yielded immunity.

Tolerance has been readily established to the weak male Y-chromosomal antigen by a single injection of viable cells (Mariani *et al.*, 1959) or with a variety of tissue homogenates (Linder, 1961; Martinez *et al.*, 1963; Harvard *et al.*, 1964; Kelly *et al.*, 1964). Tolerance across the H-1, H-3 or the H-3 difference alone could be established by parenteral

administration of cells by Guttman and Aust (1961) and by Martinez *et al.* (1963) but not by Brent and Gowland (1962) and Gowland (1965). Once spleen cells were demonstrated to induce tolerance across the H-2 barrier, disrupted spleen cells were employed to establish tolerance across this genetic difference (Shapiro *et al.*, 1961).

Medawar (1963) has pointed out that subcellular antigenic preparations are more favorable for the induction of transplantation tolerance than intact cells since (*1*) cells tend to produce sensitization, (*2*) cells raise the spectre of runt disease, and (*3*) cells are capable of recolonization and competition with host cells. Medawar (1963) disrupted pooled lymphoid cells with water and macerator, then ultrasonicated the suspension at 20 kc./second. The DNA was precipitated with sodium chloride and a final 30,000 G. antigenic sediment was then partially solubilized by alkalinization to pH 7.5–7.6. Semisoluble antigenic material given by any route other than the intravenous yielded immunity. Approximately 2.5 mg. of intravenous antigen prolonged the life of donor-specific grafts. He further demonstrated that immunosuppressive agents had a synergistic effect with antigen in prolonging graft survival. The combination of 15 to 20 mg./kg. of Amethopterin, a folic acid antagonist, administered 20 hours after grafting combined with antigen pretreatment prolonged the graft survival to 23 days. Combination with X-irradiation prolonged graft survival to 30 days when antigen was given 24 hours after radiation with 400 rads. However, there appeared to be no synergistic effect when antigen administration was combined with trypan blue treatment.

Monaco *et al.* (1965) found that one spleen equivalent of homogenate administered intravenously on the day before and 2 days following allografting yielded prolonged survival. Also when one equivalent was administered every third day for 4 weeks, followed by allografting, there was prolonged survival. He found that repeated injections produced sensitization, no effect, or prolonged survival depending upon the number of injections and the route of administration. Halasz *et al.* (1964) found that pretreatment of the donor with subcutaneous injections of donor blood yielded modest prolongation of canine skin and kidney allografts. E. R. Owen (1968) felt that he had succeeded in desensitizing the host by daily pretreatment of rabbits with crude ultrasonicated donor liver preparations for 5 weeks prior to renal transplantation. Munster *et al.* (1967) found some prolongation of murine skin grafts across the H-2 incompatibility.

In general, in the above studies, one cannot be assured that the hosts were, indeed, paralyzed against transplantation antigens, rather than

having mounted an ineffective, a deviated, or an enhancing immune response. The immune deviation phenomenon of Asherson (1966) may be the key to obtaining an ineffective host response. White *et al.* (1963) and Wilkinson and White (1966) reported that antigen administered in incomplete Freund's adjuvant tended to yield a γ_1 antibody response, whereas antigen administered in complete Freund's adjuvant induced a mixed γ_1, γ_2, and delayed-type hypersensitivity response. Asherson (1966) found that preimmunization with alum-precipitated protein induces only γ_1 antibody production upon challenge with antigen, but immunization with antigen in complete Freund's adjuvant induces not only γ_1, but also γ_2 and delayed-type hypersensitivity responses.

The procurement of true transplantation tolerance will probably require the application of immunosuppressive measures, since the threshold for the induction of immunity with these materials is very low (see above). Based upon the work of Schwartz (1966), metabolic and mitotic inhibitors have been employed to procure prolonged survival. Floresheim (1967) noted that 20–30% of mice pretreated with donor cells and methylhydrazine were permanently tolerant to skin grafts. Seifert *et al.* (1966) found the 6-mercaptopurine and methylprednisolone synergized with antigen to prolong renal allografts in dogs. In the work of Lance and Medawar (1969) and A. Monaco and Franco (1969), antilymphocytic serum (Woodruff and Anderson, 1963) appeared to synergize with donor antigen pretreatment in prolonging allograft survival. Other immunological procedures yielding prolonged survival include thymectomy (Miller and Osboa, 1967), radiation (Taliaferro, 1957), chronic thoracic duct fistula (McGregor and Gowans, 1964), administration of γ_2-globulin (Kamrin, 1959; Mowbray and Hargrave, 1966), and nonspecific paralysis by antigenic competition (Adler, 1964; Liacopoulos and Perramant, 1966), and corticosteroids.

The ability of an extract to induce permanent survival is a critical test of its antigenic composition, since graft survival demands that all of the disparate antigenic determinants between donor and host are present in the extract. On the other hand, immunization can be obtained even if a relatively small number of antigenic determinants are present in the material. Thus the tolerance assay has not only an extremely significant clinical import but also a unique biological significance.

B. Delayed-Type Hypersensitivity Responses

Although the assay systems that employ tissue transplants have a unique significance in that they directly reflect the effect of isolated

antigens on foreign grafts, they are cumbersome and time-consuming. It requires at least 2 weeks to complete the administration of antigen, the skin grafting of the recipient, and the gross and histological inspection of the test grafts. Furthermore, these procedures are quite wasteful of material, since the immunogenic dose of antigen is usually several-fold greater than the amount needed to detect serological or delayed-type hypersensitivity (DTH) activity. In addition, there are probably additional requirements for immunogenicity as opposed to those necessary to demonstrate antigenicity in the DTH or serological techniques. It may be that during purification there are changes in the physical properties of the antigenic material such that the purified material would contain less of the more immunogenic material. Dresser believes that immunization actually results from the small amount of aggregated, denatured bovine γ-globulin in commercial preparations of bovine γ-globulin. Furthermore, during purification other contaminant substances which may function as adjuvants might be eliminated from the preparation, thus making the purified material less immunogenic than the cruder material. Although final statements in regard to the actual transplantation activity of extracts can only be made after careful grafting procedures, the DTH and serological techniques may be useful and conserving of antigen. The application of these techniques is dependent upon the assumption that these three systems are mediated by the same antigenic determinant or by distinct determinants which are closely allied in an extract. In crude mixtures, Brent *et al.* (1962a) found a correlation between the immunogenic activity and the inhibition of hemagglutination, and, in relatively homogeneous preparations of guinea pig transplantation antigen, there appeared to be a correlation between immunogenic activity and the ability of the material to elicit DTH responses (Kahan and Reisfeld, 1969a).

A considerable body of evidence supports the hypothesis that transplantation immunity is mediated by a DTH mechanism, i.e., by the effects of cell-bound or cell-dependent antibody. It would, thus seem theoretically preferable to employ assay systems that depend upon the immune mechanism which mediates transplantation rejection, rather than systems reflecting humoral antibody which has an uncertain role in the allograft reaction. However, the DTH methods are best applied in a species who can express cutaneous delayed-type hypersensitivity responses. The guinea pig has been extensively studied in these systems and is probably the best species for such studies. The mouse, on the other hand, appears to be markedly less capable of expressing cutaneous DTH, although Crowle and Hu (1967) have documented these responses to dextrans. Dekaris and Allegretti (1968) showed a direct reaction upon

challenge of presensitized hosts with whole donor cells but not with extracts. These investigators used Evans blue to show increased vascular permeability accompanying DTH reactions. Transfer reactions were noted when sensitized spleen cells were inoculated intradermally. The rabbit tends to develop an Arthus-type response to transplantation antigens, although this work has not been performed with soluble derivatives. Man represents an excellent species for the investigation of DTH responses; however, the necessity for repetitive testing in a genetically well-defined situation may not be feasible in human beings.

The guinea pig system was developed by Wright in 1906 to study the effects of inbreeding on vigor, fertility, and growth (Wright, 1922). Loeb (1945) demonstrated that subcutaneously implanted organ fragments survived longer among syngeneic donor–recipient pairs than among individuals who were more distantly related and, thus, contributed significantly to the development of our knowledge about the genetic basis of allograft rejection. Significant support for the development of inbred guinea pig lines came through Freund to Bauer, who evaluated histocompatibility between two inbred lines, strain 2 and strain 13, derived from the initial thirty-five pairs of guinea pigs used by Wright. Intrastrain grafts were accepted for the lifetime of the recipient; interstrain grafts were destroyed within 6 to 9 days. Second-set challenge grafts applied from 7 to 180 days after primary immunization were rejected violently, usually as white grafts (Bauer, 1958). Estimates of the minimum number of histocompatibility genes were derived from determination of the proportion of parent-strain grafts surviving in F_2 and back-cross animals and the proportion of F_2 animals retaining both parent-strain grafts. These results suggested that no less than six genes control the rejection of strain 2 grafts applied onto strain 13 hosts and that no less than four genes control the rejection of strain 13 grafts applied onto strain 2 hosts (Bauer, 1960). There did not appear to be a sex-linked histocompatibility factor.

There are numerous documented immunological differences between members of strain 2 and members of strain 13. Strain 13 animals are more susceptible to the development of systemic (Wright and Lewis, 1921) and of local (Chase, 1960) tuberculous lesions, to the elicitation of disseminated tuberculous granulomata (Chase, 1959), to the induction of experimental allergic encephalomyelitis (Stone, 1962), and to the expression of hemorrhagic phenomena to ovalbumen (Stone, 1962). On the other hand, strain 2 but not strain 13 animals form antibody after exposure to minimal amounts of antigen (Lewis and Loomis, 1928), to poly-L-lysine (Levine *et al.*, 1963), and to certain determinants of insulin (Arquilla and Finn, 1963). These differences undoubtedly reflect genetic

factors in the sensitivity of antibody induction, the type of immune response, and the reactivity of the sensitized cells.

Just as in mice, rabbits, chickens, and humans, the guinea pig develops humoral antibodies reactive with homologous leukocytes. Walford *et al.* (1962) demonstrated that strain 2 animals that had been grafted with strain 13 skin developed antibodies that reacted strongly against the lymphocytes and only weakly against the neutrophiles of the donor strain. Repeated skin grafting produced an anemnestic rise in antibody titer after the first 3 days. In their test system, leukocytes from the donor animal were preincubated with the serum of the animal who received the graft, and then the washed cells were exposed to radio-iodinated antiglobulin. The attached alloantibody on the target cells was detected by counting the radio-iodinated fixed antiglobulin.

In summary, the guinea pig appears to respond in a similar fashion to other species following challenge with allografts, namely, by the induction of cellular immunity and the production of humoral antibodies. Although the genetic systems determining the antigenic factors eliciting allograft immunity have not been isolated as congenic lines of animals, it is to be expected that this system would conform to those found in all other similar species investigated to date [mouse (Gorer, 1955), rabbit (Terasaki *et al.*, 1961a), rat (Bauer, 1960), chicken (Terasaki, 1959), and human] i.e., one strong and multiple weak histocompatibility loci coding for individual products.

1. The Direct Reaction

Brent *et al.* (1962a) demonstrated that allograft sensitivity could be expressed as an intradermal DTH reaction. Hosts presensitized with allogenic skin grafts were challenged with intradermal inoculation of cells derived from the original donor. The reactions began at 5 to 8 hours and were maximal at 24 to 36 hours—a pattern consistent with a delayed-type response. The reactivity of the animals was unchanged at 120 days after sensitization; however, at 1 year, the reactions could not be elicited, although it is known that allograft immunity is in force at this time since second-set grafts are destroyed in accelerated fashion.

Brent *et al.* (1962a) found that 1 million whole cells gave a perceptible immune response and that 10 million cells were required for a definite positive response. On the other hand, 3 mg. of a sediment of ultrasonically disrupted cells (1.0 mg. sediment/100 mg. wet tissue weight) gave a perceptible response, and 12.5 mg. gave a clearly positive response. The authors thus concluded that extracted antigen was far more potent than living cells in eliciting DTH skin responses. Histological examination of the skin reactions showed a mononuclear infiltrate suggestive of a DTH response.

The direct cutaneous hypersensitivity reaction has proved to be a simple, rapid, and sensitive method to discern fractions possessing alloantigenic activity in the course of the purification of the active principal in cell extracts prepared by sonication. Delayed-type hypersensitivity responses were elicited in guinea pigs by challenge of presensitized hosts with 10 μg. of Sephadex Fraction I or with 0.1 μg. of acrylamide-purified component 15 (the component that had induced accelerated rejection of allografts in dosages of 1 to 3 μg.). The cutaneous reactions had the temporal and histological characteristics of delayed-type responses. Furthermore, direct DTH reactions could be elicited by challenge of hosts that had been adoptively immunized by isogeneic lymphoid cells transfers from animals previously immunized with donor-strain skin grafts (Kahan, 1967; Kahan *et al.*, 1968a). Similar DTH responses have been elicited in the human being by challenge of presensitized hosts with peripheral lymphocytes (Martin *et al.*, 1957).

In contradistinction to the delayed-type cutaneous hypersensitivity phenomena are the Arthus reactions which can also be elicited against transplantation antigens. Chutna *et al.* (1961) found that rabbits immunized with whole epidermal cells emulsified in complete Freund's adjuvant developed immediate, Arthus-type reactions upon challenge with donor-type cells. A similar pattern of reactivity was noted in rabbits known to be producing alloantibody. Guinea pigs immunized with allogeneic spleen cells emulsified in complete Freund's adjuvant also developed Arthus-type reactions upon challenge with homologous soluble Sephadex Fraction I antigen. Since complete Freund's adjuvant is known to direct the immune response toward the production of cytotoxic antibody, the Arthus reactivity probably reflects the ability of the solubilized antigen to induce several types of immune response directed against cell-bound transplantation antigens.

Arthus reactions were elicited by challenge of human beings producing monospecific alloantisera with purified transplantation antigen solubilized by sonication (Kahan *et al.*, 1968b). These individuals displayed well-defined, deep erythematous reactions with a violaceous hue and central discoloration specifically upon challenge with antigen possessing the specificity against which they were producing alloantibody. This reaction was employed to detect the active component following discontinuous acrylamide gel electrophoresis. The gel was sliced, the cuts were eluted in pairs, and the eluates inoculated into a panel of donors some of whom had been preimmunized with donor spleen cells. Figure 6 reveals (*a*) that reactions were only elicited in preimmunized donors (BIA, SGH, CAP) and not in the nonimmunized individuals (ROC and BOT) and (*b*) that the antigenic activity was well localized on the gel at cuts 9–10 corresponding to R_f 0.80.

2. *Transfer Reactions*

Brent *et al.* (1962a) further demonstrated that the local passive transfer of lymphocytes into allogeneic skin produced a delayed-type immune response—the transfer reaction. They proposed that this reaction was due to the recognition by the immunocompetent cells of the foreign transplantation antigens either present in the skin at the site or in the host leukocytes drawn to the site of inoculation. This response has been employed to assess the antigenicity of cell extracts. Intradermal third-party local possive transfers (Metaxas and Metaxas-Buchler, 1955) of presensitized cells admixed with donor allogeneic antigen into the skin of a member of the recipient strain permit the sensitized immunocompetent cells to respond to their homologous allogeneic antigen in isogeneic, immunologically neutral soil. Although Brent *et al.* (1962b) obtained only feeble but definitely positive reactions with this method, sonicated Sephadex Fraction I yielded intense cutaneous reactions (Kahan *et al.*, 1968a). The admixture of 50 to 100 μg. of this antigen with 10 million cells produced an inflammatory reaction which progressively increased in severity over 36 hours. After several days of persistent inflammation, only an area of alopecia marked the site of the transfer. The alopecia reflected the necrosis of entrapped host isogeneic hair follicular elements, analogous to the cytotoxic effects of lymphocytes incubated with antigen upon neutral cellular elements, presumably mediated by release of a humoral factor (Ruddle and Waksman, 1968).

On the other hand, immune reactions could not be induced when normal lymphocytes were transferred with antigen into isogeneic skin. The failure of the normal lymphocyte transfer test in these circumstances was attributed either to an inherent requirement for cell-bound antigen to induce the normal lymphocyte transfer reaction (Brent and Medawar, 1966) or to an insufficient amount of antigen employed in the studies to induce a primary immune response.

Ramseier and Streihlein (1965) have shown that the dorsal skin of the irradiated hamster survives as an immunologically inert but extremely sensitive milieu for the interaction of immunocompetent cells with antigen. In this local third-party xenogeneic transfer system, 2×10^7 lymph node cells obtained from individuals presensitized against allogeneic tissue were admixed with 75 μg. of the corresponding donor antigen (Kahan *et al.*, 1968a). The cutaneous sites were then scored for the appearance of hard nodules. Nodules were only detected at sites containing sensitized cells and the homologous allogeneic antigen. The sites that contained soluble antigen and sensitized cells reacted in a manner similar to those in which normal donor cells were deposited as

antigen to react with sensitized cells. These tests developed a strong reaction at 24 hours which rapidly declined until 96 hours. On the other hand, admixtures of two normal nonsensitized cell populations developed positive, but weaker, reactions which persisted over a 96-hour period (Kahan *et al.*, 1968a).

Although the transfer reactions were able to distinguish specific alloantigenic activity in putative extracts, they have not proven to be flexible or sensitive methods for the routine screening of antigenic fractions.

3. *The In Vitro Blastic Transformation System*

Morphological transformation of lymphoid cells into blastic elements which are capable of DNA synthesis and mitosis occurs following exposure to phytohemagglutinin (Nowell, 1960), to staphylococcal filtrate (Ling and Husband, 1964), to streptolysin S (Hirschhorn *et al.*, 1964), to rabbit antihuman leukocyte antiserum (Grasbeck *et al.*, 1964), to allogeneic cells (Bain *et al.*, 1964), or to subcellular antigens against which the cultured cells have been specifically presensitized (Mills, 1966). The transformation of lymphocytes obtained from two different donors admixed in culture—the mixed lymphocyte culture reaction—presumably reflects the recognition of foreign transplantation antigens. Some investigators believe that the strength of the mixed culture reaction is directly correlated with the genetic disparity between two cell donors (Bain and Lowenstein, 1964; Moynihan *et al.*, 1965; Bach and Amos, 1967), and a mathematical model has been constructed to quantitate the relatedness coefficient between two cell donors based upon the degree of blastic transformation in mixed culture (Alling and Kahan, 1969). Other investigators have not found a correlation between the mixed cell reaction and histocompatibility (Elves and Israels, 1965; Eijsvoogel *et al.*, 1967; Festenstein, 1966). Festenstein (1966) has proposed that this lack of correlation is due to the interaction of two opposite processes related to histocompatibility, namely, primary immune recognition and allogeneic inhibition.

The stimulation of blastic transformation of sensitized cells by exposure to their homologous sensitizing antigen has been observed in the rabbit, guinea pig, and human (Lycette and Pearmain, 1963; Mills, 1966; Oppenheim *et al.*, 1967; Benezra *et al.*, 1967), and this immune responsiveness has been demonstrated to depend upon a DTH mechanism (Silverstein and Gell, 1962; Mills, 1966; Oppenheim *et al.*, 1967). One would, therefore, predict that it should be possible to isolate the events of induction and expression of allograft immunity *in vitro* by exposure of immunocompetent cells to subcellular transplantation antigen.

The first step in this direction was taken in the guinea pig system. Strain 13, guinea pig, peripheral lymphocytes presensitized against strain 2 skin grafts were admixed with strain 2 water-soluble antigen prepared by sonication and gel filtration. At the third day of incubation, tritiated thymidine pulsing revealed more incorporation into the DNA of presensitized cells preincubated with allogeneic antigen than into the DNA of cells incubated with isogeneic antigen. The marked reactivity of animals presensitized with tissue grafts to this soluble material reenforced the observations obtained with the direct reaction, i.e., during the course of induction of allograft immunity against transplants, hosts are specifically sensitized against this antigen. There was greater blastogenesis evident after the third day of incubation than at the seventh day, which is the peak response for mixed cultures of normal lymphocytes. Primary responses of normal, guinea pig, peripheral lymphocytes to allogeneic antigen occurred less regularly (Kahan *et al.*, 1968a).

In subsequent work, Viza *et al.* (1968) added HL-A antigenic fractions prepared by autolytic solubilization to cultures of nonimmune allogeneic human peripheral lymphocytes. They measured blast transformation only at the fifth day, solely employing morphological criteria of blastogenesis. The authors claimed up to 20% greater degree of blastogenesis when 200–300 μg. of antigen, containing the HL-A 1, 2, 8 specificities, were incubated with cells lacking these specificities than when the antigen was incubated with the donor's peripheral lymphocytes. There were highly variable, entirely unexplained patterns of stimulation, depending upon the donor of the cultured lymphocytes and independent of the results of excellent tissue typing. In addition, there was no evidence that the cells which had been exposed to antigen had either achieved an immune state or that they had become tolerant after being exposed to high dosages of antigen, as had been hypothesized by the authors.

In elegant work, Manson and Simmons (1969) cultured lymph node cells in the presence of allogeneic, microsomal, membranous lipoprotein transplantation antigen. There was a 200% increase in blastogenesis, as determined by thymidine pulsing, at the third day. However, because of the lack of reproducibility of the primary response with the thymidine system, the authors employed an assay that directly determined the immune capabilities of the exposed lymphocytes. Allogeneic normal lymphocytes of the C57BL strain which had been "immunized" by exposure to allogeneic DBA/2 microsomal antigen *in vitro,* killed allogeneic donor-type DBA/2 spleen cells which were plaque formers in a sheep erythrocyte hemolytic system.

In summary, the delayed-type hypersensitivity systems offer unique opportunities for the study of the induction and expression of allograft

immunity. Although they have had limited application because of the facility of the serological techniques, they do provide vital information for our understanding of the physiology of the transplantation antigens.

C. Serological Techniques

1. Hemagglutination

Although transplantation immunity appears to be dependent upon a cellular effector mechanism, humoral antibody is produced in a number of species following allografting. Initially, Hildemann and Medawar (1959) found that crude membranous or nuclear antigenic matter which readily sensitized allogeneic animals to reject donortype skin grafts did not specifically absorb hemagglutinins directed against the antigen donor's cells. Brent *et al.* (1961) later succeeded in developing a serological assay for transplantation antigens based upon the absorption of hemagglutinating activity as detected in the assay of Gorer and Mikulska (1954). Prior to the development of the Gorer–Mikulska assay method, strong agglutination of erythrocytes suspended in saline was only rarely observed, and the sera showed rather unpredictable behavior upon freezing, presumably related to their transformation to incomplete antibodies (Gorer, 1947). Gorer and Mikulska (1954) found that certain agents that increased the erythrocyte sedimentation rate, namely, sera from patients with myelomatosis, ovarian cyst pseudomucin, and dextran, were effective in developing hemagglutinating reactions. Thus, dextran and normal human serum were used to detect the incomplete antibodies of the H-2 system which dominate hemagglutination reactions. Alloantisera were diluted in 1% dextran, and a 1% erythrocyte suspension was prepared in a 1:2 dilution of inactivated normal human serum. The agglutination was read microscopically after incubation at 37°C. for 90 minutes.

Crude A-line subcellular antigen prepared by ultrasonication inhibited the hemagglutinating and cytotoxic activities of CBA anti-A alloantisera (Brent *et al.*, 1961). The alloantisera were exposed to the antigen for 20 to 25 minutes at 25°C., the insoluble material was removed by ultracentrifugation, and the activity of the absorbed alloantiserum was tested against apropriate target cells. Because of the strong anticomplementary activity of the antigenic preparations, Brent *et al.* (1961) felt that the agglutination system was preferable to the lytic system. Antigen extracted from 15 mg. wet weight of tissue perceptibly decreased the hemagglutination titer, but antigen from 60 mg. was required to abolish all activity. On the other hand, 500–700 mg. of recipient strain tissue, e.g., CBA line tissue, was required to absorb the alloantisera, demonstrating a tenfold specificity ratio. Comparisons of the absorptive power of various fractions

with their ability to induce accelerated rejection suggested that it was possible to correlate these activities. Furthermore, Brent *et al.* (1961) showed that six injections of material derived from 250 mg. wet weight of original donor tissue provoked the production of hemagglutinins at a titer of 1:1000 to 1:4000. The findings of Brent *et al.* were confirmed by Davies and Hutchison (1961).

Hemagglutination methods have been applied by Palm and Manson (1966). After three 5-μg. injections of microsomal membrane lipoprotein, hemagglutinins were detected in the host. Furthermore, mice presensitized with 50×10^6 whole spleen cells responded to 1 μg. of crude antigen with the production of specific antibody. These investigators found that 250 μg./ml. of antigen inhibited homologous alloantibody, whereas 2 mg./ml. were required for a nonspecific serum.

In a similar fashion, Graff and Kandutsch (1966) raised hemagglutinating antibody by immunization with detergent-solubilized lipoprotein.

2. *Leukoagglutination*

Because the alloantigenic specificities are not well expressed on the surface of erythrocytes, methods were devised to employ leukocytes as the target cells. In the initial work of Amos (1953), isoantisera, prepared by inoculation of hosts with allogeneic tumor cells on two occasions 30 days apart, were incubated for 1.5 hours with allogeneic leukocytes derived from peritoneal exudates. There was good agreement between the leukoagglutination data and the results of Gorer and Mikulska (1954) employing hemagglutination.

The recent advances in this technique by Amos and Peacocke (1963-1964) have been incorporated into antigen assays by a number of workers (see below).

3. *Mixed Agglutination*

Abeyounis *et al.* (1964) treated strain 929L C3H mouse fibroblasts with alloantibody and then demonstrated their cell surface histocompatibility antigens with a developing reagent of Group O human erythrocytes precoated with mouse antihuman serum at subagglutinating concentrations in the fashion described by Faegraeus and Epsmark (1961). Adsorption of the marker erythrocytes to the target cells identified those cells that had complexed alloantibody. Metzgar *et al.* (1968) employed this method as well as the agglutination technique of Amos and Peacocke (1963-1964) to assay the 4^a and 4^b HL-A antigenic determinants present in detergent-solubilized preparations. In accord with the phenotype of the donor, they were able to demonstrate specific inhibition at an agglutinating titer 4 times greater than the minimum concentration required for the detection of antigenic activity (Metzgar *et al.*, 1968).

D. Cytotoxic Antibody

It is well known that following skin grafting or the intradermal injection of leukocytes, there is the formation of cytotoxic antibody (Phelps, 1937). The method of Gorer and O'Gorman (1956) described for the assay of mouse cytotoxins was applied by Amos *et al.* (1963b) to compare the potency of several antigenic preparations. They found that the antigens inhibited both agglutinating and cytotoxic antibodies but that they tended to exert a nonspecific interference with agglutination reactions. Although studies on sera with allospecific agglutinating and cytotoxic activities (Engelfriet, 1966; Walford *et al.*, 1965a,b; J. Bodmer *et al.*, 1966; Zmijewski and Amos, 1966; Payne *et al.*, 1967) suggested that both activities could be found directed against the same antigenic specificity, leukoagglutinins occurred in only a small number of cases following skin grafting (Colombani *et al.*, 1964), compared to the frequent incidence of cytotoxins. Furthermore, cytotoxic methods tend to have greater sensitivity in the antigen assay and to have less nonspecific interference than do agglutination techniques and are, therefore, more generally applied to this problem.

Antibodies mediating cytotoxic effects have been studied by changes in morphology (Kalfayan and Kidd, 1953; Shrek and Preston, 1955; Ellem, 1957; Goldberg and Green, 1959; Reif and Norris, 1960), motility (Nossal and Lederberg, 1958; Terasaki *et al.*, 1960), permeability (Green *et al.*, 1959; Green and Goldberg, 1960; Terasaki *et al.*, 1961a,b), metabolic processes (Flax, 1956; Landschuetz, 1956; Bickis *et al.*, 1959), electric potential across the skin (Merrill and Hanan, 1962), elicitation of muscular contraction (Feigen *et al.*, 1961), and interference with cell division and virus multiplication (Roizman and Roane, 1961).

One of the most widely applied methods is the use of supravital dyes, including the exclusion of negrosin (Kaltenbach *et al.*, 1958) or eosin (Hanks and Wallace, 1958) by viable cells. An automated system (Melamed *et al.*, 1969) employs a spectrophotometer measuring the absorption of trypan blue by dead cells at 5900 Å. A decrease in the total light transmitted through the system is correlated with the extent of cell death; conversely, living cells scatter light and cause increased transmission. There are systematic errors related to the presence of erythrocytes which are not affected by these cytotoxic antibodies but yet scatter light, and to certain cell types, e.g., thymocytes, which are small and exhibit peculiar optical properties. Amos (1969) has employed trypan blue in a micromethod to detect cytotoxic antibody. In principle this would afford a more efficient, flexible system than previous assay techniques depending upon trypan blue.

Rotman and Papermaster (1966) have demonstrated that fluorescein

diacetate can be hydrolyzed by viable cells to yield the fluorescent dye and serve as a method of supravital staining: only cells with intact membranes retain the fluorescent product of hydrolysis; lysed or injured cells lose the fluorescent material. This method has been employed for lymphocyte typing by Celada and Rotman (1967), by Tosi *et al.* (1968), and by W. Bodmer *et al.* (1968). The method of Tosi *et al.* (1968) represented a valuable modification for it required a minimal amount of antiserum and because it could be performed as a two-stage reaction, i.e., diffusion of the serum into an agar layer containing a uniform distribution of the target cells and washing of the surface of the slide to eliminate all unfixed material, followed by final addition of complement. This separation of the stages of antibody–cell interaction from the final mediation with complement overcomes the problem of the anticomplementary action of some of the materials present in isoantisera which might be, in part, responsible for the prozone phenomena observed with some sera and of some of the antigenic preparations. Two-stage reactions cannot be performed in the more sensitive assay of W. Bodmer *et al.* (1968) wherein 3000–5000 cells are used in microdroplets. However, it is possible in this system to perform a microabsorption test in which flourescein-diacetate labeled cells are used to detect free alloantibodies in sera that had been previously absorbed in the same microdroplet with unlabeled cells.

Edidin and Church (1968) quantitated this system by measuring the fluorescence of 100,000 cells that had been extracted with water–acetone, 3:1 (0.01 *N* sodium hydroxide). They expressed the percentage of viable cells after treatment with the antiserum as the ratio of fluorescence of the treated cells over that of the cells incubated in normal mouse serum.

The serological studies employing fluorochromasia have the advantage that dead cells preexisting in a suspension prior to treatment with antiserum do not appear in the field and, thus, are not counted and that prozone phenomena, which are common with dye exclusion methods, are not observed. However, fluorochromasia requires pure lymphocyte preparations since granulocytes take up the fluorogenic substrate and erythrocytes can be reactive as an enzyme system to release the fluorescein and increase the background, thus presenting a special problem with spectrofluorometric determinations.

Application of the fluorogenic method to other problems by Lederberg *et al.* (1964) demonstrated that the β-galactopyranoside activity of individual bacterial cells and of ribosomes could be demonstrated using fluorescein–di-β-galactopyranoside, a nonfluorescent substrate, which upon cleavage of its glycosidic bonds yields either fluorescein-monogalactoside or fluorescein. This system was adapted by Sercarz and

Modabber (1968) who prepared splenic cell extracts following immunization to β-galactosidase and determined the content of antibody-producing cells by incubating the cell suspension with β-galactosidase. After thorough washing of the unbound enzyme and addition of the fluorogenic substrate, the fluorescent products were measured microfluorometrically. This method was believed to be able to detect 100–200 antibody molecules and to measure the origin of a response as early as 12 hours and its persistence at 6 months after immunization, times at which fluorescent antibody staining methods failed to reveal any antibody.

The interaction of antibody and complement on the cell surface produces functional 150 Å. holes through which even ribosomes can escape (Goldberg and Green, 1960). The denatured cell membrane components resulting from the antibody's action on the surface of the target cell are then susceptible to digestion by trypsin, whereas normal cell membranes are unaffected by this agent. Thus, Hirata (1963) and later Terasaki and Rich (1964) employed differential cell counting with a cell particle Coulter counter to detect the cytotoxic action of alloantisera on cells. A sigmoidal curve was obtained with increasing concentrations of antibody, permitting application of a 50% lysis coefficient with only a 3% error in the range of 24 to 78% lysis. Although the Coulter counter method achieved a high degree of reproducibility and objectivity, this technique had not yet been successfully employed in the inhibition assays with solubilized histocompatibility substances.

Cytotoxic action of alloantisera can also be detected by the loss of intracellular isotopic markers from labeled cells. The application of ^{14}C-thymidine (G. Klein and Perlmann, 1963) or ^{32}P (Ellem, 1957; Forbes, 1963) incorporated into DNA showed that the isotope did not leak from cells exposed to trypsin or deoxyribonuclease but could be released by heat-killing the cells or by exposure of antibody-damaged cells to trypsin. The radiochromium technique was developed by Goodman (1961) to demonstrate immune cytolysis of cells other than erythrocytes in the rabbit–antimouse system. However, the blank count of 25% at the 50% cytolysis point was excessively large. The method was modified by Sanderson (1964, 1965a,b). Fifty thousand lymphocyte target cells that had been intracellularly labeled with sodium dichromate-^{51}Cr were treated with alloantiserum and guinea pig complement. The cytotoxicity of the serum was then detected as the liberation of chromium in a nonsedimentable form—the radioactivity present in the supernate of the cells was counted and appeared to correlate with the cytotoxic activity of the alloantiserum. Wigzell (1965) found a linear relation between the uptake of radiochromium, suggesting that the uptake per cell did not vary with the cell concentration. He found that of the total isotope that

fixed to the cells following five washings, 75% could be released by the cytotoxic action of the alloantisera. He compared the isotopic and supravital techniques concluding that the sensitivity of the methods was similar and that the isotopic method might have a somewhat smaller standard deviation.

In the chromium assay as employed by Nathenson and Davies (1966), equal volumes of putative inhibitor are admixed with an antiserum–complement mixture and then 100,000 target cells are added. The antiserum is employed at 80% lytic death, as measured by chromium release, which is thus 80 × 75% (chromium fraction × cell death detected with chromium = actual cell death) or about 60% actual cell death. This level corresponds to less than zero cytotoxicity units, thus representing a zone in which antisera are most vulnerable to nonspecific effects. These authors judge their substance to inhibit an antiserum if it reduces the cell death by 30%, i.e., in practice by 18% actual cell death, which may be just outside the range of the standard deviation of their method.

In addition, chromium techniques, although offering apparent objectivity, have a lower sensitivity since a large number of cells are required in order to have a sufficient amount of radioactivity incorporated into the cells.

The development of the microcytotoxicity test of Terasaki and McClelland (1964) represented a real advance in the detection of cytotoxic alloantibody. Because this method requires only minute quantities of serum and lymphocytes, it gains greater than a tenfold increase in sensitivity over previous techniques. Since the sensitivity of a method is inversely proportional to the number of target cells employed (C. Jensen and Stetson, 1961; Boyse *et al.*, 1962; Wigzell, 1965), an appreciable increase in sensitivity was obtained by using only 500–3000 cells. Microdroplets containing 0.003–0.00001 ml. of antiserum with 0.003 ml. of rabbit complement, and 2000 target cells were incubated for 4 hours at room temperature. The cytotoxic reactions were terminated with 0.002 ml. of 37% formalin and the viability of the target cells assessed by morphological criteria under inverted phase-contrast microscopy. Although tests employing other techniques had detected cytotoxic alloantibodies in but 6–20% of women who had had multiple pregnancies (Van Rood *et al.*, 1959; Payne, 1962; K. G. Jensen, 1962), the Terasaki–McClelland microdroplet technique revealed that 49% of women having five or more pregnancies and 16% of women with one to four pregnancies contained alloantibodies.

In the authors' hands the inhibition of alloantibody in microdroplets has proved to be a sensitive, flexible technique for the detection of alloantigenic activity. In this assay, 0.001 ml. of alloantiserum diluted at

concentrations of 0, 2, or 4 cytotoxicity units is preincubated with 0.001 ml. of serial dilutions of antigen for 1 hour at room temperature. After this time, 3000 target peripheral lymphocytes and 0.003 ml. of rabbit complement are added and an additional 4-hour incubation performed at room temperature. The reactions are stopped with 0.001 ml. of 36% formalin and read under inverted phase-contrast microscopy for the number of dead cells. Control readings of alloantiserum preincubated with Hank's buffer alone yield 95% death. Sigmoidal curves of alloantibody inhibition are obtained with the addition of serial amounts of antigen in the preincubation mixture. The inhibitory pattern is specific—antigenic preparations impair only the activity of alloantisera directed against antigenic specificities present on the cells of the antigen donor. Thus, in Fig. 5, alloantisera Torino-11.03 and Torino-32.19 are inhibited by the addition of antigen possessing the corresponding HL-A2 and HL-A7 determinants, but alloantisera recognizing the HL-A1,3,5,8 antigenic specificities are unaffected by preincubation with antigen from the same donor, who lacks these determinants. The specificity ratio (Sanderson and Batchelor, 1968), i.e., the concentration of antigen required to inhibit a nonspecific serum vs. that required for the homologous serum, is about 300 for the illustrated alloantigen.

Employing antigens possessing various mosaics of the HL-A specificities to determine the potency and specificity of alloantisera, it has been possible to characterize three operationally monospecific sera, Torino-11.03 (anti-HL-A2), Chayra (anti-HL-A8), and Hutter (anti-HL-A7). In each case the antiserum is only inhibited by the antigenic preparation possessing the corresponding specificity (Figs. 7, 8, and 9).

The specificity ratio of the antibody is then defined as the concentra-

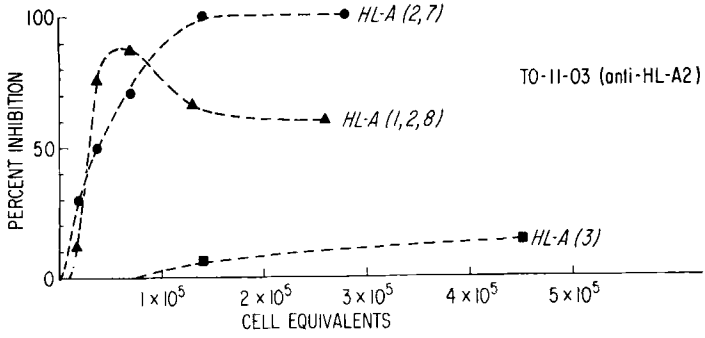

FIG. 7. Inhibition of cytotoxic reactions of Torino-11.03 (anti-HL-A2 typing antiserum employed at 2 cytotoxic units) by Sephadex Fractions I from three different cell lines: ●—● RPMI 1788 (HL-A2,7); ▲—▲RPMI 7249 (HL-A1,2,8); ■—■ RPMI 4098 (HL-A3).

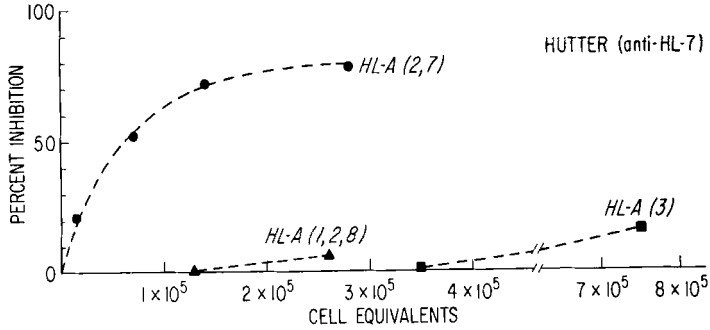

FIG. 8. Inhibition of cytotoxic reactions of Hutter (anti-HL-A7 typing antiserum employed at 2 cytotoxic units) by Sephadex Fractions I from three different cell lines: ●—● RPMI 1788 (HL-A2,7); ▲—▲ RPMI 7249 (HL-A1,2,8); ■—■ RPMI 4098 (HL-A3).

tion of antigen not possessing the homologous antigenic determinant which is required for 50% inhibition vs. the concentration of homologous antigen required for this degree of cytotoxic inhibition. Depending upon the alloantibody, these ratios vary from 2.5 to 50 and seem to be an excellent criterion of the specificity of the serum as a reagent for antigen detection and tissue typing.

It has recently been proposed that the criterion of specificity ratio of the antigen be employed to define International Units of Alloantigenic Activity and that the specificity ratio of the antibody be employed in standardizing the cytotoxic activity of these reagents (Reisfeld *et al.*, 1970b).

Other assay systems which have been employed to detect cytotoxic

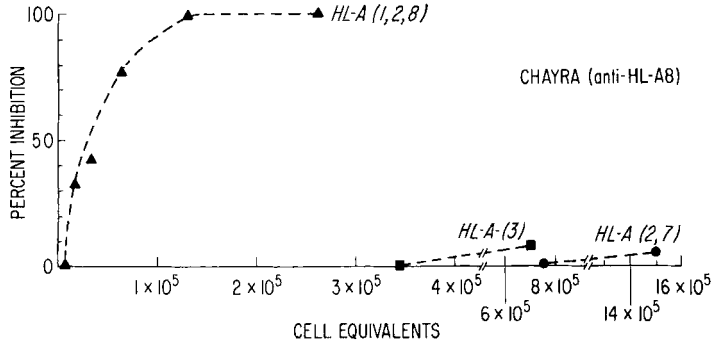

FIG. 9. Inhibition of cytotoxic reactions of Chayra (anti-HL-8 typing antiserum employed at 2 cytotoxic units) by Sephadex Fractions I from three different cell lines: ●—● RPMI 1788 (HL-A2,7); ▲—▲ RPMI 7249 (HL-A1,2,8); ■—■ RPMI 4098 (HL-A3).

antibody but not yet applied for the detection of putative transplantation antigens may be worthy of investigation. Holm and Perlmann (1967) and Perlmann *et al.* (1968) found that the addition of phytohemagglutinin or specific antigen to sensitized lymphocytes caused them to destroy target cells and that this cytotoxic effect could be measured with chromate released from the target cell erythrocyte. These authors further showed that the lymphocytes of Mantoux-positive individuals were cytotoxic for unrelated tissue culture cells following stimulation with purified protein derivative and that premixture of these lymphocytes with allogeneic human lymphocytes made them cytotoxic for tissue culture cells. The specificity of the activated lymphocyte for the target cell is not determined by histocompatibility differences alone, for there are some factors that control the reaction and dictate the type of target cell. This phenomenon might be related to the emission of macrophage-inhibiting factor from sensitized lymphocytes upon contact with antigen (Bloom and Bennett, 1966; David, 1966) or to the release of leukotactic factors from allogeneic lymphocytes in mixed cultures (Ramseier, 1967).

V. Perspectives

It has been well documented that genetic constitution plays a major role in determining the responsiveness of an individual to an antigen as demonstrated by experiments which indicate that the fate of tissue grafts depends upon the genetic relation between donor and host (Little and Johnson, 1922; Loeb, 1930; Snell, 1948). The large amount of experimental work done to investigate the genetic factors involved in the immune response, in particular the response of inbred mice and guinea pigs to synthetic polypeptide antigens, has been recently reviewed (McDevitt and Benacerraf, 1970).

The ability of these inbred animals to produce antibodies to synthetic polypeptides was shown to be controlled by an autosomal dominant gene (McDevitt and Sela, 1967). It is of interest that in mice it was demonstrated that this gene (Ir-1) is linked to one or the other allele of the H-2 locus which determines the pattern of mouse histocompatibility antigens. In fact, there is no convincing evidence thus far indicating that Ir-1 and H-2 are not identical. Furthermore, it was shown that antibodies to synthetic polypeptides produced by two inbred strains of mice differed in their activity to a cross-reacting antigen. This difference in specificity patterns was determined by a gene linked to the H-2 locus (McDevitt and Sela, 1967). Thus, in addition to their obvious role in allograft rejection and their postulated effects on cell contact and recognition, histocompatibility antigens may also be of primary importance in some of the most basic immunological phenomena.

In this regard, Jerne (1970) has recently proposed some provocative

hypotheses which link the generation of antibody diversity with the establishment of tolerance to "self" antigens. Jerne (1970) proposes that antibody v-genes present in the germ line of a species are structural genes for antibodies directed against all histocompatibility antigens of this same species and that such antibodies directed against self-antigens on cell surfaces may have an important function in embryonic life. Self-tolerance and antibody diversity are postulated to be achieved by the same process which Jerne believes to occur in the following manner.

An individual's v-genes code for antibodies against all the histocompatibility alleles of the species in early developing cells which, subsequently, express antibodies against histocompatibility antigens of the individual and are then stimulated by these antigens to proliferate. At some later stage, antibodies against self-antigens are no longer desirable and a mechanism evolves for suppressing these antibody-producing cells. This process of suppression permits the survival of mutant cells that express antibodies of a different specificity. If such antibodies retain enough affinity to self-antigens, the clone of mutant cells producing them will eventually be suppressed and double mutants may emerge. Thus, self-tolerance is established together with the generation of antibody diversity among clones of mutant cells. However, this antibody diversity is restricted by the individual's histocompatibility pattern since it determines the set of v-genes available for modification by mutation. The other set of v-genes codes for antibodies that are directed against those histocompatibility antigens of the species which the individual lacks. Cells expressing this set of v-genes are neither stimulated nor suppressed but are thought to be available for elimination of cancer cells and the rejection of transplants. Jernes hypothesis suggests that the set of v-genes which will undergo stimulation, suppression, and selection for mutants is determined by the pattern of histocompatibility antigens on the lymphocytes of an individual. Consequently, it may be possible that both the responsiveness to given types of antigen and the specificity range of the antibody produced are indirectly under the control of dominant histocompatibility genes.

VI. Summary

One of the key problems in transplantation immunology is to determine the function of histocompatibility antigens on cell membranes and, thereby, to elucidate the nature of the recognition phenomena which is experimentally challenged by allotransplantation. In man the importance and practical significance of achieving immunological tolerance to these alloantigens to attain prolonged allografts survival is only too obvious. Additional challenging research problems remain to be solved

such as the mode of attachment of histocompatibility antigens to cell membranes and the cellular origin and rate of their biosynthesis. The elucidation of these phenomena will certainly contribute to an understanding of the role that these antigens play in the molecular organization of the cell membrane.

Many of these intellectually challenging problems can be intelligently approached only after biologically and chemically well-characterized histocompatibility antigens become available in relatively large quantities. In this regard, the successful isolation of soluble alloantigens from large amounts of cells in continuous culture derived from normal human donors is, indeed, highly promising. Advances made in solubilizing, purifying, and characterizing these alloantigens are such that it is now feasible to obtain them in reasonable quantities. Electrophoretically homogeneous alloantigens with different specificities solubilized by physical and chemical means can now be fragmented by chemical and enzymatic methods to ascertain which portion of the molecule has antigenic specificity. Amino acid sequence analyses hopefully will give some insight into the chemical nature of alloantigenic determinants and contribute to an understanding of the relationship between the genetic control of allotypic specificities and the biosynthesis and structure of alloantigens.

Problems such as the elucidation of the role of histocompatibility antigens in basic phenomena such as the generation of self-tolerance and antibody diversity remain a challenge for future research.

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The Role of Bone Marrow in the Immune Response

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I. Introduction

The consideration of the cellular aspects of the immune response—namely the types of cells engaged in the mediation of the immune response both *in vivo* and *in vitro*, the cell(s) affected in the immune tolerant state, and the role of the thymus and the bursa of Fabricius (or its homolog)—has been the subject of a large number of reviews in the past few years (Chase, 1959; Smith, 1961; Hasek *et al.*, 1961; Stavitsky, 1961; Miller *et al.*, 1962; Cochrane and Dixon, 1962; Rowley, 1962; Nossal, 1962; Good and Papermaster, 1964; Makinodan and Albright, 1966; Metcalf, 1966; Uhr and Finkelstein, 1967; Dutton, 1967; Cooper *et al.*, 1967; Mackaness and Blanden, 1967; Leskowitz, 1967; Stone, 1967; Miller and Osoba, 1967; Wilson and Billingham, 1967; Uhr and Möller, 1968; Dresser and Mitchison, 1968; Sell and Asofsky, 1968; Hess, 1968; Fishman, 1969). It is, therefore, not our intention to simply abstract from these reviews and the references cited in the reviews as this would be redundant. Instead, it is our aim to attempt to integrate and interrelate the various, sometimes irreconcilable findings and present a unified scheme of cellular interactions and pathways resulting in the humoral immune response which is all-inclusive and amenable to experimental verification in the laboratory. This scheme is compatible with the experimental findings of the past few years. The role of bone marrow will be especially stressed as it has only recently gained prominence, in an immunological sense, and, therefore, its function vis-a-vis those of the established central lymphoid organs (thymus and bursa of Fabricius) and peripheral lymphoid organs (lymph nodes and spleen) must be analyzed and placed in perspective.

One of the objectives of this review is to present an alternative to the previously held view of a static *in vivo* system comprising a single pluripotential lymphoid cell (unitarian hypothesis; Sterzl and Silverstein, 1967; Siskind and Benacerraf, 1969; Makela *et al.*, 1969). Instead, we propose a dynamic system, the functions of which are served by a number of morphologically identical but functionally heterogeneous lymphoid cells. The probability that an immune response can be similarly expressed by multiple pathway systems, rather than by a single cell system, will be discussed. A better understanding of the immune processes and the cellular events and interactions that lead to the humoral and cell-mediated immune responses will greatly facilitate our comprehension of the etiology, pathogenesis, and management of the immune deficiency syndromes, autoimmune states, and related diseases, and will hasten the evolution of more rational and scientifically based approaches to prevent the graft-versus-host reaction and the graft rejection by the host.

The literature covered in this review extends to that published up to November, 1969.

A. Abbreviations

The abbreviations used in the text are as follows:

AFC —antibody-forming cell(s)
ARC —antigen-reactive cell(s)
ASU —antigen-sensitive units
BGG —bovine γ-globulin
BSA —bovine serum albumin
CFU —colony-forming unit
DNA —deoxyribonucleic acid
GARIG—goat antirabbit immunoglobulin
GVHR —graft-versus-host reaction
HGG —human γ-globulin
HRBC —horse red blood cells
HSA —human serum albumin
KLH —keyhole limpet hemocyanin
MLC —mixed leukocyte culture
PFC —plaque-forming cell(s)
PPD —purified protein derivative
RFC —rosette-forming cell(s)
RNA —ribonucleic acid
SRBC —sheep red blood cells

II. A Brief Survey of the Techniques Used for the Detection of Immunocompetent Cells

A. Transfer of Normal or Immune Lymphoid Cells to Normal or Immunoincompetent Recipients

The cell-transfer technique, wherein immune responsiveness is conferred to immunoincompetent irradiated or tolerant recipients by the administration of immunocompetent lymphoid cells, has been extensively used for the study of (*1*) the kinetics of the primary and secondary immune responses; (*2*) the identity and organ source of the immunocompetent cells; (*3*) cell migration pathways, and (*4*) the extent of participation of cells of the donor and recipient animals in antibody formation. These investigations have centered around the immune response of the recipients injected with either normal, immune, or *in vitro* antigen-incubated syngeneic or allogeneic lymphoid cells (T. N. Harris and Harris, 1957; T. N. Harris *et al.*, 1956, 1967; Sainte-Marie and Coons, 1964; Gray, 1962; Cochrane and Dixon, 1962; Makinodan and Albright, 1966; Strober and Mandel, 1969; Miller and Mitchell, 1969; Davies, 1969; Claman and Chaperon, 1969; Taylor, 1969). Both primary and secondary immune responsiveness have been successfully transferred with thoracic

duct cells, spleen cells, lymph node cells, thymus cells, and bone marrow cells, either individually or in combination. Recently, Davies *et al.* (1966, 1967) demonstrated an antigen-induced *in vivo* proliferative response of thymic antigen-reactive cells which was not accompanied by antibody formation but which was antigen-specific and, therefore, immunological in nature. It must, therefore, be appreciated that the use of antibody formation as the sole criterion of the immune response permits the detection of antibody-forming immunocompetent cells only and not of other immunocompetent cells that are incapable of antibody formation but which also participate in the immune response.

B. Transfer of Bone Marrow Cells to Immunoincompetent Recipients

The classic technique utilized to study the immunocompetence of bone marrow cells was devised by Till and McCulloch (1961). When syngeneic bone marrow cells were injected intravenously into irradiated mice, macroscopic nodules apparently derived from single cells appeared in the spleens of the recipients (Till *et al.*, 1964). These nodules consisted of clones, the majority of which were composed either of erythrocytes, granulocytes, or megakaryocytes. A small percentage of the clones were composed of mixed populations of cells (Mekori and Feldman, 1965). The clones retained the capacity to form further colonies if transferred to other irradiated recipients (Siminovitch *et al.*, 1963). The cloning capacity of chromosomally marked bone marrow cells was utilized to demonstrate the ability of bone marrow cells to populate the thymus and spleen (Till *et al.*, 1967) and to show that cells of both the hematopoietic and immune systems are derived from the same stem cell (Wu *et al.*, 1968b). A good correlation was observed between the number of dividing chromosomally marked cells in draining lymph nodes in response to the foot-pad injection of sheep erythrocytes and the number of plaque-forming cells detected *in vitro* (Wu *et al.*, 1968b). However, no direct chromosone analyses were made of the actual plaque-forming cells. Fractionation of normal bone marrow cells by equilibrium density gradient ultracentrifugation in BSA resulted in the recovery of a fraction of nucleated cells which contained up to a thirty-fold greater proportion of CFU as compared to an uncentrifuged control (Turner *et al.*, 1967). Bone marrow cells were studied extensively by various *in vitro* techniques for their myelopoietic potential (reviewed in Piscoitta and Brody, 1968) and for their ability to form colonies *in vitro* (Bradley and Siemienowicz, 1968; Imamura and Moore, 1968). Cells responsible for colony formation *in vitro* have, in fact, been found to be the same cells as those which form colonies in the spleen *in vivo* (Wu *et al.*, 1968a). The ability of the

stem cells to form CFU is dependent on the donor's immunological status, since the CFU were depressed by pretreatment of the donor mice with irradiation, endotoxin, and vinblastine (Fred and Smith, 1968). On the other hand, more CFU were formed if the donors were given spleen cells along with mouse leukemic sera (Metcalf and Foster, 1967; Foster *et al.*, 1968) or spleen cells plus cellfree extracts of embryonic tissue (Pulznik and Sachs, 1965).

C. Hemolytic Plaque Assay (Localized Hemolysis in Gel)

The Jerne plaque technique (localized hemolysis in gel) (Jerne and Nordin, 1963; Ingraham and Bussard, 1964) has improved our knowledge of the cellular events of the immune responses since a population of lymphoid cells can be screened for antibody producers. The original technique detected only those lymphoid cells that produced IgM antibodies capable of lysing erythrocytes in the presence of complement but not the IgG antibodies that could agglutinate or bind to erythrocytes but not lyse the cells in the presence of complement. Other investigators have shown that these latter antibodies will fix complement and form plaques if a proper antiglobulin developing serum is added (Dresser and Wortis, 1965; Sterzl and Říha, 1965).

The plaque technique has been used (*a*) to study the kinetics of the immune response (Hege and Cole, 1966) in relationship with the differentiation of antibody-forming cells (Kind and Campbell, 1968; Shearer *et al.*, 1968) and cell division during antibody formation (Szenberg and Cunningham, 1968; Claflin and Smithies, 1967), (*b*) as an indicator of transplantation immunity (Friedman, 1964) and the GVHR (Gengozian *et al.*, 1967), (*c*) to demonstrate the immune tolerant state *in vitro* (Hraba and Merchant, 1969; Friedman, 1965), and (*d*) for the study of cell–cell interactions required for antibody production initiated *in vitro* (primary immune response). The latter has been demonstrated by growing lymphoid cells on dialysis membranes (Marbrook, 1967) and on glass in petri dishes (Mishell and Dutton, 1967; Mosier, 1967).

D. Hemolytic Focus Assay

Kennedy *et al.* (1965a,b) and Playfair *et al.* (1965) have described a technique that can be used to enumerate the precursors of hemolytic PFC. The method is based on the observation that when normal mouse spleen or lymph node cells are transplanted together with sheep erythrocytes into heavily irradiated mice, clusters of hemolysin-producing cells arise in the spleens of the recipients. These clusters of cells may be detected by their ability to produce large foci of hemolysis in a layer of sheep erythrocytes immobilized in agar. These clusters are referred to as

antigen-sensitive cells or units and were shown to proliferate in response to antigen administration (Syeklocha *et al.*, 1966). A similar model was described by Armstrong and Diener (1969) in which the administered lymphoid cells formed colonies in agar that produced antibodies capable of immobilizing indicator bacteria.

The work of Playfair *et al.* (1965) has shown that PFC do not belong to a single clone of antibody-producing cells but are derived from multiple clones which are recruited at different times after immunization. This conclusion is derived from the demonstration that the development of PFC occurs in colonies of different sizes in anatomically different sites in the spleen. Celada and Wigzell (1966) have demonstrated that animals simultaneously injected with two antigens have antibody-producing cells against each antigen in discrete anatomical sites in the spleen, thus demonstrating that the immunocompetent clones which form are antigen-specific and are not mixed.

E. Other *in Vitro* Techniques

The various *in vitro* immunological reactions involving two cell types have been reviewed in depth recently by Coombs and Franks (1969). These reactions depend on lattic formation by the interacting cells both of which carry the same antigen resulting in mixed agglutination in the presence of the antibody. Examples of such reactions are mixed agglutination (Coombs *et al.*, 1956a), mixed antiglobulin reaction (Coombs *et al.*, 1956b), and mixed conglutination (Lachmann *et al.*, 1965). Another group of reactions is that in which sensitization of lymphoid cells with antibody and possible uptake of complement results in immune adherence (reviewed by Nelson, 1963) or phagocytosis. A third group of reactions consists of those in which sensitized lymphoid cells interact with antigens on a target cell, resulting in death of the cell. Receptors on actively sensitized lymphoid cells (lymphocytes obtained from animals immunized with foreign erythrocytes or lymphoid cells) have been shown to interact specifically with membrane antigens on the foreign erythrocytes resulting in rosette formation (Biozzi *et al.*, 1968), or with allogeneic tissue cells in monolayer cultures (Rosenau and Moon, 1961), respectively.

The correlation between the RFC and the PFC is not clear. Although Zaalberg *et al.* (1968) found that some RFC could give zones of lysis, Shearer *et al.* (1968) consider that the RFC, the direct PFC, and the indirect PFC each represents a distinct population of cells. Both RFC and PFC have been found to be lymphoid cells of different sizes (Storb and Weiser, 1967; P. F. Harris *et al.*, 1966).

The induced transformation of a lymphoid cell into a blast cell by

antigen *in vitro* has been shown to be immunologically specific (Naspitz and Richter, 1968; Richter and Naspitz, 1968a; Cowling and Quaglino, 1965; J. A. Mills, 1966; Vischer and Stastny, 1967; Benezera *et al.*, 1967, 1969) and is probably due to an interaction between the antigen with its specific receptors on the lymphoid cell (Sell, 1969). This is probably followed by the liberation of factors that may result in the further transformation of other "uncommitted" cells (recruitment) (Valentine and Lawrence, 1969; Dumonde *et al.*, 1969). In the MLC (Bain *et al.*, 1964), receptors on the leukocytes of one of the donors probably react with allogenic determinants on the leukocytes of the other donor resulting in mutually induced blastgenesis. A factor released by the cells in the MLC has been shown to be capable of inducing blastogenesis in cultures of cells obtained from only one of the original donors or from an unrelated donor (Kasakura and Lowenstein, 1965; Gordon and MacLean, 1965).

Cell suspensions, tissue slices, or organ fragments have been used in tissue culture systems designed to study the competence of lymphoid cells (reviewed in Dutton, 1967). Immunocompetence has been evaluated by the degree of antigen-stimulated DNA synthesis (Dutton and Eady, 1964), incorporation of isotopically labeled amino acids into immunoglobulin (Fahey *et al.*, 1966), and by other antibody-detection tests such as phage neutralization (Saunders and King, 1966).

All the above techniques demonstrate the presence of the precursors of and/or the actual antibody-forming cells. It is only through the use of specific markers that the identity of other cell types involved in the initiation of the immune response can be ascertained. The works of Nossal *et al.* (1968) utilizing a chromosome marker, of Miller and Mitchell (1968) using anti-H_2 sera in mice, of Lubaroff and Waksman (1968b) using fluoresceinated antisera in rats, and of Richter and Abdou (1969) utilizing antiallotype sera in rabbits have greatly clarified the source, identity, and function of the various cell types engaged in the immune response.

III. Bone Marrow As a Source of Immunocompetent Cells

A. Organ of Origin

1. The Lymphoid Cell

The source of lymphoid cells in the various lymphoid tissues has been clarified by the use of chromosomally marked bone marrow cells. These latter cells, but not labeled lymphoid cells of other organs, are capable

of populating the host's thymus and peripheral lymphoid organs for long periods when infused into syngeneic lethally irradiated mice (C. E. Ford *et al.*, 1956; C. E. Ford, 1966; Till *et al.*, 1967; Micklem *et al.*, 1966, 1968; Wu *et al.*, 1968a,b). The bone marrow of the recipients contains cells of recipient origin for the first few days but these cells then decrease in number rapidly and are replaced by donor bone marrow cells so that by days 10 to 20 almost all of the marrow cells are of donor origin. Contrary to the above, Tyan and Cole (1965) have shown in mice that, although bone marrow cells primarily populate the recipient's thymus and bone marrow, the peripheral lymphoid pool is not populated by lymphoid cells of bone marrow origin. The major criticism against these experimental protocols is the abnormal situation created by the rapid intravenous injection of a large number of hemopoietic stem cells into lymphomyeloid-depleted lethally irradiated recipients while ignoring the architectural and stromal changes which may have taken place as a result of the irradiation. The pressing requirements for granulocytes, platelets, and red cells in irradiated hosts undoubtedly influence the stem cells to differentiate along these lines rather than into lymphocytes (P. F. Harris *et al.*, 1966; Hellman and Grate, 1967).

Lymphocytes are widely distributed in the bone marrow and constitute about one-fourth of all marrow cells in rodents (Yoffey, 1966) and about one-tenth of marrow cells in man (Wintrobe, 1967). Experiments involving bone marrow cells labeled with ^{3}H-thymidine in rats (Schooley *et al.*, 1959), guinea pigs (Osmond and Everett, 1964), and dogs (Keiser *et al.*, 1964) have not provided conclusive results regarding their origin and fate. Transitional cells in the bone marrow are closely related to lymphocytes and by transfusion and shielding experiments it was shown that they are the probable source of stem cells (Cudkowicz *et al.*, 1964a; Moffatt *et al.*, 1967; Morrison and Toepfer, 1967).

Whether the bone marrow lymphocyte is the progeny of a bone marrow cell (myelogenous origin) or a circulating blood lymphocyte (hematogenous origin) or from both is still a controversial subject. Several recent studies have shown that the majority of marrow small lymphocytes are formed *in situ* (Osmond and Everett, 1964; Hudson and Yoffey, 1967; Everett and Caffrey, 1967). By injecting ^{3}H-thymidine into adult guinea pigs and rats and following the degree of labeling of the cells in the various tissues by means of radioautography, labeled dividing lymphoid cells were seen in the bone marrow prior to their appearance in the peripheral lymphoid tissues. A mechanically depleted segment of rabbit bone marrow has been found to be regenerated by cells resident in the marrow (Maloney and Patt, 1969). Surgical ablation of peripheral lymphoid tissues does not affect the kinetics or degree of labeling of the lymphoid cells in the bone marrow (Bierring, 1961;

Bierring and Grunnet, 1964a,b). By electron-microscopic studies, lymphocytes have been seen crossing the sinusoidal endothelium of guinea pig bone marrow (Hudson and Yoffey, 1967).

Bone marrow lymphocytes which are morphologically indistinguishable from lymphocytes elsewhere in the body are different in their developmental potentialities and function. Whereas peripheral lymphocytes are unable to protect lethally irradiated mice (Gesner and Gowans, 1962), bone marrow lymphocytes obtained by filtration of bone marrow through glass wool columns are capable of doing so (Cudkowicz *et al.*, 1964b; P. F. Harris and Kugler, 1963). Bone marrow lymphocytes are probably not end stage cells since they have been shown to undergo blastogenesis in response to stimulation with phytohemagglutinin (Bishun *et al.*, 1965; Singhal *et al.*, 1968a; Pegrum *et al.*, 1968) and various antigens (Singhal and Richter, 1968; Singhal *et al.*, 1968b) *in vitro*.

2. *Immunocompetent Cells*

It has been demonstrated (Tyan and Cole, 1963, 1964, 1965) that immunocompetent cells are derived from bone marrow or fetal liver cells during embryonic life. The source of these lymphoid precursor cells during adult life is not known. Cells obtained from a number of marrow-derived syngeneic hematopoietic spleen colonies, upon transfer to irradiated mice, can establish a large population of immunocompetent cells reactive to a variety of antigens. The lymphoid cells of the repopulated lymphoid organs in these mice are of donor origin as shown by the presence of the T6 chromosome marker of the donor bone marrow cells in 100% of the dividing cells in the spleen, mesenteric nodes, and bone marrow (Trentin *et al.*, 1967).

The relationship between the bone marrow cells that form colonies in the spleens in lethally irradiated recipients and the cells that constitute the immune cell system is not well understood. It has been shown that when chromosomally marked cell suspensions of various adult lymphoid tissues are injected into lethally irradiated mice, only those cells derived from the bone marrow are found in the thymus of the host in significant numbers. Although cells from other lymphoid organs readily proliferate in the peripheral lymphoid tissue, they are rarely found in the thymus (C. E. Ford and Micklem, 1963). These thymic cells, when transplanted into other irradiated syngeneic recipients, are capable of forming spleen colonies and of reconstituting immune competence (Doria and Agarossi, 1967, 1968). This latter property cannot be instituted with the transfer of normal thymus cells. These data, therefore, indicate that the hematopoietic colony-forming cells and the immunocompetent cells are derived from the bone marrow stem cells.

That the transplanted chromosomally marked bone marrow cell is

the cell responsible for antibody production was demonstrated by Wu *et al.* (1968a,b). They infused syngeneic chromosomally marked bone marrow cells into lethally irradiated recipients and observed that the chromosome marker was found in the cells of the hematopoietic colonies in the spleen, in the thymus, and in the popliteal lymph nodes. Following the foot-pad injection of SRBC, more than 50% of the dividing cells in the regional lymph node carried the donor chromosome marker and were capable of forming hemolytic plaques *in vitro*. On the other hand, chromosomally marked popliteal lymph node cells of mice not given SRBC did not divide and formed the average number of background hemolytic plaques.

Further support for the bone marrow origin of the antibody-forming cell is derived from the work of Miller and Mitchell (1968) (see below) and from *in vitro* experiments with human bone marrow. Human bone marrow cells cultured *in vitro* have been shown to secrete immunoglobulins and antibodies (Lombos *et al.*, 1963; Van Furth *et al.*, 1966a). Bone marrow plasma cells, but not bone marrow lymphocytes, showed positive fluorescent staining to immunoglobulins IgG, IgM, and IgA. Furthermore, bone marrow cells of patients with the cold hemagglutinin syndrome can synthesize cold hemagglutinins *in vitro* (Van Furth and Den Dulk, 1966). By combining *in vitro* culture studies and immunofluorescent staining of bone marrow cultures obtained from patients with multiple myeloma and Waldenstrom's macroglobulinemia, it was observed that the plasma cells of the bone marrow can synthesize monoclonal immunoglobulins with the same electrophoretic mobility and the same characteristics of heavy and light chains as the circulating immunoglobulin (Van Furth *et al.*, 1966b).

Bone marrow cells taken from hyperimmunized mice and rabbits have been known for a long time to be capable of secreting antibody *in vitro* (Ludke, 1912; Reiter, 1913; Schilf, 1926). Thorbecke and Keuning (1953) observed antibody production in culture fluids when bone marrow fragments from rabbits immunized to paratyphoid B vaccine were cultured in roller tubes. Several investigators have shown that the antibody response can be successfully obtained in the irradiated mouse if the latter is injected with bone marrow obtained from a hyperimmunized donor (Hobson *et al.*, 1959; Stoloff, 1960). Lesser amounts of antibody are formed if the genetic strain difference is increased between the donor and the recipient (Gengozian *et al.*, 1961; Doria *et al.*, 1962; Stoner and Bond, 1963).

In the adult rabbit, it has been shown that the bone marrow is the main source of the ARC, both *in vivo* (Abdou and Richter, 1969a; Richter *et al.*, 1970a) and *in vitro* (Singhal and Richter, 1968). In experi-

ments involving the restoration of immune competence of irradiated adult rabbits with cells obtained from a number of lyphoid organs, Richter *et al.* (1970a) observed that only bone marrow cell suspensions could restore immunocompetence to a variety of antigens. Rabbit lymphoid cells obtained from organs other than the bone marrow (thymus, blood, appendix, sacculus rotundus, lymph node, and spleen) failed to do so. Sacculus rotundus cells, mesenteric lymph node cells, and peripheral leukocytes were able to confer antibody formation in the irradiated recipients with respect to SRBC but not with respect to any of the other antigens. Evidence has been presented indicating that this response is probably due to the presence of potential antibody-forming cells in these organs as a result of prior sensitization with Forssman antigen or other antigens which cross-react with SRBC present in bacterial flora of the gut. The bone marrow of the rabbit, on the other hand, does not contain any antibody-forming cells (Richter and Abdou, 1969).

3. *The Macrophage*

The bone marrow has been shown to be the main source of precursors of macrophages in inflammatory reactions and in peritoneal exudates. Volkman and Gowans (1965), applying ^{3}H-thymidine labeling to the "skin-window" technique, established that the exudate macrophages in foci of sterile inflammation are derived from a rapidly dividing precursor present in the bone marrow. The failure to change the character of the exudate by previous thoracic duct drainage or whole-body X-irradiation accompanied by bone marrow shielding excluded the possibility that invading macrophages could have been derived to any appreciable extent from thoracic duct lymphocytes. Spector *et al.* (1965), using a combination of tritium and colloidal carbon labeling techniques to label dividing precursors of macrophages, identified the highly phagocytic bone marrow-derived circulating monocytes as the antecedent of the majority of peritoneal exudate macrophages. Further evidence in favor of the bone marrow origin of the macrophage stems from the work of Virolainin (1968). Radiation chimeras were injected with bone marrow cells carrying the T6-T6 marker and lymphoid cells from a genetically different donor carrying a different marker. Peritoneal macrophages as well as those present in bone marrow, spleen, lymph node, and thymus of the chimera carried the T6-T6 chromosome marker only. All of these data indicate that the bone marrow is the main source of macrophages and that lymphoid tissues outside the marrow do not contain precursor cells of macrophages (Virolainen, 1968; Volkman, 1966; Balner, 1963; Goodman, 1964). Furthermore, it has been demonstrated, using an *in vitro* culture system, that not only bone marrow cells but also

hematopoietic spleen colonies can give rise to macrophages (Virolainin and Defendi, 1968).

The origin of macrophages in the visceral organs is still controversial. Pulmonary alveolar macrophages (Bowden *et al.*, 1969; Virolainin, 1968; Pinkett *et al.*, 1966) and liver macrophages (Kupffer cells) (Boak *et al.*, 1968; Volkman and Gowans, 1965; Kinsky *et al.*, 1969) have both been shown to be at least partly derived from bone marrow precursors. In mouse chimeras in which the hematopoietic cell could be identified by a marker chromosome, it was found that approximately two-thirds of the dividing cells in the lung washings arose from the bone marrow precursor and one-third were derived from a pulmonary parenchymal cell (Pinkett *et al.*, 1966). Still to be resolved, however, is whether the macrophage precursor in the bone marrow is a hematogenous or a lymphoid cell. Recent findings by Howard *et al.* (1969), Vernon-Roberts (1969), and Boak *et al.* (1968) imply a lymphoid cell precursor of the macrophage. Although mouse spleen, lymph node, and thoracic duct lymphocytes could all apparently give rise to pulmonary alveolar and peritoneal macrophages in recipient F_1 hybrids (Howard *et al.*, 1969), it is possible that the responsible lymphocytes transferred could have been bone marrow-derived. Obviously, our knowledge of the functional heterogeneity, origin, and development of the macrophage system is too incomplete to warrant any further serious discussion.

B. Differentiation of Bone Marrow Cells

The identity of the bone marrow stem cell responsible for hematopoiesis is still unknown. Morphological studies of the developing lymphomyeloid tissues of chick embryos have suggested that stem cells may be blasts with heavily basophilic cytoplasm and a prominent nucleolus (Moore and Owen, 1967). Studies in rodents have shown that monocytoid cells (Barnes and Loutit, 1967a) and lymphocytes (Moffatt *et al.*, 1967) are the probable stem cells. Indirect quantitative and morphological evidence has shown that the transitional cell in the lymphoid bone marrow compartment is the hematopoietic stem cell (Moffatt *et al.*, 1967). During recovery from irradiation, it was noted that the bone marrow transitional cells decrease in number but not in DNA synthetic activity, suggesting that these cells are actively dividing and leaving the bone marrow at a rapid rate.

Stem cell differentiation has been shown to vary according to the degree of erythropoiesis and granulopoiesis required of the bone marrow. Suppression of erythropoiesis in guinea pig marrow results in an increased lymphocyte content of the bone marrow (Osmond, 1967). Stimulation of granulopoiesis, as occurs after the injection of vaccines (H. *pertussis*

or *Staphylococcus aureus*) results in an initial rise and then a gradual drop in the lymphocyte content of the bone marrow (Yoffey, 1955).

Whether the bone marrow stem cell is pluripotential or unipotential is still controversial. By transplanting labeled adult mouse bone marrow cells into lethally irradiated recipients, evidence has been obtained suggesting that the stem cell can differentiate into any one of a number of cell pathways, with resultant colonies composed of erythropoietic, myelopoietic, lymphopoietic, or plasmacytopoietic elements (Trentin *et al.*, 1969). Moreover, transplantation of each type of colony into a secondary irradiated host can give rise to any type of colony (Curry *et al.*, 1967), further attesting to the pluripotential nature of the bone marrow stem cell. Contrary to these findings, Bennett and Cudkowicz (1967, 1968) showed only a unipotential role for mouse bone marrow cells by showing that there exist separate progenitor cells for erythropoiesis and leukopoiesis. They found no evidence for the shunting of the transferred bone marrow lymphocytes from or into the production of nonerythroid cells if they enhanced or depressed erythropoiesis in recipient mice (Bennett and Cudkowicz, 1968). The differentiation of the bone marrow stem cell along any of the hematopoietic lines was shown to be dependent on the microenvironment in which it proliferates (Wolf and Trentin, 1968). In irradiated mice bearing hematopoietic transplants, the erythroid–granuloid (E:G) colony ratio in the spleen was about 3 whether the spleen was kept *in situ* or transplanted subcutaneously. The E:G colony ratio in the bone marrow stroma was less than 1 irrespective whether the marrow stroma was *in situ* or trocar-transplanted into the spleen. Dissected erythroid or granuloid spleen colonies produce all types of colonies upon transplantation, with the E:G colony ratio determined by whether the colonies develop into the spleen or in the bone marrow. This would indicate that differentiation of stem cells within the bone marrow is determined by the bone marrow microenvironment and not by the stem cell itself. The nature of the inducing factor is still unknown.

IV. Cells Involved in the Humoral Immune Response

In recent years, several cytokinetic models have been described to illustrate the cellular events in the immune response (Makinodan and Albright, 1966; Siskind and Benacerraf, 1969; Papermaster, 1967; Sercarz and Coons, 1963b). In general, these models postulate that when an immunologically uncommitted progenitor cell is stimulated with antigen, it gives rise to specific AFC which differentiate along an irreversible pathway and, at the same time, produce a new set of progenitor cells (memory cells) committed to the immunizing antigen. Upon second

contact with the same antigen, these memory cells give rise to AFC in the so-called secondary antibody response as well as to another generation of memory cells. This scheme probably represents an oversimplification of a very complex series of inter- and intracellular interactions. The recent work of a number of investigators (Miller and Mitchell, 1968; Davies *et al.*, 1966; Claman *et al.*, 1966; Raidt *et al.*, 1968; Moiser and Coppleson, 1968; Richter and Abdou, 1969) indicates that the cellular system responsible for the production of antibodies consists of at least two and probably three separate cell types. These are the antigen-processing cell or the macrophage, the ARC, and the AFC.

The various cells that participate in the immune response have been given different names by different investigators: "X-Y-Z" by Sercarz and Coons (1963b), "auxiliary" and "effector" cells by Claman *et al.* (1966), "PC_1" and "PC_2" by Makinodan and Albright (1966), "Antigen-reactive cell" (ARC) and "antibody-forming cell" (AFC) by Miller and Mitchell (1968), "antigen-sensitive unit" or "precursor of plaque-forming cell (P-PFC)" by Shearer and Cudkowicz (1969), "reactor cell" by Davies *et al.* (1966), and "antigen-sensitive cell" by Kennedy *et al.* (1965a,b).

Judging from the profusion of terms cited above, it is obvious that the terminology and the state of knowledge concerning the cells is in a state of confusion. Cells have acquired names irrespective of their actual function or the manner in which they manifest themselves in the immune reaction. Furthermore, the schemes cited above do not take into account the mechanism of antigen recognition by the immunocompetent cells—a stage which must necessarily precede that of overt antibody production—nor do they suggest which cell type might possess the property of antigen recognition (or, in fact, cognition). The following sections will, therefore, deal with each of the cell types individually in order to define better the specific functions and properties of these cells and to clarify the sequence of intercellular reactions in the immune response.

A. Antigen Recognition

Since the response to antigen is characterized by the extreme specificity of the reaction, it is likely that a subpopulation of lymphocytes reacts with the appropriate antigen because of the presence of membrane-associated recognition sites which are structurally complementary to the antigen (Siskind and Benacerraf, 1969; Mitchison, 1967; Cinader, 1968; Wigzell, 1969). These sites are considered to be "natural" cell-bound antibody molecules or the active fragments of the antibody molecules. The binding of antigen by the cell through its reaction with this anti-

body receptor probably initiates the sequence of cellular and intercellular events leading to antibody formation.

There is ample evidence in the literature indicating the immunoglobulin nature of these receptors on the surface of *normal* lymphoid cells:

1. Rabbit peripheral lymphocytes can be stimulated *in vitro* to transform into blasts by incubation with antiserum directed against allotypic and other Ig determinants (Sell and Gell, 1965). Transformation was shown to be strictly specific to identifiable allotypic determinants.

2. Chicken spleen lymphocytes were shown to have receptors with μ-chain specificity, as demonstrated by their capacity to undergo blastogenesis when incubated by rabbit anti-μ-chain antiserum, and not with anti-γ-chain antiserum (Skamene and Ivanyi, 1969).

3. Human thoracic duct and peripheral small lymphocytes show weak immunofluorescence for IgM (Van Furth, 1969) and can be stimulated to transform to blast cells when cultured *in vitro* with anti-IgM and anti-IgG sera (Van Furth, 1969; Oppenheim *et al.*, 1969). It has also been demonstrated that IgG-, IgM-, or IgA-sensitized human erythrocytes form rosettes with normal peripheral human lymphocytes upon the addition of the appropriate anti-immunoglobulin serum (anti-γ or anti-μ or anti-α) (Coombs *et al.*, 1969). Thus, about 10% of the peripheral lymphocytes were found to carry immunoglobulin receptors on their surface.

4. Bert *et al.* (1968) have demonstrated changes in the physical properties of normal peripheral lymphoid cells after exposure to species-specific anti-immunoglobulin serum. A marked reduction in the random migration of the cells *in vitro* with reduction of electrophoretic mobility (Bert *et al.*, 1969) was noted following incubation of human lymphoid cells with goat antihuman immunoglobulin serum.

5. Daguillard and Richter (1969) have demonstrated that normal rabbit lymphoid cells can be induced to transform into blast cells *in vitro* during incubation with goat antirabbit immunoglobulin serum.

6. Incubation of human circulating lymphocytes with submitogenic concentrations of rabbit antihuman light-chain antisera suppresses the mixed leukocyte reaction *in vito* (Greaves *et al.*, 1969).

The evidence suggesting that the recognition site on the normal lymphoid cell surface is, in fact, an antibody or an immunoglobulin fragment with antibodylike activity is based on the finding of Abdou and Richter (1969b) and Singhal and Wigzell (1969) who were able to separate *normal* rabbit bone marrow ARC committed to a specific antigen (i.e., HSA) by passing the cells through a column of glass beads sensitized with the particular antigen (HSA). The ARC retained by the

glass bead column could be eluted from the beads by vigorous shaking. These cells were found to be capable of undergoing specific blastogenesis and responding with increased DNA synthesis upon incubation with the antigen originally used to sensitize the glass beads, but not with any other antigen (Singhal and Wigzell, 1969). Furthermore, the eluted cells could transfer antibody-forming capacity only with respect to the antigen used to sensitize the glass beads and not with respect to other antigens (Abdou and Richter, 1969b). On the other hand, the bone marrow lymphocytes which passed through the antigen-sensitized glass bead column could transfer immunocompetence to all antigens tested but not to the antigen used to coat the glass beads (Abdou and Richter, 1969b). It was, therefore, concluded that normal rabbit bone marrow lymphoid cells (ARC) possess antibodylike recognition sites on their surface and that these sites are antigen-specific (Abdou and Richter, 1969b; Singhal and Wigzell, 1969).

Investigations conducted with *immune* lymphoid cells corroborate the findings with normal cells and strongly imply the presence of an antibody on the cell surface.

1. Stimulation of immune cells by hapten-carrier molecules can be inhibited by prior reaction of these cells with free hapten (Mitchison, 1967; Plotz, 1969; Segal *et al.*, 1969). Furthermore, such a hapten-immune cell complex will not stick to hapten-sensitized glass beads (Mitchison, 1967). Similar observations have been reported by Naor and Sulitzeanu (1969) using BSA as antigen.

2. Extracts of immune human tonsilar small lymphocytes have been shown to possess properties of immunoglobulins with immunological specificity for the antigens to which the donors had previously been immunized (Merler and Janeway, 1968).

3. It has been observed that immune rabbit lymphoid cells are incapable of transferring specific antibody-forming capacity if they are incubated with antirabbit immunoglobulin serum prior to their injection into the irradiated recipient along with the specific antigen. These cells preincubated with antiimmunoglobulin antiserum also fail to undergo blastogenesis in responses to stimulation with the specific antigen *in vitro* (Daguillard and Richter, 1970a). These findings strongly suggest that the antigen and the anti-immunoglobulin antibody compete for the same site on the immunocyte surface and that the site is an antibody.

4. Wigzell and Andersson (1969) were able to purify specifically directed mouse lymph node AFC by passing them through a glass bead column sensitized with the original immunizing antigen. All the cells capable of transferring immunocompetence with respect to this antigen were retained by the column, much in the same way as normal ARC are

retained by an antigen-sensitized glass bead column (Abdou and Richter, 1969b; Singhal and Wigzell, 1969).

5. Cruchaud and Frei (1967) demonstrated that circulating lymphocytes obtained from allergic individual form mixed clusters when incubated with SRBC sensitized with the allergen. Clusters were not formed when unsensitized SRBC were incubated with the immune lymphocytes.

6. It has been observed that the concentration of antigenic hapten required to induce optimal blastogenesis of immune guinea pig lymphocytes *in vitro* can be correlated with the affinity of the serum antibodies produced by these guinea pigs for the hapten (Paul *et al.*, 1967). Thus, the requirement of the cells for a high concentration of the hapten in order to be stimulated to undergo blastogenesis is directly related to the finding of antibodies of low affinity for the antigen in the circulation of the cell donor, and vice versa.

7. Biozzi *et al.* (1969) found that pretreatment of immune lymph node cells, obtained from guinea pigs immunized with SRBC, with antisera against guinea pig γ-globulin abolished their capacity to form rosettes in the presence of SRBC *in vitro*, implying that the antiglobulin antibody could successfully compete with the antigen for the same site on the lymphocyte surface.

8. McConnell *et al.* (1969) observed that mouse lymph node cells obtained from mice subsequent to immunization with SRBC could form rosettes when incubated with the red cells *in vitro*. However, rosette formation could be inhibited by treatment of the immune cells with anti-heavy-chain antisera. This finding strongly suggests that the specific receptor on the immune lymphocyte is, in fact, an antibody immunoglobulin molecule or the active segment of the antibody molecule.

However, it must be stated that, in spite of the evidence in favor of the presence of immunoglobulinlike and antibodylike receptors on the surface of the *immune* lymphoid cells, the actual demonstration of antibodies similar in composition to circulating antibodies, either on or eluted from the *normal* cell, has yet to be reported.

B. The Macrophage

The exact site of action of the macrophage in the induction of the immune response is still obscure. As will be seen below, two cell types, the ARC and AFC, have been definitely implicated as participants in antibody formation. The question to be answered is not only whether the macrophage plays an essential role but also where it participates along the sequence of reactions and whether it acts in a specific or a nonspecific manner. Is the immune function of the macrophage apparent to the in-

vestigator only because of the artificially contrived nature of the experimental protocols or does it have an immunological role under normal conditions *in vivo*. Furthermore, if it does have a specific role, does its reaction with the antigen precede or follow that of the ARC? Is it also a necessary participant with respect to all antigens as is the AFC?

The importance of the macrophage system in the processing of antigens, with subsequent release of more potent immunogenic entities and/or "nonantigenic information" is suggested by the work of Mitchison (1969) and Unanue and Askonas (1968). In comparing the immunogenicity of free and peritoneal exudate cell-bound forms of protein antigens, such as KLH, HSA, and BSA, it was shown that antigens taken up by macrophages, both *in vitro* and *in vivo*, are more potent than the native, nonprocessed forms in the *in vivo* induction of primary immunity in mice.

W. L. Ford *et al.* (1966) observed that rat macrophages which had ingested SRBC *in vivo* were capable of transferring specific information *in vitro* to normal rat thoracic duct lymphocytes since these latter cells, following their separation from the macrophages, could then be transferred to irradiated immunoincompetent recipients with resultant antibody formation directed toward SRBC. Argyris (1967) has also observed that macrophages which had ingested antigen (SRBC) *in vivo* could successfully induce specific antibody formation following their administration into normal syngeneic, but not lethally irradiated, recipients.

The wholly *in vitro* experiments tend to corroborate the above findings. Cells possessing the property of sticking to glass, presumably macrophages, are essential for the induction of the primary humoral immune response *in vitro* to sheep erythrocytes in the presence of other immunocompetent nonadherent lymphoid cells (Pierce and Benacerraf, 1969; Mosier, 1969). Cellfree extracts of macrophages incubated with antigen *in vitro* are capable of inducing the formation of antibodies in cultures of syngeneic normal lymphoid cells *in vitro*, some of which may, in fact, bear the allotype of the macrophage donor (Adler *et al.*, 1966). It was also shown that this reaction of antigen with the macrophage precedes that with the immunocompetent lymphocyte (Fishman, 1961, reviewed in Fishman, 1969). Many other investigations have shown that the RNA fraction(s) extracted from peritoneal exudate cells which had been incubated with the antigen *in vitro* is immunogenic *in vivo* and *in vitro* (Fishman and Adler, 1963; Askonas and Rhodes, 1965; Gottlieb *et al.*, 1967; Feldman and Gallily, 1967; Unanue and Askonas, 1968; Pinchuck *et al.*, 1968; Mosier and Cohen, 1968).

Bendinelli (1968) induced primary immune response to SRBC with

mouse peritoneal exudate cells and observed that the PFC were, in fact, lymphoid cells. This finding would appear to run counter to that of Holub and Houser (1969) who observed that more than 20% of the PFC in alveolar exudates from rabbit lungs, following the intrapulmonary and intratracheal administration of the antigen (SRBC), were histiocytes or monocytes by microscopic and ultrastructural criteria.

There is evidence in the literature that macrophages obtained from immune animals possess the capacity to initiate antibody formation when transferred to normal irradiated recipients. Immune mouse peritoneal macrophages were capable of transfering immunocompetence upon their injection into irradiated syngeneic recipients, with or without the administration of antigen, provided that the cell donors had been repeatedly immunized by the intraperitoneal route (Kornfeld and Weyzen, 1968). However, Argyris and Askonas (1968) have observed that within a population of immune peritoneal exudate cells, the cells not adhering to glass (lymphoid cells) were shown to be the cells responsible for the transfer of antibody-forming capacity, thus suggesting that Kornfeld and Weyzer (1968) were, in fact, transferring antibody-forming lymphoid cells in their peritoneal cell exudate. The findings of Bendinelli (1968) tend to confirm this suspicion (see above). Furthermore, macrophages are not required for the blastogenic response of immune cells by antigen *in vitro* (secondary response) (M. J. Simons and Fitzgerald, 1969).

The findings presented strongly suggest a role for the macrophage in the primary, if not in the secondary, immune response. Nevertheless, the exact role of the macrophage in the normal induction of the immune response has been questioned. Möller (1969) observed that normal mouse peritoneal exudate cells could hemolyze both syngeneic and allogeneic mouse red blood cells *in vitro*. In view of the speed of the reaction (0.5–1 hour) and the failure of complement to potentiate the reaction, it was suggested that the reaction was not mediated by antibody and complement but represented a reaction governed by unknown immunological mechanism, if the reaction is immunologically mediated. This finding would, therefore, tend to cast some doubt on the significance of findings of other investigators who have demonstrated that macrophages are immunocompetent cells, using the hemolysis in gel technique. Roelants and Goodman (1969) observed no correlation between the degree of RNA–antigen complex formation by peritoneal macrophages *in vitro* and the immunopotency of the antigen *in vivo*. Although blockade of the reticuloendothelial system in mice (Sabet *et al.*, 1968) and in rabbits (A. Cruchaud, 1968) with carbon particles prior to immunization resulted in the failure of antibody production, treatment of mice or guinea pigs with the species-specific antimacrophage serum fails to

suppress the primary antibody response although the antisera used are capable of lysing the macrophages *in vitro* (Loewi *et al.*, 1969). However, since macrophages have been shown to be both bone marrow-derived (Virolainin and Defendi, 1968; Virolainin, 1968; Volkman, 1966; Balner, 1963; Goodman, 1964; Pinkett *et al.*, 1966; Van Furth and Cohn, 1968) and lymphocyte (lymph node and thoracic duct)-derived (Howard *et al.*, 1969; Boak *et al.*, 1968), it may be that they are antigenically different in the sense that antiserum directed toward the bone marrow-derived macrophages may not affect the lymphocyte-derived macrophages, and vice versa (Unanue, 1968). Obviously, such an explanation raises more problems than it solves, particularly the veracity of the functional criteria used to classify the macrophage system of cells (Howard *et al.*, 1969).

Is the macrophage antigen-specific in its reactivity or does it act in a nonspecific manner? Does it, in fact, "recognize" antigen? It has been shown by Unanue *et al.* (1969) that antigens stick to and remain on the surface of normal macrophages. Moreover, human monocytes and hepatic and splenic macrophages were demonstrated to have an IgG receptor on their surface that could bind to red cells sensitized with an IgG anti-Rh_0 antibody (Huber *et al.*, 1969). However, it does not appear that the γ-globulin site on the macrophage is antigen specific since it has been observed that peritoneal macrophages from normal mice and those undergoing primary or secondary responses can ingest more than one antigen (Rhodes and Lind, 1968). Furthermore, following the ingestion of two antigens, HSA and ferritin, it was observed that both antigens were localized to the same intracellular lysosomes within the macrophage (Rhodes *et al.*, 1969). Although receptor sites possessing antibodylike activity have been demonstrated on the surface of the normal human monocyte capable of reacting with human C'3 and immunoglobulin (Huber *et al.*, 1968), it is highly improbable that it is cytophilic antibody since, by the criteria of Nelson and Boyden (1967), the cells synthesizing cytophilic antibody would be unipotential and not pluripotential. These findings would infer that macrophages are not specifically committed to interact with antigens, in the sense that the normal ARC (Abdou and Richter, 1969b) and immune memory cell (Wigzell and Anderson, 1969) are.

Unfortunately, mainly peritoneal exudates have been used as sources for macrophages in the studies cited above, and it has been assumed that these cells are, in fact, macrophages. These cells, which accumulate in the peritoneum in response to the intraperitoneal injection of a number or reagents, such as casein, mineral oil, thioglycollate, starch, and peptone, may not, in fact, be macrophages but cells that masquerade as macrophages under these abnormal conditions. In fact, it has been

shown by Vernon-Roberts (1969) that bone marrow small lymphocytes migrate to the peritoneal cavity where they transform into cells which morphologically appear to be macrophages. We have observed the transformation of Bayol F-induced peritoneal exudate cells into lymphocytes in cell culture *in vitro* (Likhite and Richter, 1970). Furthermore, populations of macrophages obtained from different organs as well as individual cells within populations exhibit marked heterogeneity of function, structure, and metabolism (McIntyre *et al.*, 1967; Blanden, 1968). Whereas bone marrow macrophages have a long life-span and are able to divide and incorporate tritiated thymidine, peritoneal macrophages and blood monocytes do not divide and are considered to be end cells (Van Furth and Cohn, 1968).

Macrophages certainly appear to play an important role in cell-mediated immunity (reviewed in Cohn, 1968, and in Mackaness and Blanden, 1967). They are required for the blastogenic reaction in the mixed leukocyte culture (primary response) (Gordon, 1968). Sensitized macrophages exhibit increased adhesiveness in the presence of the antigen (Nelson and North, 1965), play an important defensive function in acquired microbial resistance (Mackaness, 1969), and are the effector cells in allograft immunity (Pearsall and Weiser, 1968), probably through contact with target cells by means of a specific cytophilic antibody (Granger and Weiser, 1966). In an allograft system in mice, macrophage membrane-bound antibodies are implicated in the specific adherence to and destruction of target cells. These antibodies are also considered to be responsible for the inhibition of migration of normal macrophages in the presence of PPD (Weiser *et al.*, 1969).

The macrophage does not appear to be a cell that is affected in the immunologically tolerant state. Macrophage obtained from tolerant hosts were shown to be immunologically active in that they could take up the antigen *in vitro* and stimulate DNA synthesis in immune spleen cells *in vitro* (G. Harris, 1967) and transfer specific immunocompetence (Mitchison, 1969). However, normal antigen-containing macrophages could not restore immunocompetence if injected into tolerant recipients (Mitchison, 1969). Normal adult macrophages can increase the number of antibody-forming cells if injected into immunologically immature syngeneic recipients (Argyris, 1968a,b, 1969a), suggesting that the failure of such animals to synthesize antibody may be attributed to a deficiency of the immune macrophage system rather than to an unresponsive lymphoid system.

In summary, it may be stated that the mechanism by which macrophages function in the initiation of the humoral immune response is still essentially unresolved. Whether they deliver a highly immunogenic

processed antigen, a messenger RNA, or both, to the ARC or whether by ingesting and degrading the antigen, they act to decrease the circulating antigen concentration until the latter reaches an immunogenic threshold level for the ARC are questions which still await experimental verification (Fishman, 1969; Mitchison, 1969). The literature does not help to clarify the problem but does suggest a well-defined role for the macrophage, probably in the antigen-processing step and the transfer of "information" to the ARC (reviewed in Cohn, 1968; Gottlieb, 1968; Fishman, 1969).

C. The Antigen-Reactive Cell

The necessity to invoke recognition of, and interaction with, the antigen by the immunocompetent cell is basic to the evolution of the immune response. The question raised, therefore, is not whether an immunocompetent cell must react with antigen but rather whether the cell that reacts with the antigen is a distinct cell or cell type of which the sole *raison d'être* is its ability to interact with the native macrophage-processed antigen and to transfer information, in the form of a highly immunogenic form of the antigen, antigen–RNA complex, or just specific RNA, to the AFC; or whether the same immunocompetent cell processes the antigen and synthesizes antibody. If two cell types are involved, an ARC and an AFC, then the immune system may lend itself to extensive manipulation since these cells may possess different properties and may originate in different organs. Such, in fact, appears to be the case. Recent investigations in the mouse, rat, and rabbit leave no doubt as to the dual cell nature of the immune system.

In the mouse, it has been demonstrated that neither the normal bone marrow cells nor the normal thymus cells by themselves can transfer antibody-forming capacity with respect to SRBC following their injection into nonimmunized, syngeneic, irradiated, immunoincompetent recipients. However, the recipients of a combination of bone marrow and thymus cells can respond vigorously with humoral antibody formation (reviewed in Miller and Mitchell, 1969; Davies, 1969; Claman and Chaperon, 1969; Taylor, 1969). Since it has been demonstrated that the thymus cells must interact with the antigen prior to reaction with the bone marrow cell (Miller and Mitchell, 1968) and since the bone marrow has been shown to be the organ of origin of the AFC (Miller and Mitchell, 1968; Nossal *et al.*, 1968), it would appear that the thymus is the source of the ARC. Further support for this interpretation is provided by the results of Davies *et al.* (1966, 1967) and R. K. Gershon *et al.* (1968) who demonstrated that thymus cells and not bone marrow cells can respond to antigenic stimulation with blastogenesis and mitosis, but not with anti-

body formation. Simliar results, demonstrating a necessary interaction between the thymus and bone marrow cells, have been presented by Taylor (1968) with respect to BSA as antigen.

In the rat, the ARC have been found to reside in a number of organs, depending on the type of antigen used and the detector system employed. Thoracic duct cells from normal donors can restore the primary immune response to sheep red cells but not to diptheria toxoid in irradiated recipients (Strober and Law, 1969). On the other hand, spleen cells were found to be capable of transferring immune responsiveness to tetanus toxoid (Strober, 1968, 1969).

With respect to the rabbit, it has been demonstrated that normal bone marrow lymphoid cells, but not lymphoid cells of the other normal lymphoid organs (thymus, spleen, lymph node, sacculus rotunds, and appendix), could be stimulated by a variety of antigens *in vitro* to undergo blastogenesis and mitosis (Singhal and Richter, 1968). Furthermore, only normal bone marrow cells could transfer immunocompetence following their administration into irradiated (800 r) allogeneic recipients (Abdou and Richter, 1969a; Richter *et al.*, 1970a). The finding that the antibody-forming cell in the irradiated recipient is of host and not donor origin (Richter and Abdou, 1969) is taken as evidence for the antigen-reactive nature of the bone marrow cells transferred. The finding that bone marrow cells obtained from a donor 24–48 hours following immunization (primed bone marrow) are incapable of transferring antibody-forming capacity with respect to the antigen used to immunize the cell donor but can do so with respect to all other antigens tested, indicates that the ARC migrate out of the bone marrow following their interaction with the antigen and transfer information to the AFC in some other organ (Abdou and Richter, 1969a). The bone marrow ARC has also been shown to be radiosensitive to 800 R total-body irradiation since they could not be detected in the bone marrow of irradiated rabbits (Abdou *et al.*, 1969). A good correlation was observed between the recovery of the ARC in the bone marrow of irradiated rabbits and the ability of those animals to synthesize humoral antibodies.

These results demonstrate that the ARC in different species of animals responsive to the same antigen (SRBC) can be normally found in different organs. They are present in the thymus, spleen, and thoracic duct in the mouse (Miller and Mtichell, 1968), in the thymus (Gowans and McGregor, 1965) the thoracic duct (Strober, 1968), and spleen (Strober and Law, 1969) in the rat, but only in the bone marrow in the normal rabbit (Abdou and Richter, 1969a; Richter *et al.*, 1970a). The organ source of the ARC in the other species of animals, including man, is unknown.

Knowledge as to the mono- or pluripotential nature of the ARC is of paramount importance in order to elucidate better the cell pathways concerned with the immune response. The existing evidence indicates that the ARC is a unipotential cell, that is, it is precommitted, to interact with and be stimulated by one antigen only. Abdou and Richter (1969a) have demonstrated that rabbit bone marrow cells obtained 24–48 hours following immunization with SRBC failed to confer antibody-forming capacity with respect to SRBC upon their transfer into an irradiated (800 r) immunoincompetent allogeneic recipent, although the response of the recipient to a non-cross-reacting antigen, horse erythrocytes (HRBC), was of the same degree as that given by a recipient of normal bone marrow cells. Thus, only the ARC precommitted to respond to SRBC vacated the bone marrow following immunization, leaving behind all the ARC directed to HRBC and all other antigens. The rabbit ARC could also be fractionated by passage through an antigen-sensitized (i.e., HSA) glass bead column. All the ARC directed to HSA were retained by the column, whereas the ARC directed toward all the other antigens tested were found in the effluent (Abdou and Richter, 1969b). Thus, the cells eluted from the column could transfer antibody formation in an irradiated recipient only to HSA, whereas the effluent cells could transfer antibody formation to all the antigens tested but not to HSA. Shearer *et al.* (1969a,b) have also demonstrated the unipotential nature of the ARC by transferring mouse thymus ARC, at limiting dilutions, and determining the types of immune responses detected. They concluded that the ARC is unipotent with respect to the type of immune response it will stimulate in the AFC, i.e., direct (19 S) PFC, indirect (7 S) PFC, or cluster-forming cells. Talmage *et al.* (1969) have also presented sound theoretical arguments in favor of the unipotential nature of the ARC.

Mouse (Raidt *et al.*, 1968) and rat (Haskill, 1969) spleen cells have been separated into a number of cell fractions, using a density gradient. The cell fractions that could transfer antibody-forming capacity with respect to SRBC, either *in vitro* (Raidt *et al.*, 1968) or *in vivo* (Haskill, 1969), were unable to respond to stimulation with a non-cross-reacting antigen.

Ada and Byrt (1969) incubated normal mouse spleen cells with a highly radioactively labeled preparation of *Salmonella adelaide* flagellin antigen. These incubated cells were subsequently unable to confer antibody formation with respect to this antigen upon transfer of the cells to irradiated syngeneic recipients. However, the immune response to other antigens was normal. The authors present this finding as evidence for the unipotential nature of the ARC; however, since this antigen, S.

adelaide flagellin, is a thymus- and ARC-independent antigen (see below), it is probable that the cells which were inactivated by interaction with the labeled antigen were, in fact, unipotent AFC and not ARC.

Trentin *et al.* (1967, 1969) have presented evidence in favor of the pluripotential nature of the ARC. They transferred spleen cells to heavily irradiated recipient syngeneic mice and observed that the transferred cells formed discrete clones in the spleens of these mice. They then transferred cells obtained from these clones in secondary irradiated recipients whose spleens, containing four to fourteen clones, were now used to repopulate other tertiary irradiated, immunoincompetent recipients. These latter animals responded well to stimulation with a number of common antigens, thus suggesting that all unipotential ARC (and AFC) were transferred in the original spleen cell suspension or that the ARC is, in fact, pluripotential. A likely possibility is that the cells passaged through the intermediate irradiated hosts had dedifferentiated into stem cells, which may be pluripotential in nature, and that these cells can then give rise to all of the more mature unipotential ARC in the final recipient.

The majority of the antigens used may be considered, at least insofar as the mouse and rat are concerned, to be thymus-dependent antigens since they require interaction with a thymus cell for the successful induction of the primary immune response. However, since the immunocompetent thymus cell in the mouse has been shown to be the ARC, since spleen cells in the mouse can also exhibit ARC activity as they can successfully substitute for the thymus cells, and since in the rabbit these same antigens do not require a thymus cell for antibody formation but do require the mediation of a bone marrow ARC, it would appear to be more scientifically correct and less confusing to refer to the antigens as *ARC-dependent*, rather than *thymus-dependent*. Even with respect to SRBC, the ARC dependency in the mouse is related to the dose of antigen and to the strain of mouse used (Sinclair, 1967; Taylor and Wortis, 1968). The immune response in the mouse to S. *adelaide* flagellin antigen (Armstrong *et al.*, 1969) and to KLH, horse ferritin, and pneumococcal polysaccharide (Humphry *et al.*, 1964; Fahey *et al.*, 1965) are thymus-independent and, therefore, may be considered to be ARC-independent as well. Similarly, the immune responses in the rabbit to the γ-globulin in GARIG serum (Daguillard and Richter, 1970b) and to KLH (Richter, 1970) are bone marrow- or ARC-independent. These antigens, therefore, appear to be capable of directly interacting with the AFC without the prior intervention of either the macrophage or the ARC. These ARC-independent antigens may be considered to possess intrinsically a high affinity for the AFC, thereby imparting to them opti-

mum immunogenicity. Other antigens must first be processed by the macrophage and/or ARC in order to increase their immunogenicity or affinity for the AFC (Richter, 1970).

Are ARC in the mouse thymus-derived but mature under bone marrow influence or are they bone marrow-derived and mature under thymic influence? The data presented do not permit for a clear-cut answer. Experimental data indicating bone marrow influence on thymic anatomy and physiology are scanty. Thymectomy performed in newborn mice (Trainin and Resnitzky, 1969) and rats (Corsi and Giusti, 1967) resulted in an increase in the number of undifferentiated blasts and impairment of the capacity of the bone marrow to form clones in lethally irradiated recipients. Although bone marrow cells can reconstitute to normal the structure and function of lymphoid-depleted thymus tissue (Gengozian *et al.*, 1957; C. E. Ford and Micklem, 1963), yet the mechanism by which this effect is mediated is unknown. Recently, Burger and Knyszynski (1969) described a dialyzable agent in the bone marrow of mice and rabbits capable of stimulating proliferation of mouse thymic cells both *in vivo* and *in vitro*. This finding is of interest in view of the recent observations (reviewed in Gabrielsen *et al.*, 1969) that in lymphopenic hypogammaglobulinemia, which is probably due to a defect at the stem cell level, the thymus is rudimentary and extremely hypoplastic. A few weeks following bone marrow transplantation in a child with that defect (Meuwissen *et al.*, 1969b), the shadow in the thymic region started to enlarge, probably indicating population of the thymus by the administered bone marrow cells and/or proliferation of thymus cells in response to a factor(s) produced by the transferred bone marrow. There are a number of possible mechanisms (Fig. 1) whereby the bone marrow in the mouse might convert an immunoincompetent ARC-deficient thymus to an immunocompetent thymus: (1) differentiation of some bone marrow cells into ARC in the thymus which, following antigenic stimulation, will induce the transformation of the other bone marrow cells into ARC; (2) differentiation of bone marrow-derived cells into ARC in the thymus but only in the presence of a functioning thymus or a thymic factor (hormone?). The existence of a thymic hormone or a factor produced by the thymus which acts as a hormone has already been demonstrated and has been shown to be capable of restoring immunocompetence in an otherwise immunoincompetent thymectomized animal (Osoba and Miller, 1963; Small and Trainin, 1967; Osoba, 1965; Law and Agnew, 1967; Goldstein *et al.*, 1966; Law *et al.*, 1968); (3) the presence of bone marrow cells in contact with thymic reticuloepithelial tissue may result in the proper microenvironment for thymic cells to become immunocompetent ARC; and (4) thymic parenchymal cells may

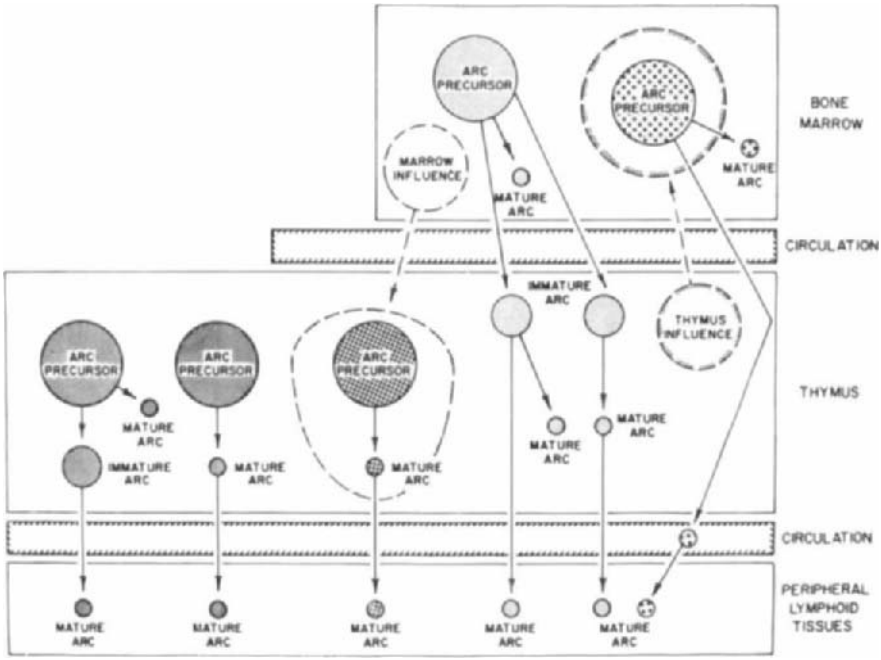

FIG. 1. A hypothetical representation of the organ source(s) and site(s) of maturation of the antigen-reactive cells (ARC).

be induced to transform into ARC under the influence of a trophic factor (hormone?) released by the bone marrow (Burger and Knyszynski, 1969).

The scheme presented in Fig. 1 is highly speculative as a great deal remains to be learned concerning the factor(s) responsible for the maturation of the ARC and its subsequent participation in the immune response. The scheme presented takes into account only the available data concerning the situation in the mouse in which a number of well-conducted investigations have unequivocally demonstrated the thymic origin of the *mature* ARC (reviewed in Miller and Mitchell, 1969; Davies, 1969; Claman and Chaperon, 1969; Taylor, 1969). However, it must be appreciated that the immature ARC may originate in a different organ and that it must mature in the thymus or under the influence of a thymic trophic factor. Furthermore, the organ of origin and habitation of the ARC may be different in other species of animals. Experiments in the rabbit suggest that the mature ARC are found in the bone marrow (Singhal and Richter, 1968; Abdou and Richter, 1969a). Where the ARC arise in man can only be speculated upon at the present time.

In summary, the usual ARC-dependent antigen interacts with the

ARC, inducing them to undergo rapid proliferation and transformation into lymphoblastoid cells *in vivo* (Davies *et al.*, 1966; R. K. Gershon *et al.*, 1968) and *in vitro* (Singhal and Richter, 1968). Administration of vinblastine inhibits this response (Syeklocha *et al.*, 1966). Once the ARC are stimulated, they vacate their original organ of residence and presumably settle in one of the other lymphoid organs, where they interact with the AFC or its precursor resulting in antibody formation by the latter cell (see below).

D. The Antibody-Forming Cell

The role of the AFC in the immune response is indisputable since, by definition, the AFC is the cell that synthesizes the antibody. Evidence was present in support of the view that AFC activity becomes manifest only after the macrophage–antigen and/or the ARC–antigen interaction has taken place with the exception of the ARC-independent antigens which do not require the mediation of the macrophage and the ARC. The interpretation of these findings is that the AFC responds not to the native antigen but only to stimulation by a highly processed form of the antigen the immunogenicity of which is directly related to its affinity for the AFC (Richter, 1970). The questions to be answered, then, are (*a*) What is the organ source of the AFC? and (*b*) Is the AFC a mono- or pluripotential cell?

There is overwhelming evidence in the literature indicating that the AFC is morphologically a lymphocyte or a lymphocyte-derived cell (plasma cell) (Cochrane and Dixon, 1962; Dutton, 1967; Sell and Asofsky, 1968). Nossal *et al.* (1967) have shown that lymphocytes from thoracic duct lymph maintained in single cell cultures can transform into AFC *in vitro*, which morphologically appear to be lymphocytes.

Mitchell and Miller (1968a,b) and Miller and Mitchell (1968) using anti-H_2 antiserum, Taylor *et al.* (1967) using antiallotype serum, and Nossal *et al.* (1968) using chromosome analysis of transferred cells, all demonstrated that the bone marrow in the mouse provides the AFC. Such is not the case in the rabbit, however, where it has been definitely shown using anti-allotype antiserum that the bone marrow does not contain the AFC (Richter and Abdou, 1969).

Evidence that organs other than the bone marrow in the mouse may harbour the AFC has been presented by Armstrong *et al.* (1969). They transferred cells of normal mouse lymphoid organs along with the antigen *Salmonella adelaide* flagellin into lethally irradiated mice and analyzed the host spleen for its capacity to form foci of bacterial immobilization *in vitro*. The bone marrow, mesenteric lymph nodes, and Peyer's patches all possessed immunocompetent cells capable of conferring im-

mune responsiveness to the irradiated host. Thus, it must be considered that all these organs harbor AFC in the mouse.

In the rat, the AFC appears to at least inhabit the thoracic duct and the spleen since both thoracic duct cells and spleen cells have been found to be capable of transferring antibody-forming capacity to irradiated immunoincompetent recipients (Strober, 1968, 1969; Strober and Law, 1969).

Although both thymus cells and bone marrow cells are required to initiate an immune response in an irradiated mouse, by providing the ARC and AFC, respectively, spleen cells by themselves are capable of transferring antibody-forming capacity *in vivo* (Claman *et al.*, 1966; Kind and Campbell, 1968; Stutman *et al.*, 1968, 1969) and of initiating antibody formation *in vitro* (Pierce and Benacerraf, 1969; Pierce, 1969; Mosier, 1969; Mishell and Dutton, 1967; Marbrook, 1968) indicating that the spleen contains both ARC and AFC. Although the thesis has been offered that the spleen contains a single cell type which, through differentiation, acquires the capacity to synthesize antibody following its interaction with the antigen, this view cannot be seriously entertained today in view of the findings that neither ARC nor AFC, by themselves, can transfer antibody-forming capacity to irradiated recipients (reveiwed in Miller and Mitchell, 1969; Davies, 1969; Taylor, 1969; Claman and Chaperon, 1969) and that the mouse spleen can be fractionated into ARC and AFC *in vitro* (Raidt *et al.*, 1968; Haskill, 1969).

The organ source of the virgin AFC in the rabbit is not known. However, it is probably not the spleen since exposure of the spleen to 10,000 r X-irradiation, with the rest of the body lead-shielded, has been found to result in an enhanced, rather than in a diminished, immune response (Taliaferro and Taliaferro, 1956). Furthermore, the appendix can also be ruled out as the source of AFC as appendectomy does not appear to affect the immune response (Sussdorf and Draper, 1956).

Is the AFC a mono- or pluripotential cell? That is, can it synthesize antibodies directed toward only one antigen or only one antigenic determinant and need the antibody molecules it synthesizes all be of the same molecular class? Attardi *et al.* (1959, 1964), Nossal and Makela (1962), and Schwartzman (1967) have observed, in single cell cultures of immune lymphoid cells, that though the majority of cells can synthesize antibodies directed to one antigen only, a few cells synthesized antibodies directed to more than one antigen. However, Makela (1967) could not detect cells capable of forming antibodies to more than one antigen using the single cell culture technique.

Playfair *et al.* (1965) have shown that PFC do not belong to a single clone of antibody-producing cells but are derived from multiple clones

which are recruited at different times after immunization. This conclusion is derived from the demonstration that the development of PFC occurs in colonies of different sizes in anatomically different sites in the spleen. However, Celada and Wigzell (1966) have demonstrated that animals simultaneously injected with two antigens have antibody-producing cells against each antigen in discrete anatomical sites in the spleen, thus attesting to the unipotential nature of the AFC.

Trentin *et al.* (1967, 1969), using the cell transfer system, observed that irradiated recipient mice of cloned populations of cells could respond with humoral antibodies against a variety of antigens i.e., SRBC, BSA, *Salmonella* "O" antigen, a finding which would be inconsistent with the assumption that each clone was derived from a single unipotential AFC. These findings do not agree with those obtained by Shearer *et al.* (1968, 1969a). The latter authors found that ASU formed in the spleens of irradiated mice by interaction of marrow and thymus cells were specialized for the type of antibody produced. By injecting graded numbers of marrow cells with a constant number of thymus cells, differentiation on the basis of the molecular class of the antibody produced (19 S, 7 S) was found to be dependent on the bone marrow (AFC) cell (Shearer and Cudkowicz, 1969; Cudkowicz *et al.*, 1969) and not on the thymic (ARC) cell (Shearer *et al.*, 1969b). The discrepancy in these findings could be attributed to the fact that the unipotentiality of the bone marrow is at the level of the class of antibody formed and not at the level of antigen recognition which is considered to be a function of the ARC.

Dutton and Mishell (1967), Haskill (1969), and Wigzell and Andersson (1969) have also presented very convincing evidence in favor of the unipotential AFC. Wigzell and Andersson (1969) separated AFC by passage of immune mouse lymph node cells through an antigen-sensitized glass bead column and demonstrated that the immune responsiveness of the eluted cells was antigen-specific. The eluted cells could transfer antibody-forming capacity to irradiated recipients only with respect to the antigen used to sensitize the column.

The findings presented, demonstrating the existence of both unipotent and pluripotent AFC, therefore, appear to be conflicting unless we assume the presence of a heterogeneous population of cells, all proliferating and differentiating in the direction of the mature AFC and becoming unipotential in their immune responsiveness only at the terminal cell stage. Thus one can reconcile all of the above findings (Table I) by postulating the presence of a cell lineage, commencing with the stem cell (P-AFC) and terminating with the actual antibody-forming cell (AFC_3). Under normal conditions, the stem cell differentiates into uncommitted AFC (U-AFC) and into cells of the hematopoetic cell lines.

TABLE I

DIFFERENTIATION AND/OR TRANSFORMATION OF ANTIBODY-FORMING CELLS (AFC)

Type of cell	Current nomenclature: Sercarz *et al.*[a]	Current nomenclature: Makinodan *et al.*[b]	Existence of cells verified experimentally by
Stem cell or precursor AFC (P-AFC) normal: normal normal: Hematopoiesis, Leukopoiesis	X-cell	PC_1	Makinodan and Albright, 1966 Wu *et al.*, 1968b Till *et al.*, 1967
Uncommitted AFC (U-AFC) normal: stress stress: Hematopiesis, Leukopoiesis	X-cell	PC_1	Makinodan and Albright, 1966 Hellman and Grate, 1967
AFC committed to antibody formation but uncommitted with respect to the specificity of immune response (AFC 1)	—	PC_2	Trentin *et al.*, 1967
AFC committed to antibody formation and committed with respect to the specificity of immune response and antibody type (AFC 2)	Y-cell or memory cell	PC_2	Cudkowicz *et al.*, 1969 Nossal, 1960 Nossal, 1962 Wigzell and Andersson, 1969
AFC (plasma cell?) (AFC 3) cell death antibody: circulation	Z-cell	—	Gowans and McGregor, 1965 Sell and Asofsky, 1968 Dutton, 1967

[a] Sercarz and Coons (1963b). Dowden and Sercarz (1967). Byers and Sercarz (1968).

[b] Makinodan and Albright (1966). Perkins and Makinodan (1964).

Under abnormal conditions, such as following irradiation, this proliferative balance is altered in favor of hematopoiesis (Hellman and Grate, 1967). It is postulated that the AFC becomes antigen-specific only once it has reached the AFC_2 stage. It is, therefore, possible that, on occasion, the AFC_1 may begin to synthesize antibody and being pluripotential might synthesize antibodies directed to two or more antigens. However, such cells would be few and far between. Although the terminology used to classify the various AFC is our own, the postulated existence of each of these functional cell types is based on findings in the references listed in Table I.

As stated above, the organ of origin of the *mature* AFC in the mouse is the bone marrow. Is this cell indigenous to the bone marrow or does it

arise in some other organ and mature in the bone marrow? Osoba's work (1968) suggests that the thymic humoral factor can convert uncommitted bone marrow AFC cells into fully competent cells. Heavily irradiated thymectomized mice grafted with marrow cells and thymus tissue enclosed in a cell-impermeable chamber could form adequate number of PFC in their spleens in response to SRBC immunization. Spleen cells of irradiated thymectomized mice which received only marrow cells failed to form plaques. The kinetics of the splenic PFC response in nonthymectomized marrow chimeras and thymectomized chimeras with thymuses implanted under the kidney capsule was identical. Doria and Agarossi (1967, 1969) and Agarossi and Doria (1968) have shown that the recovery of the immune response to SRBC in lethally irradiated mice transplanted with either syngeneic or allogeneic bone marrow cells is conditioned by the host thymus. Donor cells of bone marrow origin in mitosis were found in the thymus of mouse radiation chimeras. Thymus cells at different intervals following establishment of the chimeric state were transferred together with SRBC to lethally irradiated syngeneic mice. Thymus cells from young or syngeneic chimeras were more effective in transferring plaque-forming capacity than cells from old or allogeneic chimeras. This would indicate that both ARC and AFC are present in the thymus of antigenically- nonstimulated bone marrow-induced chimeras—a situation quite different from the normal situation when only ARC are found in the thymus.

In summary, the bone marrow but not the thymus in the mouse appears to be the source of the AFC. However, it may not be the sole source. In the rabbit, the organ of origin of the AFC has not as yet been determined but it is definitely not the bone marrow. The existing evidence also favors the unipotential nature of the AFC at the stage when it is actively synthesizing antibodies (AFC_3). However, in deference to the highly controversial and conflicting nature of the results of investigations concerned with this aspect of the problem, a new scheme and classification of the AFC has been presented (Table I) which incorporates all of the findings referred to above and which not only makes allowance for, but, in fact, justifies the various conflicting findings reported to date.

E. Properties That Distinguish the Antigen-Reactive Cells from the Antibody-Forming Cells

On the basis of the investigations cited above, it is obvious that morphological criteria cannot by themselves be used to distinguish between the various functionally different cell types, specifically the ARC and the AFC, which constitute the immunocompetent lymphoid cell system.

The following properties can be helpful in distinguishing the ARC from the AFC: (*a*) organ source, (*b*) sensitivity to irradiation *in vivo*, (*c*) sensitivity to irradiation *in vitro*, (*d*) reactivity of immunocompetent cells toward the native antigen, and (*e*) physicochemical properties.

The organ of origin is one criterion that distinguishes the ARC from the AFC in both the mouse and the rabbit. In the mouse, the primary organ of origin of the ARC appears to be the thymus, whereas the AFC arises from the bone marrow (reviewed in Miller and Mitchell, 1969; Davies, 1969; Taylor, 1969; Claman and Chaperon, 1969). In the rabbit, the bone marrow constitutes the only source of virgin ARC, whereas the organ of origin of the AFC has not yet been identified, but it is not the bone marrow (Richter and Abdou, 1969).

A second criterion is that of *in vivo* radiation sensitivity. In the mouse, both the ARC and AFC appear to be equally sensitive (Claman and Chaperon, 1969; Miller and Mitchell, 1969). In the rabbit, the ARC is sensitive to 800 r total-body irradiation, whereas the AFC is not affected until 1000 r total-body irradiation is applied (Abdou *et al.*, 1969).

The results of *in vitro* irradiation experiments corroborate the *in vivo* findings in the rabbit. The virgin ARC is inactivated if subjected to 4000 r irradiation *in vitro* (Abdou and Richter, 1970a), whereas the antigen-stimulated ARC (Abdou and Richter, 1970a) and the AFC (Daguillard and Richter, 1970b) are unaffected by this dose of irradiation.

A fourth criterion for differentiating the ARC from the AFC is their reactivity toward the native antigen. The ARC can be stimulated by interaction with the ARC-dependent antigen *in vitro* (Singhal and Richter, 1968) and *in vivo* (Davies *et al.*, 1966, 1967) to undergo blastogenesis and mitosis, whereas the AFC does not react in this fashion. Furthermore, ARC can be "activated" *in vitro* by interaction with the antigen so as to enable them to transfer antibody-forming capability to irradiated hosts (Abdou and Richter, 1970a, reviewed in Miller and Mitchell, 1969). The AFC cannot be triggered off by incubation with the native antigen *in vitro* and it cannot transfer antibody-forming capacity in the absence of stimulated ARC, with respect to the ARC-dependent antigens.

Density gradient separation of normal and immune rat spleen cells followed by testing the immune function of the different cell fractions subsequent to their transfer to irradiated syngeneic recipients has been used to study the density distribution of ARC as compared to AFC (Haskill, 1967, 1969). Haskill reported changes in the density profile of ARC in rat spleens following antigen (RBC) stimulation. This change of profile was noted as early as 10 hours after antigen administration. The AFC were found to be less dense than the ARC. Furthermore, splenic cells directed to interact with SRBC were found to have densities different

from those reactive with horse or rat red cells (Haskill, 1969). Antibody-forming cells with the same antigenic specificity obtained from different organs (spleen, lymph nodes, peripheral blood, thoracic duct cells) displayed different sedimentation profiles (Haskill *et al.*, 1969). Similar findings were reported by Raidt *et al.* (1968) who fractionated mouse spleen cells on albumin gradients and tested the cell fractions for immunocompetence *in vitro*.

F. Lymphoid Cell Migration *in Vivo*

Specific depletion of the recirculating pool of lymphocytes in the rat by thoracic duct lymph drainage results in variable degrees of lymphoid depletion in the various organs. The periarteriolar lymphoid sheaths of the spleen and the cortical zones of lymph nodes are markedly depleted of small lymphocytes, whereas the bone marrow and thymus content of lymphocytes is not affected (reviewed in W. L. Ford and Gowans, 1969). This would indicate that the bone marrow and thymus contain few or no circulating lymphocytes and that a large proportion of lymph node and spleen lymphocytes belongs to the recirculating pool. The circulation of small lymphocytes between the blood and the peripheral lymphoid tissues takes only a few hours and involves a population of nondividing small lymphocytes with an average life-span of several weeks (Gowans and Knight, 1964). Bone marrow lymphocytes continuously migrate out of the bone marrow to the peripheral lymphoid tissues. These cells, however, do not enter the recirculating pool of lymphocytes (C. E. Ford, 1966). Parrott (1967) injected labeled marrow cells into irradiated mice and showed that the most prominent site of localization of the labeled small cells is the red pulp of the spleen and not the recirculating traffic areas of the lymph nodes and spleen. Bone marrow cell migration has been shown to occur in the developing fetus, in the animal recovering from irradiation, and in the normal adult animal (Micklem *et al.*, 1968). The criticism against all these experiments is the artificial situation created, namely, irradiation of the recipients, the use of parabiosis models, and the unknown effects of the rapid intravenous administration of a large number of cells.

In the normal situation, the number of stem cells in the circulating blood is small. However, they are increased whenever there is a demand for stem cells, as during the perinatal period (Barnes and Loutit, 1967a,b). The source of these circulating stem cells has been shown to be the bone marrow. Following irradiation, a direct relationship was observed between the number of CFU in the peripheral blood and the degree of hyperplasia of the bone marrow (Barnes and Loutit, 1967a,b).

As a consequence of migration of stem cells between different com-

partments of the lymphoid tissues, the stem cells should normally be detected in the peripheral blood. This has been demonstrated in the mouse by Popp *et al.* (1958) and Goodman and Hodgson (1962), in the dog by Cavins *et al.* (1964), and in the guinea pig by Malinin *et al.* (1965).

It is not known whether bone marrow lymphoid cells migrate to the peripheral lymphoid tissues directly or by way of the central lymphoid organs, namely, the thymus and the gut-associated lymphoid tissues (bursa homolog). C. E. Ford (1966) showed that chromosomally marked bone marrow lymphoid cells injected into syngeneic irradiated mice migrate to the thymus, spleen, and lymph nodes. The time sequence for the appearance of these cells in these organs was not reported. In a similar study, it was demonstrated that dividing lymphoid cells migrate from the bone marrow to the lymph nodes via the thymus. The journey requires a period of several weeks during which time the proliferating cells probably undergo maturation and/or proliferation (Goldschneider and McGregor, 1968; Liden and Linna, 1969). Micklem *et al.* (1966, 1968) concluded from their studies that the bone marrow lymphocyte can migrate directly from the bone marrow to the lymph node without intermediary stops in the central lymphoid organs. Moreover, the same authors could not demonstrate migration of cells from the thymus to the bone marrow since no labeled donor thymus cells could be detected in the bone marrow of the recipient.

In contrast to the bone marrow, the thymus in the mouse and rat is a major site of production of recirculating small lymphocytes (Miller *et al.*, 1962). There is evidence in the literature (Sainte-Marie and Leblond, 1964; Murray and Woods, 1964; Matusuyama *et al.*, 1966) that thymus lymphocytes are released into the blood either directly or via the lymphatics. Following the infusion of tritiated thymidine directly into the thymus of the adult rat, labeled small lymphocytes were seen leaving the thymus via the blood and lymphatics and to then localize in those areas of the lymphoid tissues in which the recirculating cells predominate, namely, the Peyer's patches, the postcapillary venules in the cortical areas of the lymph nodes, and the periarteriolar areas in the white pulp of the spleen (Weissman, 1967; Linna, 1967; Goldschneider and McGregor, 1968). This migration pattern is not thymus-dependent and can be altered by prior incubation of lymphocytes with trypsin (Woodruff and Gesner, 1968).

Little is known about the migratory pathways of the ARC and AFC following administration of the antigen. The findings of Abdou and Richter (1969a) suggest that, in the rabbit, the bone marrow ARC migrates out of the marrow following contact with the antigen (Fig. 2).

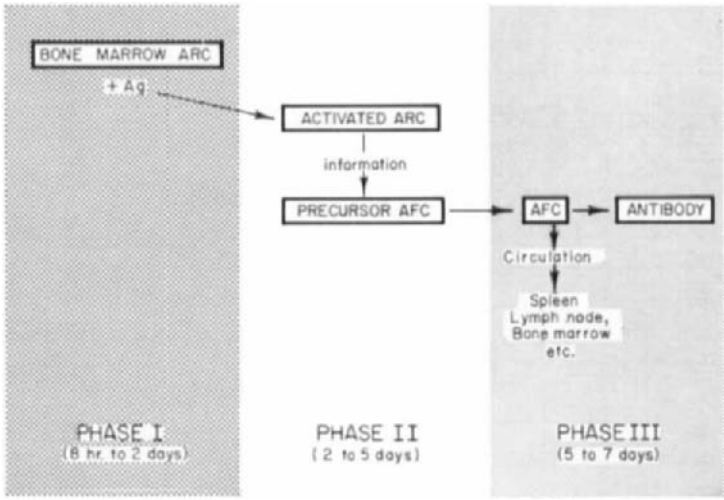

FIG. 2. The different phases and cell interactions in the immune response in the normal adult rabbit. (ARC—antigen-reactive cells; AFC—antibody-forming cells.)

The time required for the emigration of these cells from the bone marrow is directly related to the dose of antigen administered (Singhal and Richter, 1968; Abdou and Richter, 1969a). Following the injection of an appropriate amount of antigen, the ARC appear to vacate the bone marrow immediately following interaction with the antigen since the specifically committed ARC cannot be detected in the marrow within 8 to 48 hours following immunization (Phase I). These specifically activated ARC probably migrate to the organ(s) containing the AFC precursors to which it probably transfers specific information (Phase II), which probably stimulates this latter cell to differentiate into an AFC. These cells can only be detected in the spleen of the rabbit commencing 5 to 7 days following immunization (Phase III) after which time they can be detected in the circulation and other lymphoid organs (Abdou and Richter, 1969a). In the mouse, it has been observed that the ARC activity of the thymus is lost following antigen administration. As will be discussed in Section VII, these findings have been presented as evidence for the induction of tolerance in this organ. However, it may be that the ARC migrate out of the thymus following interaction with the antigen, leaving behind a specifically tolerant thymus.

The migratory habits of the AFC are not clear. The work of Chaperon *et al.* (1968) has demonstrated that, following a single intraperitoneal injection of SRBC, AFC-producing 19 S antibodies were localized to the spleen and did not recirculate; AFC-producing 7 S antibodies and the

memory cells recirculated since increasing numbers were localized to the spleen, thymus, and bone marrow at increasing intervals of time following immunization.

The initial events following antigenic stimulation appear to be increased stickiness and clumping of sensitized macrophages (Nelson and North, 1965) and the redistribution of the recirculating lymphocytes and their transformation into blasts in the lymphoid follicles of the peripheral lymphoid organs (Hall and Morris, 1965; Weissman, 1967; Austin, 1968; W. L. Ford and Gowans, 1969). The role of the recirculating lymphocyte in the induction of the humoral immune response has been dramatically displayed by the work of W. L. Ford and Gowans (1969). They observed that isolated spleens of lymphocyte-depleted rats, if stimulated with SRBC, gave no hemolysin response. However, the immune responsiveness of the spleen was restored to normal levels by perfusing it with circulating lymphocytes. It appears, therefore, that the continuous migration of lymphocytes provides cells capable of being locally stimulated by antigen even though the antigen concentration in the circulation has fallen to subimmunizing levels.

If macrophages perform an essential preliminary role by processing the antigen, then the movement of lymphocytes past the relatively sessile macrophages in the follicles would enable contact between these two cell types to take place and facilitate the transfer of information from the macrophage to the immunocompetent lymphocyte, thus initiating the sequence of events leading up to antibody formation.

V. Cell Interactions Resulting in the Induction of the Immune Response

A. Cell Interactions in the Humoral Immune Response

Evidence that more than two cell types are required for the induction of the primary immune response *in vivo* and *in vitro* stems from the work of a number of investigators (Claman *et al.*, 1966; Davies *et al.*, 1966; Miller and Mitchell, 1968; Taylor, 1968; Richter and Abdou, 1969; Talmage *et al.*, 1969; Dutton and Mishell, 1967; Mosier, 1967; Pierce and Benacerraf, 1969). Miller and Mitchell (1968) observed that viable syngeneic thymus or thoracic duct lymphocytes could reconstitute to normal levels the plaque-forming capacity of spleens of neonatally thymectomized immunoincompetent mice challenged with SRBC. No significant immunological response was achieved by giving syngeneic bone marrow cells, irradiated thymus or thoracic duct cells, thymus extracts, or yeast. Spleen cells from reconstituted mice were exposed to anti-H_2 sera directed against either the donor of the thymus or the

thoracic duct cells or against the neonatally thymectomized host. Only isoantisera directed against the host could reduce the number of hemolysin-forming cells present in the spleen cell suspensions, indicating that the AFC are of host origin and not derived from the donor thymus or thoracic duct lymphocytes. Thymectomized, irradiated recipients were also used by the same investigators. The irradiated mice were protected with syngeneic bone marrow for a period of 2 weeks and then injected with semiallogeneic thoracic duct cells together with SRBC. These mice produced a greater number of plaques than irradiated mice which received the same number of thoracic duct cells without bone marrow. By using chromosomal markers (T6) in a syngeneic system, Nossal *et al.* (1968) confirmed the above findings. When lethally irradiated mice were injected with mixtures of syngeneic thymus and bone marrow cells, one of which was chromosomally marked, all the AFC were found to be of bone marrow origin. These investigators postulated that in the mouse, the thymus or thoracic duct lymphocytes "recognize" the antigen (ARC) and interact with it, and this latter reaction triggers off the differentiation of a bone marrow-derived precursor cell to a specific AFC.

Similar findings were also reported by Davies *et al.* (1966; reviewed in Davies, 1969). In their system, chromosomally marked mouse radiation chimeras were used. Although it could be shown that thymus-derived cells responded vigorously by mitosis to antigenic stimulation, these cells were not capable of antibody production. In contrast, bone marrow-derived cells in recipients of bone marrow cells did not respond with mitosis to antigenic stimulation during the first 3 days following exposure to antigen, but they were capable of limited antibody production. Antibody was maximally produced in recipients of both thymus and bone marrow cells.

Using both the hemolytic plaque assay (Jerne and Nordin, 1963) and the hemolytic foci assay (Playfair *et al.*, 1965), Claman *et al.* (1966, 1968; reviewed in Claman and Chaperon, 1969) demonstrated that irradiated mice had to be injected with both syngeneic thymus and marrow cells in order to facilitate an adequate immune response following the injection of the antigen (SRBC). Living syngeneic thymus cells were required since sonicated or irradiated mouse thymus cells or living heterologous (rat) thymus cells were incapable of transferring immunocompetence.

Taylor (1968) using a protein antigen (BSA), also showed that a mixture of thymus and bone marrow cells has to be given to irradiated syngeneic mice in order to obtain an immune response. Reducing the number of each type of cell resulted in a diminished response. Interestingly, the author found that the administration of BSA to the donor

mouse 24 hours before sacrifice resulted in failure of the donor thymus cells to interact with normal syngeneic bone marrow cells for the induction of the immune response in an irradiated recipient (reviewed in Taylor, 1969). By using an allotype marker in mice, Taylor *et al.* (1966) showed that mouse bone marrow cells are the cells responsible for antibody production.

Abdou and Richter (1969a) have demonstrated that reconstitution of immunocompetence in an irradiated (800 r) immunoincompetent rabbit could be accomplished by the transplantation of allogeneic bone marrow cells. Lymphoid cells of other organs did not possess this capacity (Richter *et al.*, 1970a). However, by the use of specific anti-allotype serum, it was shown that the AFC in the spleens of the irradiated bone marrow recipients were of host and not donor origin, thus demonstrating the ARC nature of the transferred bone marrow immunocompetent cells (Richter and Abdou, 1969). Thus at least two cell types are definitely implicated in the immune response in the mouse and the rabbit—the ARC and the AFC.

It has been observed that a two-cell interaction is necessary for the successful induction of a *secondary* immune response with immune rabbit lymph node fragments *in vitro* (Richter and Singhal, 1970). It was found that a cell adherent to glass wool, probably a macrophage, was essential during the initial period (days 1 to 5) of the *in vitro* culture. It could subsequently be removed without affecting the immune response by the fragments. Mosier (1967, 1969) has demonstrated that three cell types—one glass adherent and two nonadherent—are required for the *in vitro primary* immune response of mouse spleen cells to SRBC. These cells form clusters in the presence of the antigen and the clusters are antigen-specific. On the basis of these findings, a three-cell model of antibody formation has been presented by Talmage *et al.* (1969). In this model an "adherent" cell would bring together two nonadherent cells, one of which, considered to be thymus-derived, delivers specific information to the second cell, considered to be bone marrow-derived, which is capable of synthesizing antibody. The recent findings of Pierce and Benacerraf (1969) strongly support this concept. The induction of a *primary* immune response to SRBC *in vitro* by mouse spleen cells involved a two-step cell interaction leading to the activation of the AFC. The first is a macrophage-dependent phase, complete in about 24 hours, followed by a macrophage-independent phase, complete in the next 24 hours. Plaque-forming cells were identified 48 hours later, the entire reaction taking 4 days.

In the mouse, both the macrophage and the AFC are of bone marrow origin, whereas the ARC is normally found in the thymus (Table II).

In the rabbit, on the other hand, the ARC is normally found in the bone marrow. The organ source of the rabbit macrophage and AFC remain to be determined (Table II). It is postulated that, depending on the type (particulate versus soluble; ARC-dependent versus ARC-independent) and state (aggregated versus aggregate-free) of the antigen, it may interact initially with either the macrophage, ARC, or the undifferentiated AFC to trigger off the inter- and intracellular events culminating in humoral antibody formation (Fig. 3). These interactions may involve the transfer of information or highly immunogenic antigen from the macrophage to the ARC which, in turn, transfer information to an antibody-forming cell, AFC_1, which is still uncommitted with regard to the specificity of the immune response (Pathway I) or the transfer of information directly from the ARC to the AFC_1 (Pathway II). A third

TABLE II
TYPES OF CELLS AND SEQUENCE OF CELLULAR INTERACTIONS MEDIATING THE PRIMARY HUMORAL IMMUNE RESPONSE

	Cells mediating the immune response					
	First cell		Second cell		Third cell	
Animal species	Functional type	Organ source	Functional type[a]	Organ source	Functional type[b]	Organ source
Rabbit	Macrophage	?	ARC	Bone marrow	AFC	?
Mouse	Macrophage	Bone marrow	ARC	Thymus	AFC	Bone marrow

[a] ARC—antigen-reactive cell.
[b] AFC—antibody-forming cell.

mechanism of antibody induction (Pathway III) may involve the interaction of the native antigen directly with the AFC_1. Pathways I and II may be characteristic of the majority of antigens used. The failure to induce tolerance with the aggregated form of the antigen in the adult animal as compared with the ease of induction of tolerance with the aggregate-free antigen (Abdou and Richter, 1970b; Biro and Garcia, 1965; Frei *et al.*, 1965) would suggest that the macrophage is bypassed by the aggregate-free antigen (Pathway II) permitting interaction with the ARC resulting in a tolerant ARC (Richter, 1970). On the other hand, the aggregate form of the antigen must probably be processed by the macrophage (Pathway I) before it can function antigenically. However, the ARC reacts with the macrophage-processed antigen not by becoming tolerant but by becoming an activated immunocompetent cell. Pathway III may be observed with the thymus (or ARC)-independent antigens,

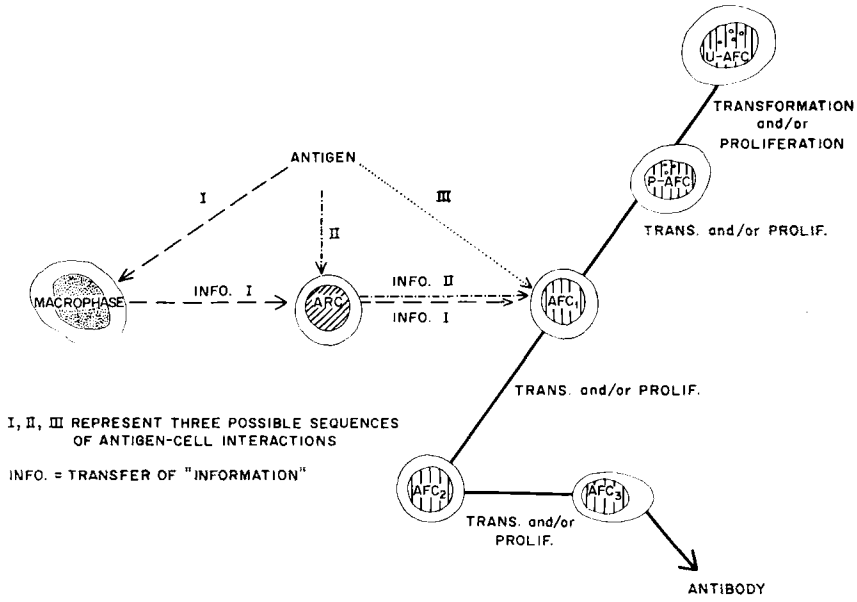

FIG. 3. The various antigen–cell and cell–cell interactions resulting in the primary humoral immune response. (U-AFC and P-AFC denote the undifferentiated and precursor states, respectively, of the antibody-forming cell; ARC, antigen-reactive cells.)

such as KLH, *Salmanella adelaide* flagellin, pneumococcal polysaccharide, ferritin, and anti-immunoglobulin serum (see Section IV,C). The interaction of the antigen with AFC_1 in any of the three pathways will render the AFC specific with respect to this antigen (AFC_2). Further differentiation of this latter cell will result in the terminal cell stage, AFC_3, which may be the plasma cell, which synthesizes and/or stores and/or secretes the antibody molecules.

Several considerations must be kept in mind before accepting as fact the synergistic effect of thymus and bone marrow cells in the immune response. Several antigens have been shown to be thymus-independent and do not require this type of synergism (see Section IV,C). Synergism has been uniformly observed in the experiments that involved the use of certain types of antigens (red cells and protein antigens) and the transfer of immunocompetent cells into heavily irradiated recipients. Radovich *et al.* (1968) transferred normal or immune spleen cells (4 days post-immunization) along with normal bone marrow cells and the original antigen, SRBC, to irradiated recipient mice. They also observed an enhanced immune response in the animals given the bone marrow and spleen cells as compared to those receiving spleen cells only. However,

they interpreted this synergistic effect of the bone marrow–spleen cell combination to be nonspecific, in the sense that the addition of bone marrow cells could enhance the immune response by providing hematopoietic precursors and, thus, in some way, prevent depletion of the precursors transferred with the immune spleen cells or, as stated by Radovich *et al.* (1968), affect in a nonspecific way the localization of antibody-forming cells in the spleen. However, the findings of Radovich *et al.* (1968) do not altogether support this assumption, since the administration of normal bone marrow cells with immune spleen cells and the antigen, SRBC, into irradiated allogeneic mice resulted in a markedly enhanced immune response as compared to that observed with recipients of immune spleen cells and SRBC only. On the other hand, the administration of normal bone marrow cells along with normal spleen cells and SRBC did not result in as enhanced a response as that observed with the transfer of normal spleen cells and SRBC only. An alternative explanation to the "nonspecific" role of the bone marrow offered by Radovich *et al.* (1968) might be that the bone marrow is supplying both hematopoietic and AFC precursors (Table I), each of which is capable of proliferating into cells of the other cell line. In time of stress, such as after irradiation, proliferation along the hematopoietic and leukopoietic cell lines will predominate. Since Miller and Mitchell (1967) observed that interaction with antigen and maturation of the thymic ARC may take as long as 7 days before the bone marrow AFC participates in the immune response, it is probable that very few AFC may be available from the transferred normal bone marrow. However, if the normal bone marrow is transferred to the irradiated recipient at a time following the ARC-reactive stage [or as Radovich *et al.* (1968) did, by transferring normal bone marrow cells and immune spleen cells], then mouse bone marrow stem cells will be stimulated by the activated ARC to differentiate into AFC rather than hematopoietic cells thus allowing for a more marked immune response.

Absence of bone marrow–thymus synergism has been observed by Craddock *et al.* (1967) using steroid-treated animals as recipients. Their findings suggest that the irradiated recipient animals, as opposed to steroid-treated recipients, are depleted of more cell types than are required for the successful mediation of the immune response and that one of these cells is probably provided by the transferred bone marrow. Furthermore, this cell would not appear to be lost in the steroid-treated animal.

B. Cell Interactions in Cell-Mediated Immune Reactions

An *in vitro* model system simulating the *in vivo* thymus–bone marrow interaction has been described by Globerson and Auerbach (1967).

Sublethally irradiated mouse spleen organ cultures required the presence of normal bone marrow cells for lymphopoiesis to occur, which was enhanced by the presence of thymus cells. Thymus and lymph node cells, in the absence of bone marrow cells, failed to induce lymphopoiesis in the irradiated mouse spleen cultures. Splenomegaly secondary to GVHR was observed when the spleen slices were grown for 2 to 3 days in the presence of thymus tissue but not when grown in the presence of a variety of other tissues (liver, kidney, and spleen). This thymic activity was demonstrated to be mediated by a humoral factor (Trainin *et al.*, 1969). When spleen slices were exposed to lethal doses of irradiation, reactivation of immuncompetence did not occur unless *both* thymus and bone marrow cells were present. Using the same *in vitro* graft-versus-host system, Umiel *et al.* (1968) found that embryonic liver cells or thymus cells were incapable of inducing splenomegaly. A combination of these two types of cells, however, was successful in inducing splenomegaly. Based on these findings, a model has been described by Talmage *et al.* (1969) in which it is assumed that the passively sensitized thymus-derived cell, which does not adhere to glass *in vitro* (nonadherent cell) is responsible for the specificity of the cell-mediated immune reaction and that its presence *in vitro* enhances the interaction between an immunocompetent adherent cell and an immunocompetent bone marrow nonadherent cell, all of which are required for the reaction to ensue.

C. Postulated Mechanisms of Cell-to-Cell Interactions

The interaction of two or more cell types is presumably required for the induction of the primary response to certain types of antigens (see Section IV,C). The interaction of the thymic ARC with the bone marrow AFC in the mouse is a requisite for the successful induction of the humoral immune response. How and where does this interaction take place? Is there a need for a third cell type? These are two questions that have not as yet been answered with respect to different types of antigens and different types of immune responses.

If the function of the macrophage is to process a highly immunogenic form of the antigen, how does the latter get transmitted to the ARC, which is the cell which presumably participates in the immune response following the macrophage? Either the immunogen is released into the circulation by the macrophage or it is transferred directly from the macrophage to another macrophage or to the ARC. This material may then be secreted by the ARC into the circulation where it will interact with the potential AFC; or the processed antigen may adhere to the surface of the ARC which itself migrates to the organ(s) containing the AFC, where it interacts physically with AFC, resulting in the passage of specific information (Richter, 1970).

Cytoplasmic bridges have been observed to occur between rabbit peritoneal cells *in vitro* (Aronson, 1963). Unanue *et al.* (1969) have observed that antigens stick to and remain on the surface of normal macrophages and speculated that these macrophage-bound antigen molecules react with specific antibodylike receptors present on the ARC, thus bringing the macrophage into close proximity with the ARC. Sharp and Burwell (1960) and Schoenberg *et al.* (1964) have, in fact, observed cytoplasmic connections between macrophages and lymphocytic cells in immune lymph nodes *in vivo*. The frequency of such interactions appeared to increase after antigenic stimulation. McFarland *et al.* (1966) have described lymphocytes interacting with macrophages by means of a cytoplasmic projection called a "uropod" in MLC. Maclaurin (1969) has demonstrated cytoplasmic bridging between normal macrophages in culture in the presence of phytohemagglutinin and between immune macrophages in the presence of the antigen, tuberculin. Richter and Naspitz (1968b) observed aggregates or rosette formation in long-term phytahemagglutinin-stimulated cell cultures composed of a central mononuclear macrophagelike cell surrounded by lymphocytes of varying sizes.

VI. Effects of Irradiation on the Immune Response[3]

The effects of irradiation on the immune response, both *in vivo* and *in vitro*, may vary according to the type, dose, and rate of irradiation used and the type of antigen. Although 750 r *in vivo* irradiation does not inactivate peritoneal exudate cells which have already taken up the antigen, a suppressive effect was described if this dose of irradiation was administered prior to the uptake of the antigen *in vivo* (Gallily and Feldman, 1967). Macrophages in mice subjected to 200 to 550 r total-body irradiation are, however, capable of taking up ingested antigen *in vivo* (Mitchison, 1967) and of taking up an aggregated protein antigen, BGG, *in vitro* (Pribnow and Silverman, 1967, 1969). In fact, irradiation of macrophages *in vitro* at doses up to 50,000 r did not prevent their capacity to engulf opsonized SRBC *in vitro* (Perkins *et al.*, 1966). Pribnow and Silverman (1967, 1969) could succesfully transfer specific antibody-forming capacity to sublethally irradiated (550 r) immunoincompetent mice by the transfer of macrophages preincubated *in vitro* with the antigen, aggregated BGG. Similar results were reported by H. Gershon and Feldman (1968) in mice with respect to *Salmonella paradysenteria* antigen. On the other hand, they (H. Gershon and Feldman, 1968) could not reconstitute the immune response to SRBC in this way. Taliaferro *et al.* (1964) observed that 500 r total-body X-irradiation of

[3] The subject matter in this section has, in large part, been contributed by Dr. Leo Yaffe, Chairman, Department of Chemistry, McGill University, Montreal, Canada.

immunized rabbits resulted in inhibition of the hemolysin response provided the antigen was not given more than a few hours following irradiation. However, Abdou and Richter (1969a) observed that 800 r was the dose of ^{60}Co irradiation which was required to depress the immune response in the normal adult rabbit.

Furthermore, it would appear that results of *in vivo* total-body irradiation are not related, in a simple way, to results of *in vitro* irradiation. Kasakura and Lowenstein (1968) and Daguillard and Richter (1970b) observed that 4000 r ^{60}Co irradiation *in vitro* was required to inactivate human and rabbit lymphocytes, respectively, with respect to their blastogenic response to stimulation with mitomycin-C-inactivated allogeneic and xenogeneic lymphocytes, and 6000 r *in vitro* irradiation was required to inhibit completely the phytohemagglutinin-induced blastogenic response (Kasakura and Lowenstein, 1967). The findings of Vann and Makinodan (1969) that immune mouse spleen cells could still respond with antibody formation following 10,000 r X-irradiation *in vitro* are supported by similar findings made by Daguillard and Richter (1970) with rabbit lymphoid cells. Why such a high dose of irradiation (4000 r and over) is required *in vitro* to inactivate normal, previously nonstimulated lymphoid cells (Abdou and Richter, 1970a), whereas exposure of the animal to a much lesser amount of irradiation (800 r) (Abdou *et al.*, 1969) will result in inhibition of the immune response, cannot be ascertained at the present time.

It is extremely important, in the intercomparison of effects obtained as a result of irradiation, to insure that all the variables have been kept constant or taken into consideration so that the results are truly comparable. Some of the variables are listed below.

1. *Units* in which the irradiation has been measured. Results are quoted in the literature in roentgens, rems, and rads. The roentgen or "rep"—roentgen equivalent physical—is defined as "the amount of radiation that will produce, in 0.001293 gm. of dry air at S.T.P., 1 e.s.u. of charge of positive ions and 1 e.s.u. of negative ions." This, when multiplied by a factor that takes account of the efficiency of the radiation in acting on mammalian tissue, is known as the "rem"—roentgen equivalent mammalian. For example, X-rays will cause 20 times as much ionization in tissue as will γ-particles and this must be taken into account. The "rad" is the unit most hightly recommended because it is defined simply as 100 ergs of energy deposited per gram of material and is independent of the medium or particle causing ionization.

2. *Type of radiation.* Even though the radiation dose may be measured in rads and thus be seemingly independent of the type of radiation, yet the type cannot be neglected. For example, 400 rads of whole-body

radiation with α-rays will affect only the skin of the animal initially, due to the low penetrating power of the α-rays. The same dosage with high-intensity γ-rays would produce a genuine whole-body effect. The same is true if one irradiates with low-intensity X-rays and attempts a comparison with an irradiation with high-energy γ-rays such as those from ^{60}Co.

3. *Time of irradiation.* Care should be taken to see that cumulative doses are really comparable. For example, a 10-hour irradiation at a certain dose rate may, physiologically or chemically, not be the equivalent of a 1-hour irradiation at 10 times the former dose rate.

VII. Cells Involved in Cell-Mediated Immunity

A. Mechanism of Antigen Recognition

As was discussed in Section IV,A, the recognition of the antigen by immunocompetent humoral-antibody-forming cells in a number of animal species is effected through the interaction of the antigen with an immunoglobulin molecule or fragment possessing antibodylike properties on the surface of the cell. In the normal unimmunized rabbit, the recognition of the antigen is a unique property of the ARC, which is incapable of synthesizing antibody (Richter and Abdou, 1969). In the immune animal, antigen recognition is a property of the AFC or memory cell (Wigzell and Andersson, 1969; Daguillard and Richter, 1970b). The findings in the normal animal are in keeping with the concept of clonal selection, which implies that there exist in the normal unimmunized animal cells precommitted to interact with any type of antigen (Burnet, 1957, 1962; Jerne, 1955). Obviously, the number of such cells precommitted with respect to any particular antigen would have to be small. Such, indeed, appears to be the case. Results of a number of investigations, with both rabbits and mice, place the number of specific precommitted cells (ARC) to be between 1 per 1,000 to 1 per 50,000 lymphoid cells (Abdou and Richter, 1969b; Ada and Byrt, 1969; Sulitzeanu and Naor, 1969; Naor and Sulitzeanu, 1967).

A similar mechanism appears to be operating in cell-mediated hypersensitivity and transplantation immunity. Incubation of human circulating lymphocytes with a submitogenic concentration of rabbit antihuman light-chain antiserum results in suppression of the blastogenic response induced by tuberculin and allogeneic leukocytes (mixed leukocyte reaction) (Greaves *et al.*, 1969). This would indicate that the cells participating in cell-mediated immunity possess on their surface a light chain or an Fab monomer which acts as a receptor site. Talmage *et al.* (1969) have postulated a model in which they attribute the recognition of the

antigen in cell-mediated immunity to a cytophilic antibody present on the surface of the reactive cell.

The clonal selection theory does not, however, appear to be applicable to an understanding of the cellular events culminating in the cell-mediated (GVHR, delayed hypersensitivity reaction) immune reaction. It has been demonstrated that the percentage of lymphoid cells capable of inducing a GVHR or of participating in the MLC-induced blastogenic reaction is much greater than can be anticipated on the basis of randomized clonal selection and is much greater than the figures cited above with respect to the humoral immune response. As many as 1–3 out of every 100 lymphoid cells appear to be capable of initiating and/or participating in the cell-mediated immune reaction induced by any one specific antigen (Nisbet *et al.*, 1969; Wilson *et al.*, 1968; Simonsen, 1967). These apparently contradictory findings (humoral versus cellular immunity) suggest that the cell mechanisms mediating the two types of immune responses are different, both qualitatively and quantitatively. Richter *et al.* (1970b) have, in fact, demonstrated that the cells in the rabbit that participate in the host-versus-graft reaction are functionally different from those that mediate the humoral immune response in that the former response does not require the ARC. It would, therefore, appear that the ARC is a unipotential cell, capable of interacting with one antigen only, whereas the comparable cell mediating the cellular immune reaction, if it functions in a manner similar to the ARC in humoral immunity, would appear to be pluripotential.

B. Graft-versus-Host and Transplantation Rejection Reactions

Lymphocytes are the effector cells in the GVHR reaction (reviewed in Gowans and McGregor, 1965; Meuwissen *et al.*, 1969a). Dicke *et al.* (1968, 1969) have conclusively shown, by cell fractionation studies, that lymphocytes are the cells responsible for the GVHR in the mouse and monkey. Mouse spleen or monkey bone marrow cells were fractionated on a discontinuous albumin gradient and the hematopoietic capacity and GVHR activity of the cell fractions obtained were studied in lethally irradiated allogeneic recipients. A cell fraction rich in blast cells showed a ten-fold increase in the concentration of CFU (index of hematopoiesis) and a more than tenfold decrease in GVHR activity as compared to the original cell preparation. No secondary disease was observed in recipients that received this fraction. A second fraction, composed mainly of lymphocytes, was very poor in reconstituting hematopoietic activity but was very active in inducing GVHR. Shortman and Szenberg (1969), using fowl peripheral leukocytes, have shown that a minor population of lymphocytes are the active cells in the GVHR. The active cells, however,

were not found to be physicochemically homogeneous since they could be obtained as a series of peaks upon fractionation in a density gradient.

The source of cells responsible for graft rejection is unsettled. Although the thymus in the mouse is essential for the induction of cellular immunity (Miller and Osoba, 1967), its role in the development of the GVHR is controversial. R. L. Simmons *et al.* (1965) have demonstrated that the GVHR induced in lethally irradiated thymectomized mice with allogeneic bone marrow cells was as intense as the GVHR induced in nonthymectomized irradiated hosts. Field and Gibbs (1965), however, have shown that thymectomy increases the susceptibility of F_1 hybrid rats to GVHR induced by the intraperitoneal injection of parental strain spleen cells. Although spleen cells taken from normal donors were shown to be effective in inducing GVHR, spleen cells taken from adult mice which had been thymectomized at birth could not induce this reaction, suggesting a thymic origin for the GVHR cell (Dalmasso *et al.*, 1963). In contrast, ablation of the bursa of Fabricius in the chicken does not decrease the capacity of the circulating lymphocytes to exert a GVHR (Warner, 1965).

The results of other investigations are conflicting since it has been observed that thymocytes are both highly effective (M. W. Cohen *et al.*, 1963; Stutman *et al.*, 1968) and poorly effective (Billingham and Silvers, 1964) in inducing GVHR in appropriate recipient hosts. In systems where thymus cells were found to be effective in inducing GVHR, no synergism was observed between thymus and bone marrow cells since the addition of bone marrow cells to thymus cells did not render the latter more efficient in inducing GVHR in the F_1 host (Stutman and Good, 1969). However, results of an opposite nature demonstrating bone marrow–thymus synergism have recently been obtained by Argyris (1969b).

Willard and Smith (1966) studied the capacity of syngeneic transplanted mouse lymphoid cells to reject allogeneic bone marrow cells in irradiated recipients. The following decreasing order of effectiveness of the transferred cells was observed: leukocytes, lymph node cells, spleen cells, and peritoneal exudate cells. Thymocytes and marrow cells were not effective. The failure of the syngeneic bone marrow to reject the allogeneic marrow in the lethally irradiated mouse could have been due to proliferation of the hematopoietic cells in the transferred syngeneic marrow along erythropoietic cell lines rather than along immunocompetent cell lines or to the absence of mature immunocompetent cells capable of mediating the GVHR reaction. Although bone marrow cells by themselves are incapable of inducing the GVHR (Billingham and Silvers, 1964; Stutman and Good, 1969; Dicke *et al.*, 1969) the lymphoid cells that are capable of inducing GVHR have been shown to be derived from

bone marrow (Goldschneider and McGregor, 1968; McGregor, 1968; Tyan and Cole, 1965). These bone marrow-derived lymphocytes were shown to be present in large numbers in the spleen (Tyan and Cole, 1965), lymph nodes (Billingham and Silvers, 1964), and thymus (Sosin *et al.*, 1966; Stutman and Good, 1969). In the rat, it has been shown that the cells responsible for GVHR are bone marrow-derived but mature elsewhere (McGregor, 1968). Thoracic duct cells were obtained from F_1 hybrid rats (intermediate host) which had been inoculated at birth with parental strain bone marrow cells. The thoracic duct cells were then transferred into a second F_1 hybrid recipient of a different genotype. A GVHR was regularly observed in these latter recipients. However, the capacity of the thoracic duct cells of the F_1 intermediate host to transfer GVHR was diminished if spleen cells and not bone marrow cells were initially injected into the intermediate host. Thoracic duct cells from normal uninoculated F_1 hybrids failed to give the reaction in the recipients. However, the bone marrow is not the source of *mature* GVHR cells since it is less potent than the thoracic duct cells in transferring GVHR to F_1 hybrids. Furthermore, thoracic duct cells from rats injected neonatally with bone marrow cells obtained from lymphocyte-depleted adult donors were as effective as thoracic duct cells obtained from rats injected neonatally with bone marrow cells obtained from normal untreated donors in transferring the GVHR to other recipients (McGregor, 1968). This would indicate that it is the bone marrow cell and not the circulating small lymphocyte or the lymphocyte residing in the peripheral lymphoid tissues which can best transfer GVHR in the rat. In the mouse, Tyan and Cole (1965) were able to show that a significant number of deaths occurred among F_1 hybrid hosts when they had received spleen cells from parental mice which had been injected with chromosomally marked, adult, bone marrow cells. Spleen cells from mice that had not been injected with the marrow or had been injected with thymus cells only were less effective.

C. The Delayed Hypersensitivity Reaction

The successful induction of the delayed hypersensitivity reaction appears to require the interaction of two cell types in the rat—a thymic-derived cell for the initial sensitization step(s) and a bone marrow-derived cell which participates in the subsequent cellular infiltrative reaction (Lubaroff and Waksman, 1967, 1968a,b). The involvement of the thymus or of the thymus-derived cells in the delayed hypersensitivity reaction is illustrated by demonstrating the effects of neonatal thymectomy. In the rat, neonatal thymectomy prior to sensitization with the antigen inhibits the subsequent delayed hypersensitivity reaction. On the

other hand, thymectomy of adult Lewis rats followed by sensitization with tubercle bacilli does not inhibit their ability to develop delayed skin reactions (Jankovic *et al.*, 1962; Miller *et al.*, 1962). Neonatally thymectomized animals can become sensitized if they are injected with sensitized syngeneic lymphoid cells prior to challenge. These data indicate that the thymus is required for the active induction of cellular immunity but not for expression of the infiltrative reaction.

The existing evidence strongly suggests that the majority of cells infiltrating the site of the delayed hypersensitivity reaction are not actively sensitized cells, but rather are circulating cells which tend to accumulate, in a random fashion, at the site of the lesion (McClusky *et al.*, 1963; Turk and Oort, 1963; S. Cohen *et al.*, 1967; Najarian and Feldman, 1963a,b). In all of these studies, sensitized radioactively labeled lymphoid cells were transferred to normal recipients or recipients sensitized with a different antigen, in whom lesions accompanied by cell infiltrates were induced by the injection of the specific (antigen used to sensitize the cell donor) and nonspecific (recipient-specific) antigen. On the basis of radioautographs of biopsy sections or radioactive analyses of tissue specimens, it is generally agreed that the majority of the mononuclear cells infiltrating the specifically sensitized sites are "nonsensitized" cells. These cells are phagocytic and resemble macrophages that appear in sites of nonspecific inflammation (Volkman and Gowans, 1965). The experiments of Lubaroff and Waksman (1968a,b) demonstrate that the successful transfer of tuberculin hypersensitivity with sensitized lymph node cells to thymectomized irradiated recipients depends on the simultaneous or prior injection of normal bone marrow cells. Normal thymus, spleen, lymph node, or peritoneal exudate cells, even at high doses, could not be substituted for the bone marrow in producing the tuberculin reaction. These experiments indicate that once sensitization has occurred, a bone marrow cell and not a thymic cell is required for the manifestation of the delayed hypersensitivity reaction. The precise origin of the cells infiltrating the skin was investigated by the administration of allogeneic bone marrow to the thymectomized irradiated rats prior to the administration of the sensitized lymph node cells. Fluorescein-conjugated antiserum against the cells of the bone marrow donor and recipient was then applied to the biopsies of the sites of skin reaction. The majority of the cells were shown to be derived from the infused (donor) marrow. The relative percentages of marrow-derived and lymph node-derived cells in the tuberculin reaction remained the same during the 9–24-hour period following the skin test (Lubaroff and Waksman, 1968b).

Histological analyses (Spector, 1967) and studies with labeled cells suggest that a similar mechanism is involved in other reactions similar to

the tuberculin-induced reaction, such as autoallergic lesions (Kosunen *et al.*, 1963), the skin homograft reactions (Prendergast, 1964), disseminated lesions of adjuvant arthritis (Burstein and Waksman, 1964), and contact allergy (McClusky *et al.*, 1963). In the latter, Liden (1967) has shown that bone marrow cells contribute to the formation of the mononuclear infiltrate at skin sites of allergic contact dermatitis induced by dinitrochlorobenzene in the guinea pig. Local *in situ* labelling of the bone marrow cells of the experimental animal with tritiated thymidine results in their emigration from the bone marrow and their accumulation in the skin lesion. The labeled cells were also detected in the regional lymph nodes and in nodes at sites unrelated to the area of sensitization (Liden and Linna, 1969), thus indicating the nonspecific nature of the bone marrow participation in this reaction. The demonstration of participation of two cell types in contact allergy was confirmed morphologically by Davies *et al.* (1969). They described a biphasic response in the regional nodes of mice painted on the skin with oxazolone. An initial paracortical proliferative response of thymus-derived cells was followed by medullary hyperplasia and with germinal center formation, composed mainly of bone marrow-derived cells.

VIII. Cells Affected in Immunological Tolerance

Immune tolerance is considered to be due to a depletion of immunocompetent cells specifically reactive to the tolerogenic antigen or to an altered reactivity of these cells so that they can no longer recognize the antigen (reviewed in Dresser and Mitchison, 1968). The failure to respond with antibody formation could be due to either interference with the access of antigen to the reactive cells (afferent limb of the immune response) or interference with the synthesis and release of antibody by the AFC (efferent limb of the immune response). Evidence for failure at the cellular level of the immune response has been shown by several investigators (Billingham *et al.*, 1956; Weigle and Dixon, 1959; Friedman, 1962; Battisto and Chase, 1963; Sercarz and Coons, 1963b). It has been demonstrated that the immune response can be induced in the immunoincompetent host by the transfer of cells of normal lymphoid tissues (lymph node, thymus, or spleen cells) (Brooke and Karnovsky, 1961) and that lymphoid tissues from the tolerant donors fail to give a response in irradiated recipients (Mitchison, 1963; Martinez and Good, 1963; McGregor *et al.*, 1967), thus demonstrating clearly that the immunoincompetent cell in the paralyzed animal is the small lymphocyte. McCulloch and Gowans (1967) have shown that populations of small lymphocytes from the thoracic duct of rats may show full immune reactivity or partial or complete tolerance toward either histocompatibility

antigens or sheep erythrocytes, depending on the immune status of the cell donor with respect to these antigens. Since thoracic duct lymphocytes contain both ARC and AFC, it cannot be concluded from this study which type of cell is tolerant in the immune tolerant state. However, recent studies, using *Salmonella adelaide* flagellar antigen (Armstrong *et al.*, 1969), SRBC (R. K. Gershon *et al.*, 1968; Many and Schwartz, 1969), BSA (Taylor, 1968), BGG (Isakovic *et al.*, 1965; Staples *et al.*, 1966), and tumor antigens (Abdou and McKenna, 1968) have shown that the thymic ARC is the site of the lesion in the immune tolerant state in the mouse and rat. This conclusion is based on the fact that the thymus in these animals is the source of the ARC and in the failure of the transferred "tolerant" thymus cells to mediate an immune response in an immunoincompetent irradiated or neonatal host with respect to the tolerogenic antigen although the response to other antigens is normal. However, it may be that the tolerant donor thymus is not tolerant due to any effect on the thymic ARC; rather the tolerance may be attributed to the ARC having vacated the thymus following interaction with the antigen, much in the same way as the rabbit bone marrow ARC has been considered to vacate the bone marrow 8–48 hours following interaction with the antigen *in vivo* (Abdou and Richter, 1969a,c).

Recently, however, Playfair (1969) has shown that bone marrow cells obtained from adult mice made tolerant to SRBC through the combined injection of SRBC and cyclophosphamide were unable to transfer immunocompetence to irradiated recipients, even when transferred along with normal thymic cells. This finding suggests that the ARC is not the cell affected in drug-induced tolerance or that its organ of habitation may be altered as a result of the administration of the cyclophosphamide.

Abdou and Richter (1969c) have demonstrated that rabbits made tolerant at birth to HSA or BGG could be rendered immunocompetent with respect to the tolerogenic antigen by the administration of normal bone marrow ARC. These results demonstrate that the AFC is unaffected in the tolerant animal. Moreover, bone marrow cells taken from tolerant rabbits failed to transfer immunocompetence to irradiated immunoincompetent rabbits, in whom the ARC had been inactivated by the irradiation but which still possessed the normal complement of AFC (Richter and Abdou, 1969). Since the AFC is still capable of responding to the tolerogenic antigen, provided normal allogeneic ARC are injected into the animal, it would appear that the lesion in the immune tolerant state is at the level of the ARC (Fig. 4). Whether the ARC are, in fact, still present in the host in an inactive state or whether they no longer exist cannot be ascertained from existing data. However, our data (Abdou and Richter, 1969c) permit for an understanding for the failure

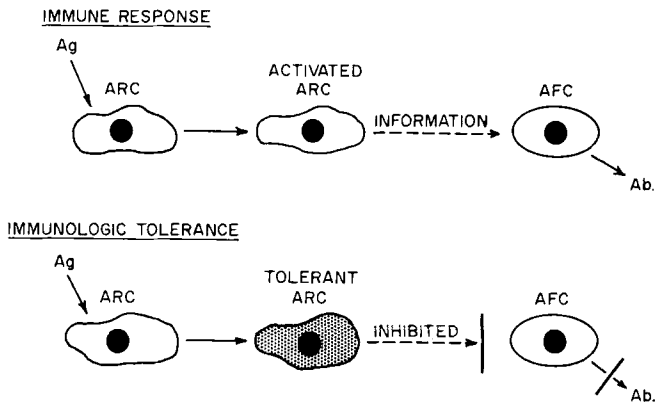

FIG. 4. Cellular interactions resulting in the induction of the immune response and immunological tolerance. (ARC—antigen-reactive cells; AFC—antibody-forming cells; Ag—antigen; Ab—antibody.)

of Sercarz and Coons (1963a,b) to detect AFC in the tolerant animal. Although the AFC is capable of responding to the antigen, it never gets the "message" since the block occurs at a stage prior to that of AFC function (Fig. 4).

That the macrophage is not the tolerant cell has been shown by the studies of Mitchison (1969) and G. Harris (1967) who observed that macrophages obtained from peritoneal exudates of tolerant animals can take up the tolerogenic antigen and can initiate a specific immune response in both tolerant and normal recipients. Macrophages from paralyzed mice were as active as normal macrophages in generating PFC *in vitro* when allowed to interact with suspensions of lymphocytes obtained from spleens of normal syngeneic mice (Forbes, 1969).

Self-recognition can be altered by contact of bone marrow cells with allogeneic red cells (Uphoff, 1969a,b). Lethally irradiated mice failed to accept syngeneic skin grafts if given syngeneic bone marrow that had been incubated *in vitro* with allogeneic erythrocytes. Moreover, the *in vitro* incubated bone marrow could protect the allogeneic but not the syngeneic host against the lethal effect of irradiation and was unable to induce secondary disease in the allogeneic host (the red cell donor)—a disease induced in 100% of irradiated mice injected with allogeneic bone marrow cells not incubated *in vitro* with erythrocytes. These results suggest that, during the *in vitro* incubation of the allogeneic erythrocytes with the bone marrow cells, the latter are modified in such a way that they no longer recognize the allogeneic strain as foreign nor the syngeneic strain as self. However, in view of the unique nature of these findings and the absence of confirmatory findings using this and other

antigens, the significance and interpretations of these findings may be presumptuous at this time.

The mechanism whereby immunological tolerance is induced must surely be related to the fact that the ARC and the AFC are two separate cell entities. This "division of labor" permits for the exclusion of ARC of a particular specificity without affecting the general immune responsiveness of the host. On the assumption that ARC exist early in fetal life, their interaction with autologous antigens to which they may be committed ("forbidden clones") may result in death of this cell population, much in the same way that fetal exposure to antiallotype serum results in subsequent failure of the immunologically mature adult rabbit to synthesize immunoglobulin of this particular allotype (Marcuson and Roitt, 1969, Mage and Dray, 1965; Lummus *et al.*, 1967). The ease with which immunological tolerance may be accomplished in prenatal and neonatal life, as compared with the difficulty encountered in attempting to induce tolerance in adult animals (Smith, 1961; Dresser and Mitchison, 1968), may be attributed to the absence of scarcity of functioning macrophages in the fetus and neonate. It has been demonstrated that the transfer of peritoneal exudate cells into isogeneic neonatal recipient mice results in a markedly enhanced immune responsive state, thus suggesting that the immunoincompetence in the neonatal state may be attributed more to a deficiency of macrophages than to an immature lymphoid cell system (Argyris, 1969a). It would, therefore, not be too presumptuous to assume that the fetus and neonate are devoid of functional macrophages, the function of which is considered to be that of antigen trapping and processing, resulting in the release of highly immunogenic forms of the original antigen. It is considered that interaction of the ARC with the native antigen results in the induction of a tolerant state in this cell, whereas interaction of the ARC with the macrophage-processed form of the antigen sets off the intra- and intercellular reactions culminating in antibody formation (Richter, 1970). In the absence of a functional macrophage population of cells, as occurs in the fetus and neonate, the native antigen can react directly with the ARC, thus rendering it permanently incompetent or resulting in cell death. In the immunologically mature animal, on the other hand, the presence of the macrophages would tend to "protect" the immunocompetent ARC from the native antigen (Nossal *et al.*, 1966; McKhann, 1969).

Recent findings using nonphagocytizable antigens support the above interpretation as to the role of the macrophage. Aggregate-free preparations of HGG or BSA, obtained by ultracentrifugation or gel filtration of the "native" antigen or filtration through the reticuloendothelial system of an intermediate animal (Biro and Garcia, 1965; Frei *et al.*, 1965, 1968),

induce a state of tolerance rather than immunity following their injection into adult recipients. The induction of tolerance in the adult animal is probably facilitated by the bypass of the macrophage, resulting in direct interaction of the native antigen with the ARC (Abdou and Richter, 1970b).

IX. Bone Marrow Transplantation—Application

The transplantation of bone marrow for the treatment of various blood dyscrasias in animals was first attempted in the late nineteenth century (referred to in Murphy, 1914). The benefits derived from it were rather equivocal. The concept of hematopoietic tissue transplantation as a postirradiation therapeutic measure has, however, endured. Shielding of the exteriorized spleen or implantation of hematopoietic tissue (infant spleen) in mice subjected to lethal irradiation results in enhanced survival (Jacobson *et al.*, 1951). Rekers and co-workers (1950) attempted marrow transplantation in irradiated dogs and obtained slightly favorable results as reflected by small differences in mortality and hematological responses between experimental and control animals. Lorenz and co-workers (1952) demonstrated the protective effects of the postirradiation injection of syngeneic bone marrow in 70 to 95% of lethally irradiated mice and guinea pigs. They subsequently extended their studies to show that allogeneic and xenogeneic bone marrow transplants in irradiated mice are less effective if compared to syngeneic marrow transplants.

By using markers, it was established that the transplanted bone marrow is accepted by the immunoincompetent host so that the recipient's hematopoietic cells became of donor type. Among the markers used are chromosome markers in man (Bach *et al.*, 1968; Gatti *et al.*, 1968) and mouse (Carter *et al.*, 1955), differences in karyotype between mouse and rat and between normal mice and those with a visibly abnormal chromosome (C. E. Ford *et al.*, 1956), blood group antigens (Lindsley *et al.*, 1955; Makinodan, 1956), histocompatibility antigens (Mitchison, 1956), histochemical differences in alkaline phosphatase activity of mouse and rat granulocytes (Nowell *et al.*, 1956), and the differences in hemoglobin concentration of progeny of stem cells of donor and recipient origin (Popp *et al.*, 1958). These investigations all demonstrated that the donor bone marrow can populate and replace the recipient's blood elements.

These initial results provided an impetus for more extensive investigations involving bone marrow transplantation in animals and man for correction of abnormal hematopoiesis or various immune deficiency syndromes. Congenital macrocytic anemia of strain WW^v mice could be

corrected by the administration of hematologically normal allogeneic (DBA/H T_6T_6) hematopoietic cells. By studying the anemic mice for red cell hemoglobin and bone marrow cells for the chromosome marker, it was shown that the hematopoietic system of the anemic mice was totally replaced by the donor stem cell (Seller and Polani, 1966, 1969). Cases of aplastic anemia in human twins could be cured by bone marrow infusion from the twin donor (Robins and Noyes, 1961; S. D. Mills *et al.*, 1964; Thomas *et al.*, 1964). Bone marrow transplants have also been used with variable degrees of success in radiation sickness, overdosage of immunosuppressants (reviewed in Loutit, 1965), and in some cases of leukemia (reviewed in Mathé, 1960; Mathé *et al.*, 1965) in animal and man. Successful treatment of leukemia and lymphoma by lethal irradiation or massive doses of immunosuppressants followed by infusion of syngeneic marrow cells in animals and man have been reported (Atkinson *et al.*, 1959; Thomas *et al.*, 1959; Floersheim, 1969). Allogeneic bone marrow transplants have proved to be unsuccessful (reviewed in Mathé, 1968).

In the combined humoral and cellular immune deficiency syndrome in man (lymphopenic or Swiss-type hypogammaglobulinemia), it has been postulated that the defect is at the stem cell level. Since graft rejection is impaired in these patients, attempts at restoration of immune competence by grafting closely matched bone marrow cells have been successful (Gatti *et al.*, 1968; Meuwissen *et al.*, 1969b; de Koning *et al.*, 1969). To avoid the risk of GVHR, special treatment was given to donor bone marrow lymphocytes either by incubating the marrow cells at 37° C. for 2 hours in the presence of antilymphoblast serum (Meuwissen *et al.*, 1969b) or by fractioning the bone marrow cells in albumin gradient and infusing cells devoid of their small lymphocyte content (de Koning *et al.*, 1969). These types of treatments were shown to reduce the number of immunocompetent cells in the transferred marrow (Mathé *et al.*, 1966; Najarian *et al.*, 1969; Dicke *et al.*, 1968). A case of Wiscott-Aldrich syndrome has also been successfully treated with a bone marrow transplant. Evidence of a chimeric state was detected a few weeks following the transplant (Bach *et al.*, 1968).

There are certain risks in transplanting bone marrow to immunoincompetent recipients. Graft-versus-host reaction is frequently seen after the establishment of an allogeneic chimera due to interaction of immunocompetent cells of the grafted marrow with the host tissues, which contain transplantation antigens absent in the cell donor. The GVHR is characterized by wasting, diarrhea, skin rash, fever, splenomegaly, and hepatomegaly (reviewed in Simonsen, 1962; Mathé *et al.*, 1967; Kretschmer *et al.*, 1969; Hathaway *et al.*, 1965; Uphoff and Law,

1959). Aplasia of lymphatic tissue and aplastic anemia are also considered to be part of the GVHR directed toward both the stem cell and lymphoid tissues of the recipient (Meuwissen *et al.*, 1969a). Syngeneic chimeras, on the other hand, do not develop the disease (Barnes *et al.*, 1962, 1964; Bach *et al.*, 1968; Meuwissen *et al.*, 1969b).

In lethally irradiated mice, lymphoid aplasia could be prevented by the transfer of syngeneic fetal liver cells or syngeneic adult marrow given in suboptimal amounts or after multiple passages of the marrow in lethally irradiated mice (Barnes *et al.*, 1962). The lymphoid tissue aplasia could, however, be prevented in irradiated mice by the transplantation of a mixture of syngeneic lymph node cells and a suboptimal number of marrow cells indicating that the latter cell preparation was deficient in lymphopoietic precursor cells.

Bone marrow grafting would appear to open a new approach in the treatment of otherwise fatal immune and hematopoietic deficiency diseases. The advances in our knowledge concerning the role of the bone marrow in the maturation of immunocompetence will certainly also prove to be of immense value in the treatment of a wide variety of diseases.

X. Conclusions

The objectives of this review were to summarize, interpret, and evaluate the various experimental findings related to the source and function of the various immunocompetent cells, to relate the functions of these cell types with respect to the induction of the humoral and cell-mediated immune responses, to evaluate the role of the bone marrow as a source of and/or as an influence upon stem cells and immunocompetent cell precursors, and to present a scheme of cellular interactions culminating in antibody formation which incorporate the findings of all investigations to date in a variety of animal species. The scheme presented specifies multicellular pathways, composed of a number of highly specialized cells, rather than the single cell pathway characterized by a single, pluripotential immunocompetent cell.

It has been demonstrated that the bone marrow is a source of either the ARC or the AFC or of both (the stem cell), depending upon the animal species investigated. Furthermore, it also appears to supply the macrophages as well as the cells that constitute the cellular infiltrations in cell-mediated immune reactions. The thymus–bone marrow axis has been discussed in detail since the thymus is considered to be a central lymphoid organ. In view of the role that the bone marrow plays in supplying and influencing the immunocompetent cells, it too must be considered to be a central lymphoid organ.

The schemes and concepts presented should not be viewed as depicting the complete or ultimate picture since other types of cell and/or mediators may be involved. We have, instead, presented a concept which is flexible, which stresses the dynamic rather than static nature of the various cell compartments participating in the immune response, and which is amenable to verification in the laboratory.

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Cell Interaction in Antibody Synthesis[1]

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I. Introduction

Immunologists have been prone to construct complex models of cell interaction and cell differentiation to explain the observations that they or their contemporaries have made on various features of the immune response (Wissler *et al.*, 1957; Ellis *et al.*, 1967; Makinodan and Albright, 1967; Sterzl, 1967; Makinodan *et al.*, 1969). The initial models were based on histological descriptions of the localization of injected antigen and led to the concept of cooperation between a macrophage, which digested and processed the antigen, and a cell, which responded to the modified antigen by making antibody (Fishman, 1959, 1961; Fishman and Adler, 1963, 1967; Adler *et al.*, 1966; Feldman and Gallily, 1967; Gallily and Feldman, 1967). This concept was supported by experiments in which extracts of cells previously exposed to antigen were shown to be capable of inducing antibody formation in normal lymphocytes (Cohen and Parks, 1964).

In the last few years it has been possible to demonstrate with separated cell populations that an interaction between two or more living cells was required for the antibody response to at least some antigens both *in vivo* (Claman *et al.*, 1966a,b, 1968; Claman and Chaperon, 1969; Davies *et al.*, 1966, 1967; Davies, 1969; Miller *et al.*, 1967; Miller and Mitchell, 1967a,b, 1968, 1969a,b; Miller and Osoba, 1967; Gershon *et al.*, 1968; Mitchell and Miller, 1968a,b; Nossal *et al.*, 1968; Radovich *et al.*, 1968; Talmage *et al.*, 1969a) and *in vitro* (Dutton and Mishell, 1967a,b; Mishell and Dutton, 1967; Mosier, 1967, 1969; Mosier and Coppleson, 1968; Raidt *et al.*, 1968; Pierce and Benacerraf, 1969; Roseman, 1969;

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Talmage *et al.*, 1969b). These findings have made it necessary to reappraise many of the widely accepted and basic concepts of immunology, such as the clonal selection theory, the role of the macrophage referred to above, and the difference between cells responding to the first and second injections of antigen.

In the present paper we shall first review the recent observations on the interactions of separate populations of cells from inbred mice and relate these to experiments with congenital dysfunction of the immune system in humans and surgical removal of the thymus in mice and the thymus and bursa in chickens. We shall then briefly review three related phenomena: antigenic competition, the carrier effect of multiple antigenic determinants, and the suppressive and enhancing effects of passively administered antibodies. Finally, we shall attempt to discuss briefly the more likely hypotheses which have survived.

II. Two Universes of Immunocompetent Cells

Birds have two clearly defined organs for differentiation of immunocompetent cells, the bursa of Fabricius and the thymus (Warner *et al.*, 1962; Warner and Szenberg, 1962, 1964; Szenberg and Warner, 1964; Warner, 1964). Surgical removal of one or the other of these organs from the newly hatched chick produces distinctly different deficiencies in immunological capacity. Loss of the bursa affects serum immunoglobulin levels, formation of secondary nodules in spleen and lymph nodes, and humoral antibody responses (Cooper *et al.*, 1965, 1966; Cain *et al.*, 1968, 1969; Meuwissen *et al.*, 1969a,b; Van Meter *et al.*, 1969). Early loss of the thymus produces a deficiency in delayed hypersensitivity and impairment of homograft rejection (Jankovich *et al.*, 1962; Aspinall *et al.*, 1963; Cooper *et al.*, 1968; Good *et al.*, 1968).

The conclusion that the role of the thymus concerns the differentiation of cells involved in delayed hypersensitivity is borne out in humans with congenital absence of the thymus (DiGeorge syndrome). These individuals have complete failure of delayed hypersensitivity, and their lymphocytes are not stimulated by phytohemagglutinin (August *et al.*, 1968; DiGeorge, 1968; Meuwissen *et al.*, 1969a,b). However, they may have normal immunoglobulin levels, secondary nodules in spleen and lymph nodes, and make vigorous antibody responses to certain antigens. On the other hand, individuals with sex-linked hypogammaglobulinemia have a normal thymus and delayed hypersensitivity (Bruton, 1952). Thus there appear to be two distinct universes of immunocompetent cells, one derived from the thymus and the other derived from the bursa of Fabricius in birds. In mammals the source of the second cell type is not established.

Although most immunologists would agree in general with the above statements, controversy develops over evidence for interaction between the two cell types. Neonatally thymectomized mice (and probably humans with DiGeorge syndrome) do not have a normal humoral response to all antigens. The response to sheep red cells and heterologous serum proteins is absent or reduced, whereas the response to bacterial and viral antigens appears normal (Miller, 1962; Miller and Osoba, 1967; Rothberg and Robert, 1967; Neselof, 1968; Armstrong, 1969; Armstrong *et al.*, 1969; Davies, 1969; Miller and Mitchell, 1969a,b). This has led to the concept that there are thymus-dependent and thymus-independent antigens. However, the separation of the two types of antigen varies with species; in the rat, the response to sheep red cells is thymus-independent (Claman *et al.*, 1966a,b; Pinnas and Fitch, 1966), whereas in the mouse this antigen is thymus dependent.

Direct evidence for interaction between the two universes of cells was first found by Claman *et al.* (1966a,b). X-Irradiated mice repopulated with thymus or bone marrow cells alone made very poor immune responses to sheep red blood cells. When both cell types were injected the response was much higher than the sum of the two individual responses. It was soon shown that the bone marrow, not the thymus, contributed the precursor of the antibody-forming cell (P cell) (Davies *et al.*, 1967; Mitchell and Miller, 1968a,b; Nossal *et al.*, 1968; Talmage *et al.*, 1969a); however, the question of the role of the thymus cell in humoral antibody production to the so-called "thymus-dependent antigens" was left unanswered.

III. The Adherent Cell

More recently, Mosier showed that an interaction between two kinds of cells could be demonstrated in the *in vitro* response of mouse spleen cells to sheep red blood cell antigen (Mosier, 1967). The spleen cells could be separated into a population adherent to glass or plastic and a nonadherent population. A synergism between the two populations could be demonstrated.

The adherent cell (A cell) does not appear to be the precursor cell (P cell) nor the thymus cell (T cell) required for the *in vivo* response in X-irradiated mice. Nonadherent spleen cells separated on a glass bead column will give an excellent response when injected into X-irradiated mice but are completely unresponsive *in vitro* (Talmage *et al.*, 1969a,b). The probable reason for this distinction was found by Roseman, who demonstrated that the adherent cell is relatively resistant to X-radiation (1969) in contrast to the marked sensitivity of the other two cells (Mosier, 1967; Mosier *et al.*, 1969; Roseman, 1969; Roseman *et al.*, 1969).

Thus the adherent cell is probably still functional in the X-irradiated mouse for at least a short time after radiation. We have confirmed the radioresistance of the adherent cell and used this radioresistance to show that there is a higher than normal concentration of adherent cells in the spleens of X-irradiated mice reconstituted with bone marrow (BMSC) (Table I). If these mice were given a second dose of X-radiation 2 hours before harvesting the bone marrow-derived spleen cells, there was little or no loss of function, but if the second dose of X-radiation was given 24 hours before harvesting, loss of the adherent cell or its function was marked. In these experiments spleens were taken from mice 7 days after radiation (1000 r ^{60}Co) and the injection of syngeneic bone marrow. A second dose of X-radiation (500 r) was given to these animals 2 or 24 hours before sacrifice. The cells from spleens (BMSC) taken 2 hours after 500 r were far superior to X-rayed normal spleen cells in supporting the *in vitro* response of a small number of immune spleen cells. However, by 24 hours after X-radiation the effectiveness of the BMSC had decreased markedly. This delayed effect of X-radiation on the adherent cell may account for the delayed effect of X-radiation on the immune response (Dixon *et al.*, 1952; Pribnow and Silverman, 1967, 1969).

It thus appears that there are two different kinds of cell interactions.

TABLE I

THE PLAQUE-FORMING CELL RESPONSE TO SHEEP ERYTHOCYTES *in vitro* USING UNTREATED AND X-IRRADIATED BONE MARROW-RECONSTITUTED SPLEEN CELLS AND X-IRRADIATED SPLEEN CELLS

Adherent cell ($\times 10^{-6}$)	Immune spleen cell ($\times 10^{-6}$)	Untreated BMSC[a,b]	BMSC[a,b] (2 hr. after 500 r)	BMSC[a,b] (24 hr. after 500 r)	Normal spleen[b] (2 hr. after 500 r)
10	3	3944/6	3639/6	250/3	—
10	1	1414/9	1389/9	169/3	6/3
10	0.3	195/9	143/9	13/2	2/3
10	0.1	38/12	5/12	—	—
10	—	11/3	1/3	—	—
20	2	—	—		1145/3
10	2	3850/3	4010/3		775/3
5	2	1545/3	1530/3		—
2.5	2	243/3	670/3		—

[a] BMSC—bone marrow (reconstituted) spleen cells.

[b] Average number of plaque-forming cells (PFC)/number of cultures. Usually the cells from two or three cultures were pooled and duplicate PFC assays performed on the pool. In these experiments, 3×10^{-6} immune spleen cell controls without added adherent cells gave from 0 to 100 PFC.

An interaction between T and P cells can be demonstrated by giving thymus and marrow cells to the X-irradiated mouse. An interaction between A cells and a mixture of P and T cells can be studied *in vitro* (Table I). Unfortunately, it has not been possible to separate the mixed population of P and T cells in the spleen, and neither thymus nor bone marrow cells work well in the *in vitro* culture system. Some evidence of interaction within the population of immune spleen cells can be seen in the data of Table I. The slope of the cell dose–response curve is considerably greater than 1.

The function of the A cell is not clear. Possibly the antigen adheres to it as to the dendritic cells of the lymph follicle. However, some immunologists have postulated that the antigen is ingested by macrophages and "processed" in some way to make it functional (Garvey and Campbell, 1957; Adler *et al.*, 1966; Feldman and Gallily, 1967; Fishman and Adler, 1967; Gallily and Feldman, 1967). We know of no convincing evidence in favor of such a concept and there are two important bits of evidence against it: (*1*) for several days after the injection of antigen, the antibody response can be inhibited by an injection of specific antibody (Möller and Wigzell, 1965; Wigzell, 1966; Dixon *et al.*, 1967; Britton and Möller, 1968) (this suggests that the functioning antigen is outside of cells); and (2) the major part of the antibody response is to antigenic determinants that are lost if the quaternary structure of the antigen is broken down (Sela *et al.*, 1967; Henney and Ishizaka, 1968).

TABLE II

RESPONSE TO SHEEP RED BLOOD CELLS OF ^{60}Co-IRRADIATED RECIPIENTS RECEIVING SPLEEN CELLS AND HORSE AND SHEEP RED BLOOD CELLS AT VARIOUS INTERVALS AFTER CELL TRANSFER[a,b]

	10×10^6 Cells transferred		50×10^6 Cells transferred	
Group[c]	Mice (No.)	Response (PFC)[d]	Mice (No.)	Response (PFC)[d]
I. S-RBC day 0	8	66 ± 14	7	218 ± 25
II. S-RBC day 4	7	181 ± 49	8	102 ± 17
III. H-RBC day 0; S-RBC day 4	11	58 ± 7	12	12 ± 5

[a] Five irradiated recipients given sheep red blood cells on day 4 but no spleen cells averaged 8 plaque-forming cells (PFC)/spleen.

[b] From Radovich and Talmage (1967).

[c] S-RBC—sheep red blood cells; H-RBC—horse red blood cells.

[d] Mean PFC for S-RBC per million cells transferred in spleen of recipient 6 days after injection of S-RBC ± S.E.

IV. Antigenic Competition

The relationship between the phenomenon of antigenic competition and cell interaction can be seen in the data presented in Table II. Here the depressive effect of one red cell antigen (horse) on the response to a second, apparently unrelated, red cell (sheep) is studied in the X-irradiated mouse reconstituted with 10 or 50 million normal spleen cells. The response with 50 million spleen cells is more than 5 times higher than with 10 million, which is probably due to the greater chance of cell interaction when cells are more concentrated. In any case, the result of the greater response to the first antigen in the 50-million group is a greater depressive effect on the response to the second antigen.

Initially, the experiment of Table II was interpreted as suggesting the existence of a suppressive humoral factor which was produced by the response to the first antigen (Radovich and Talmage, 1967). [The production of such a factor has been implicated in quite different experiments by Ambrose (1969).] However, the demonstration of an interaction between several cells requires a reassessment of this conclusion. At least one of the cells involved in the immune response must be nonspecific, and this cell may be used up in the response to the first antigen. This preemption is more likely to take place with a high concentration of interacting cells than with a low concentration.

V. Enhancing Effect of Multiple Antigenic Determinants

The importance of a carrier molecule to the immune response of simple chemical determinants (haptens) has been well known since the experiments of Landsteiner (Landsteiner and Lampl, 1917; Landsteiner, 1919, 1921, 1962). More recently it has been shown that the antigenic determinants on the carrier molecule are important in the response to the hapten. Protein molecules to which the injected animal has been made tolerant are poor carriers, whereas the response to the hapten is enhanced if the animal has been preimmunized to the carrier. Apparently, the haptenic determinant occupies a position on the carrier molecule equivalent to native determinants. If an animal is preimmunized to a haptenic determinant complexed with one carrier, this will increase the antibody response to native determinants of a completely different carrier if the latter is complexed to the same hapten; and the addition of two haptenic determinants is better than one in inducing an antibody response to the carrier (Haurowitz, 1936; Cinader and Dubert, 1955; Dixon and Maurer, 1955; Dubert, 1956; Cinader and Pierce, 1958; Salvin and Smith, 1960; Weigle, 1962, 1964, 1965a,b; Ashley and Ovary, 1965; Linscott and Weigle, 1965; Dietrich, 1966; Green *et al.*, 1966; Leskowitz

et al., 1966; Yoshimura and Cinader, 1966; Cinader *et al.*, 1967; Fronstein *et al.*, 1967; Levine, 1967; St. Rose and Cinader, 1967; Maurer and Pinchuck, 1968; Plescia *et al.*, 1968; Rittenberg and Campbell, 1968; Landy and Braun, 1969; Leskowitz, 1968; Plescia, 1969; Rajewski, 1969).

The above findings are well explained by the requirement for cell interaction in the antibody response. If the antigen is an important factor in cell interaction, such interaction will be greatly enhanced by the presence of many determinants on the antigen molecule. If this concept is correct, then the greater enhancing effect of two antigenic determinants compared to one determinant suggests that at least one of the cells involved is antigen-specific. It also suggests that cells specific for different determinants may interact and enhance each other's response.

VI. Enhancing and Suppressive Effects of Passively Administered Antibody

It is well established that passively administered antibody can both suppress and enhance the active response to an injected antigen (Jerne, 1967; Pearlman, 1967; Cerottini *et al.*, 1969). In general, the demonstration of an enhancing effect is demonstrable only with small doses of antigen which produce less than a maximal response. Although the suppressive effect of antibody is limited to the specific determinants represented in the antibody population, the enhancing effect of antibody is nonspecific and applies to other determinants on the antigen molecule (Pearlman, 1967).

If the antigen is important in achieving cell interaction, then antibody in excess, by blocking the antigenic determinants, will inhibit such interaction. An effect of antibody on blocking the aggregation of antibody-forming cells has been demonstrated *in vitro* by Mosier (1969). The prevention of clumping by this means or by mechanically reducing cell movement (Mishell and Dutton, 1966, 1967) was shown to inhibit antibody formation.

The nonspecific enhancing effect of antibody suggests that at least one of the cells involved in the interaction can fix antibody to its surface. The presence of the antibody will thus aid in the fixation of antigen and other cells coated with antibody. Lang and Ada (1967) showed that the presence of a small amount of antibody enhances the fixation of antigen to the dendritic cells of the lymph follicle.

VII. Discussion and Speculations

The finding that there is a requirement for cell interaction in the antibody response to some antigens has greatly complicated the problem of interpretation of much experimental data. A major problem is the

specificity of cell potential. The observation that single cells make only one antibody (Green *et al.*, 1967; Mäkelä, 1967) and that cells responding to one antigen may be selectively destroyed (Dutton and Mishell, 1967a) probably indicates that at least one of the interacting cells is specific. One possibility is that the two separate universes of cells, thymic and extrathymic, are both highly specific. However, in such a case there must be cells of each universe specific for different antigens and the chance of two such cells meeting and interacting is very small if they must both be specific for the same antigen.

A related question is the nature of the cell interaction and the substance that passes from the auxilliary cell (T cell) to the antibody-forming cell (P cell). If the antibody-forming precursor is highly specific, the auxiliary cell probably provides only a nonspecific stimulus. However, if the antibody-forming precursor is nonspecific, then it seems more likely that some kind of specific informational ribonucleic acid or inducer is transferred (Talmage *et al.*, 1969a).

One approach to this question has been to determine which cell is the carrier of immune memory, since this memory is presumed to result from the selective change in number of specific cells, either by selective growth [positive memory or anamnestic responsiveness] or by selective destruction [negative memory or tolerance]. Conflicting results have been obtained in experiments designed to determine the locus of immunological tolerance (Taylor, 1968; Landy and Braun, 1969; Miller and Mitchell, 1969a; Playfair, 1969), and it has not been clear whether the unresponsiveness involved was cellular (clone loss) or humoral (due to feedback immunosuppression). It has not been possible to study the locus of enhanced responsiveness since no reliable method exists for separating the two types of nonadherent cells after they have interacted.

What is the significance of thymus-independent antigens and the immune responses in neonatally thymectomized mice and in athymic humans? These responses appear to involve memory and, thus, cells with specific potential (Rothberg and Robert, 1967; August *et al.*, 1968; DiGeorge, 1968; Kretshmer *et al.*, 1968, 1969; Neselof, 1968; Rosen, 1968). It is also apparent that if cell interaction is required in these responses, one of the interacting cells need not be a thymus cell.

If extrathymic cells are able to interact and give immune responses, then the simplest explanation for the fact that they are unable to do so with some antigens is that the extrathymic tissue contains too few of the right type of specific cells to have a reasonable chance of cell interaction. If this is the case, then thymus cells will increase the chance of interaction only if they are *less* specific than extrathymic cells. There is some suggestion of this in experiments which indicate that less than 100

homologous lymphoid cells are needed to produce antigen-dependent foci on the chorioallantoic membrane or graft-versus-host reaction in chick embryos (Szenberg *et al.*, 1962; Simons and Fowler, 1966; Simonsen, 1967).

If the above line of reasoning is correct, then the two separate universes of immunocompetent cells which have evolved have different functions and different degrees of specificity. One is highly specific and highly specialized for humoral antibody production and the other is several orders less specific and adapted to cell-cell interactions. The function of the thymus would be to decrease the specificity or rather to increase the range of responsiveness of immunocompetent cells. In the process the thymic cell loses its ability to export antibody to the serum but gains a much greater ability to interact with other cells.

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The Role of Lysosomes in Immune Responses[1]

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I. Introduction

Described as a new class of subcellular organelles by de Duve *et al.* (1955), lysosomes have been at the center of many studies in tissue injury, inflammation, and immunity. Yet, as more data become available, it is clear that the bulk of the lysosomal concept was foreshadowed by the works of Elie Metchnikoff (1905). His work overlaps the borders between classical "humoral" immunology (Ehrlich) and cellular pathology (Aschoff). In formulating the principle of phagocytosis, he recognized that uptake of foreign material was a primary event, common to both immune recognition and acute inflammation. With deeper understanding of the role played by the "macrophages" and "microphages" (polymorphonuclear leukocytes and lymphocytes) in immunity, the extent of Metchnikoff's insight becomes even more impressive. Although he supposed wrongly that the complement of Bordet and Ehrlich was elaborated by microphages, he deduced correctly that phagocytosis was a necessary step for immune reaction to particulate antigens and that uptake of particles per se could initiate sterile inflammation. It is toward the recent elaboration of these concepts that this review will be addressed.

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Lysosomes can be considered parts of a vacuolar system or, more exactly, of an "exoplasmic reticulum" concerned with the digestion of heterologous and autologous material (reviewed in de Duve and Wattiaux, 1966; Weissmann, 1967; de Duve 1969). The organelles have also been studied in those cells central to immune responses: macrophages, monocytes, histiocytes, thymus cells, neutrophiles, eosinophiles, lymphocytes, etc. (see specific chapters in Dingle and Fell, 1969). But we do not plan to discuss lysosomes by cell type; instead, we have divided our subject into two major categories: (*1*) the processing of antigen by the vacuolar system—a responsibility chiefly of macrophages—and (*2*) the mediation by lysosomes of immune injury—a responsibility chiefly of microphages. These topics follow as naturally from the outlines of Metchnikoff's thought as they express themselves in terms coined by de Duve.

A. Abbreviations

A list of abbreviations used in this chapter follows:

BAA = benzoyl-L-arginine amide
BCG = Bacillus Calmette-Guérin
BSA = bovine serum albumin
cyclic AMP = cyclic adenosine 3′,5′-monophosphate
DNA = deoxyribonucleic acid
EACA = ϵ-aminocaproic acid
L-GAT = poly-(L-glutamic acid, L-alanine, L-tyrosine)
HSA = human serum albumin
LDH = lactic dehydrogenase
PHA = phytohemagglutinin
PPD = purified protein derivative
PP-L = protein polysaccharide (light fraction of bovine nasal cartilage)
PWM = pokeweed mitogen
RNA = ribonucleic acid
SRS-A = slow reacting substance of anaphylaxis
TAME = tosyl-L-arginine methyl ester
L-TG = poly-(L-tyrosine, L-glutamic acid)
D-TGA = poly-(D-tyrosine, D-glutamic acid, D-alanine)
(T,G)–A–L = poly-(L-tyrosine, L-glutamic acid)–poly-DL-alanine–poly-L-lysine
TLCK = tosyl-lysine chloromethylketone
TPCK = tosyl-phenylalanine chloromethylketone

II. Processing of Antigen by the Vacuolar System

A. A Role for Macrophages in the Afferent Limb of the Immune Response

The induction of primary immune responses against many types of antigens has been widely recognized to require the sequential interaction of foreign materials with two, or possibly three, distinct cell populations. Each of the properties of antigen-handling (Fishman, 1969), of antigen recognition, and of antibody synthesis (J. F. A. P. Miller and Mitchell, 1969; A. J. S. Davies, 1969; Claman and Chaperon, 1969; Taylor, 1969) has been attributed to an anatomically distinct cell line.

Since Metchnikoff (1899, 1905) it has been appreciated that macrophages in the liver, spleen, lymph nodes, and peritoneal cavity can ingest particulate and soluble materials and that these cells participate, at least in part, in the development of immunity. Antigen processing begins with endocytosis, which is a general phenomenon that occurs in the majority of living cells (Jacques, 1969). Little evidence is available, however, for implication of endocytic cells other than macrophages in the induction of the immune response. It is this cell type which will therefore engage our interest. Interrelationships between macrophages, the reticuloendothelial system, and immunity, have been the subject of frequent and extensive reviews (Rowley, 1962; Thorbecke and Benacerraf, 1962; Campbell and Garvey, 1963; Suter and Ramseier, 1964; Mackaness and Blanden, 1967; Nelson, 1969). There is general agreement that mature macrophages do not themselves synthesize specific antibodies (Ehrich *et al.*, 1946; Weiler and Weiler, 1965); although possible exceptions to this rule will be dealt with below. It is likely that macrophages play a more subtle and, probably, indirect role in the afferent limb of the immune response. The lines of evidence that implicate these cells in the inductive stage of immunity can be summarized as follows:

1. In a variety of experimental models, the presence of macrophages is a prerequisite for the induction of antibody formation. Although lymphocytes from adult donors failed to confer immunological competence to newborn rabbits (Dixon and Weigle, 1957), peritoneal macrophages rendered such animals responsive to a protein antigen that otherwise induced immunological tolerance (Martin, 1966). Similarly, development of immunological reactivity to heterologous red cells was accelerated in baby mice receiving macrophages from mature donors (Braun and Lasky, 1967; Argyris, 1968). Similar results were obtained

in attempts to restore immunological reactivity to bovine γ-globulin or *Shigella paradysenteriae* early after X-irradiation of rabbits (Pribnow and Silverman, 1967) or mice (Gallily and Feldman, 1967), respectively. In both cases, injection of antigen with lymph node cells failed to elicit antibody formation. However, immune responses were promptly induced if lymph node cells were transferred together with macrophages which had been incubated with the appropriate antigen *in vitro*. That pure lymphocyte suspensions could be instructed by primed macrophages, but not by antigen alone, was very elegantly demonstrated in experiments of Ford *et al.* (1966). Rat thoracic duct lymphocytes were temporarily incubated with macrophages containing sheep red blood cells and then freed of both phagocytes and antigen. Subsequently, only lymphocytes which had been exposed to macrophages were able to confer the capacity to synthesize specific antibody to irradiated, syngeneic recipients.

The initiation of hemolysin formation by spleen cell suspensions *in vitro* has been shown to depend on the presence of a macrophage-rich, surface-adherent, and relatively radio-resistant subpopulation of cells. This population fails to make antibody by itself, but upon incubation with antigen it can stimulate antibody production by nonadherent cells (Mosier, 1967; Mosier and Coppleson, 1968; Pierce, 1969; Roseman, 1969; Pierce and Benacerraf, 1969).

Analogous observations have been made by Oppenheim *et al.* (1968), who found that adherent cells were required for antigen-induced lymphocyte transformation *in vitro*. A similar role for glass-adherent cells has been claimed in lymphocyte transformation induced by PHA, or the mixed lymphocyte reaction (Gordon, 1968; Levis and Robbins, 1969; Levis *et al.*, 1970). It should be stressed, nevertheless, that certain types of immune responses, notably homograft reactions *in vivo* and *in vitro*, may not always depend on the presence of macrophages during the inductive stage (Gowans, 1965; Strober and Gowans, 1965; Wilson, 1967). Adherent cells, moreover, may well comprise cell populations other than macrophages (Bianco *et al.*, 1970).

2. The immunogenicity of several protein antigens is directly related to their state of aggregation and, therefore, to their relative palatability to macrophages. Rapidly sedimentable bovine γ-globulin was immunogenic in mice, whereas supernatant fractions induced tolerance (Dresser, 1962; Claman, 1963). Similarly, biologically "filtered," isotopically labeled BSA, which had been passaged through rabbits, was recovered from their serum and found to be tolerogenic when injected into further recipients. Equivalent amounts of unfiltered BSA were immunogenic (Frei *et al.*, 1965). Again, in contrast to the highly immunogenic native anti-

gen, cyanogen bromide-digested fragments of *Salmonella adelaide* flagellin were shown to be tolerogenic in adult rats (C. R. Parish *et al.*, 1967). Such observations, together with the finding that some macrophage-associated antigens may possess increased immunogenicity (see below), have led to the formulation of a macrophage bypass theory of immunological tolerance, which has been amply reviewed by Leskowitz (1967) and by Dresser and Mitchison (1968). On the other hand, efficient phagocytosis cannot be the sole prerequisite for immunogenicity. Undigestible or slowly metabolized antigens, such as pneumococcal polysaccharides (Felton *et al.*, 1955; Coons, 1963) or copolymers of D-amino acids (Carpenter *et al.*, 1967; Janeway and Humphrey, 1968, 1969), are very efficiently taken up by macrophages but tend to induce paralysis rather than immunity (Felton *et al.*, 1955; Janeway and Sela, 1967; Janeway and Humphrey, 1969; Howard and Siskind, 1969).

3. Several proteins have been found to be more potent immunogens when administered to recipients within macrophages than when given in free form (Mitchison, 1967, 1968, 1969a; Unanue and Askonas, 1968a). This difference, however, seems to hold only for antigens which are rather slowly taken up by phagocytic cells *in vivo*. In contrast, the immunogenicity of aggregated materials was not further increased after ingestion by macrophages *in vitro* (Mitchison, 1969a).

4. Finally, one of the more challenging claims for a macrophage step in the induction of immunity stemmed from the observation that filtrates and RNA-containing fractions of macrophages incubated with T_2 bacteriophage or hemocyanin may have elicited specific antibody formation both *in vivo* and *in vitro* (Fishman, 1959, 1961; Fishman and Adler, 1963, 1964). The presence of specific antigen in such macrophage RNA preparations was later demonstrated and confirmed (Askonas and Rhodes, 1965; Friedman *et al.*, 1965; Adler *et al.*, 1966). The significance of antigen–RNA complexes is now under intensive investigation (Gottlieb *et al.*, 1967; Gottlieb, 1968, 1969a,b; Gottlieb and Straus, 1969).

In summary, although there is a considerable body of evidence implicating macrophages in the afferent limb of the immune response, their exact role is by no means clear. Macrophages may simply prevent excess antigen from paralyzing lymphocytes, act as convenient carriers of antigen throughout the body, concentrate and retain antigen over longer periods of time, present antigen to responsive cells, degrade antigen into active subunits, convert crude antigen into a better immunogen, translate antigen into specific information, or, finally, exert some nonspecific effect on antigen-reactive cells which allows them to perform properly. Moreover, these mechanisms are by no means mutually exclusive.

B. The Localization of Antigen in Lymphoid Tissues

1. Macrophages and Dendritic Reticular Cells

Depending on the route of injection, antigen is cleared from the bloodstream, serous cavities, or lymphatic channels, and the bulk of the material is taken up by free or fixed macrophages throughout the body (Sabin, 1939; Kruse and McMaster, 1949; Coons *et al.*, 1951). Antigen concentration in phagocytic cells of liver, lung, and bone marrow may far exceed the uptake by macrophages in lymphoid tissues (Campbell and Garvey, 1961, 1963; Ada *et al.*, 1964b), but it is obvious that the functional importance of the different subpopulations may vary tremendously. This view is supported by the studies of Franzl (1962) and Cohn (1964), who demonstrated considerable differences in the retention of immunogenic material between liver and spleen and between alveolar and peritoneal macrophages. It was calculated, moreover, that only a minute proportion (less than 0.05% in the case of keyhole limpet hemocyanin) of the originally administered antigen played a significant role in the actual process of immunization (McConahey *et al.*, 1968). It is crucial for the understanding of the mechanism of antibody formation to define the anatomical pathways of *this fraction* through lymphoid tissues.

When apparently nonantigenic materials, such as titanium dioxide, isotopically labeled chromium phosphate, Thorotrast, saccharated iron oxide, or colloidal carbon, were injected intravenously into rats, mice, or rabbits, they quickly became localized in macrophages of the red pulp of the spleen—particularly in the marginal zone surrounding the follicles of the white pulp (Goulian, 1953; Baillif, 1953; Odeblad *et al.*, 1955; Nossal *et al.*, 1966; Pinniger and Hutt, 1956; Hunter and Wissler, 1965; Hunter *et al.*, 1969). In Nossal's study, some of the carbon was also found in "tingible body macrophages" of the secondary follicles. Similarly, injection of substances with little or no antigenicity (gelatin, rat hemoglobin, or rat red blood cells) into the footpads of rats was associated with uptake of the material in the corresponding locations of the draining lymph nodes, i.e., lining macrophages of the medullary and marginal sinuses (Ada *et al.*, 1964a). Also, if antigens, such as horse ferritin, BSA, diphtheria toxoid, *Salmonella adelaide* flagella, (T,G)-A-L, hemocyanin, or HSA, were administered to nonimmune recipients, they were always found to be concentrated first in the sinus lining macrophages of lymph nodes or in the marginal-zone macrophages of the spleen (Ada *et al.*, 1964a; Nossal *et al.*, 1964, 1966; McDevitt *et al.*, 1966; Humphrey *et al.*, 1967; Humphrey and Frank, 1967). Later, antigenic material appeared to associate with surfaces of nonphagocytic, dendritic

reticular cells in follicles of lymph nodes and spleen (see also White, 1963). These elements could be distinguished clearly by morphological and functional criteria from macrophages (Mitchell and Abbot, 1965; Nossal *et al.*, 1968b) which are also present in the cortex of lymph nodes and in the follicular areas of the spleen.

Follicular antigen localization was the subject of a recent review artice by McDevitt (1968). It seemed clear that specific antibody was required for efficient trapping of antigen on the surface of dendritic reticular cells. In most cases, follicular localization coincided with the appearance of circulating antibody (Humphrey and Frank, 1967; White *et al.*, 1967; French *et al.*, 1969), was more pronounced in actively or passively immunized animals (Nossal *et al.*, 1965b; McDevitt *et al.*, 1966; Mitchell and Abbot, 1965), and could be reduced or abolished by irradiation (Jaroslow and Nossal, 1966; Williams, 1966b), chronic thoracic duct drainage (Williams, 1966a), or induction of specific immunological tolerance (Humphrey and Frank, 1967). Not surprisingly, germfree rats had a diminished capacity for localization of antigen on dendritic cells (J. J. Miller *et al.*, 1968). Claims that nonantigenic material can also be localized on dendritic reticular elements of lymph node follicles (Cohen *et al.*, 1966; Hunter *et al.*, 1969) have been open to criticism (Nelson, 1969) and await further confirmation.

Trapping of antigens by reticular cells may not be the only mechanism responsible for their localization in follicles. Recently, there has been characterized a new subpopulation of lymphocytes which binds antigen–antibody complexes in the presence of modified complement (Bianco *et al.*, 1970). Since the bulk of this subpopulation, termed "complement receptor lymphocytes," resides in the follicular areas of peripheral lymphoid tissue (Dukor *et al.*, 1970), it would seem likely that they contribute to antibody-mediated retention of antigen by lymphoid follicles. Follicular antigen localization is thought to provide the substrate for the firing of *secondary* immune responses (Ada *et al.*, 1968), long-term antibody production, and the proliferative expansion of immunologically committed cells (Hanna *et al.*, 1969). It is not, however, a prerequisite for the induction of a *primary* response.

2. *Antibody-Producing Cells*

The presence of antigen in antibody-producing elements has been the subject of controversy (McDevitt, 1968). Employing highly sensitive electron-microscopic and radioautographic techniques, several authors have followed the fate of ferritin and isotopically labeled *Salmonella* flagellin, (T,G)-A-L, hemocyanin, and HSA in different species. They have failed to demonstrate any detectable antigen outside macrophages

or dendritic reticular cells (de Petris and Karlsbad, 1965; Buyukozer *et al.*, 1965; Nossal *et al.*, 1965a; McDevitt *et al.*, 1966; Humphrey and Frank, 1967). Other workers have reported considerable amounts of antigen in plasma cells, their precursors, and even in small lymph node lymphocytes, both of unprimed animals (Roberts, 1966; Han and Johnson, 1966; Han *et al.*, 1967) and to a greater degree, of specifically immunized animals (Wellensiek and Coons, 1964; Roberts, 1964). In a reevaluation of this problem, Nossal *et al.* (1967) were able to trace labeled flagellin in a minority of isolated single antibody-forming cells obtained from lymph nodes very early during primary and secondary immune responses, but never in thoracic duct lymphocytes. The authors concluded that antigen *could* enter primitive plasmoblasts, but that it was impossible to decide whether antigen entry was necessary for cell proliferation and antibody production.

Recent findings challenge the belief that phagocytic cells are incapable of antibody synthesis. Hannoun and Bussard (1966) and Bussard and Lurie (1967) identified large cells, apparently histiocytes with vesicular cytoplasm, as antibody producers *in vitro*. Pernis *et al.* (1966) described polarized epitheloid cells, obtained from granulomatous lesions, which shared the ultrastructural characteristics both of macrophages and of plasma cells. Holub *et al.* (1966) and Holub and Hauser (1969) demonstrated specific, puromycin-sensitive, hemolytic-plaque formation by aveolar histiocytes containing abundant vesicles, lysosomes, and inclusion bodies. Unlike lymphoid cells which produced antibody, many of these cells were destroyed by the uptake of silica. Moreover, plaque-forming spleen cells were recently shown to incorporate carbon particles (Noltenius and Chahin, 1969). It is not clear whether these findings indicate that primitive macrophages possess the capacity to synthesize specific antibody. Indeed, the data can also be interpreted to demonstrate that activation of the vacuolar apparatus follows exposure of lymphocytes to antigen or nonspecific mitogens (R. Hirschhorn *et al.*, 1967; Brittinger *et al.*, 1968) (see below).

C. The Fate of Antigen in Macrophages

1. Endocytosis

Cellular ingestion of particulate and soluble foreign material occurs by phagocytosis and pinocytosis. The histochemical, ultrastructural, and metabolic features of this process have been reviewed by Cohn (1968), Daems *et al.* (1969), and Jacques (1969). Cell eating and cell drinking are basically similar events involving the invagination of plasma membrane which fuses with itself and thus interiorizes extracellular com-

ponents within a phagosome or pinosome. However, the metabolic requirements of phagocytosis and pinocytosis, or rather, of the uptake of extracellular material by large and by small vesicles into mononuclear phagocytes, may differ considerably (Cohn, 1968; Casley-Smith, 1969).

The rate of endocytosis can be enhanced by a variety of stimulants. Increased phagocytic capacity of macrophages *in vitro* was induced by maintenance of the cells over prolonged periods in culture (W. E. Bennet and Cohn, 1966; Perkins *et al.*, 1967) or through nonspecific activation by BCG (Evans and Myrvik, 1967). Pinocytosis was stimulated by anionic molecules (Cohn and Parks, 1967a), by nucleosides and nucleotides (Cohn and Parks, 1967b), and by antibody directed against a macrophage membrane antigen (Cohn and Parks, 1967c).

Although specific antibody is not an absolute prerequisite for the ingestion of all types of foreign material by macrophages, recognition phenomena are involved in the initial attachment phase preceding engulfment (Rabinovitch, 1967a,b, 1968, 1969). Special significance may be attributed to a macrophage membrane coat that is sensitive to trypsin (Vaughn, 1965), chymotrypsin, and papain (Lagunoff, 1969). This coat has, indeed, been shown to determine the attachment of damaged erythrocytes and the rate of pinocytosis of foreign protein. The surface properties (Rabinovitch, 1969), state of aggregation, and relative foreigness (Perkins and Leonard, 1963) of the offered material are also decisive for its palatability to macrophages. The latter phenomenon, however, may reflect an involvement of "natural antibodies." The importance of cytophilic antibodies, complement factors, and opsonins for uptake of foreign matter by phagocytic cells was amply dealt with in a recent review (Nelson, 1969) and is beyond the scope of this article.

2. *Segregation of Foreign Material within Lysosomes*

The current concepts of granule flow and merger during endocytosis are based on light microscopic and ultrastructural observations on macrophages from different sources (Essner, 1960; Novikoff and Essner, 1960; Cohn and Wiener, 1963a,b; Cohn *et al.*, 1966; Leake and Myrvik, 1966; North, 1966). Newly formed endocytic vacuoles, which are probably devoid of acid hydrolases, flow from the plasma membrane toward the Golgi region where they fuse with primary lysosomes. The extrusion of granule contents into the phagosome is evidenced by the loss of primary lysosomes from the cytoplasmic matrix and the appearance of hydrolases in the phagolysosomes (Straus, 1964). These digestive bodies (also called "heterophagic vacuoles" or "secondary lysosomes") probably display decreased resistance to mechanical stress, which may

TABLE I

EXAMPLES OF SEGREGATION OF FOREIGN MATERIAL IN MACROPHAGE LYSOSOMES

Foreign material	Source of macrophage	Techniques used[a]	Reference
Thorium dioxide	Rat liver	EM	Wiener *et al.*, 1964
	Mouse spleen	EM, HC	Daems and Persijn, 1965
	Rabbit liver	DC, DG	Weissmann and Uhr, 1968
Plutonium dioxide	Rat peritoneum	EM	Sanders and Adee, 1969
Gold	Mouse peritoneum	LM	Cohn and Benson, 1965
Triton WR-1339	Rat liver	DC, DG, EM	Wattiaux *et al.*, 1963
	Rat spleen	DC, DG	Bowers and de Duve, 1967
Various oligo- and polysaccharides	Mouse peritoneum	LM, EM	Cohn and Ehrenreich, 1969
Dextran	Mouse spleen	EM, HC	Daems and Persijn, 1965
	Rat spleen	DC, DG	Bowers and de Duve, 1967
Various synthetic oligopeptides	Mouse peritoneum	LM, RA	Ehrenreich and Cohn, 1969
Poly-(D-tyrosine, D-glutamic acid, D-alanine)	Mouse peritoneum	DG	Kölsch and Mitchison, 1968
Poly-(L-tyrosine, L-glutamic acid)	Mouse peritoneum	DG	Kölsch and Mitchison, 1968
Horseradish peroxidase	Rat liver	LM, HC	Straus, 1964
	Guinea pig peritoneum	EM, HC	Catanzaro *et al.*, 1969
Bovine serum albumin	Mouse liver	DC	Mego and McQueen, 1965; Mego *et al.*, 1967
	Mouse peritoneum	DG	Kölsch and Mitchison, 1968
Human serum albumin	Rat lymph node	DC	Ada and Lang, 1966
	Rat lymph node	DC, DG	Williams and Ada, 1967
	Mouse peritoneum	LM, RA	Ehrenreich and Cohn, 1967
	Mouse peritoneum	LM, EM, RA	Rhodes *et al.*, 1969

Ferritin	Rat lymph node	EM	Buyukozer *et al.*, 1965
	Mouse peritoneum	LM, EM	Rhodes *et al.*, 1969
Maia squinado hemocyanin	Rat lymph node	DC	Ada and Lang, 1966
	Mouse lymph node	DC, DG	Askonas *et al.*, 1968
Bacteriophage T2	Rabbit peritoneum	EM	Aronow *et al.*, 1964
	Rabbit spleen	DC	Uhr and Weissmann, 1965
	Rabbit liver	DC, DG	Weissmann and Uhr, 1968
	Rat peritoneum	EM	Friend *et al.*, 1969
Bacteriophage ΦX 174	Guinea pig liver	DC	Uhr and Weissmann, 1965
	Rabbit liver	DC, DG	Weissmann and Uhr, 1968
Salmonella adelaide flagella	Rat lymph node	DC, DG	Ada and Williams, 1966
	Rat lymph node	EM, RA	Nossal *et al.*, 1968a
Salmonella adelaide flagellin	Rat lymph node	DC	Ada and Lang, 1966
	Rat lymph node and spleen	DC, DG	Williams and Ada, 1967
Listeria monocytogenes	Mouse spleen	EM	Armstrong and Sword, 1966
Escherichia coli	Rabbit lung and peritoneum	DC	Cohn, 1964
Sheep red blood cells	Mouse spleen	DC	Franzl, 1962
Mouse and rat red blood cells	Rat spleen	DC, DG	Bowers and de Duve, 1967

[a] Key to abbreviations:
DC = differential centrifugation
DG = density gradient analysis
EM = electron microscopy
HC = histochemistry
LM = light microscopy
RA = radioautography

account for the intracellular redistribution of lysosomal enzymes from sedimentable to nonsedimentable forms during active endocytosis (Cohn and Wiener, 1963b). Uptake and segregation of antigens within lysosomes have now been firmly established (Table I).

a. Nonantigenic Particulates. In ultrastructural studies, indigestible inorganic particulates, such as thorium dioxide, plutonium dioxide, or gold, were sequestered into phagolysosomes of Kupffer cells (Wiener *et al.*, 1964), spleen red pulp macrophages (Daems and Persijn, 1964), or mononuclear phagocytes of the peritoneal cavity (Cohn and Benson, 1965; Sanders and Adee, 1969). For weeks they remained associated with increasingly smaller and denser lysosomes which assumed the ultrastructural characteristics of residual bodies. Intravenous injection of thorotrast also resulted in an augmented density of hydrolase-rich granules from rabbit liver homogenates (Weissmann and Uhr, 1968). This new class of thorotrast-containing lysosomes could be separated from other large granules and appeared to possess the characteristic fragility of secondary lysosomes.

Triton WR-1339, a detergent, was also found to be sequestered by lysosomal fractions obtained from liver (Wattiaux *et al.*, 1963) and spleen (Bowers and de Duve, 1967). In this case, the secondary lysosomes became less dense and, again, were more fragile to mechanical trauma than acid hydrolase-rich granules from control animals.

b. Oligo- and Polysaccharides. A number of indigestible di-, tri-, and tetrasaccharides were readily taken up by macrophages *in vitro* and became associated with swollen, phase-lucent, lysosomal storage granules (Cohn and Ehrenreich, 1969). In the electron-microscopic studies of Daems and Persijn (1965) and Daems *et al.* (1969), uptake of dextran particles by phagosomes and their subsequent merger with acid phosphatase-containing lysosomes could also be visualized. In a density gradient analysis of subcellular fractions from spleen homogenates of ^{14}C-dextran-injected rats, Bowers and de Duve (1967) demonstrated localization of the label in the dense macrophage lysosome fraction. Subsequent electron-microscopic examination of red pulp macrophages revealed selective association of dextran with electron-dense lysosomes containing abundant iron-rich breakdown products (Bowers, 1969).

It would seem, therefore, that administration of appropriate materials may change both morphological and sedimentation characteristics of secondary lysosomes and thus provide a convenient label for lysosomal subpopulations.

c. Peptides and Proteins. Synthetic oligopeptides (Ehrenreich and Cohn, 1969) and polypeptides (Kölsch and Mitchison, 1968), serum

albumin (Mego and McQueen, 1965; Ada and Lang, 1966; Mego *et al.*, 1967; Ada, 1967; Williams and Ada, 1967; Ehrenreich and Cohn, 1967; Kölsch and Mitchison, 1968; Rhodes *et al.*, 1969), horseradish peroxidase (Straus, 1964; Catanzaro *et al.*, 1969), ferritin (Buyukozer *et al.*, 1965; Rhodes *et al.*, 1969), and hemocyanin (Ada and Lang, 1966; Askonas *et al.*, 1968) were all localized within phagolysosomes.

When mouse macrophages were exposed to two antigens: isotopically labeled HSA and unlabeled ferritin at the same time, both became sequestered within the same phagolysosomes (Rhodes *et al.*, 1969). Similar findings were obtained by Casley-Smith (1969). This corroborates the results of an earlier study which demonstrated uptake of two separate bacteriophages by a single, thorotrast-laden population of secondary lysosomes (Weissmann and Uhr, 1968). Ada and Lang (1966) investigated the subcellular distribution of HSA and hemocyanin in rat lymph node homogenates after local administration of the antigen. Both proteins became partly associated with the large granule fraction where they were degraded to low molecular weight products. Localization in this fraction was considerably increased when antibody-complexed albumin was injected. In subsequent studies (Williams and Ada, 1967) such complexes were shown to localize on dendritic reticular cells of lymphoid follicles, whereas heat-denatured HSA became exclusively sequestered into vacuoles of medullary macrophages. Macrophage-localized antigen banded in urografin gradients together with latent lysosomal enzymes, whereas antigen from lymphoid follicles was found to sediment at high density values. This result suggests very strongly that at least part of the antigen localizing in the "large granule" fractions from draining lymph nodes was associated with membranes from dendritic reticular cells or "complement-receptor lymphocytes."

Experiments by Kölsch and Mitchison (1968) indicate that proteins which have been ingested by macrophages are rapidly segregated into two subcellular compartments with greatly differing characteristics. Using a pulse-and-chase technique, the authors investigated the subcellular distribution of ^{125}I- and ^{131}I-labeled, heat-denatured BSA (and other protein antigens) in homogenates of mouse peritoneal macrophages. It was found that, irrespective of the antigen dose used, 90% of newly phagocytized label localized initially in a "turnover" compartment with a density of 1.19 gm./cm.^{3} This antigen was rapidly broken down within the next few hours. About 10% of the antigen, however, was associated with a "storage" compartment containing particles of higher density (1.25 gm./cm.^{3}). Label in this second localization was retained for many hours. Although ingestion of antigen was barely altered by previous X-irradiation of macrophage donors with 900 r, transfer of antigen into

the "storage" compartment seemed to be impaired. Antigen in the light particulate fraction was readily solubilized by detergent and could be precipitated by specific antiserum. Label in the dense fraction, on the other hand, resisted solubilization by most detergents and was apparently membrane-bound. Essentially similar features were established for the other antigens used, except that the slowly metabolizable synthetic polypeptides D-TGA and L-TG were initially associated with a still lighter granule fraction before moving to the 1.19 "turnover" compartment.

As judged by the enzyme distribution pattern, the two light fractions—containing specifically precipitated antigen—were rich in lysosomal hydrolases. The nature of the "storage" compartment is less clear. Antigen may become associated with very dense secondary lysosomes or residual bodies. Alternatively, membrane-associated antigens may sediment together with the heavier, nuclear-debris fractions. This possibility will be discussed further in the context of findings by Unanue and Cerottini (1969) (see below). Unfortunately, the immunological relevance of the two compartments described by Kölsch and Mitchison remains in doubt, since all of the subcellular fractions described proved to be very poorly immunogenic when tested in a highly sensitive *in vivo* system.

d. Viruses. Interactions between viruses and lysosomes were the subject of a review by Dales (1969). Few studies on macrophages have been conducted at the subcellular level, and the role of lysosomal uptake for the uncoating of the viral genome has yet to be clarified. Nevertheless, the fate of bacteriophage inside the vacuolar system of mononuclear phagocytic cells has been investigated by several workers, since antibody formation against these viruses has served as a choice model in immunology.

Electron microscopy of *in vitro* endocytosis of T2 phage by rabbit peritoneal macrophages revealed that virus particles were first absorbed to the cell membrane, then surrounded by macrophage pseudopods, and, within minutes, incorporated into phagocytic vacuoles. The phagosomes then moved centripetally towards the perinuclear region. Indirect evidence for fusion of phagosomes and depletion of primary lysosomes was also obtained (Aronow *et al.*, 1964; Friend *et al.*, 1969). In contrast to preliminary observations by Fishman *et al.* (1965), phage particles were never detected over the nucleus or outside vesicular, membrane-bounded structures in these studies.

Sequestration of intravenously injected bacteriophages T2 and ϕX 174 into the large granule fractions of guinea pig liver and rabbit spleen, but not of rabbit kidney, was demonstrated in a study by Uhr and Weissmann (1965). They assumed that phage-containing lysosome fractions were derived from reticuloendothelial cells in these organs. Indeed, plaque-

forming phage could be released from the hydrolase-rich fraction by lysolecithin. In further experiments, lysosome-rich fractions from livers of rabbits injected with bacteriophage T2, ϕX 174, and F_2 were purified on sucrose density gradients. The phage particles sedimented together with a hydrolase-rich fraction following administration *in vivo*, whereas their sedimentation properties proved to be different when phages were admixed to liver homogenates *in vitro*. Moreover, when Thorotrast injection was followed by the administration of bacteriophages, plaque-forming units were associated not only with the original lysosomal population, but also with the Thorotrast-induced, dense particles (Fig. 1). It therefore appeared likely that a population of secondary lysosomes par-

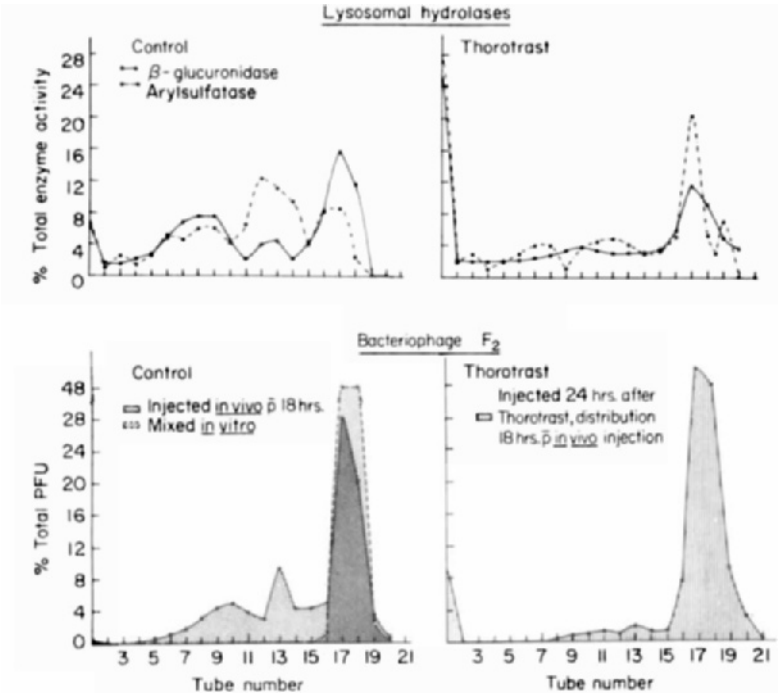

FIG. 1. Distribution of bacteriophages and lysosomal hydrolases in large-granule fractions of rabbit liver. Discontinuous sucrose gradient: tubes 1–4 = 1.8 *M*; 5–8 = 1.7 *M*; tubes 9–12 = 1.6 *M*; tubes 13–16 = 1.5 *M*; tubes 17–20 = 1.4 *M* sucrose. Upper left—distribution of β-glucuronidase and arylsulfatase activities of large-granule fraction from control animal; upper right—distribution of β-glucuronidase and arylsulfatase activities of large-granule fraction from animal injected 24 hours previously with colloidal thorium dioxide (Thorotrast); lower left—distribution of bacteriophage F_2 in large-granule fractions, after admixture *in vitro* 18 hours after injection; lower right—distribution of bacteriophage F_2 18 hours after injection into rabbit injected 24 hours previously with Thorotrast (Weissmann and Uhr, 1968).

ticipated further in the uptake of newly ingested material (Weissmann and Uhr, 1968).

e. Bacteria and Bacterial Components. Incorporation of whole bacteria and bacterial antigens into phagolysosomes of macrophages has been firmly established by many investigators (for review, see Allen, 1969). Locally injected *Salmonella adelaide* flagella is found within the phagolysosomes of medullary macrophages from draining lymph nodes, as shown by subcellular fractionation and electron-microscopic radioautography. Soon after administration of iodinated flagella, most of the radioactivity was found in the supernatant fraction of lymph node homogenates (Ada and Williams, 1966; Ada, 1967). After 1 to 2 days, however, about half of the radioactivity was present in the large granule fraction, and 15% of the label became associated with the nuclear-debris fraction. Within the following 2 months, the proportion of radioactivity in this latter fraction rose steadily to about 25%, whereas most of the remaining label stayed with the large granules. The antigen (reactive with antibody) in the large granule residue was shown to become more tightly associated with membranes. It is possible that antigen in this and in the nuclear-debris fractions is associated with membranous material derived from dendritic reticular cells or "complement-receptor lymphocytes" which so avidly attract antigen-antibody complexes.

High-resolution radioautography (Nossal *et al.*, 1968a) revealed that iodinated flagella entered into macrophages by two pathways: some of the labeled antigen was taken up by pinocytosis, whereas another fraction seemed to penetrate the plasma membrane directly. Single grains found lying, apparently free, in the cytoplasm up to 30 minutes or longer were then surrounded by small dense vesicles and, subsequently, sequestered into membrane-bounded inclusions. Evidence for fusion of antigen-laden vacuoles with electron opaque primary lysosomes was also obtained. Some of these antigen-containing bodies were very dense and closely resembled the telolysosomes described by Gordon *et al.* (1965). As time progressed, many of the labeled phagolysosomes became larger and more complex. As in the fractionation studies by Ada, radioactivity remained associated with such lysosomes for at least 6 weeks. At no time could significant labeling be demonstrated in nuclei, nor was evidence obtained for the exit of labeled antigen fragments from secondary lysosomes. Finally, entry of whole bacteria, e.g., *Listeria monocytogenes* (Armstrong and Sword, 1966) or *Escherichia coli* (Casley-Smith, 1969) into phagosomes and phagolysosomes of macrophages has been visualized by electron microscopy.

f. Metazoal Cells. Phagocytized metazoal cells, such as erythrocytes, are also sequestered into lysosomes. Franzl (1962) and Franzl and

Morello (1965) demonstrated the specific immunogenicity of lysosomal fractions, which had been isolated from spleen homogenates of mice injected with sheep red blood cells *in vivo*. When ^{51}Cr-labeled erythrocytes were administered to rats, radioactivity could be recovered in spleen homogenates. The label sedimented with the light lysosome fraction of lymphoid tissue macrophages (Bowers and de Duve, 1967).

The data reviewed in this section indicate clearly that a wide range of materials differing in size, physicochemical properties, and antigenicity become segregated by the vacuolar system. Whether antigens can totally resist intralysosomal degradation, whether a fraction of the ingested antigen can escape from the lysosomal compartment, or else, whether some of the ingested antigen is channeled into alternative pathways without ever entering the vacuolar system is less clear.

3. *Intralysosomal Degradation*

a. Oligosaccharides and Oligopeptides. Once inside the vacuolar system, ingested matter is rapidly broken down by hydrolases. In a study of the uptake and intracellular hydrolysis by mouse macrophages of carbohydrates, Cohn and Ehrenreich (1969) demonstrated that sucrose and a number of other di-, tri-, and tetrasaccharides could not be degraded by macrophage lysosomal enzymes. Consequently, these were retained inside swollen storage vacuoles over long periods of time. In such vacuolated cells, exogenous invertase and suitable hexosidases were promptly taken up by pinocytosis and found specifically to reverse lysosomal swelling. Presumably because small molecules can diffuse across lysosomal membranes, vacuolization was not induced by indigestible sugars of low molecular weight. Similar results were obtained with readily pinocytosed, indigestible D-oligopeptides (Ehrenreich and Cohn, 1969).

b. Proteins. Intralysosomal degradation of a variety of antigenic, exogenous proteins has been followed by morphological and biochemical methods. Within 2 to 3 days the histochemical reaction product of systemically administered horseradish peroxidase gradually disappeared from the phagolysosomes of Kupffer cells (Straus, 1964). In analogous experiments, Cohn and Benson (1965) demonstrated the loss of pinocytosed fluorescein-labeled proteins from cultured mouse macrophages.

Low molecular weight breakdown products of HSA and *Maia squinado* hemocyanin were recovered in highest proportions from the large granule fraction of draining rat lymph nodes within 2 to 3 days after local injection of the antigens (Ada and Lang, 1966). The breakdown of such proteins might be fairly complete. Isolated liver lysosomes containing formaldehyde-treated ^{131}I HSA were found to release monoiodotyrosine

into the surrounding medium (Mego *et al.*, 1967). Both externally labeled ^{125}I-HSA (Ehrenreich and Cohn, 1967) and internally labeled ^{3}H-rabbit hemoglobin (Ehrenreich and Cohn, 1968) were lost rapidly from phagolysosomes of cultured peritoneal mouse macrophages. Label was quantitatively recovered from the culture fluid and found to be associated with single amino acids. Peptides of low specific activity or low concentration would not have been detected in these experiments. Moreover, some 10% of the radioactivity of BSA and about 40% of hemoglobin were still present in the macrophages 40 hours after the antigen pulse (Ehrenreich and Cohn, 1968). Similar observations were made with other protein antigens: isotopically labeled *Maia squinado* hemocyanin (Unanue and Askonas, 1968b), keyhole limpet hemocyanin (Unanue, 1969; Unane and Cerottini, 1969), or BSA (Kölsch and Mitchison, 1968) were all rapidly lost from pulse-fed peritoneal macrophages, but in each case a small proportion (3–10%) of the initial radioactivity remained associated with the macrophages for a much longer time. From the experiments of Kölsch and Mitchison (1968), it would appear that such label was no longer (if ever) localized in the bulk of macrophage lysosomes; findings of Unanue (Unanue and Askonas, 1968b; Unanue and Cerottini, 1969) would suggest, instead, that the retained antigen fraction was bound to the plasma membrane.

c. Viruses. Although the role of macrophages in virus infections has been investigated frequently, (for a review, see Nelson, 1969), few data are available which directly implicate the lysosomal system of these cells. Uhr and Weissmann (1965) reported a considerable decrease in the infectivity of large granule fractions from guinea pig liver homogenates within 48 hours after injection of bacteriophage ϕX 174. However, a similar loss of infectivity was observed in the postgranular supernatant. In rat macrophages exposed to bacteriophage T2, most ingested phage particles underwent intralysosomal degradation within 45 minutes after uptake (Friend *et al.*, 1969).

d. Bacteria and Bacterial Components. The role of macrophage lysosomes in the breakdown of bacteria and bacterial antigens is well established (Allen, 1969). Degradation of Group A streptococci in phagolysosomes of mouse macrophages was followed morphologically by Gill and Cole (1965), who demonstrated an early, partial loss of an immunofluorescent complex from the bacterial surface and subsequent segregation of fluorescent M-protein in macrophage vacuoles. Low molecular weight breakdown products of *Salmonella adelaide* flagella or of soluble flagellin were localized in large granule fractions from lymph node homogenates within 1 to 3 days following local injection of the antigens (Ada and Williams, 1966; Ada and Lang, 1966). As judged by

high-resolution radioautography, substantial amounts of labeled flagellar material were detectable in phagolysosomes for at least 6 weeks (Nossal *et al.*, 1968a).

The bactericidal effect of macrophages could in some instances be correlated with the availability of lysosomal hydrolases. The development of bactericidal activity against gram-negative bacteria in rat macrophages during fetal and early postnatal life (Karthigasu *et al.*, 1965) was paralleled by the development of acid phosphatase-positive granules in such phagocytes, however, their endocytic activity remained unchanged during the critical period of observation (Reade, 1968). In rabbits, BCG-induced alveolar macrophages were found to contain much higher activities of acid hydrolases than oil-induced mononuclear phagocytes from the peritoneal cavity (Cohn and Wiener, 1963a). Correspondingly, immunogenicity of an *Escherichia coli* agglutinogen was almost completely destroyed within 2 hours by alveolar cells, but not by the peritoneal macrophage population (Cohn, 1964).

Ingested bacteria may themselves induce changes of the lysosomal apparatus. Berk and Nelson (1962) observed lowered hydrolase activities in mouse macrophages which were infected with *Pseudomonas aeruginosa*. Chronic infection with relatively resistant strains of mycobacteria might finally exhaust the enzyme complement of macrophage lysosomes (Allen *et al.*, 1965; Merkal *et al.*, 1968). Conversely, certain bacteria and bacterial components—notably BCG and endotoxins—were shown to elicit in macrophages an increased content of lysosomal hydrolases (Grogg and Pearse, 1952; Suter and Hulliger, 1960; Thorbecke *et al.*, 1961; Heise *et al.*, 1965; Saito and Suter, 1965). The "immune phagocyte" concept, which is in part based on such observations, has been repeatedly the subject of extensive reviews (Suter and Ramseier, 1964; Rowley, 1966; Mackaness and Blanden, 1967; Nelson, 1969).

This scanty evidence (Section II,C,*3,a–d*) does not permit definite conclusions as to the ultimate fate of intralysosomal antigen. It is not clear whether digestible foreign matter is uniformly degraded within the lysosomal compartment or whether, upon entering a heterogeneous granule population, fractions of antigen may be retrieved and spared from ultimate destruction. It is clearer that certain macrophage-associated antigens can escape degradation for considerable lengths of time and that such antigen may remain within lysosomes.

4. Extralysosomal Sites of Antigen Sequestration

Most investigators have failed unequivocally to demonstrate that antigens can be sequestered outside the lysosomal system. Possibly the lack of positive data may simply reflect the insufficient sensitivity of the

systems employed for the detection of antigen. Nossal *et al.* (1968a) calculated, for example, that when high-resolution radioautography was employed for the detection of ^{125}I-flagella (substitution rate of one iodine atom per molecule), the appearance of one silver grain over a cell section could have represented 4000 antigen molecules per macrophage.

Some significance should, therefore, be attached to recent investigations in which the intracellular fate of peroxidase was followed at the ultrastructural level. The system made use of the amplifying effect of an electron opaque, enzymatic, reaction product. Following administration of lactoperoxidase to mice, the tracer was detected not only in lysosomes of Kupffer cells but also in the perinuclear space and in the rough-surfaced endoplasmic reticulum (Graham *et al.*, 1969). Similar results were obtained by Catanzaro *et al.* (1969), who exposed guinea pig peritoneal macrophages to horseradish peroxidase *in vitro*. Within 15 minutes, tracer was detected in pinocytic vacuoles, but 25–30% of the cells also displayed marked enzymatic activity in the perinuclear space and in the rough endoplasmic reticulum. There was little increase of reaction product in this location and no change in the proportion of cells containing extralysosomal peroxidase after prolonged periods of incubation, although phagolysosomal sequestration of the antigen continued.

The possible spatial association of an exogenous, antigenic protein with rough-surfaced endoplasmic reticulum is particularly interesting in view of recent findings of Gottlieb (1969a) and Bishop and Gottlieb (1969). These authors incubated peritoneal macrophages with an isotopically labeled, soluble, synthetic copolymer, L-GAT. Macrophage extracts yielded RNA–antigen complexes, a part of which were bound to monoribosomes. It is, therefore, possible that certain antigens may bypass the lysosomal compartment and gain direct access to the ribosomes of the rough-surfaced endoplasmic reticulum, there to become complexed to an immunogenic RNA. Such data require further analysis and confirmation.

An altogether different lysosome bypass mechanism is suggested by experiments of Unanue and Cerottini (1969). Peritoneal macrophages which had been exposed to keyhole limpet hemocyanin retained a small fraction (3%) of the antigen on the cell surface for a period of several days. Plasma membrane-associated hemocyanin was neither ingested nor catabolized, but could be removed by trypsin. The presence of surface-associated antigen was demonstrated by specific binding of antibody to the membrane and by high-resolution radioautography. Indeed, the immunogenicity of transferred live macrophages bearing hemocyanin on their surface was abolished by passive administration of antibody.

It is not clear whether reactive antigen associated with the cell surface can pass through the lysosomal compartment. Unloading of secondary lysosomes is thought to occur mostly through membrane transport or diffusion of small breakdown products. Although reverse pinocytosis has been proposed to account for the release of hydrolases from endocytic cells (see below), no convincing evidence for lysosomal defecation by mononuclear phagocytes has yet been obtained (Cohn and Fedorko, 1969).

5. *Immunogenicity of Macrophage-Associated Antigen*

The possible immunological significance of small amounts of antigen, persisting in lymphoid tissue for prolonged periods of time, has been established by the early work of Haurowitz (1960) and of Campbell and Garvey (1961, 1963). A fraction of ingested *Maia squinado* hemocyanin may be retained by peritoneal macrophages for at least 72 hours and, upon transfer in suitable recipients, elicits a specific antibody response (Unanue and Askonas, 1968b). Protein antigens persisting in irradiated macrophages may retain their priming capacity for a period of up to 3 weeks (Pribnow and Silverman, 1969). Although, as discussed above, the immunogenic protein may be carried on the surface of the macrophage (Unanue and Cerottini, 1969), earlier data suggested an association of immunogenic antigen with lysosomal fractions. Large granule preparations from spleen, but not from liver, of mice receiving sheep red blood cells conferred specific antibody-forming capacity to primed (Franzl, 1962) and unprimed (Franzl and Morello, 1965) recipients. In either case, a critical time interval between injection of the antigen and transfer of the granule fractions was important. Analogous findings were obtained by Uhr and Weissmann (1965), who found that large granule fractions from liver homogenates of guinea pigs were specifically immunogenic. While the actual number of plaque-forming units in the lysosomal fraction decreased, immunogenicity increased. In both series of experiments, plasma-membrane contaminants could have sedimented together with the large granules.

Moreover, the pathways of antigen handling by macrophages may depend on the type of antigen involved. The priming capacity of *Maia squinado* hemocyanin (Unanue and Askonas, 1968a) and of human and bovine serum albumins (Mitchison, 1968, 1969a) when transferred in live macrophages, was up to a 1000 times greater than when the same antigens were injected in their free form. No such enhancement was found during the secondary response. Differences in priming ability of macrophage-associated and free antigen were much smaller in the case of lysozyme or ovalbumin (Mitchison, 1969a). Highly immunogenic

antigens, such as heat-aggregated or guanidated BSA (Mitchison, 1969a) or keyhole limpet hemocyanin (Unanue, 1969) were all much less immunogenic when they were transferred inside of macrophages rather than injected directly.

Finally, evidence is accumulating that antigen–RNA complexes obtained from macrophages incubated with T2 phages (Friedman *et al.*, 1965; Adler *et al.*, 1966; Gottlieb *et al.*, 1967; Gottlieb, 1968; Fishman and Adler, 1968; Fishman, 1969), *Maia squinado* hemocyanin (Askonas and Rhodes, 1965), or synthetic polypeptides (Pinchuck *et al.*, 1968; Gottlieb, 1969a) may transfer immunological reactivity very effectively to both *in vivo* and *in vitro* systems.

The protein moiety of the immunogenic complex of T2–RNA did not contain more than thirty to thirty-five amino acids (Gottlieb and Straus, 1969) but could effectively inhibit phage-neutralizing antibody induced with the complete virus (Gottlieb, 1969b). It was thus demonstrated that preservation of the native tertiary structure of tail fiber antigen was not necessary for the immunogenicity of bacteriophage T2. Nevertheless, other antigens are known to elicit the formation of antibodies directed against determinants associated with tertiary structure (Kaminski, 1965). These observations further suggest that antigen handling by macrophages (and the vacuolar system) may differ considerably, depending upon the antigen.

III. Mediators of Tissue Injury Found in Lysosomes

Mediation of immune injury has already been discussed extensively for this series by Cochrane (1968). Since that survey, the functions of leukocytes, and especially their lysosomes, have been investigated further. Whatever the role of humoral mediators in acute inflammation, structural *injury* to tissues (as opposed to vasodilation, pain, and swelling) cannot proceed without hydrolytic degradation of extracellular and intracellular macromolecules. Lysosomes appear suitably equipped for this role. Substances found in lysosomes of leukocytes or other cells have now been shown capable of degrading the following materials relevant to tissue injury: collagen, elastin, protein polysaccharides of cartilage, intact cartilage, components of the complement system, hyaluronate, chondroitin sulfates, endotoxin, histones, and nucleic acids. A summary of these actions of lysosomal enzymes (with appropriate references) is found in Table II. One note of caution: it is still not entirely clear whether all, or indeed most, lysosomal hydrolases can attack undenatured substrates at hydrogen ion concentrations likely to obtain under physiological or even pathological circumstances. The best example is the collagen molecule, which in its native, undenatured state is cleaved at neutral pH by lyso-

TABLE II

HYDROLYSIS OF MACROMOLECULES RELEVANT TO TISSUE INJURY BY LYSOSOMAL HYDROLASES

Substrate	Enzyme	Source	Reference
		Extracellular structures	
Collagen	Collagenase	Leukocytes	Lazarus *et al.*, 1968
Protein polysaccharides	Hyaluronidase	Liver	Weissmann and Spilberg, 1968
Protein polysaccharides	Neutral protease	Leukocytes	Weissmann and Spilberg, 1968
Protein polysaccharides	Cathepsin D	Liver cartilage	Barret, 1969
Hyaluronate, chondroitin sulfate	Hyaluronidase	Liver, bone	Aronson and Davidson, 1967; Vaes, 1967
Cartilage matrix	Cathepsin D	Cartilage	Ali (1964)
Elastin	Elastase	Leukocytes	Janoff and Zeligs, 1968
Arterial walls	Elastase	Leukocytes	Janoff and Scherer, 1968
Basement membranes	Cathepsins D, E	Leukocytes	Cochrane and Aikin, 1966
Erythrocyte membranes	?	Liver	Desai and Tappel, 1965
		Intracellular structures	
Mitochondria	?	Liver	Tappel *et al.*, 1963
Deoxyribonucleic acid	Deoxyribonuclease	Liver, etc.	Bernardi, 1968
Ribonucleic acid	Ribonuclease	Liver, etc.	de Duve *et al.*, 1955
Histones	Histonase (pH 7.4)	Leukocytes	P. Davies *et al.*, 1970
Histones	Cathepsins D, E	Leukocytes	P. Davies *et al.*, 1970
		Circulating materials	
C'1s, C'1$\bar{s}$	Neutral protease	Leukocytes	Taubman *et al.*, 1970
C'3, C'5	Neutral protease	Leukocytes	Taubman *et al.*, 1970; Ward and Hill, 1970
Kinins	Acid peptidases	Leukocytes	Melmon and Cline, 1968
Fibrin	?	Leukocytes	Riddle *et al.*, 1965
Thyroglobulin	Cathepsins D, E	Spleen	Weigle *et al.*, 1969
γ-Globulin	Neutral, acid proteases	Beef, human spleen	Fehr *et al.*, 1968
Endotoxin	?	Liver	Filkins, 1970
"Cytotaxigens"	? Neutral proteases	Leukocytes, macrophages	Borel *et al.*, 1969
Plasminogen	Urokinase	Kidney	Ali and Lack, 1965

somal collagenase (Lazarus *et al.*, 1968) to yield two distinct subunits corresponding to 25 and 75% of the molecule. Subsequently, it may be attacked by less specific proteases, such as those of macrophages (Woessner, 1965). When native collagen is exposed to acid pH, it becomes partially denatured and susceptible to the latter group of enzymes. In similar fashion, proteins such as PP-L (Weissmann and Spilberg, 1968), thyroglobulin (Weigle *et al.*, 1969), or histones may be denatured either during isolation or assay, so as to render them prey to the known lysosomal proteases, rather than to specific enzymes. Furthermore, a series of nonenzymatic factors have been isolated from lysosomes. These induce capillary permeability (Burke *et al.*, 1964), release histamine from mast cells (Janoff *et al.*, 1965), provoke fever (pyrogen) (Herion *et al.*, 1966), and kill bacteria (Zeya and Spitznagel, 1966). It is beyond the scope of this review to discuss these in detail; instead, we shall focus on the mechanisms that govern their release in immune reactions.

IV. Lysosomes in Four Types of Immune Injury

One convenient way of classifying immunologically induced tissue injury is according to Coombs and Gell (1963), who suggest four categories into which such reactions can be placed. *Type I* reactions are acute allergic reactions, mediated by IgE and the release of vasoactive amines from basophiles and/or mast cells which have been passively coated by antibody. *Type II* reactions are mediated by complement after circulating antibodies have been attached to the surface antigens of target cells. In *Type III* reactions, circulating antigen–antibody complexes activate complement in the fluid phase. *Type IV* reactions are mediated by lymphocytes that have become transformed and rendered capable of tissue injury after an encounter with antigen or foreign tissue. None of these categories, of course, is either exclusive or comprehensive. Thus anaphylaxis is mediated by both Type I and Type III reactions, homograft rejection can result from Type II and Type IV reactions; etc. Furthermore, autoimmunity is such a complex phenomenon that it may well fall into all 4 categories. The role of lysosomes in autoimmunity is discussed elsewhere (Weissmann, 1964, 1965, 1966) and is not detailed below.

A. Type I Reactions

The cell types which are prominent in these reactions are the *blood basophile,* the tissue *mast cell,* and the *eosinophile.* From the work of Terry *et al.* (1969), of Fedorko and Hirsch (1965), and of Sampson and Archer (1967), clearly the blood basophiles contain a uniform population of granules, which resemble those of other polymorphonuclear leukocytes only in their biogenesis from cisternae of the Golgi apparatus

(Terry *et al.*, 1969). The granules can be shown to possess a crystalline material disposed as a hexagonal lattice, although a hexagonal array of microfilaments is not excluded. The basophiles are poorly phagocytic, and their granules contain few acid hydrolases, but are rich in histamine. There are, as yet, no compelling reasons to classify the granules as lysosomes. Mast cells, which bear some relationship to basophiles have been more extensively studied. Their specific granules contain histamine, serotonin, heparin, and a chymotrypsin-like enzyme (Selye, 1965). The granules can be isolated in intact form without an adherent membrane (Lagunoff *et al.*, 1964); indeed, their membranes may form a sort of cytoplasmic syncytium (Padawer, 1969). Since it is presumed that the overall integrity of mast cell granules is governed by electrostatic interaction, there is little reason to regard them as true lysosomes. The cells are clearly phagocytic—they take up colloidal thorium dioxide, zymosan, etc.—but the merger of endocytic vacuoles with mast cell granules is somewhat different from that described with true lysosomes (neutrophile, macrophages) (Padawer, 1969). Eosinophiles contain granules that differ only in enzymatic content (lack of lysozyme, high peroxidase activity) from neutrophiles. Their granules are true lysosomes, and they merge with endocytic vacuoles in a manner strictly comparable to that of the neutrophile, after uptake of particles, cells, or immune complexes (Archer and Hirsch, 1963a,b). They are attracted chemotactically to antigen–antibody complexes (Litt, 1964).

Indeed, it has been clearly shown that Type I reactions *in vitro* do not involve lysosomes. Thus, Pruzanski and Patterson (1967) studied the subcellular distribution and release of histamine and lysosomal hydrolases in human leukocytes. Both the amine and lysosomal β-glucuronidase were found in large granule fractions. When leukocytes were obtained from sensitized individuals and exposed to antigen *in vitro* histamine was released into cell supernatants and surrounding media *without* concomitant release of β-glucuronidase. In contrast, uptake of starch particles was associated with release of β-glucuronidase, but not of histamine. Crowder *et al.* (1969) obtained similar dissociation between release of histamine and two lysosomal enzymes after challenge by opsonized staphylococci or a protein antigen. We have not only confirmed these data but also demonstrated that procedures that can raise the intracellular level of cyclic 3′,5′-adenosine monophosphate can inhibit release both of histamine and of β-glucuronidase (May *et al.*, 1970) (Tables III and IV). These experiments indicate that the mechanisms that govern release of histamine (from specific granules of blood basophiles) and of β-glucuronidase (from azurophile granules of neutrophiles) (Baggiolini *et al.*, 1969) can be affected by similar pharmacological means (see below).

TABLE III
EFFECT OF THEOPHYLLINE AND/OR CYCLIC ADENOSINE 3′,5′-MONOPHOSPHATE ON RELEASE OF HISTAMINE FROM HUMAN POLYMORPHS

Agent added	No. of Experiments	Total histamine released (%)
None	9	0–3
Zymosan	9	3.6
Ragweed 6 μg./ml.	6	74.0[a]
Ragweed + theophylline 10^{-3} *M*	2	14.0[b]
Ragweed + cyclic adenosine 3′,5′-monophosphate 10^{-3} *M*	2	60.5
Ragweed + cyclic adenosine 3′,5′-monophosphate 10^{-3} *M* + theophylline 10^{-3} *M*	4	0.0[b]

[a] p (vs. control) < 0.01.
[b] p (vs. ragweed) < 0.01. Mann-Whitney "U" test (modified from May *et al.*, 1970).

Moreover, they also clearly demonstrate that reactions mediated by IgE result in histamine release without an effect upon extrusion of enzyme from lysosomes, whereas, phagocytosis has the opposite action. These considerations further remove the basophile granule from the general category of lysosomes.

Other mediators of immediate reactions are the kinins (reviewed by Kellermeyer and Graham, 1968) and SRS-A (Orange *et al.*, 1969). Whereas kinins are generated from plasma by neutral and acid proteases, acting on circulating kininogen, they can be degraded by acid

TABLE IV
EFFECT OF THEOPHYLLINE AND/OR CYCLIC ADENOSINE 3′,5′-MONOPHOSPHATE ON RELEASE OF β-GLUCURONIDASE FROM HUMAN POLYMORPHS

Agent added	No. of Experiments	Enzyme release (μg.Φ/ml./hr.)
None	9	4.7
Triton X-100 (0.2%)	9	47.0[a]
Ragweed 6 μg./ml.	6	5.0
Zymosan	9	13.6[a]
Zymosan + theophylline 10^{-3} *M*	5	8.3[b]
Zymosan + cyclic adenosine 3′,5′-monophosphate 10^{-2} *M*	3	12.2
Zymosan + cyclic adenosine 3′,5′-monophosphate 10^{-2} *M* + theophylline 10^{-3} *M*	4	5.2[b]

[a] p (vs. control) < 0.01.
[b] p (vs. zymosan) < 0.01. Mann-Whitney "U" test (modified from May *et al.*, 1970).

proteases (kininases) of leukocyte lysosomes (Melmon and Cline, 1968). Hegner (1968) has found both kinin-forming and kinin-destroying enzymes localized in lysosomal fractions of horse leukocytes. The SRS-A appears to be formed by polymorphonuclear leukocytes (Macmorine *et al.*, 1968; Orange *et al.*, 1969), but its subcellular distribution has not been studied extensively. Release of SRS-A from leukocytes can also be influenced by agents which modify intracellular levels of cyclic AMP (Ishizaka *et al.*, 1970).

Since the description by Janoff and Zweifach (1964) of a cationic protein component of neutrophile lysosomes, capable of provoking capillary permeability, several studies have implicated this and related proteins in acute inflammation. This subject was reviewed by Cochrane (1968). Briefly, the cationic proteins of neutrophiles are quite heterogeneous. One well-defined protein fraction (Seegers and Janoff, 1966) induces inflammation by virtue of the disruption of mast cells; other fractions induce inflammatory responses by less well-defined means (Cochrane, 1968).

B. Type II Reactions

When heterologous antibodies are added to living cells, the consequences to lysosomes depend upon the presence of complement. Thus, Dumonde *et al.* (1965) added heterologous antibody to ascites tumor cells in the absence of complement and found (by histochemical means) alterations in the access of lysosomal acid phosphatase to substrate at discrete granular sites in the cytoplasm. In the presence of complement, however, the granules were no longer observed. Cell death had been accompanied by disruption of lysosomes, the enzymes of which became diffusely distributed throughout the cytoplasm. Weiss and Dingle (1964) raised antiserum in rabbits to lysosome-rich fractions of rat liver. Heated antiserum had no effect upon their test systems, i.e. lysosome-rich subcellular fractions of rat liver, slices of rat liver, cultured fibroblasts. In contrast, unheated serum induced release of lysosomal hydrolases from, and altered the staining of, lysosomes, and released enzymes from intact liver slices. However, no effect of the serum upon isolated organelles was detected. Dorling and Loewi (1965) observed altered staining of lysosomes in rat kidney cells exposed to rabbit antisera. Fell and Weiss (1965) prepared rabbit antiserum against fetal mouse cells. The changes induced by unheated sera in cultured mouse limb bones near term resembled closely those provoked by other agents which caused release of lysosomal enzymes from cells, i.e., retinol, sucrose. Each of these experiments suggested that complement-sufficient antisera directed against components of cell membranes acted to "labilize" lysosomes by an

indirect action mediated by primary events at the cell surface. In contrast, Quie and Hirsch (1964), using purer, absorbed, heterologous antisera directed against leukocyte lysosomes, were able to induce intact leukocytes to degranulate—with apparent discharge of acid hydrolases into cytoplasm. Such antisera provoked release of three lysosomal hydrolases from isolated leukocyte lysosomes *in vitro*. This apparent discrepancy awaits resolution. The group in Cambridge (Fell *et al.*, 1966, 1969; Dingle *et al.*, 1967; Coombs and Fell, 1969) investigated in detail the action of complement-sufficient antisera upon cartilage and bone in culture. Their results may be summarized as follows:

1. Both antifowl erythrocyte and anti-Forssman sera caused dissolution of cartilage matrix, when added to fetal chick bones in culture.

2. Only the cells at the surface of the explants were killed by antisera, whereas the bulk of osteocytes and chondrocytes remained viable in the absence of solid cartilage matrix.

3. Complement was necessary for the production of these changes by both purified IgG and IgM fractions of antisera. Addition of C'6 to serum deficient in this factor conferred the ability to activate antibody; the activity of fresh serum could be abolished by absorption with antigen–antibody complexes or zymosan.

4. Breakdown of cartilage matrix was associated with increased synthesis and release into the media of lysosomal acid protease identified largely as cathepsin D. Antisera directed against the purified enzyme prevented cartilage matrix breakdown induced by a variety of procedures (Weston *et al.*, 1969).

5. The effects of complement-sufficient antisera upon cartilage matrix and enzyme release may be overcome by cortisol (a stabilizer of lysosomes; Weissmann and Dingle, 1961) or EACA (an inhibitor of the acid protease of cartilage; Ali, 1964). These experiments suggest that Type II reactions result in the activation of complement at the cell membrane and secondary events cause the lysosomal system to extrude its enzymes into extracellular matrix. The subsequent hydrolysis by cathepsin D of the major constituent of cartilage matrix (PP-L) leads to loss of metachromasia. Two points warrant elaboration, however. First, whereas EACA clearly inhibits crude acid protease activity of cartilage and the breakdown of tissue (Fell *et al.*, 1966) this inhibitor is ineffective upon more purified cathepsin D (Barrett, 1969), even from liver. Furthermore, antisera directed against cartilage cathepsin D (antigenically identical to that of liver) do not entirely abolish cartilage breakdown, especially over longer periods (Weston *et al.*, 1969). These points may indicate that lysosomes contain other proteases, inhibited by EACA, which can degrade PP-L. Indeed, such activity has been identified in granulocyte

lysosomes that contain a neutral, EACA-inhibited protease active on PP-L (Weissmann and Spilberg, 1968).

Another Type II reaction which has been extensively studied is nephrotoxic nephritis. The contributions of neutrophile lysosomes to the lesions of this model glomerulitis were reviewed in detail by Cochrane (1968). In brief, heterologous antibodies directed against glomerular basement membrane become fixed to glomeruli, complement is bound and activated, factors chemotactic for neutrophiles are elaborated, and the white cells come into direct contact with the naked basement membrane. The subsequent dissolution of the membrane can be monitored by analysis for its degradation products in urine. Cochrane and Aikin (1966) suggest that cathepsins D and E are responsible because the capacity for degrading basement membrane of lysates prepared from *rabbit* neutrophiles parallel their capacity to split denatured hemoglobin at pH 2.5 (pH optimum of cathepsin E = 2.5, of cathepsin D = 3.4). Janoff and Scherer (1968), studying lysates of *human* leukocyte lysosomes, have presented evidence that their elastolytic activity, at netural pH, could account for hydrolysis of elastic fibers in human kidney *in vitro* and dog aorta *in vivo*. The elastase has been partially purified and shown capable of hydrolyzing benzyloxycarbonyl-L-alanine *p*-nitrophenyl ester—a synthetic elastase substrate at near-neutral pH (Janoff, 1969). Several real problems remain unsolved in this model system. The neutrophiles possess granule enzymes capable of hydrolyzing basement membranes, and degraded fragments of the latter escape in the urine. Neutrophiles are directly apposed to the basement membranes, but there has been no suggestion as to how the enzymes might be released. The neutrophiles are *not* degranulated, their membranes have not been shown to be compromised, nor has direct exocytosis of granule contents been observed (Cochrane, 1970). Further work on this problem should do much for our understanding of tissue injury, and an *in vitro* model utilizing micropore filters impregnated with immune reactants has already shown that neutrophiles release azurophile enzymes upon contact with the prepared surface (Henson, 1970).

C. Type III Reactions

The union of antigen with certain types of antibody results in the formation of immune complexes, which in soluble or insoluble form, can enter cells by endocytosis. The major contributions to uptake of immune complexes are made by cells of the reticuloendothelial system and by leukocytes (Fennel and Santamaria, 1962; Dixon, 1963; Grant *et al.*, 1967). Thereafter, the consequences of uptake to endocytic cells are due to (*1*) enhanced endocytosis per se and (2) the nature of the material

taken up, which, in the case of immune complexes, includes components of the complement sequence (Müller-Eberhardt, 1968). Daems and Oort (1962) first showed that immune complexes were taken up by leukocyte lysosomes, the ultrastructural appearance of which became altered in the process. Thomas (1964) then demonstrated that isolated neutrophile lysosomes could substitute for intact neutrophiles in reversed passive Arthus reactions induced in leukopenic rabbits. One interpretation of these experiments is that neutrophile granules, presumably taken up by histiocytes, etc., supplied the missing inflammatory substances. Such studies suggested that the uptake of immune complexes by lysosomes containing neutrophilic materials was followed by inflammation in the immediate vicinity of the endocytosing cell. This was not entirely surprising, since Metchnikoff (1905) had first described this phenomenon:

"The leucocytes, having arrived at the spot where the intruders are found, seize them after the manner of the amoeba and within their bodies subject them to intracellular digestion. This digestion takes place in the vacuoles in which usually is a weakly acid fluid which contains digestive ferments (cytases); of these, a very considerable number are now recognized. The cytases must be grouped with soluble ferments which are not thrown off by the phagocytes so long as these remain intact. Immediately these cells are injured, however, they allow a part of their cytases to escape."

In a series of studies, Movat and collaborators (Lovett and Movat, 1966; Movat *et al.*, 1964, 1968; Taichman and Movat, 1966) have established that immune complexes follow the route in phagocytic cells of other particulates. They are endocytosed in phagosomes of leukocytes and platelets. The phagosomes merge with primary lysosomes to form secondary lysosomes. Consequently, and by less well-studied mechanisms, lysosomal enzymes are released extracellularly. Following immune complex formation *in vivo*, at a time when uptake of microprecipitates of antigen and antibody had been demonstrated by ultrastructural means, levels of acid protease (denatured hemoglobin, pH 2.5), β-glucuronidase, and acid phosphatase became elevated significantly in the serum (Movat *et al.*, 1968). *In vitro* studies of passive cutaneous anaphylaxis in the guinea pig showed that neutrophiles could endocytose microprecipitates of antigen (ferritin) and antibody (hyperimmune antiferritin) into secondary lysosomes. Later stages of particle uptake were associated with "degranulation" of the endocytic cell (Lovett and Movat, 1966). Furthermore, it was found that substances released from leukocytes that had ingested immune complexes were capable of inducing local capillary permeability (Burke *et al.*, 1964). By means of protease inhibitors, this permeability factor could be distinguished from the basic, inflammatory

proteins described by others (Janoff and Zweifach, 1964; Golub and Spitznagel, 1966; Ranadive and Cochrane, 1968). Grant *et al.* (1967) found that union of antigen (ferritin) with antibody need not necessarily proceed in the circulation or in vessel walls and have emphasized the extravascular events which may be early triggers of the local Arthus reaction. Astorga and Bollet (1965) showed that leukocytes could endocytose complexes of rheumatoid factor with γ-globulin; three lysosomal hydrolases became redistributed in these cells, presumably due to formation of more fragile, secondary lysosomes.

Not only neutrophiles are involved in particle uptake. Treadwell (1965) has conducted several studies of the role of Kupffer cells in systemic anaphylaxis in the mouse. Within 15 to 20 minutes after challenge of sensitized animals by antigen or complexes, plasma acid phosphatase showed six- to sevenfold increases; the liver content of the enzyme was decreased concordantly. Further studies showed that these changes were due to the disruption of Kupffer cells after these had endocytosed immune precipitates (Santos-Buch and Treadwell, 1967). Recently, Treadwell (1969) established that increments of lysosomal acid phosphatase in the plasma—and decrements in enzyme content of the liver—could be used as sensitive indexes of genetic susceptibility to anaphylaxis in some mouse strains (W-BRVS, SJL/J) vs. others (C57BL/6J).

Such studies suggest not only that immune complexes are taken up by lysosomes but also that the encounter of complexes with the organelles leads to escape into the circulation of previously sequestered enzymes and biologically active materials. Possibly, under these abnormal situations (hyperimmunization, heterologous antibody), cells are killed by some other means and lysosomal hydrolases are released *pari passu* with other cellular constituents. Since none of the above studies was monitored with appropriate enzyme markers for other organelles or the cell sap itself, we must turn to data on phagocytosis of particulates in general. From data in Tables III and IV, it appears that polymorphonuclear leukocytes extrude a portion of their hydrolases when forced to take up zymosan (May *et al.*, 1970). These observations are in accord with other studies (Baehner *et al.*, 1969; Pruzanski and Patterson, 1967; W. E. Parish, 1969; Holmes *et al.*, 1969; Crowder *et al.*, 1969) on extrusion of lysosomal hydrolases during particle uptake. These, too, are open to criticism, since release of hydrolases after ingestion of inert particles can be explained on the basis of diminished viability, nonspecific damage to the plasma membrane during endocytosis, contamination by endotoxin, etc. In two of these studies (Crowder *et al.*, 1969; May *et al.*, 1970), viability was assayed by dye exclusion. Baehner *et al.* (1969) found that a soluble cytoplasmic enzyme (catalase) was extruded from human poly-

morphs during uptake of starch particles, although to a lesser extent than the concomitantly extruded lysosomal hydrolases. In investigating this problem, we have exposed mouse peritoneal macrophages to zymosan (Dukor and Weissmann, 1970) and found no effect upon viability or extrusion of lactate dehydrogenase (a cytoplasmic enzyme) under circumstances in which considerable amounts of lysosomal β-glucuronidase were released (Table V). Release of lysosomal hydrolases, at least with zymosan, is *not* associated with nonspecific release of LDH, although dead cells readily release both enzymes. Furthermore, it is also possible to block release of β-glucuronidase from macrophages by addition of cyclic AMP. The latter point needs to be explained. The work of Lichtenstein and Margolis (1968), Levy and Carlton (1969), and Malawista (1968) suggests that two types of agents can block release of histamine from blood basophiles by antigen. One group (cyclic AMP, methyl xanthines) apparently acts by raising the intracellular level of cyclic AMP; whereas, the other group includes modalities (colchicine, cold temperatures, D_2O) that interfere with the function or aggregation of microtubules. Now the relationship that these two groups bear to each other is by no means clear, but it has become common to attribute to microtubules a function in the directed flow and merger of cytoplasmic organelles (Malawista, 1965; Porter, 1966; Lacy *et al.*, 1968; R. Hirschhorn *et al.*, 1970a). It may well be, therefore, that microtubules regulate

TABLE V

ENZYME RELEASE BY ZYMOSAN (OR FREEZING AND THAWING) FROM CULTURED MOUSE PERITONEAL MACROPHAGES[a]

Culture conditions	% Cells with ingested particles	β-Glucuronidase released (%)[b]	Lactic dehydrogenase released (%)[c]	% Cells excluding eosin Y
Controls	—	1.0 ± 0.3	0.2 ± 0.5	99.2 ± 0.4
Freezing and thawing[d]	—	60.3 ± 8.9	38.9 ± 6.4	0
Zymosan[e]	83.2 ± 5.7	8.9 ± 0.4	0.1 ± 0.3	98.5 ± 0.9
Zymosan and adenosine 3′,5′-monophosphate 10^{-3} *M*	88.3 ± 2.0	3.9 ± 0.3	ND	98.7 ± 0.6

[a] Thioglycollate-induced peritoneal mononuclear phagocytes cultured for 24 hours in medium "199" containing 10% fetal calf serum (2×10^6 cells in 2 ml. per culture). Particle uptake, viability, and enzyme release determined after a further 2-hour period; 6–12 cultures per group.

[b] Activity of supernatant expressed in per cent of total enzyme activity of culture after six cycles of freezing and thawing.

[c] Activity of supernatant expressed in per cent of total enzyme activity of culture after addition of 0.1% Triton X-100.

[d] One cycle at the beginning of the 2-hour period.

[e] Particles per culture, 4×10^7.

the flow of phagosomes to primary lysosomes, a site sensitive to colchicine, as Malawista and Bensch (1967) suggest. If this site were also sensitive to the intracellular level of cyclic nucleotides, the release or extrusion of hydrolases from neutrophiles might be modified by those agents that, in basophiles, modify release of histamine. This discussion has focused upon the zymosan model, but since immune complexes provoke the release of hydrolases from intact cells (a point that deserves still further critical evaluation), the situation should hold for uptake of antigen–antibody particulates.

There is no question (Cochrane, 1968) that immune complexes can generate chemotactic factors from complement components in the fluid phase. Thus they call forth the cellular machinery for their own disposal by the endocytic process. But do components of the complement system play a role in subsequent events within the endocytic cell or its environment? Is the system capable of self-amplification? Following experiments in which leukocytes (Hurley, 1964) or their lysosomes (Cornely, 1966; Borel *et al.*, 1969) were shown to generate chemotactic factors from fresh serum, Ward and Hill (1970) identified a neutral protease in rabbit leukocyte lysosomes. The enzyme was shown capable of generating chemotactic activity by means of a complement component. Indeed, lysates of the granules cleaved C′5 to C′5a fragments which varied in molecular weight from 4 to 15,000. The enzyme, which had a pH optimum of 7.2 to 7.3, was inhibited by soybean trypsin inhibitor, EACA, TAME, and BAA methyl ester. Hill and Ward (1969) had previously shown that a neutral protease from heart tissue could generate chemotactic activity from C′3; this protease was also inhibited by trypsin inhibitor, TAME, and BAA. Taubman *et al.* (1970) have recently confirmed the generation of chemotactic activity from C′5 and, furthermore, demonstrated that neutrophile lysosomes can cleave C′3 into large and small fragments—as yet not biologically active. Additionally, lysosomal fractions can both activate C′1s to C′1$\bar{s}$, and inactivate C′1$\bar{s}$. Such studies indicate that lysosomal enzymes—in the absence of immune reactions—can induce alterations usually associated with sequential, immunological activation of the complement system. It is thus likely that when leukocytes in plasma endocytose inert particles or immune precipitates they may also take up components of the complement system in various stages of activation. Recent studies with model membranes, liposomes (reviewed by Sessa and Weissmann, 1968), have shown that the activated complement sequence C′1 → C′9 can disrupt these lipid models for lysosomes or other biomembranes (Haxby *et al.*, 1969) and that C′5–9, acting via a phospholipase C disrupts liposomes (Lachmann *et al.*, 1970). It is of interest, therefore, to determine whether one or another of the lipid-

disruptive components (C'8, C'9), activated *inside* secondary lysosomes during endocytosis, is involved in the damage to cells following uptake of immune complexes.

One further mechanism for tissue injury in immune complex disease must be mentioned. When leukocytes are injured in Arthus reactions, some or many of their lysosomal granules are extruded in intact form (Lovett and Movat, 1966). Now, when leukocytes are exposed to particles such as latex or zymosan, they can take up many of these without a major loss of viability. But when neutrophiles were permitted to ingest isolated (heterologous) leukocyte granules, they underwent prompt and rapid cell lysis (Wiederman *et al.*, 1966; R. Hirschhorn and Weissmann, 1967). This was not brought about when cells were exposed to lysates of the granules and, presumably, was due to the activation of one or another granule product during ingestion. Macrophages do *not* undergo destruction after uptake of neutrophile granules. It remains to be determined whether isologous granules will induce similar degrees of cell death.

D. Type IV Reactions

Lymphocytes, presumably derived from the thymus (Mitchison, 1969b), undergo transformation into cells capable of inflicting tissue injury after their encounter *in vitro* and ? *in vivo* with (*1*) antigens to which the donor has become sensitized (Pearmain *et al.*, 1963) (*2*) allogeneic cells (Dutton, 1965) (*3*) nonspecific mitogens from *Phaseolus vulgaris* (PHA) (Nowell, 1960), or PWM (Farnes *et al.*, 1964), (*4*) antisera against allotoypic determinants of immunoglobulins (Sell and Gell, 1965), and (*5*) antisera against surface antigens of the lymphocytes themselves (Grasbeck *et al.*, 1963). The transformed lymphocyte in culture has been shown to elaborate several biologically active factors into the medium. These include a macrophage inhibitory factor (David, 1966), a factor capable of inducing transformation and mitosis of resting lymphocytes (Maini *et al.*, 1969), a factor capable of inducing local inflammation (B. Bennett and Bloom, 1968), a transfer factor (Lawrence, 1969), a factor capable of inducing responsiveness to specific antigens in unsensitized lymphocytes (Valentine and Lawrence, 1969), a factor chemotactic for other macrophages (Ward *et al.*, 1969), and a factor which is cytotoxic to target cells (Granger and Kolb, 1968). This subject has been reviewed extensively in a recent volume (Lawrence and Landy, 1969). The relationship of these factors to lysosomes and the vacuolar system is by no means established, but each of them is released at a time when the vacuolar system is activated. The transformed lymphocyte is rich in lysosomes (Allison and Malucci, 1964; Parker *et al.*, 1965;

R. Hirschhorn *et al.*, 1965), whereas there is a paucity of these organelles in normal or resting blood lymphocytes. Within 36 to 48 hours after cells are stimulated by PHA or antigen, new, acid phosphatase-positive granules apppear. The cells' total content of acid β-glycerolphosphatase, acid phenolphthalein phosphatase, and aryl sulfatase undergoes significant increases (R. Hirschhorn *et al.*, 1967). It must be pointed out, however, that other organelles (Douglas *et al.*, 1967; Chapman *et al.*, 1967; Halpern *et al.*, 1968; Clausen and Bouroncle, 1969) such as the endoplasmic reticulum and the Golgi apparatus (and their associated enzymes), undergo similar development. Histochemical studies of transformed lymphoid cells engaged in the destruction of target tissues have shown that the invading lymphocyte is rich in a variety of primary and secondary lysosomes; indeed, the latter show residual evidence of earlier endocytosis (Weiss, 1968; Brandes *et al.*, 1969). Resting lymphocytes do not attach to target cells and are poorly phagocytic (Robineaux *et al.*, 1969). In contrast, the plasma membranes of transformed cells are closely opposed to (Weiss, 1968) or even joined with (Brandes *et al.*, 1969) those of target cells. Uptake of materials such as endotoxin, immunoglobulins, peroxidase, or neutral red is considerably enhanced in the transformed cell (Hirschhorn *et al.*, 1968; Robineaux *et al.*, 1969). *In vitro* transformation of lymphocytes is followed by mitosis, and many investigators have presented evidence that lysosomes of lymphocytes and other cells (Robbins and Gonatas, 1964; Allison and Malucci, 1964; R. Hirschhorn *et al.*, 1965; Kent *et al.*, 1965; Bastos *et al.*, 1967) undergo morphological changes at mitosis. Perhaps their hydrolases are required for the remodelling of cells during division (R. Hirschhorn *et al.*, 1965; Allison, 1969). Critical evidence to this point is still lacking.

Lysosomes and the vacuolar system are clearly involved *early* in the course of lymphocyte transformation. Allison and Malucci (1964) described that, within a few hours after PHA stimulation of lymphocytes, there was an increased permeability of their membranes to the substrate for acid phosphatase. In a detailed biochemical study of these events, Brittinger *et al.* (1968) and R. Hirschhorn *et al.* (1968) established that within 2 to 4 hours after stimulation of lymphocytes by PHA, lysosomal hydrolases (but not malate dehydrogenase) become redistributed from granular to less-sedimentable fractions of cell homogenates (Table VI). These changes are accompanied by enhanced permeability of lymphocyte membranes to substances in the ambient medium such as neutral red. Furthermore, Brittinger *et al.* (1969) were able to demonstrate that a nonaggregating mitogen (PWM) also induced redistribution of lysosomal hydrolases. Other studies had shown that changes in the nuclei of transformed cells [acetylation of histones (Pogo *et al.*, 1966), turnover of

TABLE VI

DISTRIBUTION OF ENZYMES AMONG SUBCELLULAR FRACTIONS DERIVED FROM LYMPHOCYTE CULTURES INCUBATED WITH AND WITHOUT PHYTOHEMAGGLUTININ-P FOR 120 MINUTES AT 37°C.

Enzyme	Treatment[b]	No. of experiments	Fraction[a]					
			Debris	Nuclei	Granules	$P \pm$ SED[c]	Supernatant	$P \pm$ SED[c]
β-Glucuronidase	Control	13	5.6 ± 1.0	13.2 ± 1.1	69.3 ± 1.4	<0.001	12.0 ± 0.87	<0.001
	PHA-P	13	3.6 ± 0.5	18.7 ± 2.8	57.8 ± 2.3	±2.45	19.9 ± 0.89	±0.774
Acid phosphatase	Control	8	4.9 ± 1.6	15.7 ± 2.0	61.6 ± 2.7	<0.05	17.8 ± 1.4	<0.001
	PHA-P	8	3.7 ± 0.8	18.5 ± 3.3	53.9 ± 2.4	±3.21	23.9 ± 1.4	±1.10
Malate dehydrogenase	Control	5	3.0 ± 2.2	13.0 ± 2.2	27.5 ± 1.9	Not significant	56.6 ± 2.5	Not significant
	PHA-P	5	3.5 ± 0.9	16.3 ± 4.0	31.3 ± 2.9	±3.44	49.0 ± 2.0	±2.41
Protein	Control	10	9.6 ± 1.7	10.0 ± 1.2	16.6 ± 1.7	Not significant	63.9 ± 3.2	Not significant
	PHA-P	10	7.2 ± 1.4	11.5 ± 1.5	16.2 ± 0.9	±1.80	65.0 ± 2.3	±2.28

[a] Values are given as per cent of the total recovered activity ± SEM.

[b] PHA-*P* = phytohemagglutinin-P.

[c] Paired t-test ± standard error of differences between paired samples (Hirschhorn *et al.*, 1968).

phosphoproteins (Kleinsmith *et al.*, 1966), and enhanced binding of actinomycin D (Darzynkiewicz *et al.*, 1969) or acridine orange (Killander and Rigler, 1969)] might indicate that controls of the transcription of DNA were affected by PHA. One possible sequence of events, therefore, appeared amenable to further study. The encounter of resting lymphocytes with PHA (or antigens in the case of sensitized cells) might lead to enhanced endocytic activiy (R. Hirschhorn *et al.*, 1968). The redistribution of lysosomal hydrolases which accompanied these events might render available to the resting nucleus a neutral protease that would remove repressor proteins (?histones) from DNA, making pre-

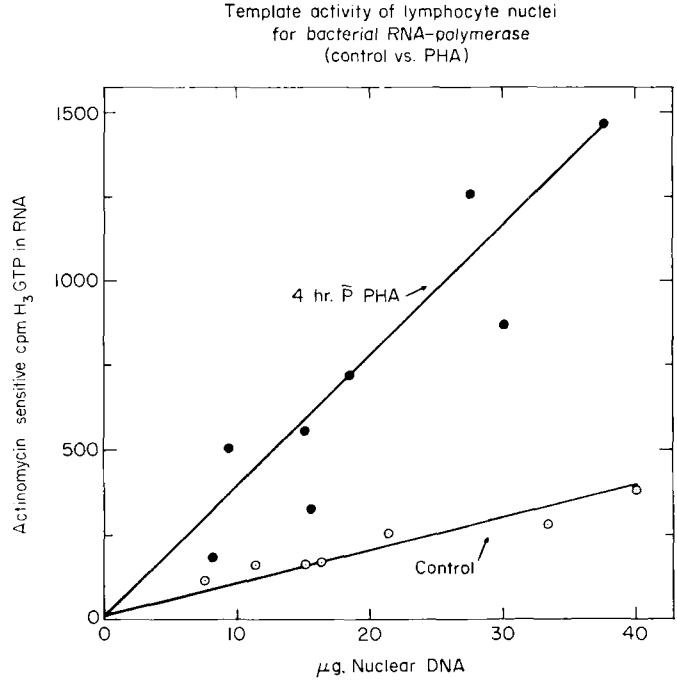

FIG. 2. Effect of stimulation of lymphocytes with phytohemagglutinin (PHA) for 4 hours on template capacity of the isolated nuclei. Lymphocytes were incubated for 4 hours with (●) and without (○) PHA; nuclei were isolated and incubated for 10 minutes at 37°C. in 0.5 ml. containing 58 μmoles Tris buffer (pH 7.5), 1.2 μmoles spermidine HCl, 1.17 μmoles $MnSO_4$, 1 μmole $MgCl_2$, 0.450 μmole each of the four nucleoside triphosphates, 3.33 μcuries ^{3}H-guanosine triphosphate (GTP) (~3 mμmoles), 2–50 μg. nuclear DNA, and 50 units RNA polymerase (*Micrococcus lysodeikticus*, Miles Laboratories); and ^{3}H-GTP incorporation was determined. Each value is the average of duplicate determinations of counts per minute (c.p.m.) incorporated in the absence of actinomycin D minus the counts per minute incorporated in the presence of actinomycin D (R. Hirschhorn *et al.*, 1969).

viously repressed sites available for transcription by RNA polymerase. Histochemical evidence for dissociation of DNA from histones has, in fact, been obtained by Zetterberg and Auer (1969) and Rigler and Killander (1969). A further precedent for this sequence can be adduced from the studies of Allfrey *et al.* (1963), who found that treatment with trypsin augmented the template capacity of nuclei for endogenous RNA polymerase. To test this hypothesis, nuclei were isolated from cells 4 hours after PHA stimulation and from controls, and the template capacity of the former was found considerably enhanced (Fig. 2) (R. Hirschhorn *et al.*, 1969). Furthermore, experiments with frozen–thawed and detergent-treated nuclei excluded the possibility that changes in the nuclear membrane accounted for the augmentation of template activity. Perhaps most persuasive were experiments in which trypsin was added to nuclei from control and stimulated cells. Although the enzyme clearly augmented the template capacity of both nuclear preparations (Table VII), the *relative* enhancement of template activity by trypsin was clearly diminished in nuclei from PHA-stimulated cells. One explanation for these findings, in line with the above hypothesis, was that a neutral

TABLE VII

EFFECT OF TRYPSIN ON THE TEMPLATE CAPACITY OF NUCLEI ISOLATED FROM PHYTOHEMAGGLUTININ-STIMULATED AND NONSTIMULATED HUMAN LYMPHOCYTES[a]

Trypsin (μg./ml.)	No. of experiments	PHA	Control	*P/C* ratio
0	9	1,370 ± 225	370 ± 24	3.56 ± 0.507[b]
1.25	2	10,050 ± 550	5,320 ± 343	1.89 ± 0.071
10.7	3	14,100 ± 251	8,880 ± 156	1.60 ± 0.102
12.5	2	13,250 ± 150	10,450 ± 1,909	1.29 ± 0.150
25.0	2	25,500 ± 4,500	12,200 ± 1,300	2.08 ± 0.145
			Average with trypsin	1.7 ± 0.110[c]

[a] Nuclei isolated from cells cultured with and without phytohemagglutinin (PHA) for 2 hours were incubated for 20 minutes at 37°C. with trypsin (1.25–25 μg./ml.) and the reaction stopped by addition of soybean trypsin inhibitor (2.5–50 μg./ml.). The capacity of treated nuclei to prime for incorporation of ^{3}H-guanosine triphosphate (GTP) into RNA in the presence of exogenous RNA polymerase was determined. Values represent actinomycin D-sensitive (c.p.m.) ^{3}H-GTP incorporated/100 μg. DNA ± SEM. The amount of DNA per assay was varied between 10 and 20 μg. for trypsin-treated nuclei and between 10 and 40 μg. for nontrypsin-treated nuclei. *P/C* ratio = incorporation ^{3}H-GTP into RNA by nuclei from PHA-treated cells/incorporation of ^{3}H-GTP into RNA by nuclei from untreated cells.

[b] $P < 0.005$ (paired t-test) PHA versus control.

[c] $P < 0.005$ (t-test) *P/C* ratio without trypsin versus trypsin average (R. Hirschhorn *et al.*, 1969).

protease had preemptively acted upon protease-sensitive sites in stimulated cells. Indeed, Uyeki and Llacer (1968) were able to enhance DNA synthesis of spleen cell cultures by exposing them to trypsin. To test further the general hypothesis, inhibitors of protease action were added to cells exposed to PHA, and it was found that EACA, TAME, TLCK, and TPCK inhibited the expected response of lymphocytes to the mitogen (R. Hirschhorn *et al.*, 1970b). Furthermore, in experiments analogous to those discussed above with neutrophiles and macrophages (R. Hirschhorn *et al.*, 1970a), it was found that cyclic AMP ($>10^{-3}\,M$), its dibutyryl derivative ($>10^{-5}\,M$), and theophylline ($>10^{-4}\,M$), each diminished the response of cells (DNA, RNA synthesis) to PHA. When lower concentrations of cyclic AMP (between 3.3×10^{-5} to $3.3 \times 10^{-4}\,M$) were added to resting lymphocytes, *augmented* incorporation of tritiated thymidine was observed. These biphasic responses suggest that protein kinase may be the crucial enzyme involved (Miyamoto *et al.*, 1969); the enzyme is activated by low, and inhibited by high, concentrations of cyclic AMP. Since the transformation process is sensitive to variations in the level within cells of cyclic AMP, the hypothesis is reinforced that the merger and flow of components of the vacuolar system display similar sensitivity.

Because some of the agents that nonspecifically stimulate lymphocytes also disrupt isolated lysosomes, e.g., streptolysin S, staphylococcal α-toxin, Hg^{2+}, ultraviolet irradiation, and because some of the agents that stabilize lysosomes also inhibit lymphocyte transformation, e.g., cortisone or chloroquine, it was suggested tentatively that release of hydrolases from lysosomes within cells initiates lymphocyte transformation (K. Hirschhorn and Hirschhorn, 1965). Reevaluation of these data, together with the recent demonstration that the lysosome-disruptive factor in streptolysin S could be dissociated from its mitogenic activity (Taranta *et al.*, 1969) suggest that this concept is no longer tenable in its original form. By virtue of the possible role of the adenyl cyclase system (see above) and the inhibition by ouabain of lymphocyte transformation (Quastel *et al.*, 1969), it now becomes likely that nonspecific mitogens in *all cells*, and antigens in *some* cells, perturb the surface of the cell. Most agents active on lysosomes *in vitro* have similar effects at the plasma membrane (review by Weissmann, 1969). Surface membrane alterations, by means as yet unexplored, subsequently induce rearrangement of intracellular vacuoles. Fisher and Mueller (1968) have indeed shown increments of the incorporation of $^{32}PO_4^{2-}$ into phosphatidyl inositol shortly after PHA. Critical experiments are still lacking, however, to test the hypothesis that lysosomal proteases are responsible for changes in the nucleus after the primary stimulus to the vacuolar system has acted at the cell periphery.

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Molecular Size and Conformation of Immunoglobulins

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I. Introduction

One of the most exciting problems in molecular biology today is the elucidation of the relationship between the biological properties of immunoglobulins and their organization at the several levels of protein structure.

Significant advances have been made in the determination of the amino acid sequence of immunoglobulin subunits, particularly from myeloma proteins. The results of sequence studies have been extensively reviewed with particular reference to the genetic and evolutionary information they are thought to convey (Lennox and Cohn, 1967; Cohen and Milstein, 1967; Edelman and Gall, 1969). It is not our intention to reiterate these findings in detail except where they have yielded information on the particular structural feature under discussion. The present review is primarily concerned with studies on the higher levels of organization in immunoglobulin molecules; their size and shape as well as the more intimate aspects of their internal folding.

[1] A portion of the work described in this review was carried out during the tenure of a Wellcome Travelling Fellowship, 1966 and 1967, at Duke University.

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II. Molecular Size of Immunoglobulins and Subunits

The molecular size of the various immunoglobulins and their subunits are discussed separately in this section. However, to aid comparisons some of the most definitive data (in our opinion) are collected together in Table I.

TABLE I

MOLECULAR WEIGHTS OF IMMUNOGLOBULINS AND THEIR POLYPEPTIDE CHAINS[a]

Immunoglobulin or polypeptide chain	Species studied	Mol. wt. × 10^{-3}	
		Total	Polypeptide portion[b]
γG-Globulin	Human, rabbit, horse	143–149	—
γ Chain	Human, rabbit, horse	52–54	49–51
Light chain[c]	Human, rabbit, horse	22–23.5	—
γM-Globulin	Human, rabbit, shark, lamprey	800–950	—
γM_s Subunit	Human, rabbit	175–185	—
μ Chain	Human, rabbit	65–70	56–60
μ Chain	Shark, lamprey	70–77	—
γA-Globulin	Human serum, 7 S	158–160	—
	Human serum, 9–10 S	318	—
	Human, rabbit secretory	370–390	—
α_1 Chain	Human	56–58	50
α_2 Chain	Human	52–53	46
γA-Globulin	Mouse	115–120	—
α Chain	Mouse	<50	—[d]
γD-Globulin	Human	175–180	—
δ Chain	Human	60–65	54–59
γE-Globulin	Human	185–190	—
ε Chain	Human	71–73	62–63

[a] These figures reflect the authors' prejudices regarding the reliability of different molecular weight determinations. See test for full listing of results.

[b] The mass of the carbohydrate has been subtracted from the molecular weights of all heavy chains to facilitate comparison of the lengths of the polypeptide chains per se.

[c] All available evidence indicates that the same light chains are utilized in the assembly of all immunoglobulins, regardless of heavy-chain category, and that the different classes of light chain have nearly the same molecular weight.

[d] Level of carbohydrate has not been determined.

A. γG-Globulins

Estimates of the molecular weight of γG-globulins prior to 1960 have been tabulated by Porter (1960). The values obtained ranged from 150,000 to 190,000 and, in general, were above those obtained more recently (see below). Ultracentrifugal studies provided most of the early data although osmotic pressure, light scattering, and low-angle X-ray scattering were also used.

In recent years attention to the concentration dependence of the molecular weight values and the effects of aggregates in the γG preparations has resulted in lower values for the molecular weight. Cammack (1962) obtained a value of 137,000 for rabbit γG using sedimentation and diffusion, taking into account the concentration dependence of the parameters used. Failure to do this resulted in higher values, e.g., 179,000 at 10 mg./ml. The same sample examined by the Archibald approach to equilibrium method gave a value of 172,000 at 9.2 mg./ml. The effect that small amounts of polymerized γG can have on molecular weight values has been well illustrated by Pain (1965). A sample of equine γG, apparently free of aggregates, gave a value of 151,000 by sedimentation and diffusion analysis. However, sedimentation equilibrium studies gave a weight-average molecular weight (M_w) of 160,000 and a z-average value (M_z) of near 170,000 indicating mass heterogeneity. Gel filtration on Sephadex G-200 eliminated trace amounts of polymer and the M_z dropped to 151,000. Similarly, rabbit γG gave a value of M_z near 141,000 immediately following gel filtration, but after concentration and storage for a few days the value had increased to 157,000, presumably owing to aggregation.

The development of the high-speed sedimentation equilibrium method of Yphantis (1964) has resulted in more consistent and reproducible molecular weights, at least for γG preparations. The use of low protein concentrations in this method (0.1–1.0 mg./ml.) largely eliminates the concentration dependence problem. Marler *et al.* (1964) obtained a value of 145,000 for rabbit γG from sedimentation equilibrium measurements. A similar value (151,000) on the same sample was obtained from sedimentation and viscosity data, using the equation of Scheraga and Mandelkern (1953) (Noelken *et al.*, 1965). Small and Lamm (1966) obtained a value of 140,000 for rabbit γG in 5.0 M guanidine hydrochloride by sedimentation equilibrium. Equilibrium sedimentation studies by one of us (K.J.D.) on human normal and myeloma γG-globulins have yielded values from 143,000 to 148,000 both in dilute salt solution and 6.0 M guanidine hydrochloride.

The problems of aggregation and limited solubility of the heavy chain of γG-globulin (and other immunoglobulin classes) in neutral aqueous solvents have meant that reliable values for their molecular size have only been obtained in dissociating solvents. In practice this has also been true for the light chain. Marler *et al.* (1964) deduced the molecular weights of the chains of rabbit γG without prior separation. The γG was reduced with 0.1 M 2-mercaptoethanol in 6.0 M guanidine hydrochloride and subjected to equilibrium sedimentation at various protein concentrations in the same solvent. Values of number, weight, and z-average

molecular weights were obtained and compared with similar values for calculated equimolar mixtures of chains of assumed sizes. The results were compatible only with a four-chain model; the heavy chains having a molecular size between 50,000 and 55,000 and the light chains between 20,000 and 25,000. This conclusion was further tested by calculating the solute distribution in the centrifugal field for equimolar mixtures of the chains assuming the above molecular weight values and comparing it with the actual distribution—within the experimental error the distributions were identical. The molecular sizes for heavy and light chains deduced by Marler *et al.* were in close agreement with those of Pain (1963) and Small *et al.* (1963) who used the more conventional approach of separating the chains prior to the molecular weight determinations. A more precise study has been reported more recently by Small and Lamm (1966) on rabbit γG chains. They separated the heavy and light chains from extensively reduced and alkylated γG on Sephadex G-200 in 5.0 *M* guanidine hydrochloride. The yields of heavy and light chains were 68 and 32%, respectively, consistent with a four-chain model. The rationalized "best" values for the molecular weights of the heavy and light chains were 53,000 and 22,000 in a γG molecule of 140,000 molecular size. Some evidence was presented suggesting that complete reduction of the γG was not achieved. However, the yields of heavy and light chains appeared to be independent of the method used for reduction, so that the molecular weight values are probably unaffected.

Equine γG can be resolved into three antigenically distinct components (Rockey *et al.*, 1964). The molecular size of the polypeptide chains of γG_{ab} have been determined by Montgomery *et al.* (1969). Sedimentation equilibrium in 6.0 *M* guanidine hydrochloride and gel filtration on calibrated columns of G-200 in 8.0 *M* urea–0.05 *M* propionic acid gave values between 52,300 and 53,900 for the heavy chain and 22,300 and 23,100 for the light chain.

Comment on the values of the partial specific volume ($\bar{v}$) chosen for the protein under investigation might be appropriate here. Sedimentation equilibrium provides estimates of $M(1 - \bar{v}\rho)$, where ρ is the solvent density. The final accuracy of M depends on the precision of the $\bar{v}$ value used; a 1% error in $\bar{v}$ gives a 5–6% uncertainty in M in guanidine or 3% uncertainty in dilute aqueous salt solution. Although direct measurements of $\bar{v}$ can be easily made pycnometrically in the relevant solvent, large amounts of protein (250 mg.) are required compared to the amount needed for the actual molecular weight determination (ca. 1 mg.). Determination of $\bar{v}$ with less protein requires sophisticated apparatus. For proteins in dilute salt solution (ca. 0.1 *M*), a reasonable estimate of $\bar{v}$ can be obtained from the $\bar{v}$'s of the constituent amino acids if an amino

acid analysis is available (Cohn and Edsall, 1943). However, for glycoproteins (e.g., immunoglobulins) some estimate of the contribution of the carbohydrate to the overall $\bar{v}$ must be made in view of their low $\bar{v}$ values (ca. 0.6–0.65 ml. gm.$^{-1}$) compared to amino acids. It is necessary for the subunit molecular weight determinations in the presence of guanidine hydrochloride or urea to estimate a value of the "effective specific volume" of the anhydrous protein in the mixed solvent. This quantity includes the actual $\bar{v}$ of the protein plus a correction term which depends on the interaction of the protein with the solvent components (Hade and Tanford, 1967). As discussed by a number of workers (Hade and Tanford, 1967; Schachman and Edelstein, 1966) the preferential interaction of protein with water (preferential hydration) would lead to a value for the "effective $\bar{v}$" larger than the $\bar{v}$ of the protein in dilute salt solution. On the other hand, preferential guanidination would cause little change and the effective $\bar{v}$ would closely approximate the $\bar{v}$ of the native protein in dilute salt. Hade and Tanford (1967) have demonstrated preferential guanidination for several proteins, and a number of workers have shown that the effective $\bar{v}$ for anhydrous, salt free γG-globulin in concentrated guanidine hydrochloride, determined according to the method of Cásassa and Eisenberg (1961), is only slightly lower than the corresponding quantity in dilute salt solutions (Marler *et al.*, 1964; Small and Lamm, 1966). The problems associated with molecular weight determinations in three-component systems have recently been discussed in thermodynamic terms by Reisler and Eisenberg (1969).

With the determination of the complete sequence of the heavy chain of one γG molecule (Edelman *et al.*, 1969) we can calculate its molecular weight to be 48,600 (not including carbohydrate which would bring it up to 50,100) without the ambiguities due to uncertainties in $\bar{v}$. The light chain of this protein has a molecular weight of 23,400, and the molecular weight of the whole molecule is 144,000 (polypeptide portion only) or 147,000 (carbohydrate included).

B. γM-Globulins

The γM-globulins are extensively reviewed by Metzger in a separate chapter of this volume, and the discussion here will therefore be confined to new molecular weight data by Dorrington and Mihaesco (1970). These workers have determined the molecular size of two intact Waldenström macroglobulins together with their μ chains and the various fragments produced by papain and pepsin. Sedimentation equilibrium (Yphantis, 1964) of intact γM in dilute salt and in 6 *M* guanidine hydrochloride yielded a value of 891,000 (S.D. $\pm$20,000). Provided care was taken to remove any polymers of the 19 S species, the observed molecular

weight was insensitive to changes in rotor speed and initial protein concentration. The μ chains were prepared from mildly reduced and alkylated γM and subsequently fully reduced in 6 *M* guanidine hydrochloride and purified by gel filtration. The molecular weight was found to be 65,200 (S.D. $\pm$1800) independent of initial protein concentration and rotor speed, using values of the partial specific volume calculated from the amino acid and carbohydrate compositions. In addition, it has been possible to determine the molecular size and relative location of regions of the μ chain corresponding to distinct antigenic determinants from the molecular size of μ-chain fragments within various proteolytic fragments. A fuller discussion of these data will be deferred until Section III.

It seems established from the above and other data (see Metzger, this volume) that the molecular size of the μ chain is greater than the γ chain of γG. This can only be partially accounted for by the higher carbohydrate content of γM (7–11%) compared to γG (2–3%). For the γM-globulins referred to above, of the total mass of 65,200 for the μ chain, some 9000 gm./mole was due to carbohydrate leaving a polypeptide portion of 56,200. This compares with nearly 48,600 for the polypeptide of the γ chain (Edelman *et al.*, 1969). It would seem, therefore, that the μ chain is some 65 to 70 residues longer than the γ chain. Since there is some evidence that the μ chain appeared before the γ chain in evolution, the apparent deletion in γ chain compared to μ chain has stimulated some interesting discussion (Hill *et al.*, 1967; Lennox and Cohn, 1967).

C. γA-Globulins

The γA-globulins were reiewed by Tomasi (1968) in Vol. 9 of this series, and only the recent work on the molecular size will be discussed.

The molecular size of several monomeric and dimeric γA-globulins have been determined by Dorrington and Rockey (1970a). All the proteins studied were isolated to a high degree of immunochemical purity. The monomeric γA-globulins had sedimentation rates ($s^{\circ}_{20,w}$) between 6.5 S and 6.7 S and the dimeric γA, 9.6 S. The molecular weights of the γA monomers were between 158,000 and 160,000 as determined by sedimentation equilibrium in either dilute salt or 6.0 *M* guanidine hydrochloride solutions. The γA dimer had a molecular weight of 318,000 (S.D. $\pm$7500) close to the value expected for a dimer of a 160,000 basic unit. The molecular size originally determined in dilute salt solution was unaffected by the presence of 6.0 *M* guanidine hydrochloride, clearly indicating that the monomer units are covalently linked in the dimer. Sedimentation velocity studies in the presence of low concentrations of 2-mercaptoethanol showed that under these conditions the

dimer is converted to the monomeric species.

It is now established that two subclasses of human γA are present in serum and certain external secretions (see below) and that the antigenic differences between γA_1 and γA_2 are associated with differences in the α chains (D. Feinstein and Franklin, 1966; Kunkel and Prendergast, 1966; Vaerman and Heremans, 1966). This differentiation was originally detected on the basis of the antigenic deficiency of γA_2 when tested with antisera prepared against γA_1. In addition to its antigenic uniqueness, γA_2 has been shown to possess distinct chemical properties compared to γA_1. The α_2 and the light chains of γA_2 are not linked to each other by disulfide bridges and can be separated, in the form of stable disulfide-linked dimers, in dissociating solvents without prior reduction (Grey *et al.*, 1968). The linking of the two light chains in the native molecule by a disulfide bridge is an intriguing feature of the γA_2 structure not previously encountered in human immunoglobulins although a similar situation occurs in certain murine γA-globulins (Abel and Grey, 1968).

The molecular size of the heavy and light chains of the γA_1- and γA_2-globulins have been determined by Dorrington and Rockey (1970a,b) by sedimentation equilibrium in 6.0 *M* guanidine hydrochloride. The α_1 chain obtained from extensively reduced and alkylated γA by gel filtration on Sephadex G-200 in 8.0 *M* urea–0.05 *M* propionic acid had a molecular weight of 56,300 (S.D. ±1700). None of the preparations examined showed any dependence of *M* on initial protein concentration or rotor speed. The light chain had a molecular weight of 22,700 (S.D. ±1000). The α_2 and light chains of γA_2-globulin were examined by sedimentation equilibrium in the form of disulfide-linked dimers obtained by dissociating γA_2 in concentrated urea solution without prior reduction and also as fully reduced and alkylated chains. Fully reduced α_2 chain had a molecular weight of 52,200 (S.D. ±700) and the light chain, 22,800 (S.D. ±2400). As anticipated the α_2 and light chain dimers had molecular sizes approximately twice those of the monomeric chains, i.e., α_2 dimer, 104,600 (S.D. ±2000); L dimer, 45,400 (S.D. ±1500). These data strongly suggest that the α_2 chain is significantly smaller than the α_1 chain by approximately 4000 gm./mole. Assuming this difference is due to changes in amino acid content rather than to variation in the mass of carbohydrate, it represents a deletion of some thirty to thirty-five residues. Work on the structure of γA_2 has not reached a stage where one can say how far this deletion is responsible for the unique properties of this subclass. It is not known whether the cysteine residue involved in the heavy–light —S—S— bridge in γA_1 is absent in γA_2 or forms an intrachain —S—S— bridge. It may well be, however, that the deletion occurs in the extended ("hinges") region of the α_2 chain.

There seems to be an inconsistency in the molecular size of serum γA α chain determined by Dorrington and Rockey (1970a,b) and the α-chain from rabbit colostral γA (Cebra and Small, 1967). Although it is possible that this difference is a real one, it seems more likely to be principally due to the choice of $\bar{v}$ used in the calculations. Dorrington and Rockey used a value of 0.718 calculated from amino acid and carbohydrate analyses and adjusted for the effects of guanidine binding, whereas Cebra and Small (1967) used a value of 0.732 based on direct density measurements in 5.0 *M* guanidine hydrochloride. The latter value seems somewhat high considering the values measured for intact γA (0.685) and light chain (0.703) in 5.0 *M* guanidine hydrochloride by the same authors. The interest in determining the real molecular size of the α chain lies in whether it is larger than the γ chain. If the value of 56,300 (Dorrington and Rockey, 1970a,b) is adjusted for the bound carbohydrate, we calculate 49,900 for the polypeptide portion of the α chain, i.e., approaching the value for the γ chain (48,600). The value of Cebra and Small (1967) gives a polypeptide portion larger than the γ chain, which implies differences at the level of the genome, as suggested for the μ chain.

D. Other Immunoglobulins

Rowe and Fahey (1965a,b) demonstrated that a unique myeloma protein was a representative of a new class of immunoglobulin (γD) present in low, but variable, levels in normal human serum. Several γD myeloma proteins have been shown to have sedimentation coefficient ($s^{\circ}_{20,w}$) between 6.1 S and 6.2 S (Rowe *et al.*, 1969). Molecular weight determinations, by the Archibald approach to equilibrium method, yielded values near 183,000 (Rowe *et al.*, 1969). A γD myeloma protein, studied in the laboratory of one of the authors (C.T.) by Elizabeth Rowe and R. Griffith was found to have a molecular weight of about 180,000 by sedimentation equilibrium. The heavy chain (δ chain), by sedimentation equilibrium in 6 *M* guanidine hydrochloride, had a molecular weight of 69,000. Another γD protein, studied by Dorrington and Bennich (1970) gave molecular weights of 175,000 and 63,000 for the whole protein and the δ chain, respectively. An unusual feature of these proteins is the remarkably *low* sedimentation coefficient, in the light of the relatively *high* molecular weight. The protein also seems to be unusually susceptible to the action of proteolytic enzymes (Griffith and Gleich, 1970). A possible interpretation is that γD is less compactly folded than other immunoglobulin molecules.

Bennich and Johansson (1971) have reviewed the current knowledge of γE, the most recently described class of immunoglobulins.

Therefore we will only briefly mention the most recent work on the molecular size of γE and ε chain.

Dorrington and Bennich (1970) found the molecular weight of a γE myeloma protein to be 188,100 (S.D. ±3000) from high-speed sedimentation equilibrium studies. This is somewhat lower than the value of 196,000 obtained earlier by Bennich and Johansson (1967). The size of the ε chain was found to be 72,500 (S.D. ±2400) by sedimentation equilibrium in 6 *M* guanidine hydrochloride, of which 14% was accounted for by carbohydrate. The polypeptide portion of the ε chain (63,350) is, therefore, significantly greater than for the heavy chains of other human immunoglobulins.

III. Conformation of Immunoglobulins and Subunits

A. Overall Shape and Flexibility

1. γG-Globulin

Two types of topographical model have been proposed for γG-globulin, incorporating the available information on the size and shape of the molecular envelope, the localization of the antigen-binding sites, and the contribution of the heavy and light chains to these sites. Edelman and Gally (1964) considered the molecule to be a rigid rodlike structure with the antibody-combining sites at the extreme ends of the rod. Noelken *et al.* (1965), however, favor a model in which most of the polypeptide chains are incorporated into three compact globular regions, corresponding to the Fab and Fc fragments, linked by a flexible extended portion of the heavy chain. The models are compared in Fig. 1. The properties of γG upon which the evidence for which models are based will be discussed in this section.

When the shape of a molecule is inferred from hydrodynamic data, it must be realized that such data can only provide evidence that the molecule is not globular. It cannot distinguish between an increase in hydrodynamic radius due to a segment of randomly coiled polypeptide chain and that due to a nonspherical shape with the retention of rigidity. If the shape is nonspherical, it could be any regular or irregular shape at all. Equations relating hydrodynamic properties to equivalent ellipsoids of revolution have until recently been the only available equations for quantitative interpretation, and ellipsoidal axial ratios, based on these equations, have been frequently reported. These cannot be expected to have much significance in terms of the real molecular shape.

From intrinsic viscosity, osmotic pressure, and sedimentation velocity

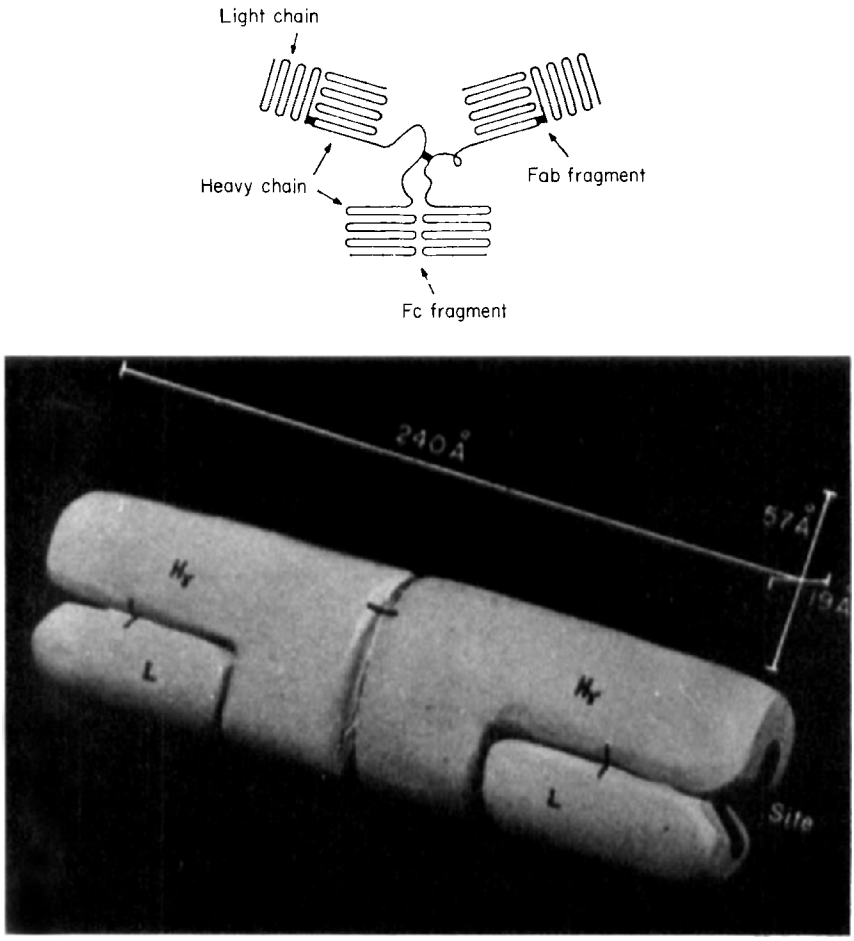


FIG. 1. A diagrammatic comparison of (a) the flexible model of γG of Noelken *et al.* (1965) and (b) the rigid-rod model of Edelman and Gally (1964). In both models the short, heavy lines represent the interchain disulfide bonds.

measurements, Oncley *et al.*, (1947) proposed that human γG was approximately by a prolate ellipsoid with dimensions of 235 by 44 Å., assuming hydration of 0.2 gm. water per gram protein. A similar molecular length (230 Å.) was calculated from flow birefringence studies on human γG in 60 to 70% glycerol solutions by Edsall and Foster (1948). This observation tends to support the rigid rod model since the Noelken-type structure should not show significant alignment along the flow lines of a viscous liquid. However, we do not know whether the conformation of γG would be the same in concentrated glycerol solu-

tion as in dilute salt and whether the presence of aggregates might account for the birefringence properties. A series of low-angle X-ray scattering studies by Kratky's group (Kratky *et al.*, 1955, 1963; Kratky, 1963) suggested a cylindrical model for human γG, with an elliptical cross section and dimensions of $240 \times 57 \times 19$ Å. and total volume 2.0×10^5 Å.^{3}.

The X-ray scattering method can distinguish between a molecule that is completely randomly coiled and a molecule that is a completely rigid ellipsoid, but whether it can distinguish the Noelken-type model from a rigid rod is open to question. A recent paper, representing the combined efforts of Kratky's and Edelman's laboratories (Pilz *et al.*, 1970), attempts to answer this question on the basis of new measurements using a homogeneous myeloma γG. Theoretical scattering curves for eight different models are compared to the experimental scattering curve, and it is concluded that the best agreement is obtained with a rigid T-shaped model, in which there is no central region of relatively low density of scattering material, as is shown in the model of Noelken *et al.* It is not easy to decide how seriously this conclusion should be taken, especially as the molecular weight determined from the same data is in error by 10%. The myeloma protein used for this study is that for which the complete amino acid sequence has been determined (Edelman *et al.*, 1969), and the true molecular weight is 148,000. The experimental value was 162,000.

A comparison of the hydrodynamic properties of intact γG and the Fab and Fc fragments produced during limited proteolysis with papain, provided some of the evidence for the "flexible" model of Noelken *et al.* (1965). The fractional coefficient ratios (f/f_{min}) were calculated for γG and the fragments to give a measure of deviation from a compact globular shape. The ratio, f/f_{min}, is a measure of the combined effects of hydration and shape on hydrodynamic properties. For typical globular proteins f/f_{min} lies in the range of 1.10 to 1.25, indicating that both hydration and deviation from a spherical shape are small. Fragments Fab and Fc behave as typical globular proteins ($f/f_{min} = 1.21$–1.24), whereas intact γG does not ($f/f_{min} = 1.47$). Therefore either γG is more asymmetrical or possesses a large amount of hydration. Hydration in this context includes hydrodynamically trapped solvent, so that a molecule with regions of flexibly coiled polypeptide chain would appear to have anomalously large amounts of hydration when examined by hydrodynamic methods. The same conclusion is reached from intrinsic viscosity measurements. Intact human and rabbit γG has $[\eta] = 6.0$ cc./gm. (Jirgensons, 1963; Noelken *et al.*, 1965), whereas the values for the papain fragments are near 4.0 cc./gm. These findings are compatible with a structure consisting

of compact Fab and Fc regions linked by a flexible, extended portion consisting of a part of each heavy chain but does not exclude a rigid linear arrangement of the fragments (i.e., Fab-Fc-Fab).

The most convincing evidence in favor of the flexible model is the observation that the heavy chains of γG can be cleaved in a relatively limited region by a variety of proteolytic enzymes and cyanogen bromide. The region of the heavy chain in which the flexibility proposed by Noelken *et al.* must occur (so-called "hinge" region) has been delineated by the amino acid sequence studies of Smyth and Utsumi (1967) and of Givol and De Lorenzo (1968). These workers have defined the cleavage points for papain, pepsin, typsin, and cyanogen bromide (Fig. 2). The region of the chain containing these cleavage points must be much more freely accessible to cyanogen bromide and to the active sites of the proteolytic enzymes than similar susceptible bonds elsewhere in the γG molecule. It is interesting that the region involved contains three consecutive prolyl residues, immediately carboxy terminal to the inter-heavy-chain disulfide bond, on each heavy chain. Such a prolyl tripeptide is unique among known sequences and may accout, in part, for the extended nature of this region of the heavy chain. Givol and De Lorenzo (1968) limit the flexible region of the heavy chain, accessible to proteolytic enzymes, to between 25 and 30 residues on each chain.

A flexible model for γG-globulin seemed to provide an explanation for the low values of the rotational relaxation time obtained from fluorescence depolarization studies. Dimethylaminonaphthalene sulfonyl chloride (DNS) conjugates to γG have been studied by a number of workers (Churchich, 1961; R. F. Steiner and Edelhoch, 1962; Chowdhury and Johnson, 1963; M. H. Winkler, 1965) and have provided values of less than 100 nsec. for the relaxation time. On the basis of the

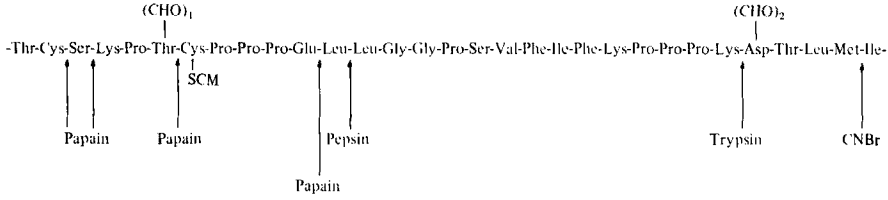


FIG. 2. The amino acid sequence of the "hinge" region of rabbit γG-globulin showing the cleavage points for papain, pepsin, trypsin, and cyanogen bromide (CNBr). Cys.SCM represents the blocked half-cystine residue involved in the inter-heavy-chain disulfide bond. Two types of rabbit heavy chain are known, one, shown in the figure, has two sites at which carbohydrate (CHO) is found, whereas the second does not have $(CHO)_1$. In the presence of $(CHO)_1$, the Thr-Cys. SCM bond is not attacked by papain. (Adapted from Smyth and Utsumi, 1967, and from Givol and De Lorenzo, 1968.)

molecular dimensions obtained from other measurements, when interpreted in terms of a rigid ellipsoid, a value near 220 nsec. would have been anticipated. Indeed, measurements of the rotational relaxation time for horse γG by dielectric dispersion (Oncley, 1943) and human γG by electric birefringence relaxation (Krause and O'Konski, 1965) have given values of 220 and 200 nsec., respectively. Electric birefringence studies on bovine γG gave rotational relaxation times of 215 nsec. (Ingram and Jerrard, 1963.) The discrepancies in the relaxation times provided by fluorescence depolarization measurements and other methods was interpreted to mean that different regions of the γG molecule could rotate independently; a situation which could be envisaged in the Noelken *et al.* model.

More recently this interpretation of the fluorescence depolarization data has been challenged (Weltman and Edelman, 1967; Wahl and Weber, 1967). These workers suggest that thermally activated rotations of the covalently bound DNS groups can occur independently of the rotation of the region of the molecule to which they are attached thus leading to low values for the rotational relaxation time of the protein. The use of fluorescence depolarization to determine molecular relaxation times depends on the requirement that the fluorescent label should be immobilized by strong interactions with the protein molecule. Weltman and Edelman (1967) and Wahl and Weber (1967) showed that the slopes of the fluorescence depolarization curves obtained isothermally at varying viscosities (sucrose isotherms) are smaller than the slopes obtained when the temperature is changed to alter the viscosity of the solvent without sucrose. Values of the rotational relaxation time calculated from sucrose isotherms at different temperatures ranged from 191 to 244 nsec., whereas heating curves gave values below 100 nsec. in agreement with earlier studies. These results seemed to indicate that under experimental conditions where free rotation of the dye molecules is minimized, values of the rotational relaxation time can be obtained from fluorescence studies which are apparently consistent with values obtained by other methods and previously calculated molecular dimensions. This does not appear to be the whole story according to a recent paper by Zagyansky *et al.* (1969). The latter have shown that dimethylaminonaphthalene sulfonyl chloride (DNS) conjugates of human and rabbit γG have different fluorescence properties from DNS conjugates of serum albumin and ovalbumin. Of particular relevance was the finding that the lifetime of the excited state for DNS γG was shorter (7.3 nsec.) than for DNS albumin (12.1 nsec.). Since the value of the lifetime of the excited state (τ) is required in the depolarization calculations, exact knowledge of it is of great importance. Zagyansky *et al.* (1969) calculated the rotational

relaxation times of the DNS γG to be near 60 nsec. using sucrose isotherms and $\tau = 7.3$ nsec. Recalculation of the data of Weltman and Edelman (1967) and Wahl and Weber (1967) gives values of 100 to 130 nsec. using the lower lifetime value. The even lower value of 60 nsec. obtained by Zagyansky *et al.* may have been due, in part, to their removal of aggregates from the DNS γG preparations before the fluorescence measurements. These authors also provide some experimental evidence that the shorter lifetime of the excited state in DNS γG may be explained either by the substitution of DNS onto different amino acid side chains, the alternative conjugates having different spectral properties, or by differences in the hydrophobic nature of the environment of the DNS groups between DNS γG and DNS BSA. DNS–aspartate transaminase seems to have similar fluorescence lifetime properties to DNS γG (Polyanovksy *et al.*, 1970).

It seems from the above discussion that, even allowing for free rotation of the dye molecules, the rotational relaxation time for DNS γG is lower than expected for a rigid molecule. Thus it would seem that the units of the γG molecule with some rotational freedom are smaller than the whole molecule, and it is tempting to suggest that they correspond to the Fab and Fc fragments.

Two recent papers have reported fluorescence polarization measurements by direct measurement of the relaxation process (time span in the nanosecond range) following excitation by very short light pulses. This technique is free from some of the ambiguities that apply to previous results obtained by steady state measurements under constant illumination. Wahl (1969) employed DNS γG, with the DNS coupled covalently to the protein. His results were analyzed in terms of two relaxation processes, with relaxation times of 370 and 23 nsec. The first, which accounts for 65% of the overall relaxation process, is ascribed to rotation of the whole molecule and the second to internal Brownian movements of a globular region of the molecule. (The relaxation time, however, is much too short to represent a globular region of the size of the Fab region of the molecule.)

The second paper using the nanosecond fluorescence technique is by Yguerabide *et al.* (1970). They coupled the DNS chromophore to γG noncovalently by using γG that was an antibody directed against it. They also observed two relaxation times, one of 500 nsec., which is of the same order of magnitude as Wahl's longer relaxation time. The shorter relaxation time, however, was found to be 100 nsec., i.e., four times as large as Wahl's. When the same measurements were carried out with the complex between the DNS hapten and the Fab fragment of the antibody, only a single relaxation time of 100 nsec. was observed. The 100 nsec. relaxation

time is consistent with expectation for a rigid ellipsoid, molecular weight 50,000 plus hydration of 0.32 cc./gm. with an axial ratio of about 2. These are the probable dimensions of the Fab fragment, which thus appears to rotate as an essentially rigid unit. The results as a whole, including additional data for $F(ab')_2$, are completely consistent with the flexible model, but inconsistent with a rigid ellipsoid model for the native γG molecule.

Electron microscopy has been widely used to study the size and shape of γG-globulins despite the uncertainties inherent in the extrapolation from the dehydrated state to proteins in solution. Observations have been made either directly on immunoglobulin preparations or on antibodies attached to large particles which can be clearly seen on negative staining. A review of the subject has appeared recently (Green, 1970) and we will concern ourselves only with certain aspects of the data.

Recent electron-microscopic studies have provided evidence that γG is made up of three linked globular regions. A. Feinstein and Rowe (1965) first suggested, from negative contrast studies on ferritin–antiferritin complexes, that when cross-linking of antigen occurs the antibody molecule "clicks open" to varying degrees about a hinge point located at one end. A significant technical advance was made by Valentine and Green (1967) who studied complexes of purified high-affinity antibody to 2,4-dinitrophenyl (DNP) with a bifunctional DNP hapten (DNP·NH·$(CH_2)_8$·NH·DNP). On mixing equivalent amounts of the antibody and hapten, symmetrical cyclic polymers are formed. The number of antibody molecules in the polymer depends on the angle between the combining sites (e.g., an angle of 60° gives trimers of three divalent molecules and 90° gives tetramers). The advantages of this symmetrical arrangement are many: the shape of the polymer indicates the arrangement of the binding sites and the common features of each unit can be distinguished from the random distortions caused by the negative staining. In addition, the units are coplanar and are viewed in the same orientation, relative to the electron beam. From such studies, Valentine and Green (1967) and Valentine (1967) conclude that the antibody molecule is flexible since a wide range of different shapes are formed. The overall morphology of the γG molecule is Y-shaped made up of three rigid rods representing the Fab and Fc fragments (Fig. 3). The angle between the two Fab fragments is apparently variable between nearly 0° and 180°. On the electron micrographs the Fc fragment is seen as a conspicuous projection at the corners of the various figures (Fig. 3). Incubation with pepsin leaves the geometrical figures intact but removes the projections, thus confirming that they are Fc fragments. The antigen-binding sites lie at the extreme ends of the Fab fragments since the various shapes show no distortion where the molecules are linked by the hapten. The

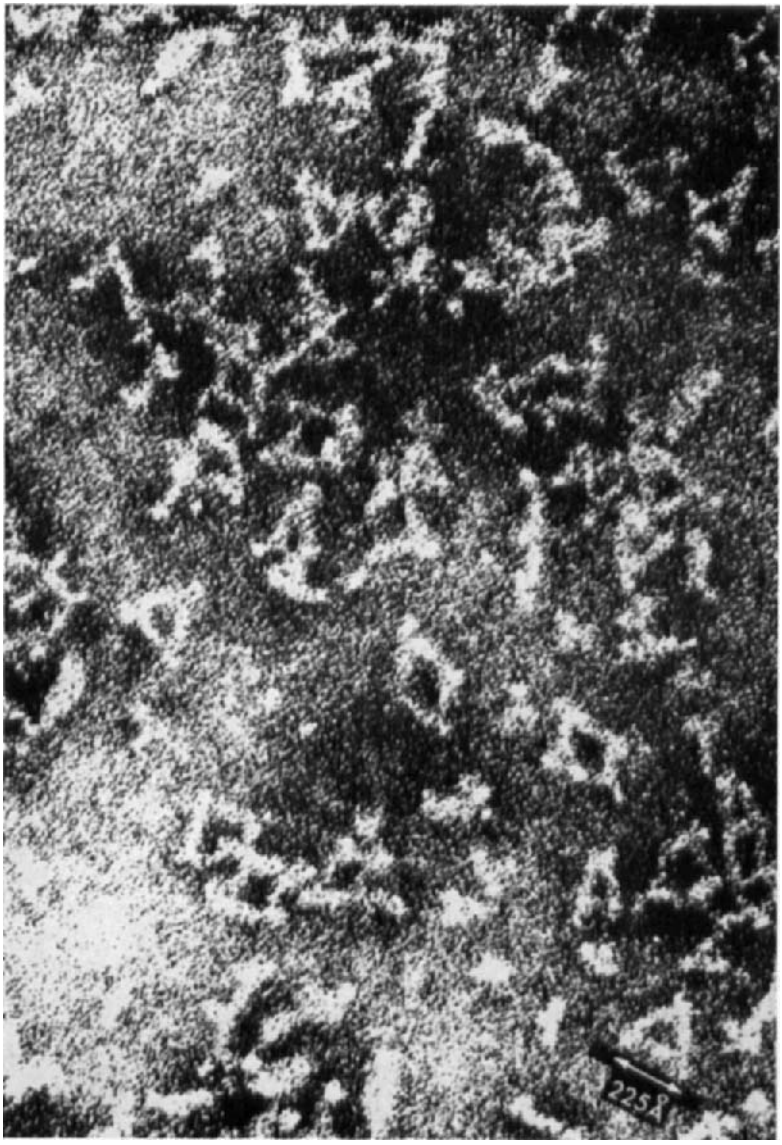


FIG. 3. Electron micrograph of complexes of rabbit γG antibody, directed against the 2,4-dinitrophenyl (DNP) group, with the bifunctional reagent bis-*N*-DNP octamethylenediamine (Valentine and Green, 1967).

maximum length of the Fab fragments was 70 Å., thus making the maximum span of the molecule approximately 140 Å.

Pilz *et al.* (1970) point out that the actual dimensions of the Valentine–Green model, which lead to a calculated radium of gyration of 46 Å., are incompatible with the radius of gyration (76 Å.) determined in solution from X-ray scattering measurements. They suggest that the discrepancy arises from dehydration and partial "collapse" of the original antibody–hapten complex on the surface of the grid used to support the sample for electron microscopy. Such a constriction of the molecule would seem to be most likely to occur if the greater extension of the molecule in solution arises from the presence of a region of the polypeptide chain that is loosely coiled. It is more difficult to visualize at what portion of the molecule the constriction occurs if one accepts the model for the structure in solution preferred by Pilz *et al.*

Further evidence for a Y-shaped model for γG has recently come from transient electric birefringence studies on a rabbit high-affinity anti-DNP by Cathou and O'Konski (1970). The birefringence of an antibody solution was measured in the presence of zero, one, and two moles of a tribasic hapten, α-dinitrophenyl glutamyl aspartate, per mole of antibody and found to be the same in all three cases. The results were not compatible with a rigid-rod model. On the basis of comparisons of calculated and experimentally determined specific Kerr coefficients, the authors favor a Y-shaped model where the angle between the Fab fragments lies between 130 and 180 degrees.

It has been recently established that there are additional positions of preferred cleavage by proteolytic enzymes at positions halfway between the hinge region of the γG molecule and the chain termini. Although the rate of cleavage at these points is much slower than at the hinge region, it is measurably fast. Thus fragments of light chain (molecular weight 11,000–12,000) occur in the urine of persons having Bence-Jones proteins, and presumably correspond to the variable and constant halves of the chain (Deutsch, 1963b; Solomon *et al.*, 1966; Williams *et al.*, 1966; Cioli and Baglioni, 1966; Van Eyk and Myszkowska, 1967; Tan and Epstein, 1967). Solomon and McLaughlin (1969) have demonstrated splitting of Bence-Jones proteins by endogenous urinary endopeptidases at acid pH, and also showed that light chains could be split into V and C regions by papain, pepsin, trypsin, and subtilisin. Björk (1970) has shown that incubation of isolated rabbit heavy chains with papain results in the production of small amounts of a fragment with a molecular weight near 12,000. In addition fragments corresponding to the carboxy terminal half of the Fc fragment have been isolated from pepsin and papain digests of γG (Turner and Bennich, 1968). Karlsson *et al.* (1969) have shown that

the halves of light chains either occurring naturally in urine or produced by proteolysis *in vitro* have compact globular structures. Such fragments have molecular weights of 10,000 to 11,000, from sedimentation equilibrium studies, and sedimentation rates near 1.6 S, which, when combined, yield a frictional ratio near 1.1. A Stokes radius of 16 Å was calculated from their behavior on gel filtration.

This information suggests that the Fab and Fc fragments are not the basic compact domains of the γG molecule but that the compact domains correspond to the partly homologous segments of about 110 residues that constitute the building blocks of the amino acid sequence. Each segment is folded into a tight globular structure, but there are relatively unstructured regions between segments, smaller in extent and less accessible at the junction points within the Fab and Fc fragments than at the hinge region in the center of the molecule. In our opinion, an extension of the flexible model, proposed by Noelken *et al.* (1965), to incorporate the probable existence of smaller compact domains and additional "loose" connecting regions, would account in the most satisfactory way for all

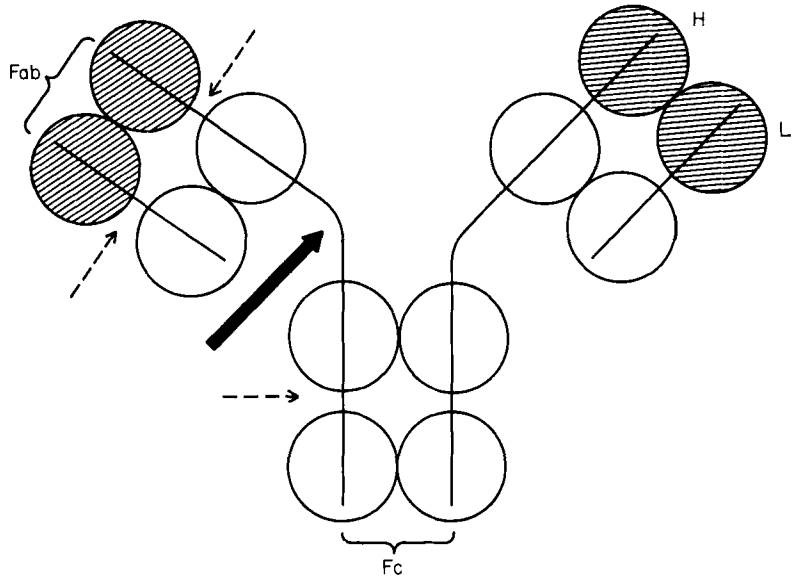


FIG. 4. The "compact domain" model for γG. Each circle represents a compact, globular region of polypeptide chain of about 11,000 molecular weight and containing a single intrachain disulfide bond. The heavy arrow indicates the region (hinge) of the heavy chain most susceptible to enzymatic and chemical cleavage. The interrupted arrows indicate the sites on both the heavy and light chains which are sensitive to cleavage but to a much lesser extent than the hinge region. (Adapted from Edelman and Gall, 1969.)

the experimental results cited in this section. A representation of the molecule on this basis is shown in Fig. 4, taken from a recent review by Edelman and Gall (1969). In view of the fact that these authors are also co-authors of the paper by Pilz *et al.* (1970), cited earlier, it is likely that their opinions as to the exact nature of the connecting regions differ somewhat from those of the authors of this review. However, it is clear that the major difference in interpretation inherent in the two models shown in Fig. 1 no longer exists.

2. *γM-Globulin*

Hydrodynamic studies on Waldenström macroglobulins clearly indicate that γM, like γG, shows gross deviations from a compact globular structure. Calculations of the frictional coefficient ratio (f/f_{min}) from sedimentation and diffusion constants for intact γM and 7 S γM_s gave values of 1.92 and 1.69, respectively (Miller and Metzger, 1965a). Values of the intrinsic viscosity for γM have varied from 6.0 (Kovacs and Daune, 1961) to 20.0 cc./gm. (Jirgensons *et al.*, 1960). In a careful study Jahnke *et al.* (1958) obtained a range from 10.6 to 15.3 cc./gm. for four different γM preparations. Miller and Metzger (1965a) obtained 16.2 cc./gm. for their γM which dropped to 8.0 cc./gm. upon mild reduction to γM_s. These high values for the intrinsic viscosity are consistent with the elevated f/f_{min} ratios. The wide range of values for the intrinsic viscosity may reflect various degrees of polymerization of the 19 S species in the γM preparations used. Aggregation may also account for the variations in $s^{\circ}_{20,w}$ and $D^{\circ}_{20,w}$ values found for different γM-globulins (Suzuki and Deutsch, 1967).

The high values of the frictional ratios and the intrinsic viscosities of γM can be accounted for by either a rigid or a flexible model for γM as was the case for γG. Metzger *et al.* (1966) used fluorescence depolarization studies with DNS conjugates of γM in an attempt to distinguish between these two alternatives. A mean rotational relaxation time of 1700 nsec. was calculated for a hypothetical prolate ellipsoid with an axial ratio of 18 (i.e., consistent with the hydrodynamic data). However the experimentally determined relaxation time for the DNS γM was only 80 nsec., nearly an order of magnitude lower than the theoretical minimum value for a sphere of equivalent mass (730 nsec.). The short relaxation time of γM was not simply due to independent rotation of the γM_s units, since these units had a relaxation time of 69 nsec. which is less than that expected of a sphere of equivalent mass. This suggests that γM_s also has considerable internal flexibility. The relaxation time of the tryptic fragment of γM, Fab μ, was greater than expected for an equivalent

sphere, suggesting that the internal flexibility of this unit is negligible. The relaxation times of Fab μ and intact γM were closely similar suggesting that the regions corresponding to Fab μ represent the largest rotational subunit of γM. As in some of the earlier studies on the fluorescence depolarization of γG (Section III,A,*1*), Metzger *et al.* make the assumption that the dye molecule interacts sufficiently strongly with the region of the protein to which it is attached so that it truly reflects the rotational properties of that region. The objections raised concerning the interpretation of the depolarization studies of γG because of thermally activated rotations of the dye molecule, independently of the protein, may be relevant to the studies on γM. Reinvestigation of the relaxation properties of γM under isothermal conditions at different solvent viscosities, together with measurements of the lifetime of the excited state, seem to be indicated for γM.

Some interesting observations regarding the ultrastructure of γM have appeared recently from electron micrographs (Svehag *et al.*, 1967; Chesebro *et al.*, 1968; A. Feinstein and Munn, 1969). Examination of negative contrast preparations of γM from a variety of species revealed a high concentration of stellate structures (Fig. 5). These structures appear to be composed of five "legs" connected to a central ring. The legs are of variable length, apparently bent in different configurations, thus giving particles of different diameters. The average diameter or span is 300 Å. and each leg has maximum dimensions of 100 $\times$ 25 Å. The central ring has an outer diameter of 100 Å. and a hole of 40 Å. diameter. A. Feinstein and Munn (1969) provide evidence that the legs are divided into two

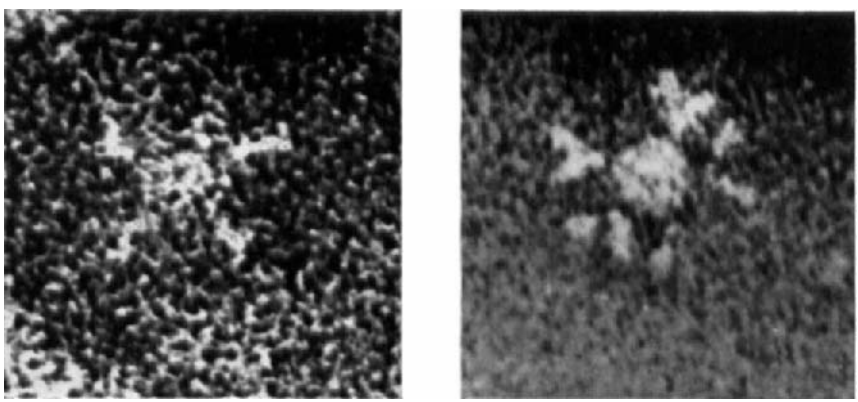


FIG. 5. Electron micrographs of individual γM molecules visualized by negative staining. On the left is the more commonly seen figure showing the five "legs" joined to the central ring; on the right, a figure with each leg divided into two. (Reproduced by kind permission of Drs. Feinstein and Munn.)

along part of their length (55–70 Å.). These two regions of each leg are thought to represent the Fab μ fragments, and where they join represents a flexible hinge region which accounts for the variable disposition in space of the legs seen in the micrographs. Further evidence for the flexibility of this region has been provided by these workers: electron micrographs of antibody–antigen complexes (*Salmonella* flagellum–γM antiflagellum) show γM molecules with all their legs bent around and attached to the flagellum.

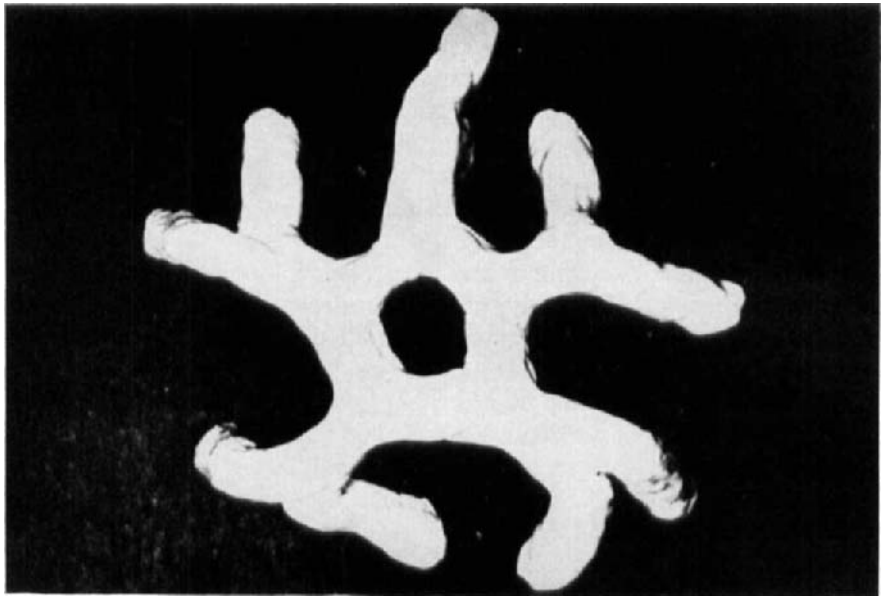
The electron-microscopic evidence together with the chemical information available on γM can be used to construct a schematic model for this molecule (Fig. 6). The model is based on the following chemical evidence:

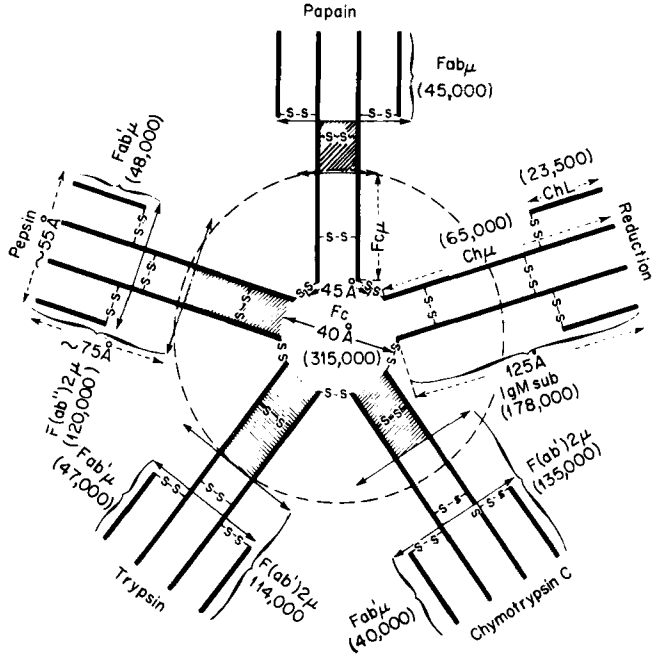
a. Molecular weight and other evidence indicates that γM is made up of five units (γM_s) of molecular size 180,000 (Table I).

b. Enzymatic cleavage with papain produces an Fab μ fragment, molecular weight 48,000, composed of one light chain and part of the amino-terminal region of the μ chain (Mihaesco and Seligmann, 1968a; Dorrington and Mihaesco, 1970). Each Fab μ fragment has a single antigen-binding site. Papain also produces an Fc μ fragment, molecular size 320,000, which yields, on reduction in 6 *M* guanidine hydrochloride, fragments of mass near 32,000 (Dorrington and Mihaesco, 1970) indicating that it is made up of the carboxy-terminal regions of the ten μ chains. It seems likely that the central ring of γM seen in the electron micrographs represents this polymerized Fc μ fragment.

c. Pepsin and trypsin produce a fragment of molecular size near 120,000 ($F(ab'')_{2}\mu$) with two antigen-binding sites (Mihaesco and Seligmann, 1968b; Miller and Metzger, 1966; Metzger, 1967). This fragment is composed of two light chains and a region (Fd'') of each of the heavy chains of molecular size near 36,000. The $F(ab'')_{2}\mu$ is further digested by pepsin to give two Fab'' μ fragments of similar molecular size and chain composition to papain Fab μ (Mihaesco and Seligmann, 1968b; Miller and Metzger, 1966). Chymotrypsin C digestion yields a larger fragment, of mass 135,000 (Chen *et al.*, 1969).

d. The mass of γM can be accounted for by the polymeric Fc μ and five pepsin ($F(ab'')_{2}$) fragments. Dorrington and Mihaesco (1970) have delineated three antigenically distinct regions of the μ chain; Fd μ (or Fd' μ) with a mass near 24,000, Fc μ with a mass of approximately 32,000, and a region not included in these two fragments which is very sensitive to proteolytic attack. This latter region is reminiscent of the extended, flexible hinge region of γG. In addition to its sensitivity to proteolysis this region shows only a low level of conformational antigenic determinants (Mihaesco and Seligmann, 1968b). The putative hinge region of γM is




Papain
Fabμ
(45,000)
Fab'μ
(48,000)
Pepsin
~55Å
~75Å
(23,500)
ChL
(65,000)
Chμ
Reduction
Fcμ
45Å
Fc
40Å
(315,000)
125Å
IgM sub
(178,000)
F(ab')2μ
(120,000)
Fab'μ
(47,000)
Trypsin
F(ab')2μ
114,000
F(ab')2μ
(135,000)
Fab'μ
(40,000)
Chymotrypsin C

approximately 80 to 90 residues, significantly longer than the corresponding region of the γ chain. The Fd μ regions are probably compact and globular since they are fairly resistant to proteolytic attack. The Fc μ is probably not as tightly folded since proteolysis can occur fairly readily in this region of the μ chain, Fc μ being obtained in low yield. A fragment corresponding to Fc μ is not obtained from γM_s suggesting that much of the resistance to papain is a function of the close apposition of the subunits in intact γM.

e. The γM_s units are joined by a single disulfide bond, which is shown as the most carboxy-terminal half-cystine residue of the μ chain in Fig. 6. More recent evidence indicates that the adjacent (more amino-terminal) half-cystine is involved in the inter-γM_s disulfide bond (Beale and Feinstein, 1969, 1970). The cyclic arrangement of the γM_s subunits in intact γM is clearly shown in the electron micrographs but was suggested by earlier studies on the disulfide bonds of γM (Miller and Metzger, 1965b). It is also difficult to understand why simple linear polymers of γM_s should be restricted to five units unless a cyclic structure is formed. Presumably a pentameric ring structure is the most thermodynamically stable. During the biosynthesis of γM, the polymerization of γM_s seems to occur just prior to or simultaneously with secretion from the cell since only γM_s can be detected within the cell (Parkhouse and Askonas, 1969). There is no evidence for the secretion of intermediate polymers of γM_s.

Considerable attention has been focused on the intriguing question of the valency of γM antibodies. A number of studies indicate that γM antibodies possess five binding sites—one associated with each γM_s subunit (Onoue *et al.*, 1965; Lindqvist and Bauer, 1966; Metzger, 1967; Stone and Metzger, 1968; Coligan and Bauer, 1969). However, it has been demonstrated for one such antibody, a Waldenström macroglobulin with antihuman γG activity, that each tryptic Fab μ fragment can bind antigen with equal affinity (Metzger, 1967; Stone and Metzger, 1968). Clearly, then, this antibody had ten potential binding sites, only half of which were available in the intact γM. By contrast, other γM antibodies have been described where ten binding sites were detectable in the 19 S molecule (Merler *et al.*, 1968; Onoue *et al.*, 1968). The antihapten γM studied by Onoue *et al.* (1968) had two groups of binding sites, five with affinities 100-fold greater than the other five. Each γM_s appeared to possess one high and one low affinity site. In the antibody studied by

FIG. 6. A model for γM based on the electron-microscopic evidence (upper) and chemical evidence (lower). The upper part of the figure is a clay scale model constructed from dimensions obtained from the electron micrographs. The lower part of the figure is based on a variety of chemical observations (for discussion see text).

Merler *et al.* (1968), all ten sites were of equal affinity. The reason for the unavailability of one of the two paired sites on γM_s is not known at this time. It is possible that the phenomenon might be explained by steric hindrance; antigen bound to one site preventing access to the neighboring site. This may well account for the situation in antibodies to macromolecular antigens (e.g., γG) but is not so convincing for low molecular weight haptens. Alternatively, the quarternary folding of γM might lead to a situation where only half the binding sites are in a suitable configuration to allow significant interaction with the antigen.

It is interesting to note in this connection that the 7 S and 19 S γM of elasmobranchs differ in valency. The naturally occurring 7 S-γM antibody is apparently bivalent since it can agglutinate antigen-coated erythrocytes and the activity is resistant to further mild reduction. However the 7 S γM_s obtained following mild reduction of the 19 S species shows no activity in the same test system (Clem and Small, 1967). The structural and biosynthetic bases for this difference in valency between the two 7 S species remain to be determined.

B. Internal Folding

At the present time X-ray crystallography provides the ultimate in protein conformational analysis, and its usefulness has been admirably demonstrated over the past several years (Davies, 1967; Stryer, 1968). It is possible with this technique, under optimal conditions, to describe with a high degree of accuracy the secondary and tertiary structure of a protein. Until very recently the possibility of performing such an analysis on immunoglobulins seemed very remote; the heterogeneity of these proteins seemed to preclude the preparation of stable, suitably sized crystals for analysis. The crystallizability of rabbit γG Fc fragment has resulted in some preliminary observations of its crystal properties (Poljak *et al.*, 1967; Goldstein *et al.*, 1968). However, while Fc has a number of interesting biological properties, studies on this fragment are unlikely to answer the vital questions regarding the relationship between protein structure and antibody activity.

The recent discoveries of crystalline γ-globulins, a human γG myeloma protein (Terry *et al.*, 1968), a myeloma γG Fab fragment (Rossi and Nisonoff, 1968), and a rabbit antiazobenzoate antibody (Nisonoff *et al.*, 1967) suggest that X-ray examination of intact immunoglobulins may be possible. In fact a preliminary report has appeared on a human myeloma γG cryoglobulin (Terry *et al.*, 1968). Unit cell dimensions have been measured and evidence obtained for half a molecule per unit cell, indicating that γG has a twofold axis of symmetry. The presence of a diad axis is consistent with the chemical data showing that the molecule is made up

of two identical pairs of chains linked by disulfide bonds. Also the Fc fragment has been shown to have twofold symmetry (Goldstein *et al.*, 1968). The crystalline Fc can be enclosed in a parallelepiped 50 × 40 × 70 Å. When these dimensions are corrected for the water content of the crystal, they are consistent with those obtained from the electron micrographs.

In the absence of detailed X-ray analysis, much of the information on the conformation of immunoglobulins in solution, although speculative, has come from optical rotatory dispersion (ORD) and, more recently, circular dichroism (CD) studies. Discussion of the theory and measurement of these two related phenomena will not be attempted here since excellent reviews have appeared elsewhere (Urnes and Doty, 1962; Yang, 1967; Beychok, 1967, 1968).

Much of the ORD data on immunoglobulins has been performed on γG-globulin. The spectra of γG from a variety of species are essentially

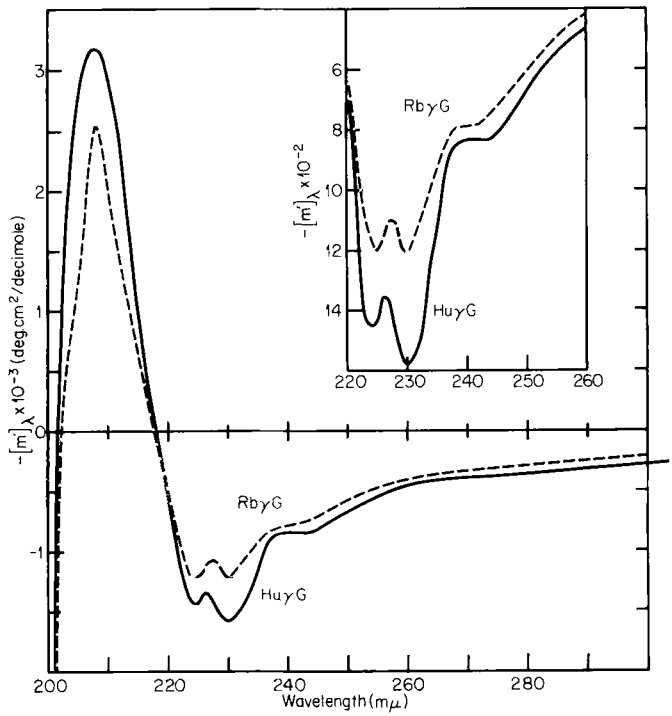


FIG. 7. The optical rotatory dispersion spectra of a human γG myeloma protein (Hu-γG) and a sample of pooled rabbit γG (Rb-γG) between 300 and 200 mμ. Inset shows the 260–220-mμ. region in more detail. These two spectra illustrate the kind of variability found in the optical rotation of different preparations of γG.

similar (L. A. Steiner and Lowey, 1966; Dorrington *et al.*, 1967; Cathou and Haber, 1967; Ross and Jirgensons, 1968; Rockey *et al.*, 1970). Basically the ORD of γA and γM show similar features to the spectra of γG (Dorrington and Rockey, 1968; Dorrington and Tanford, 1968). The spectra of these immunoglobulin classes are characterized by (*a*) a low level of rotation throughout the wavelength range, (*b*) Cotton effect minima near 230, 225, and 198 mμ., (*c*) a single maximum between 204 and 210 mμ. with a crossover (zero rotation) near 220 mμ., and (*d*) only in γG a Cotton effect of low rotatory strength at 240 mμ. (Figs. 7–9). The absolute levels of rotation at the maximum and minima show considerable variation between different proteins within any immunoglobulin class.

More recently, attention has been focused on the circular dichroic

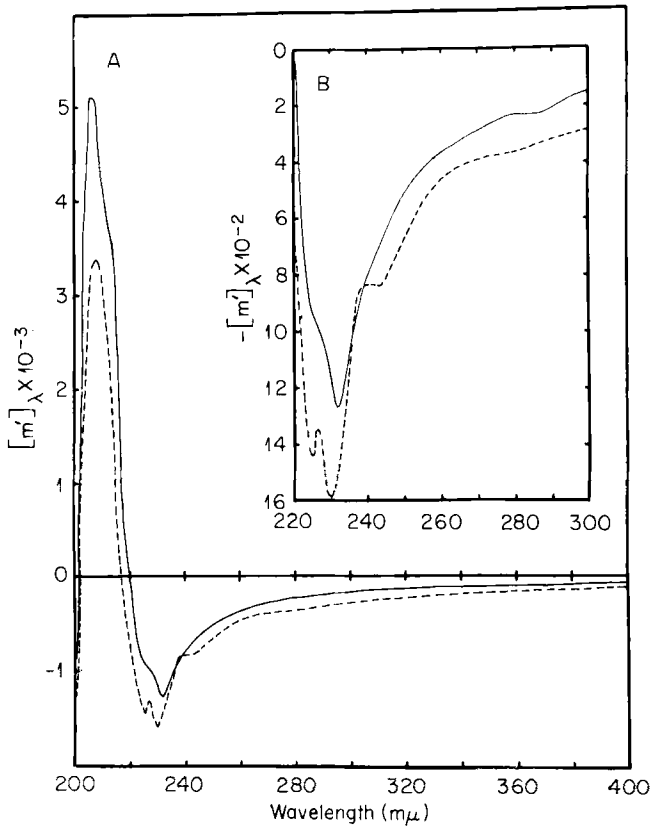


FIG. 8. The optical rotatory dispersion spectrum of human γM-globulin (solid line) compared with human γ G(dashed line) between 200 and 400 mμ. Inset shows the 220–300-mμ. region in more detail. (From Dorrington and Tanford, 1968.)

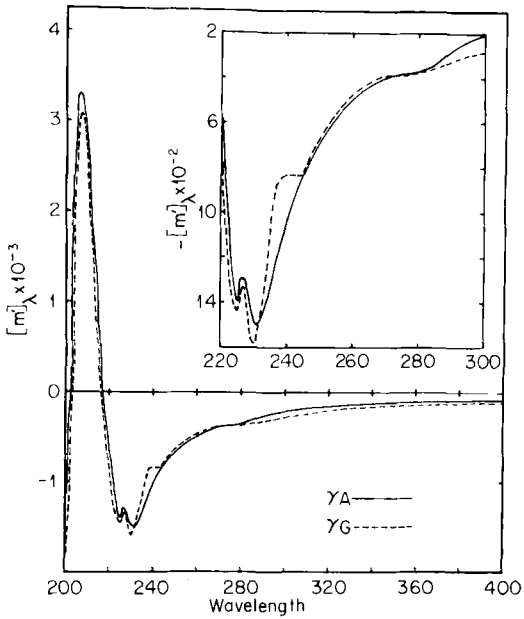


FIG. 9. The optical rotatory dispersion spectrum of a human γA myeloma protein (solid line) compared with human γG (dashed line) between 200 and 400 mμ. Inset shows the 220–300-mμ. region in greater detail. (From Dorrington and Rockey, 1968.)

properties of γG (Cathou *et al.*, 1968; Ikeda *et al.*, 1968; Ross and Jirgensons, 1968). Although circular dichroism is closely related to ORD, the optically active transitions are seen as discrete bands usually set against a background of zero optical activity. The component bands are more easily resolved than the individual Cotton effects from the ORD spectrum. The most extensive study of the CD of γG has been performed by Cathou *et al.* (1968) using rabbit high-affinity anti-DNP. The CD spectrum of γG (Fig. 10) exhibits negative bands at 192, 217, and 240 mμ.; positive bands are seen at 202 and 232 mμ. and in the 260–300-mμ. region. The transitions of low rotatory strength above 260 mμ. can be partially resolved at 275 to 280 and 290 mμ.

The ORD of γG and γM can be reproduced by an appropriate combination of the spectra of their respective Fab and Fc fragments indicating that little change in conformation occurs during proteolysis (L. A. Steiner and Lowey, 1966; Dorrington and Tanford, 1968). The ORD spectra of Fab and Fc from γG are shown in Fig. 11. The 225- and 240-mμ. Cotton effect minima of γG are due to Fab, whereas the 230–232-mμ. minimum can be attributed to the Fc region. These minima of

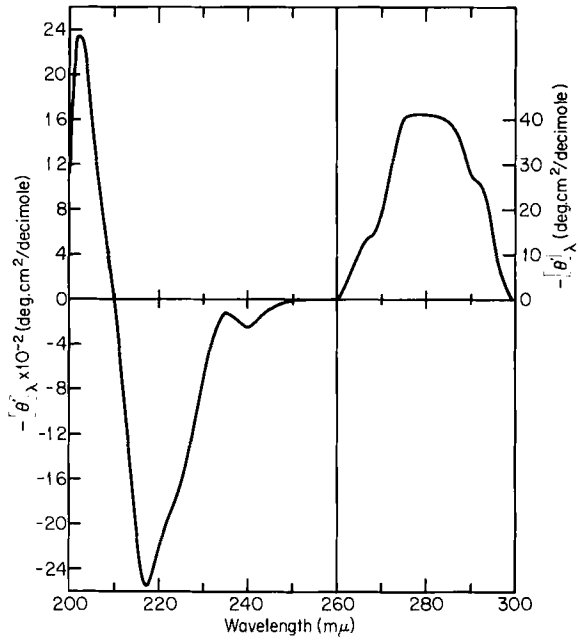


FIG. 10. The circular dichroism spectrum of a human γG myeloma protein, between 200 and 300 mμ., in 0.1 *M* NaCl–0.01 *M* phosphate buffer, pH 7.2. Note the difference in scale between the right- and left-hand ordinates.

Fab and Fc between 225 and 235 mμ. probably represent optical activity associated with the $n \rightarrow \pi$ electronic transition of the peptide chromophore, by analogy with synthetic polypeptide spectra. The reason for the marked blue shift of this transition in Fab is not understood. The additivity of the spectral properties of Fab and Fc fragments in intact γG has been confirmed by CD (Cathou *et al.*, 1968). It is worthy of note that although the ORD spectra of intact γM and γA do not exhibit the small Cotton effect at 240 mμ. (Dorrington and Tanford, 1968; Dorrington and Rockey, 1968) the Fab and $F(ab')_2$ fragments derived from these proteins clearly show this feature. In the case of γM the failure to detect the 240-mμ. minimum was due to the larger contribution of the Fc region to the total rotation of the whole molecule. The 240-mμ. Cotton effect seems to be a common, characteristic feature of all the immunoglobulin classes studied so far (all except γD) with the exception of equine γT (Rockey *et al.*, 1970). The optical transition responsible for the 240-mμ. Cotton effect is not known at this time but the possible chromophores which may be involved have been discussed (Cathou *et al.*, 1968; Rockey *et al.*, 1970).

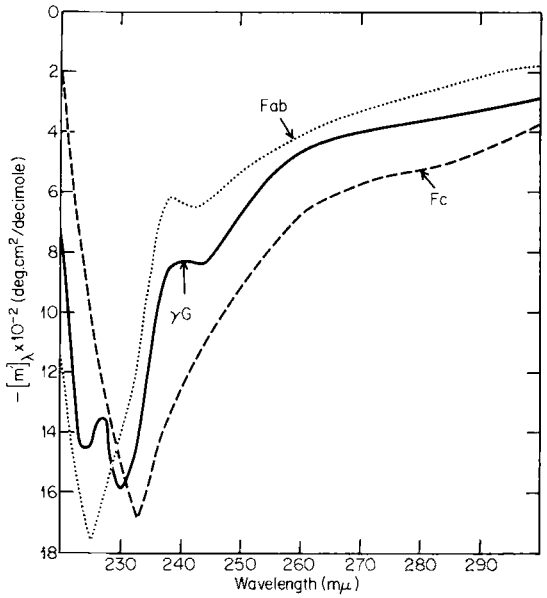


FIG. 11. The optical rotatory dispersion spectra of a human γG myeloma protein and the Fab and Fc fragments isolated from a papain digest of the same protein.

The contributions of the heavy and light chains to the ORD spectrum of γG will be discussed in Section III,C. The results suggest that the 225-mμ. minimum of the whole molecule and its Fab fragment arises principally from the light chain, whereas the 240-mμ. Cotton effect comes from the heavy chain. The 232-mμ. minimum is seen in heavy-chain preparations, which is consistent with its presence in the Fc fragment.

Although the ORD and CD properties of immunoglobulins are reasonably well established over the experimentally accessible wavelength range, interpretation of these features in terms of specific conformations is impossible at this time. It has become fashionable to try to account for the ORD and CD properties of globular proteins in terms of fractional contents of α helix, β structure, and random coil on the basis of the ORD and CD of synthetic polypeptides known to be in these conformations (Greenfield *et al.*, 1967; Magar, 1968; Greenfield and Fasman, 1969). These computed curves, although yielding fairly reliable results for synthetic polypeptides, fail to account satisfactorily for the ORD and CD spectra of those globular proteins of which the structure is known from X-ray analysis (e.g., myoglobin and lysozyme). The present authors feel that this type of approach is without value

or justification. For example, neither myoglobin nor lysozyme has any random coil. Nonhelical and non-β-structure regions of the backbone are not random but fixed in space. There is no reason to believe that such regions will have optical properties comparable to randomly coiled polypeptides. In addition, globular proteins have nonpeptide chromophores (aromatic amino acids and disulfide bonds) which are known, from model compound studies, to have complex ORD and CD spectra which in many instances are highly dependent on the environment of the chromophore. The day seems far off when the contribution of such chromophores to the ORD and CD of a protein can be unambiguously evaluated. Such an analysis would probably have to be supported by X-ray data which would render the ORD or CD analysis redundant. The immunoglobulins illustrate the problems associated with the above approach since no combination of the ORD or CD characteristics of the three conformations listed above would account for the spectra of immunoglobulins.

The one structural feature of protein molecules that can be recognized with fair certainty from ORD or CD data is the presence of a high content of α-helical regions. The reason for this is that the magnitude of the optical activity near 230 mμ., and the curvature of ORD plots at higher wavelength, as represented by the b_0 parameter of the equation of Moffitt and Yang (1956), are much greater for α-helical polypeptides than for synthetic polypeptides in other conformations. Quantitatively similar results have been obtained for every protein known to have a high α-helix content on the basis of other information, but in no case for any protein known not to have a predominantly helical structure. These features, therefore, seem to be specific indicators of the α-helical conformation.

On the basis of the experimental results, it seems fairly certain that immunoglobulins do not have significant amounts of α-helix. The parameter, b_0, of the Moffitt-Yang equation, consistently has zero or slightly positive values for immunoglobulins. The maximum magnitude of levorotation near 230 mμ. is only 1200–1800 deg.cm.^{2}/decimole, whereas, for the polypeptides in 100% helical form, $[m']_{233\,m\mu}$. has a value near $-15{,}000$ deg.cm.^{2}/decimole. Also helical polypeptides have a maximum at 198 mμ., whereas immunoglobulins have a minimum at the same wavelength. The peptide bond absorption of γG does show marked hypochromicity in the 190–205-mμ. region (Gould *et al.*, 1964; Ross and Jirgensons, 1968) as has been shown for α-helical polypeptides (Imahori and Tanaka, 1959; Rosenheck and Doty, 1961). However, Gould *et al.* attribute this decreased absorption to vicinal effects of the side-chain chromophores or to the existence of some periodic structure distinct from the α-helix.

C. Properties of Separated Heavy and Light Chains

There is now good evidence that isolated heavy chains from γG antibody can bind homologous antigen or hapten (Fleischman *et al.*, 1963; Metzger and Singer, 1963; Utsumi and Karush, 1964; Haber and Richards, 1966). The equilibrium constant for combination with antigen or hapten is, however, usually one or two orders of magnitude smaller than that of each binding site of the whole molecule or of the Fab fragment. In some instances, light chains have also been shown to possess small, but measurable affinity for homologous antigen (Goodman and Donch, 1965; Mangalo *et al.*, 1966; Yoo *et al.*, 1967). There are two possible explanations for the observation of specific affinity with reduced binding constant —one being that the binding site of the native antibody involves portions of both light and heavy chains, the other being that only one chain carries the binding site but that the other chain is required to maintain it in a reactive conformation. Decision between these possibilities would contribute significantly to an understanding of the generation of antibody specificity, and this has, therefore, stimulated interest in the conformational properties of the isolated heavy and light chains.

In order to separate the heavy and light chains, it is first necessary to break the interchain disulfide bonds and to protect the resulting SH groups against reoxidation. This can be done under relatively mild conditions, but does not significantly affect the cohesion between the chains, which is primarily due to noncovalent interactions. Quite drastic conditions are required to break these interactions: 1 *M* propionic acid is needed for rabbit γG, though 1 *M* acetic acid or very low pH alone will suffice for human γG. The chains can be separated by gel filtration in the dissociating media and then returned to more benign conditions for examination. The heavy chains tend to form aggregates or even to precipitate from solution, but stable preparations can be maintained at pH 5.5 at low protein concentrations if optimal procedures are followed. Appropriate procedures for rabbit heavy chains are described by Björk and Tanford (1970) and for human chains by Stevenson and Dorrington (1970). Even under the best conditions, aggregates tend to form slowly and must be removed from solutions that have been stored for an appreciable length of time. The requirements for obtaining stable preparations of light chains are less stringent than for heavy chains.

Björk and Tanford (1970) have shown that rabbit heavy chains have a molecular weight of 108,000 to 117,000 by sedimentation equilibrium, i.e., they exist as dimers (H_2). They are dissociated to monomers at low pH or in 6 *M* guanidine hydrochloride, showing that they were held together by noncovalent forces as is to be expected from the fact that

the thiol groups derived from the original interchain disulfide bonds were blocked. The sedimentation coefficient of H_2 was found to be $s^{\circ}_{20,w} = 5.7$ S, leading to a frictional coefficient ratio, $f/f_{min} = 1.40$, similar to that observed for whole γG. The dimer could be digested with papain at pH 7, leading to a product with molecular weight of 47,000 to 50,000. In 6 *M* guanidine hydrochloride this product dissociated to yield about 85% material with molecular weight 25,000 and smaller amounts of material with molecular weights of 12,000 and of about 1000. Both the high value of f/f_{min} and the susceptibility to papain digestion suggest that the hinge region of γG is preserved in H_2. The product of papain digestion is presumably a mixture of Fd and Fc, and the most interesting aspect of the results is the finding that Fd, like Fc, exists as a dimer. The noncovalent interactions that bind Fd to the light chain in Fab and in γG are evidently replaced by similar interactions between adjacent Fd segments when the light chain is absent.

The molecular weights obtained for the papain-cleaved product in 6 *M* guanidine hydrochloride are consistent with this explanation. When noncovalent interactions are disrupted half-H chains should remain, the portions derived from the Fc and Fd regions being indistinguishable on the basis of molecular weight. The material with molecular weight 12,000 presumably indicates that some cleavage occurred at the secondary cleavage points shown in Fig. 4. (In the absence of guanidine hydrochloride this additional cleavage need not affect the molecular weight.) Cleavage of Fc by papain has been reported by other authors. Whether the 12,000-molecular weight product observed in this study came entirely from Fc or whether some cleavage also occurred in the Fd region is not known.

Stevenson and Dorrington (1970) have shown that heavy chains from human γG also exist as dimers at pH 5.5. They have a sedimentation rate ($s^{\circ}_{20,w}$) of 5.3 S and a molecular weight of 105,000 in 4 m*M* acetate buffer. In 6.0 *M* guanidine hydrochloride, the molecular weight drops to half this value.

Similar studies have been carried out with light chain preparations. Light chains from nonspecific rabbit γG exist as a mixture of monomer and dimer (Björk and Tanford, 1970; Stevenson and Dorrington, 1970), which can be separated by gel filtration chromatography and retain their identity on rechromatography. Human light chains also exist as mixtures of monomers and dimers, but the proportion of monomer is much less than in rabbit γG (Stevenson and Dorrington, 1970).

The integrity of the internal folding of H_2 and L_2 has been examined by ORD measurements. Initial studies (Dorrington *et al.*, 1967) indicated that there were gross differences between the conformations of the

separated chains and the parent molecule—the magnitude of rotation was much more negative and the characteristic Cotton effect at 240 mμ. was no longer evident. More recent studies have shown that such drastic changes in the ORD are not seen if stable preparations freed of aggregated material are examined. Figure 12 shows results obtained inde-

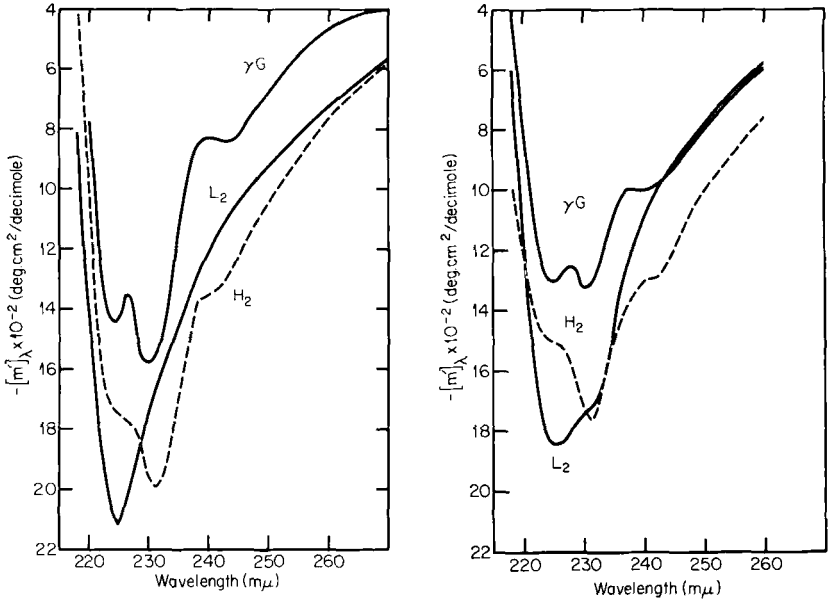


FIG. 12. The optical rotatory dispersion spectra, between 220 and 270 mμ., and γG and of heavy- and light-chain dimers derived from it following mild reduction and alkylation. The solvent is acetate buffer, pH 5.5. Portion A represents a human myeloma protein (Stevenson and Dorrington, 1970); portion B represents pooled rabbit γG (Björk and Tanford, 1970).

pendently in two laboratories for preparations consisting of essentially pure H_2 and pure L_2, from human and rabbit γG. They show that changes in conformation have accompanied chain separation. If no conformational change had occurred, the residue rotation for the native protein would be a weighted average of the residue rotations of the two chains, i.e., with the relative weights of H and L chains

$$[m']_{\gamma G} = 0.68[m']_H + 0.32[m']_L \quad (1)$$

This relation clearly does not hold true. (The corresponding relation does hold true for the ORD curves of γG, Fab and Fc, shown in Fig. 11.)

Although Fig. 12 thus indicates that conformational changes accompany chain separation, the *distinctive* features of the dispersion curve are preserved. The 225-mμ. trough of γG appears in the ORD curve

for L_2, the 230-mμ. trough and the 240-mμ. Cotton effect are seen in the curve for H_2. It can be concluded that substantial regions of the ordered structure of H and L chains in γG are preserved in the isolated chains, in the dimeric form in which they are present at pH 5.5.

The ORD patterns of H and L chains under conditions where chain separation occurs, i.e., at low pH or in propionic acid, are, of course, quite different. The polypeptide chains are in a denatured state under these conditions.

Since it is not possible to assign any feature of the ORD pattern uniquely to those portions of the polypeptide chains that constitute the binding site for antigen, these experiments cannot resolve the question posed at the beginning of this section regarding the underlying cause for the diminution in binding constant for antigen that accompanies chain dissociation. Additional evidence concerning this question is, however, provided by the recombination experiments described in Section IV,C.

IV. Recovery of Native Conformation Following Chain Dissociation and Unfolding

Basically, two types of experiment have been performed on the unfolding of immunoglobulins, almost exclusively with γG-globulin. In the first type of experiment, γG is completely unfolded to the random-coil form in which state it loses all its biological and antigenic properties. Recovery of some specific property (e.g., antibody activity) is assessed following refolding of the molecule. Such experiments have been used to determine whether or not all the information required to generate a specific three-dimensional structure resides in the amino acid sequence. The second type of experiment aims principally at an understanding of the interaction between the constituent polypeptide chains, e.g., the chains are separated and hybrid molecules with chains from functionally different parent molecules are studied. Since chain separation cannot be achieved without partial unfolding of the internal structure of each chain, these experiments necessarily also involve reversible disruption of the three-dimensional structure, but not to the same extent as when a random coil is formed.

A. Reversible Random-Coil Formation

Buckley *et al.* (1963) unfolded Fab fragments, derived from rabbit antibovine serum albumin (anti-BSA) antibody, with 6 *M* guanidine hydrochloride. Under such solvent conditions, no noncovalent bonds remain and the only restrictions on random-coil formation are those from the intact disulfide bonds. (For review, see Tanford, 1969.) The unfolding of Fab could be reversed by the slow removal of the guanidine

hydrochloride by dialysis. The combining ability of the native antibody fragment and the refolded fragment was assessed by sedimentation velocity studies in the presence of antigen (BSA). Seventy-five per cent of the combining ability of the native Fab was recovered. Physical studies clearly showed that the native and refolded fragments were indistinguishable.

Noelken and Tanford (1964) carried out essentially similar studies with Fab fragments from high-affinity DNP antibody. This system has the advantage that a sensitive fluorescence quenching technique can be used to assess antibody activity. Approximately 70% of the native Fab activity was recovered upon refolding of protein unfolded in 6.3 *M* guanidine hydrochloride.

Haber (1964) was the first to report a successful recovery of specific antibody activity upon refolding and reoxidation of completely reduced and unfolded Fab fragment, derived from rabbit antiribonuclease. The Fab fragments were unfolded in the presence of 2-mercaptoethanol in 8 or 10 *M* urea or 6 *M* guanidine hydrochloride. The protein was randomly coiled in the latter two solvents as judged by ORD, but considerable residual ordered structure was present in 8 *M* urea. Refolding and reoxidation (in air) of Fab was achieved by dialysis, first, against low concentration (10^{-3} *M*) of buffered 2-mercaptoethanol (pH 8) and, second, against buffer alone at pH 8. Under optimal conditions, 20–27% of the original binding activity, as measured in a radioimmunoassay system, was recovered following refolding and reoxidation of protein reduced in 10 *M* urea or 6 *M* guanidine hydrochloride, or 56% from protein reduced in 8 *M* urea.

Whitney and Tanford (1965a) performed similar experiments on anti-DNP Fab fragments unfolded in 6 *M* guanidine hydrochloride and reduced with 0.1 *M* 2-mercaptoethanol. Oxidation and refolding of the protein resulted in the recovery of 14 to 24% of the original activity as judged by fluorescence quenching. Reoxidation in the presence of excess antigen enhanced the amount of activity recovered to a small extent. In a separate study, Whitney and Tanford (1965b) assessed how far the reoxidized Fab was comparable to the native protein using several physical techniques. Antigenic analysis, sedimentation velocity, and ORD studies indicated that the reoxidized protein was very similar to native Fab. The principal difference was seen in the ORD where the Cotton effect near 240 mμ., clearly seen in native Fab, is only partially recovered in the reoxidation product.

Freedman and Sela (1966a,b) have reoxidized completely reduced and unfolded rabbit γG and assessed the recovery of both antibody activity and antigenic determinants. They utilized an earlier finding

(Fuchs and Sela, 1965) that the attachment of DL-alanine peptides to γG enhances its solubility properties to the extent of yielding a soluble molecule even when completely reduced. Initial studies on the recovery of antigenic determinants were performed on nonspecific poly-DL-alanine γG (Freedman and Sela, 1966a) using a goat antiserum against rabbit nonalanylated γG. It had been demonstrated previously that the poly-DL-alanylation did not significantly affect the antigenic structure of γG (Fuchs and Sela, 1965) and the use of nonalanylated antigen in the goat avoided the production of antibodies against the DL-alanine peptides. The poly-DL-alanine γG was reduced with 2-mercaptoethanol either in 8 to 10 *M* urea or in 6 to 8 *M* guanidine hydrochloride. Optmial conditions for reoxidation were determined, and these allowed recovery of almost all the antigenic determinants, as judged by quantitative precipitin analysis, although up to 4 times more reoxidized protein was required. Since the antigenic determinants on γG are predominantly conformational (as opposed to sequential), recovery of antigenic activity following reoxidation is a good index of the recovery of the native conformation. As 95–100% of the reoxidized poly-DL-alanine γG was soluble at pH 8, compared with 5 to 10% of unsubstituted γG, recovery of antigenic activity could be assessed in essentially all the molecules. It was found that the recovery of the antigenic determinants was more complete and the efficiency of precipitation was greater when the heavy and light chains were first reoxidized separately, suggesting that the reoxidation process involves the initial formation of intrachain disulfide bonds followed by the formation of interchain disulfides. This observation is consistent with the current experimental evidence on the biosynthesis of immunoglobulins.

In their second paper, Freedman and Sela (1966b) studied the recovery of specific antibody activity following reoxidation of completely reduced rabbit poly-DL-alanine anti-BSA. The immunospecifically purified poly-DL-alanine antibody was reduced with 2-mercaptoethanol in 8 *M* guanidine hydrochloride. Upon reoxidation, 25% of the antigen-binding activity was recovered in an assay measuring ^{131}I-BSA-binding capacity. A second assay system involving the inhibition of the homologous BSA–anti-BSA precipitin reaction indicated a 50% recovery of activity. The difference in the estimates of antibody activity recovered was probably a function of the incomplete recovery of the antigenic determinants of the poly-DL-alanine γG. The ^{131}I-BSA-binding assay depends on the precipitation of the antibody–antigen complex with goat anti-γG, whereas the inhibition reaction does not involve this type of interaction. The reoxidation conditions giving maximum recovery of antigenic determinants did not yield optimal recovery of antibody activity. Freedman and Sela

(1966b) interpreted this as indicating that re-formation of the combining site is an intrachain event, whereas recovery of antigenic determinants involved more than one polypeptide chain. This conclusion was supported by the observation that the antibody activity recovered was independent of whether the heavy and light chains were reoxidized separately or together, in contrast to their findings regarding the recovery of antigenic determinants.

In a recent report from Sela's group (Jaton *et al.*, 1968), recovery of specific antibody activity upon reoxidation of completely reduced heavy chain and Fd fragment has been studied. Poly-DL-alanine heavy chain and poly-DL-alanine Fd were prepared by mild reduction of poly-DL-alanine anti-DNP and cyanogen bromide cleavage of the poly-DL-alanine heavy chain, respectively. The polyalanylated heavy chain and Fd were soluble in aqueous solvents at neutral pH (Fuchs and Sela, 1965) and possessed an average of 0.26 and 0.16 hapten binding site, respectively. The average association constants (K_A) for these sites on both proteins was $2.4 \times 10^6\ M^{-1}$, approximately two orders of magnitude lower than the intact antibody. Complete reduction was achieved with 0.4 *M* 2-mercaptoethanol in 8 *M* guanidine hydrochloride. Following reoxidation, 37% of the hapten binding sites on the poly-DL-alanine heavy chain were recovered, and 59% of those on the alanylated Fd. The K_A for the reformed sites on the heavy chains was $1.3 \times 10^6\ M^{-1}$ and $0.6 \times 10^6\ M^{-1}$ for the Fd fragment, i.e., the same order of magnitude as before reduction. These results support the earlier conclusions of Freedman and Sela (1966b) that the formation of the binding site per se is an intrachain event since recovery of binding activity can occur in the absence of the light chain. Further, formation of the site requires only a portion of the heavy chain (Fd). This does not, however, invalidate other evidence that the light chain has an, as yet, undetermined role in influencing the binding characteristics of the combining site (Section IV,C).

The results of the experiments described above have bearing on two important areas of protein chemistry:

1. It has been proposed, principally by Anfinsen (1962, 1967), that the folding and cross-linking of polypeptide chains occurs spontaneously, directed solely by thermodynamic forces dependent on the amino acid sequence alone. This concept fits well current observations on the synthesis of proteins which provide for a transfer of sequential information from the base triplets of the nucleic acids to the amino acids of the protein without provision for the transfer of conformational information. Experimental verification of Anfinsen's hypothesis has been obtained with a number of single-chain proteins (for references, see Neumann *et al.*, 1967). However, recovery of biological activity following complete

reduction of proteins with more than one polypeptide chain has been less convincing. The results with Fab and intact γG clearly show that levels of activity significantly greater than expected from random recombination can be achieved with multichain proteins.

2. Broadly speaking, theories of antibody production fall into two principal categories (Burnet, 1969). Selective theories propose that each potential antibody-producing cell is programmed to make only a single type of immunoglobulin with a defined sequence. The antigen selects, by some poorly understood mechanism, the cell or cells able to produce an antibody showing good binding characteristics for itself. Proliferation subsequently takes place to form a clone of antibody-producing cells. The antigen does not induce major, irreversible conformational changes in the antibody-combining site.

In the second type of theory the conformation of the antibody-combining site is controlled by the antigen during biosynthesis of the immunoglobulin. In other words the antibody molecule is adapted to fit the particular antigenic determinant. The adaptive conformational change remains stable while the molecule is in its native state but is lost on unfolding. Recovery of the binding site would, therefore, require the presence of the antigen during refolding. The reoxidation–refolding experiments are compatible only with a selective mechanism since specific binding sites are formed in the absence of antigen. Further, in experiments in which reduced nonspecific γG (or Fab) has been reoxidized in the presence of antigen, no evidence of induced binding ability could be detected (Whitney and Tanford, 1965a).

B. Reversible Dissociation into Half-Molecules

Rabbit γ Gcan be dissociated into half-molecules (i.e., one heavy and one light chain) by reduction of the single inter-heavy-chain disulfide bond and disruption of the noncovalent interactions between the Fc portions by lowering the pH to 2.5. The process can be carried out without alteration in the Fab portion of the molecule, and, since the antibody-binding site is contained wholly within the Fab portion, recombined half-molecules are indistinguishable from native antibody (Palmer *et al.*, 1963; Palmer and Nisonoff, 1964). Hybrid molecules containing two binding sites with different specificities are readily obtained (Nisonoff and Hong, 1964).

These experiments constitute control experiments for those described in the following section. The corresponding experiments cannot be carried out with human γG because disruption of the noncovalent interactions between the Fc portions of human heavy chains occurs simultaneously with disruption of the Fab portions of the molecule.

C. Dissociation into Heavy and Light Chains and Its Reversal

This section is concerned with reversal of the lesser perturbations in conformation that accompany dissociation of mildly reduced γG to heavy and light chains at low pH or in propionic or acetic acid solution. It was noted earlier that the separated chains may be returned to pH 5.5 and that they exist there as dimers (H_2 and L_2), which retain many of the conformational features seen in γG. However, there are also conformational differences, notably a close association between the Fd portions of the two heavy chains, which appears to have replaced the close association between Fd and L chains in native γG. In the experiments described here, dissociated and separated H and L chains are mixed in equimolar proportions, and dialyzed to nearly neutral pH in an aqueous medium, or, alternatively, a mixture containing separated chains is returned to nearly neutral pH without actual fractionation into solutions containing only H and only L chains.

It has been demonstrated that renaturation of this type leads to reformation of a 7 S molecule (Edelman *et al.*, 1963; Olins and Edelman, 1964; Roholt *et al.*, 1964), although the extent of recombination shows considerable variation among proteins (Gordon and Cohen, 1966). The recombined molecules, their subunits held together by noncovalent forces alone, resemble the original immunoglobulin as judged by sedimentation velocity, molecular weight, antigenic structure, and electrophoretic properties. Recombination can be achieved between chains derived from different classes of immunoglobulin (Gally and Edelman, 1964; Grey and Mannik, 1965) and different species (Fougereau *et al.*, 1964).

Significant recombination of heavy and light chains does not occur when the chains are mixed at neutral pH, under which conditions light chains exist as dimers and heavy chains as dimers and insoluble aggregates. The apparent requirement for mixing of the chains in organic acid solution at low pH prior to recombination has been interpreted to mean that monomeric chains are the species involved in the recombination reaction. (Both heavy and light chains are principally monomers at pH 2.5.) However, Stevenson has shown that rapid recombination of heavy and light chain dimers occurs at pH 5.5 (Stevenson, 1968; Stevenson and Dorrington, 1970), the overall reaction being $\gamma_2 + L_2 \rightarrow \gamma_2L_2$. It is likely that the mechanism involves prior dissociation of L_2 to monomeric L chains, i.e., $L_2 \rightleftharpoons L$, $L + \gamma_2 \rightarrow L\gamma_2$, $L\gamma_2 + L \rightarrow L\gamma\gamma L$. Disulfide-bonded dimers of light chains do not combine at all with γ_2.

The most interesting aspect of the recombination process is its specificity. Measurements of affinity between antigen and antibody have

always demonstrated requirement for a high degree of specificity. The heavy chain of an antibody, in the absence of light chain, generally combines with specific antigen, but with an affinity much less than that of the native antibody molecule or its Fab fragment. Recombination with L chain invariably results in marked enhancement of the affinity only if homologous light chains are used. Heterologous light chains lead to little if any enhancement (Edelman *et al.*, 1963; Franek and Nezlin, 1963; Metzger and Mannik, 1964; Roholt *et al.*, 1964, 1965a,b; Hong and Nisonoff, 1966; Lamm *et al.*, 1966; Haber and Richards, 1966). Maximal recovery of activity in recombined γG requires that the light chains are derived from the same animal as well as from antibody of the same specificity (Roholt *et al.*, 1965a,b). Zappacosta and Nisonoff (1968) have shown however that the binding activity of rabbit anti-DNP heavy chain was significantly enhanced by light chains from antibody of the same specificity raised in other rabbits of the same allotype. Hong and Nisonoff (1966) showed that when anti-DNP antibody from a single rabbit was fractionated into populations of high and low binding affinity, preferential enhancement was exhibited by light chain from the same fraction as the heavy chain.

Further evidence for specificity in the association of heavy and light chains has come from studies with myeloma proteins. Grey and Mannik (1965) showed, using ^{131}I-labeled light chains, that the heavy chain of a γG myeloma protein preferentially recombined with its autologous light chain in the presence of heterologous light chain. However, considerable variation was exhibited among light chains from different myeloma proteins in their ability to replace autologous light chains. The competition between various light chains for a particular heavy chain has been studied in more detail by Mannik (1967). The preferential recombination of autologous heavy and light chains was confirmed but seemed to be variable among different myeloma proteins. In some instances, preferential recombination could still be demonstrated in the presence of eighty-fold excess of heterologous light chain. As the proportion of heterologous light chain increased the preferential recombination decreased. Normal γG light chains were shown to contain populations of molecules that could replace autologous myeloma light chains in recombination with their heavy chains. The ability of heterologous light chains to substitute for autologous light chain was not related to either antigenic type (κ or λ) or electrophoretic mobility of the parent protein or to the banding of isolated light chains in alkaline starch-gel electrophoresis. Roholt *et al.* (1967) have devised a convincing demonstration of the preferential recombination of antibody subunits. They prepared heavy and light chains from two high affinity rabbit antihapten antibodies and made

heterologous recombinants which showed little or no antibody activity. Both types of recombinant were mixed in propionic acid and dialyzed to neutrality to re-form 7 S molecules. When assayed for the original anti-hapten activity the molecules showed activities much higher than would be anticipated as a result of the random re-formation of specific sites. These observations tend to suggest that noncovalent interaction between heavy and light chains involves the variable region of the light chain. Ruffilli and Givol (1967) have presented some evidence that both the variable and constant halves of light chain are involved in the interaction with heavy chain.

The integrity of the conformation of reformed γG has been tested by ORD measurements, and the results at first appeared to provide an intriguing parallel with antibody activity measurements, i.e., full restoration of the ORD spectrum of native γG occurred only when heavy chains were recombined with autologous light chains (Dorrington *et al.*, 1967). These results, and speculations about the role of H-L interactions in the generation of antibody specificity based on them (Tanford, 1968), have now been shown to be at least partly incorrect. Improvements in technique which led to isolation of heavy chains with much less loss of characteristic conformational features than in earlier experiments (Sec-

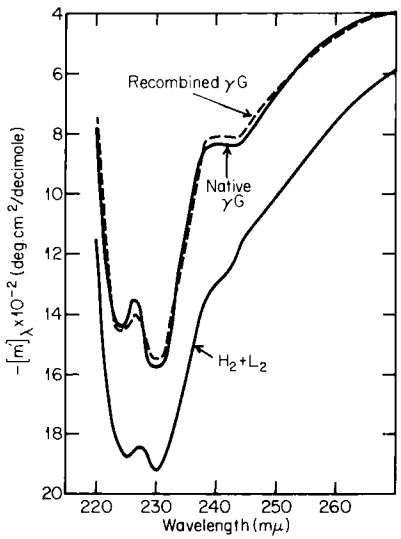


FIG. 13. The optical rotatory dispersion spectrum (ORD) of purified, recombined, human myeloma γG compared with the native, untreated protein between 220 and 270 mμ. The third curve represents the ORD spectrum for an equimolar mixture of heavy and light chains, calculated according to Eq. (1).

tion III,C) have also led to the conclusion that full restoration of the native ORD spectrum may not require that a heavy chain be combined with a uniquely matching light chain.

Figure 13 (Dorrington and Stevenson, 1970) shows the ORD curves of a native human myeloma γG, the weighted average of the ORD curves of the separated chains of the same protein, calculated according to Eq. (1), and the ORD curve for the reconstituted protein. The latter is clearly identical, within experimental error, with the curve for the native protein, showing that no irreversible conformational change accompanies chain separation when the H and L chains are autologous. Figure 14 (Dorrington and Stevenson, 1970) shows similar results for pooled normal human γG. In this case the separated chains must constitute a highly heterogeneous mixture, and the concentration of autologous light chains for a given heavy chain must be very small, so that the bulk of the recombined molecules must contain heterologous chains. The yield of 7 S γG was not 100% in this experiment, some unrecombined chains remaining in the mixture. They were removed before the ORD measurements were made, so that the results reflect the properties of the reconstituted protein alone. It is seen that the native ORD spctrum is substantially, but not fully recovered. However, similar experiments

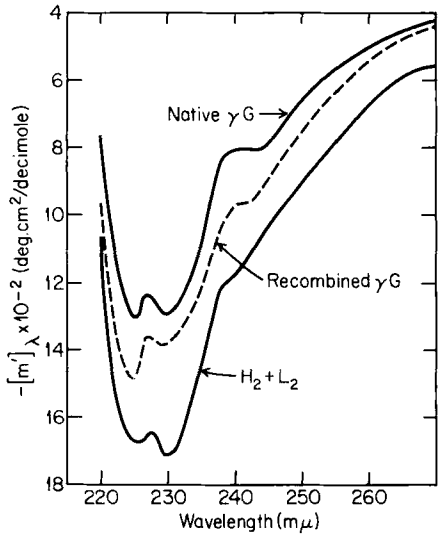


FIG. 14. The optical rotatory dispersion spectrum (ORD) of purified, recombined, pooled, normal human γG compared with the native, mildly reduced and alkylated protein, between 220 and 270 mμ. The third curve represents a calculated ORD spectrum for an equimolar mixture of heavy and light chains from the same γG.

with pooled normal rabbit γG (Björk and Tanford, 1970) have yielded a product (about 75% yield) which was indistinguishable from native γG by ORD measurements as well as by other techniques. The reason for the discrepancy between the two results has not yet been established. It is evident, in any event, that heterologous H-L combinations can differ only minimally in conformation from autologous combinations.

Taken at face value, these experiments suggest that the failure to obtain full biological activity when the H chain of an antibody is combined with a heterologous L chain is not due to failure to achieve restoration of conformation. The possibility exists that there are important conformational features which do not contribute significantly to the ORD pattern in the wavelength range examined, and it is obviously necessary to examine the conformational integrity of the renatured γG by other methods. However, if other methods yield similar results, it must be concluded that the H-chain conformation in the reconstituted γG is independent of the nature of the L chain with which it is combined, and in that event the effect of the nature of the L chain on the specific affinity for antigen can only be interpreted as indicating that portions of both chains form part of the antibody-binding site.

Even this conclusion must be regarded as very tentative, however. The foregoing results have shown that the conformation of recombined γG (and, as shown in Section III,C, that of the the separated H chains) is very sensitive to the exact experimental procedure used to separate and recombine the polypeptide chains. To be certain that H and L combinations exist which appear to have *both* full conformational integrity *and* diminished affinity for specific antigen, it is necessary to monitor conformation and activity on the same sample. This has not been done to date.

V. Conclusions

It is apparent from the work described in this review that we are still a long way from a complete analysis of the structure of immunoglobulins in three-dimensional terms. We are, however, only a little over a decade away from the first realization that immunoglobulins are multichain proteins. Since that time tremendous advances have been made in the sequence analysis of these biologically essential molecules. One suspects that correlations of amino acid sequence and antibody specificity are just around the corner. In addition, we are able to propose topographical models, at least for γG and γM, with a reasonable degree of confidence. Refining these models to the atomic level still seems a long way off. The reasonable assumption is that such knowledge can come only from X-ray crystallography. As mentioned earlier the first

steps in this direction have already been taken. The bulk of the time-consuming work, such as the preparation of suitable heavy metal isomorphous replacements, has still to be attacked. With appropriate commitments of resources and manpower we could have an atomic picture of an antibody or at least an antibody Fab fragment before the end of the decade.

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