ADVANCES IN CANCER RESEARCH

Volume 20

George Klein & Sidney Weinhouse

ADVANCES IN CANCER RESEARCH

VOLUME 20

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ADVANCES IN CANCER RESEARCH

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TUMOR CELL SURFACES: GENERAL ALTERATIONS DETECTED BY AGGLUTININS

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I. Introduction

One of the most fundamental questions concerning malignant cells is: Why do they proliferate beyond normal limits and become invasive? This is the question posed in its most simple form by both layman and scientist.

Even though a host of researchers have been applying themselves to

this question for a number of years, it is still far from being answered. One concept that has emerged is that malignant cells show great alterations in their relationships, not only toward cells in the surrounding tissue which they may invade, but also toward each other. Kalckar (1965) has spoken of an altered "cell sociology" of malignant cells, stemming at least in part from their altered surface characteristics, and Pardee (1964) has pointed out that alteration of such membrane functions as transport could play an important part in control of growth.

Let us list briefly here some of the characteristics of tumor cells which are indicative of their having altered surface membranes:

A. *In the organism*

1. Tumor cells, whether they are part of a malignant or a benign tumor, multiply beyond normal limits, without being influenced by neighboring cells.
2. Most tumor cells do not cooperate with each other to form a tissue specialized for a certain function; individual cells thus become autonomous.
3. Although the chemical structure of cell surfaces is still poorly known, serological studies have shown the antigenic makeup of tumor cells to differ in several respects from that of normal cells:
   a. Appearance of tumor-specific transplantation antigens (TSTA) as well as virus-specific antigens on the surface of virally transformed cells (Klein, 1969),
   b. Appearance in many cases of carcinoembryonic antigens or oncofetal antigens (Abelev, 1971; Gold and Freedman, 1965a,b),
   c. Tumor cells are less differentiated than their normal counterparts; one manifestation of this is the decrease, or in some cases the total loss, of tissue-specific antigens (Weiler, 1959).

B. *In vitro*. The possibility of transforming cells *in vitro* (by oncogenic viruses, or by chemical carcinogens) or the ability to select spontaneously transformed cells allows a comparison between normal and transformed cells originally stemming from the same tissue.

1. A characteristic of untransformed tissue culture cells is their density-dependent inhibition of growth (DDI)[1] (Stoker and

[1] Abbreviations: cAMP, cyclic AMP = adenosine 3′,5′-monophosphate; CMP, cytidine 5′-monophosphate; cGMP, cyclic GMP = guanosine 3′,5′-monophosphate; cIMP, cyclic IMP = inosine 3′,5′-monophosphate; cUMP, cyclic UMP = uridine 3′,5′-monophosphate; DDI, density-dependent inhibition (usually of growth); diBcAMP, dibutyryl cAMP; DNP, dinitrophenol; Ig, immunoglobulin; PGE$_1$, prostaglandin E$_1$; RSV, Rous sarcoma virus; TAME, tosyl-arginine methyl ester (a protease inhibitor); TLCK, tosyl-lysyl-chloromethylketone (a protease inhibitor); TPCK, tosyl-phenylalanylchloromethylketone (a protease inhibitor). For abbreviations of lectin names, see Table I.

Rubin, 1967): At the stage where growing cells become confluent they cease dividing and do not continue to grow beyond the monolayer stage. Tumor cells escape this DDI of growth: contact does not prevent them from proliferating for a certain time, and they usually form disorganized multiple layers.

2. As first reported by Coman (1944) transformed cells are less adhesive toward each other and toward an external support. They can thus more easily grow in suspension, a property which in the organism would certainly be of advantage for their invasive and metastasizing growth (see also Abercombie and Ambrose, 1962).
3. Chemical and serological studies of surface membranes isolated from untransformed and from the corresponding transformed cells have shown a number of differences. Some of these studies will be briefly reviewed in Section VI.

C. *Increased agglutinability*. Based on some previous reports by Easty *et al.* (1960), Aub and his collaborators reported in 1963 that a lipase preparation from wheat germ agglutinated tumor cells preferentially to normal cells. Until that time phytohemagglutinins or lectins, proteins usually isolated from plant seeds, had been used mainly for blood typing and for carbohydrate studies. This rested on Boyd's demonstration in 1945 (see Boyd, 1970) that the specificity of various agglutinins for different blood groups depends on a specific reaction between the lectin and the blood group carbohydrates located on the surface of erythrocytes, which was reminiscent of antigen–antibody reactions. The differential behavior of normal and tumor cells with an agglutinin was another indication for the existence of surface differences between these cells, and this agglutinin reaction has become a very useful tool for the study of cell surfaces. Attempts to find out how the transition from a normal and nonagglutinable cell to one that is transformed and agglutinable takes place, should yield insights into molecular alterations and rearrangements occurring with the transformation process. Studies along these lines have been going on for the last few years in our own and in several other laboratories. A few questions have been answered, but many more have been raised.

This review will be concerned with studies on the agglutinin reaction, its use for the investigation of normal and of tumor cell surfaces, and with the process of transformation. It may not be amiss to stress at this point that this agglutination reaction is an artificial system since lectins are not present in the organism to interact with tumor cells. This should not detract from the usefulness of this reaction as a tool for the study of cell surfaces in general and of malignant transformation in particular.

It should be emphasized already at the beginning of this review that even though some correlations exist between changed surface characteristics of tumor cells and their neoplastic behavior, a causal relationship between these properties as well as an irrefutable proof of a direct involvement of the cell membrane in malignancy have yet to be conclusively demonstrated.

II. Correlation between Lectin Binding, Agglutinability, and the Transformed State

Following Aub's initial observation (Aub *et al.*, 1963) that a crude wheat germ lipase preparation selectively agglutinated malignant cells, the active principle from such preparations, the protein wheat germ agglutinin (WGA), was isolated (Burger and Goldberg, 1967). This lectin did indeed agglutinate leukemia cells and cells transformed by the tumorigenic polyoma virus better than the corresponding untransformed cells. From carbohydrate inhibition studies it was concluded that the reactive group at the cell surface, probably a glycoprotein, contained β-1,4-di-*N*-acetylglucosamine (Burger and Goldberg, 1967). These initial studies opened the way to a large number of investigations in many laboratories, using various plant agglutinins, such as concanavalin A (Inbar and Sachs, 1969), soybean agglutinin (Sela *et al.*, 1970), and a number of others (see Table I).

A review of these studies, which we shall present here, and which is summarized in Table I, leads to the conclusion that in general there is indeed a good correlation between tumorigenicity of cells and their ability to be agglutinated by plant lectins; like all generalizations, however, this one suffers a number of exceptions, and these will also be discussed here.

A. Agglutination Studies

Long before the discovery of the affinity of agglutinins for tumor cells, plant lectins were known to agglutinate red blood cells and Boyd found in 1945 (see Boyd, 1970) that they could be used for blood group typing [for historical review about this discovery, and for description of a number of lectins and their carbohydrate specificity, see Boyd (1970), Lis and Sharon (1973), and Sharon and Lis (1972)]. The ability of many lectins to agglutinate red blood cells from certain species shows that they are not exclusively tumor-specific (it should, however, be remarked in passing that human erythrocytes are not typical somatic cells). Aub himself followed his initial observations on the agglutinability of tumor cells (Aub *et al.*, 1963) by a series of tests on normal and tumor cells, and he found that although tumor cells consistently gave a

higher degree of agglutination than normal cells, the latter were often also agglutinated to a slight degree (Aub *et al.*, 1965a,b).

Although in the great majority of cases it is tumor cells and transformed cells that are agglutinated by lectins, a few cases have also been reported where the normal cells, not their transformed counterparts, agglutinated better. This, however, seems so far to be a particularity of the interaction of lentil (*Lens culinaris*) agglutinin with normal liver cells and with rat hepatoma cells (but not with other types of cells, Borek *et al.*, 1973; see also Section III,B,4).

In several instances no difference in agglutinability could be observed between normal and tumor cells (see Table I). In these cases transformation has not changed the availability of a certain receptor site; this, however, does not preclude modification of sites on the same cells for other lectins. An example of this phenomenon is the agglutinability of normal lymphocytes as well as lymphoma cells by the red kidney bean agglutinin PHA (*Phaseolus vulgaris* agglutinin), whereas concanavalin A (con A) agglutinates the lymphoma cells only (Inbar *et al.*, 1973a). It should also be kept in mind that different types of cells differ in their surface components and consequently in their ability to interact with different lectins [L1210 leukemia cells,[2] for instance, are very well agglutinated by WGA but not by Con A (Burger, 1973), whereas Yoshida sarcoma cells are agglutinated by Con A and by PHA, but poorly by WGA (Tomita *et al.*, 1972b); cells from a rat ascites hepatoma are agglutinable by WGA, but practically not by Con A (D. F. Smith *et al.*, 1973)].

Interesting are also the cases where cells have become agglutinable after infection with nononcogenic viruses: Infection of baby hamster kidney (BHK) cells by Newcastle disease virus resulted in a thinning of the cell coat and agglutinability of the cells by Con A and WGA; a viral mutant which did not produce this thinning of the cell coat did not render the infected cells agglutinable (Poste and Reeve, 1972). Becht *et al.* (1972) showed that cells infected with a variety of nononcogenic enveloped RNA viruses, such as Sindbis, vesicular stomatitis, or influenza virus, became agglutinable by Con A concomitantly with the appearance of hemagglutinin on their cell surface; cells infected in

[2] Description of cells mentioned in this review: 3T3, mouse embryo fibroblasts; Py3T3, polyoma virus-transformed 3T3 cells; SV3T3, simian virus 40 transformed 3T3; RSV3T3, Rous sarcoma virus-transformed 3T3; BHK (or BHK21), baby hamster kidney fibroblasts; CHO, Chinese hamster ovary cells (a dedifferentiated, epithelioid cell line); HeLa, a line derived from a human epithelioid carcinoma (cervical); KB, a line derived from a human carcinoma (oral), epithelioid; L cells, mouse fibroblasts; L1210, mouse leukemia cells; NIL, hamster fibroblasts.

TABLE I*
SOME AGGLUTININS AND THEIR PROPERTIES
(Numbers in parentheses correspond to references at bottom of table. Complete bibliographical references will be found at end of review.)
Part A

Lectin and source	Isolation and purification	Specific inhibitor	Agglutination: Normal cells (other than erythrocytes)	Agglutination: Normal cells + proteases or in mitosis	Agglutination: Transformed cells	References	Other properties
Concanavalin (Con A), from jack bean (*Canavalia ensiformis*)	Isolation, purification, and characterization (1, 17, 22, 38, 59, 60, 74, 75, 82) Crystallization (74, 75)	α-D-Glucopyranosides, α-D-mannopyranosides, α-D-fructofuranosides (α-methylmannose generally used (29))	0, but binds to normal cells	+	+	5, 6, 8, 10, 12, 14–16, 19, 20, 25–28, 33, 36, 40, 42, 45, 48–50, 54, 61, 68, 71, 76–79, 83	Isolation of cell membrane receptor (3, 4, 72, 86) Agglutination is temperature dependent (35, 37, 39, 53, 80) Agglutination depends on low cellular ATP content (81) Con A is mitogenic (43, 44, 56, 64) Stimulation of lymphocytes by Con A raises cellular cGMP content (31) Lymphoma cells agglutinated better than normal lymphocytes (37) Membrane-bound Con A has relatively high mobility (70)
	MW of tetramer 100,000–120,000 (46, 82)		Some lines +, some lines −		Some lines + some lines −	26, 45, 47, 66, 67	
	Con A not a glycoprotein (2, 59)				Temperature-sensitive mutants agglutinable only at permissive temperature	10, 13, 21, 53, 55, 65	
					Cells having regained growth control are less agglutinable	16, 34, 41 62, 69	

Normal, confluent cells + protein synthesis inhibitors are agglutinable	6, 12	Fetal cells are agglutinable (24, 48, 51, 73, 84) Cells infected with some nononcogenic RNA viruses are agglutinable (7, 11, 63, 76, 89)
Normal cells treated with urea are agglutinable	85	Covering of Con A sites on transformed cells (by monovalent Con A) restores growth control (14)
CHO cells converted to fibroblasts are less agglutinable	32	Agglutination depends on intact microtubules (9, 23, 88) Con A bound to lymphocytes restricts Ig receptor mobility (30, 87) Tumor cells incubated with Con A prior to injection are more tumorigenic and invasive (18) Con A does not block hamster egg fertilization (58) Binds to sea urchin egg and inhibits normal cell division (57)

* Parts A–F of Table I appear on pages 6–17.

(*Continued*)

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4. Allan *et al.* (1972)
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84. Weiser (1972)
85. Weston and Hendricks (1972)
86. Wray and Walborg (1971)
87. Yahara and Edelman (1972)
88. Yin *et al.* (1972)
89. Zarling and Tevethia (1971)

TABLE I (*Continued*)

Part B

Lectin and source	Isolation and purification	Specific inhibitor	Agglutination			References	Other properties
			Normal cells (other than erythrocytes)	Normal cells + proteases or in mitosis	Transformed cells		
Soybean agglutinin (SBA), from soybean (*Glycine max*)	Isolation, purification, and characterization (2, 5–9, 11, 17) MW of tetramer: 110,000 (6, 8, 11, 17) Is a glycoprotein (8, 17) Four isolectins isolated (7)	*N*-Acetyl-D-galactosamine (D-galactose is a weaker inhibitor) (10)	0, but binds to normal cells	+	+	3, 12, 13	Agglutination is not temperature dependent (3, 4) Agglutination does not depend on cellular ATP content (16) Is not mitogenic (4) Normal lymphocytes and lymphoma cells are agglutinated to same degree (4) Trypsinization of erythrocytes increases binding and agglutinability (1, 15) Membrane-bound lectin has relatively low mobility (14)
			0	+	Transformed hamster embryo cells agglutinated only after protease treatment	12	

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TABLE I (*Continued*)
Part C

Lectin and source	Isolation and purification	Specific inhibitor	Agglutination: Normal cells (other than erythrocytes)	Agglutination: Normal cells + proteases or in mitosis	Agglutination: Transformed cells	References	Other properties
Wheat germ agglutinin (WGA), from wheat germ (*Triticum vulgaris*)	Isolation, purification, and characterization (1, 2, 8, 10, 25, 27, 30–32, 40)	Di-*N*-acetylchitobiose; *N*-acetylglucosamine; ovomucoid (2, 8, 10, 16, 45)	0, but binds to normal cells	+	+	3–5, 8–10, 13, 14, 17, 19, 22–24, 29, 35, 36, 39 41, 46, 48	Isolation of cell membrane receptor (7, 21, 44, 50) Agglutination not temperature dependent (19, 20) Agglutination not dependent on cellular ATP content (47) Nonmitogenic (20) Normal lymphocytes and lymphoma cells agglutinated to same degree (20) Membrane-bound lectin has relatively low mobility (43)
	Crystallization (30, 32) MW of dimer: 35,000 (2, 31) Not a glycoprotein (1, 2, 31) Three isolectins separated (2, 27)		Some lines +, some lines −		Some lines +, some lines −	4, 15, 26, 28	
					Temperature-sensitive mutants agglutinable only at permissive temperature	11, 12, 33, 38	
					Cells having regained growth control are less agglutinable	36, 39	
			Normal, confluent cells + protein synthesis inhibitor are agglutinable			6	

		CHO cells converted to fibroblasts are less agglutinable	18	Fetal cells not agglutinated, but have cryptic receptors (23, 29, 49) Cells infected with some nononcogenic RNA viruses are agglutinable (37) Synthetic antigen with chitobiose determinant can protect against tumor growth (42) Binds to hamster egg membrane and blocks fertilization (34)

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42. Shier (1971, 1973)
43. Shinitzky *et al.* (1973)
44. D. F. Smith *et al.* (1973)
45. Uhlenbruck *et al.* (1968)
46. Vlodavsky *et al.* (1972)
47. Vlodavsky *et al.* (1973)
48. Weber (1973)
49. Weiser (1972)
50. Wray and Walborg (1971)

TABLE I (*Continued*)

Part D

Lectin and source	Isolation and purification	Specific inhibitor	Agglutination		References	Other properties
			Normal cells (other than erythrocytes and leukocytes)	Tumor cells		
Phytohemagglutinin of red kidney bean (PHA), from *Phaseolus vulgaris*	*N.B.* Several lectins have been isolated from red kidney bean, having erythro- or leuko-agglutinating ability and/or mitogenicity for lymphocytes Isolation, purification, and characterization (1, 3, 6, 14–18, 22) MW of subunits: 29,000–36,000 (1, 3, 6, 14, 16–18, 22)	*N*-Acetyl-D-galactosamine (4)	0 Trypsinized normal cells are agglutinable (5)	+	5, 12, 20, 21	Isolation of erythrocyte membrane receptor (9) Isolation of lymphocyte membrane receptor (2) PHA is mitogenic (2, 3, 10, 13, 19) A mitogenic fraction practically devoid of leuko- and erythroagglutinating activity has been isolated (14) *N.B.* Numerous reports on the mitogenic action of PHA

The subunits apparently differ in their biological properties, and can be associated in a variety of ways, frequently as tetramers. All are glycoproteins. (1, 3, 6, 14, 15, 17, 18, 22)	have appeared, but they are out of the scope of this review Agglutination of lymphocytes is temperature sensitive (8) Normal lymphocytes are agglutinated as well as lymphoma cells (8) PHA binds to platelets, causes their aggregation, and has effect analogous to that of thrombin (11) Binding of PHA to platelets causes exposure of additional lentil agglutinin receptors (11) Stimulation of lymphocytes by PHA raises cellular cGMP content (7)

Key to references for Table I,D:

1. Allan and Crumpton (1971)
2. Allan and Crumpton (1973); Allan *et al.* (1971)
3. Allen *et al.* (1969)
4. Borberg *et al.* (1966)
5. Borek *et al.* (1973)
6. Dahlgren *et al.* (1970)
7. Hadden *et al.* (1972)
8. Inbar *et al.* (1973a)
9. Kornfeld and Kornfeld (1970)
10. Lindahl-Kiessling and Peterson (1969a,b)
11. Majerus and Brodie (1972)
12. Metz (1973)
13. Nowell (1960)
14. Oh and Conard (1971, 1972)
15. Rigas and Head (1969)
16. Rigas and Johnson (1964)
17. Rigas and Johnson (1967)
18. Rigas *et al.* (1966)
19. Robbins (1964)
20. Tomita *et al.* (1970)
21. Tomita *et al.* (1972b)
22. Weber (1969)

TABLE I (*Continued*)
Part E

Lectin and source	Isolation and purification	Specific inhibitor	Agglutination: Normal cells (other than erythrocytes and leukocytes)	Agglutination: Tumor cells	References	Other properties
Lentil agglutinin (*Lens culinaris* or *Lens esculenta*)	Isolation, purification, characterization (5, 6, 7, 8, 12, 13, 22, 26) MW of dimer: 42,000–49,000 (7, 8, 22) Is a glycoprotein (8, 22, 26) Two isolectins separated (8, 12, 22)	α-D-Mannosides, α-D-glucosides (5, 6, 7, 20, 26, 28) A number of glycopeptides are good inhibitors (12)	0	+	3, 23	Characterization of erythrocyte membrane receptor (12) Isolation of lymphocyte membrane receptor (6) Is mitogenic (1, 21, 26–28) Mitogenicity of one of isolectins is increased by simultaneous binding of nonmitogenic lectin from *Agaricus bisporus* (1) Binds to platelets but does not cause their aggregation, nor has it other thrombin-like effects (15) (cf. PHA, Table I,D)
			+, normal rat liver cells	0, rat hepatoma cells	3	
				0, Yoshida sarcoma cells	25	

Castor bean agglutinin (*Ricinus communis*)	Isolation, purification, characterization (4, 9, 14, 18, 24) MW: 60,000 = RCA_{60}; 120,000 = RCA_{120} (18) The 2 isolectins RCA_{60} and RCA_{120} differ in composition, specificity, and biological properties (18, 24)	D-Galactose inhibits both lectins (4, 18) *N*-Acetylgalactosamine inhibits only RCA_{120} (18)	0 Growing, sparse, 3T3 cells are moderately agglutinable (18) Trypsin-treated normal cells are agglutinable (3, 18)	+ Only RCA_{120} has high agglutinating activity (18, 24) 0, Yoshida sarcoma cells (25)	3, 10, 16, 17, 18, 23, 24	Agglutination of ascites tumor cells is temperature-dependent (10) Embryonic neural retinal cells are agglutinable (11) Cells infected by the non-transforming sindbis virus are agglutinable (2) Binds to hamster egg membrane and blocks fertilization (19)

Key to references for Table I,E:

1. Ahmann and Sage (1972)
2. Birdwell and Strauss (1973)
3. Borek *et al.* (1973)
4. Drysdale *et al.* (1968)
5. Entlicher *et al.* (1969)
6. Hayman and Crumpton (1972)
7. Howard and Sage (1969)
8. Howard *et al.* (1971)
9. Kabat *et al.* (1947)
10. Kaneko *et al.* (1973)
11. Kleinschuster and Moscona (1972)
12. Kornfeld *et al.* (1971)
13. Landsteiner and Raubitschek (1908)
14. Lin *et al.* (1970)
15. Majerus and Brodie (1972)
16. Metz (1973)
17. Nicolson (1973)
18. Nicolson and Blaustein (1972)
19. Oikawa *et al.* (1973)
20. Stein *et al.* (1971)
21. Stein *et al.* (1972)
22. Tichá *et al.* (1970)
23. Tomita *et al.* (1970)
24. Tomita *et al.* (1972a)
25. Tomita *et al.* (1972b)
26. Toyoshima *et al.* (1970)
27. Toyoshima *et al.* (1971)
28. Young *et al.* (1971)

TABLE I (*Continued*)

Part F

Lectin and source	Isolation and purification	Specific inhibitor	Agglutination		References	Other properties
			Normal cells (other than erythrocytes and leukocytes)	Tumor cells		
Dolichos agglutinin, from horse gram (*Dolichos biflorus*)	Isolation, purification, characterization (5, 7) MW: 140,000 (5) Is a glycoprotein (5, 7)	*N*-Acetyl-D-galactosamine (5)	0	+ Agglutination only after 20 minutes incubation	11	Nonmitogenic (1, 7) Binds to normal and tumor (ascites and hepatoma) cells, but has no effect on them (1) Binding of *Dolichos* agglutinin increases agglutinability by other lectins (11) Cells infected by nontransforming RNA viruses are agglutinable by Con A, but not by *Dolichos* agglutinin (2) Binds to hamster egg membrane and blocks fertilization (12)
Lotus agglutinin, from *Lotus tetragonolobus*	Isolation, purification, characterization (9, 18) MW of dimer: 107,000 (18) Is a glycoprotein (9) Either 2 or 3 isolectins separated (8, 9)	L-Fucose (8, 9, 18)	0	0	8	Does not bind to or agglutinate either normal or transformed cells, even after trypsin treatment (8)

Pea agglutinin, from *Pisum sativum* L.	Isolation, purification, characterization (4) MW: 53,000–54,000 (4) Two isolectins; possibly glycoproteins (4)	α-D-Glucosides, α-D-mannosides, α-D-fructose (4, 13, 17)	0 Trypsinized normal cells agglutinable	+	16, 17 17	
Robinia agglutinin, from black locust (*Robinia pseudo-acacia*)	Isolation, purification, characterization (3, 6) MW: 90,000–100,000 (3, 6) Is a glycoprotein (3, 6)	No simple sugar inhibitor (10) Is inhibited by a urinary sialo-glycopeptide (10)				Is mitogenic (1, 10) Stimulates growth of normal rat liver cells in culture, but inhibits growth of ascites and hepatoma cells (1)
Wax bean agglutinin, from yellow wax bean (*Phaseolus vulgaris*)	Isolation, purification, characterization (14, 15) MW of tetramer: 125,000–132,000 (14, 15) Two isolectins separated; both are glycoproteins (14)	No simple sugar inhibitor found (14) Fetuin is a good inhibitor; ovomucoid and bovine submaxillary mucin also inhibit (14)	0 Trypsinized normal cells are agglutinated	+	14 14	Is mitogenic (14) Inhibits development of polyoma virus—or chemically induced tumors *in vivo* (14)

Key to references for Table I,F:

1. Aubéry *et al.* (1972)
2. Becht *et al.* (1972)
3. Bourrillon and Font (1968)
4. Entlicher *et al.* (1969, 1970)
5. Etzler and Kabat (1970)
6. Font and Bourrillon (1971)
7. Font *et al.* (1971)
8. Inbar *et al.* (1972b)
9. Kalb (1968)
10. Lemonnier *et al.* (1972)
11. Metz (1973)
12. Oikawa *et al.* (1973)
13. Paulová *et al.* (1970)
14. Sela *et al.* (1973)
15. Takahashi *et al.* (1967)
16. Tomita *et al.* (1970, 1972b)
17. Veselý *et al.* (1972)
18. Yariv *et al.* (1967)

conditions which did not allow hemagglutinin production remained non-agglutinable. Both these examples, chosen among several others (see Table I), show that nononcogenic viruses which do not induce malignant transformation of a host cell may nevertheless induce alterations of this cell surface, enabling it to interact with agglutinins. In a restricted sense this type of infection may be compared to abortive transformation by an oncogenic virus where the agglutinable state is also expressed although the cell is not permanently transformed. For permanent transformation the agglutinable surface alteration, as well as other changes, will have to be fixed by a second process.

Another special group of cells are those from embryonic tissues: they are often agglutinable by lectins, but they lose this property on further differentiation. This will be taken up in more detail in Section VI, where the dedifferentiated state of tumor cells will be discussed.

In this brief survey of agglutination studies, we have taken care to list negative as well as positive correlations between agglutinability by lectins and neoplastic transformation, and the reader may well wonder whether a strong positive correlation between these properties does in fact exist. Table I should, however, indicate that positive correlations do indeed outnumber the negative cases. A few words of caution should be added concerning the interpretation of the data summarized in Table I: a fully quantitative assay for cell agglutination has not yet been devised, and results from different laboratories using different assay conditions are not strictly comparable. Since agglutination assays usually involve macroscopic or microscopic observation of cell clumping, they inevitably retain a certain degree of subjectivity which prevents them from being fully quantitative [for description of agglutination assay, see Benjamin and Burger (1970) and Burger (1974)]. Furthermore many experiments have been performed with crude lectins whose properties and agglutinating power may differ considerably from those of the pure material.

In spite of exceptions and divergences, what clearly emerges is that transformation of cells is accompanied by changes in their surface membranes, often resulting in an altered agglutinability. That these surface changes are indeed part of the transformation process has been demonstrated with the aid of conditional (host range, temperature-sensitive) mutants: cells infected with certain viruses under permissive conditions (usually the lower temperature) were neoplastically transformed: they lost their DDI and became agglutinable; under the nonpermissive conditions, however, the viruses did not transform the cells: these did not grow beyond a low saturation density, and were only poorly agglutinated by lectins (see Benjamin and Burger, 1970; Biquard and Vigier, 1972a,b;

Burger and Martin, 1972; Eckhart *et al.*, 1971; Noonan *et al.*, 1973b; Renger, 1972; Renger and Basilico, 1972). In some cases the cells were transformed under permissive conditions and only then exposed to nonpermissive conditions, whereupon they agglutinated much less or bound less agglutinin. Similarly, variant or revertant clones of cells could be isolated from transformed cells which had regained some DDI of growth; these so-called flat variants had concomitantly lost their agglutinability (Inbar *et al.*, 1969; Pollack and Burger, 1969). In other cases transformed cells were selected for loss of agglutinability and were found to have regained some of their DDI of growth (Culp and Black, 1972; Ozanne and Sambrook, 1971b).

B. Relation of Lectin Binding to Agglutinability

In earlier agglutination studies, it was assumed as a matter of course that ability of certain cells to become agglutinated by a given lectin meant that these cells possessed specific surface lectin receptor sites; the surface of nonagglutinable cells would be essentially devoid of such sites, so that the lectin could not be bound in large enough amounts to provoke agglutination.

Unexpectedly we found in 1969 that untransformed cells also contain lectin receptor sites, but in cryptic form: a very mild trypsin treatment of normal cells rendered them as agglutinable by WGA as were transformed cells (Burger, 1969). Inbar and Sachs, also in 1969, showed that receptor sites for another agglutinin with a different specificity, Con A, also lie in cryptic form in normal cells and could be uncovered by mild trypsin treatment. Another demonstration of cryptic receptor sites in normal cells was made in our laboratory by Fox *et al.* (1971): we observed that fluorescent WGA bound not only to polyoma-transformed mouse fibroblasts, but to normal fibroblasts during a short part of the cell cycle, namely at mitosis. These experiments will be further discussed in Section V.

The cryptic site hypothesis (Burger, 1970b) had to be modified, however (see also Section III), when it was discovered that normal cells were also capable of binding either fluorescein-labeled or radioactively labeled agglutinin (Arndt-Jovin and Berg, 1971; Ben-Bassat *et al.*, 1971; Birdwell and Strauss, 1973; Cline and Livingston, 1971; De Salle *et al.*, 1972; François *et al.*, 1972; Greenaway and LeVine, 1973; Mallucci, 1971; Ozanne and Sambrook, 1971a; Sela *et al.*, 1971).

Quantitative studies on the relative number of lectin binding sites on different cells should be interpreted with caution; it should be borne in mind that transformed cells are usually smaller than their normal counterparts, and therefore only a comparison of the amount of lectin bound

per cell surface area, not per cell, is valid; most, but not all of the authors quoted above did take this size difference into consideration. Another pitfall to avoid is the partial internalization by pinocytosis of agglutinin which can take place when lectin binding assays are performed at room temperature or higher, thus giving falsely high levels of bound lectin. To avoid these difficulties a binding assay at 0°C (Noonan and Burger, 1973b) was developed. Under these conditions, it was found that polyoma-transformed 3T3 (mouse fibroblasts) cells bound 2.5 to 5 times more Con A-^{3}H per milligram of cell protein than did nontransformed 3T3 cells. R. S. Turner (unpublished observations, 1973) also found that at low concentrations of fluorescent Con A somewhat more label was bound to polyoma-transformed 3T3 cells than to untransformed 3T3 cells at 0°; at higher Con A concentrations or at 37°C, however, all cells were labeled. Smith and Revel reported (1972) that SV40-transformed 3T3 cells bound twice as much Con A as did 3T3 cells—a small, but not necessarily insignificant difference.

It is evident that further careful assays need to be done before one can reliably assign to different types of cells a definite number of specific receptor sites for different lectins. What already emerges at this point, however, is that the difference in binding sites between transformed and untransformed cells is not large and that the cryptic site hypothesis is probably insufficient to explain all situations of increased agglutinability. Various models have been proposed, all attempting to explain what modifications are brought to the cell surface in the course of neoplastic transformation which could simultaneously explain the increased agglutinability. These models will be discussed in the next section.

III. Discussion on Possible Mechanisms of Agglutination

A. Generalities about the Agglutination Process

Most lectins are plant or invertebrate proteins that interact with carbohydrate components on the surfaces of cells, and some of these lectin receptor sites have been partially characterized [Allan *et al.* (1972): Con A receptor; D. F. Smith *et al.* (1973) and Wray and Walborg (1971): Con A and WGA receptors; Burger (1968b), Jansons and Burger (1973) and Jansons *et al.* (1973): WGA receptor site; Hakomori and Murakami (1968): WGA receptor; Kornfeld and Kornfeld (1970): PHA receptor]. Lectin receptor sites form part of the antigenic makeup of the cell surface, and specific antibodies can be prepared against them (Jansons and Burger, 1973; Jansons *et al.*, 1973).

The exact mode of interaction of a given lectin with its particular cell surface receptor and the mechanism of the subsequent agglutination is not known, but in some regards it is analogous to an antigen–antibody reac-

tion. A decrease in valency by chemical alteration or a partial degradation (Albrecht-Bühler *et al.*, 1973; Burger and Noonan, 1970) leads to a lectin that still can bind to the receptor site, but can no longer agglutinate—a situation comparable to that with monovalent antibodies.

A summary of views and models concerning the agglutination process is given in Section III,E, and illustrated in Figs. 1–4 of that section.

B. Architectural Alterations in the Surface Membrane

We shall examine in this section some of the models that have been proposed to explain what changes neoplastic transformation might bring to the cell surface that would make the transformed cells agglutinable.

1. *Possible Increase in Number of Available Lectin Receptor Sites on Transformed Cells*

The most obvious explanation for increased agglutinability would be an increase in the number of lectin binding sites, and transformed cells have indeed been shown in several cases to bind more agglutinin than did their normal counterparts. As already discussed in Section II,B, however, the results of binding studies must be interpreted with great caution, as only those that avoid unspecific binding and minimize internalization of the lectin can be judged reliable.

In cases where transformed cells do have an increased number of receptor sites, the pertinent question is to know where these new sites come from. One might think that new receptor sites could be coded for by genes of a transforming virus, or that the virus might cause proliferation of a few preexisting host-coded sites. If this were the case, it would imply that all malignant transformations, including chemical or spontaneous transformations, or those induced by radiation, must ultimately depend on viruses, a possibility that as yet has been neither proved nor ruled out. It is very unlikely, however, that some of the small viruses containing very little DNA could code for the several different lectin receptor sites which are often observed to appear on one cell. A further argument against the formation of new receptor sites during transformation is the fact, already mentioned in Section II, that normal cells also contain lectin receptor sites, and that protease treatment of these cells renders them as fully agglutinable as are transformed cells (Burger, 1969; Inbar and Sachs, 1969).

Such experiments with proteases form the basis for the cryptic site hypothesis (Burger, 1970b), which assumes that lectin receptor sites are present, but in cryptic form, in normal cells and are then made available as a result of the neoplastic transformation.

It still is not known how protease treatment makes lectin sites available, but recent experiments suggest that the enzymes, rather than alto-

gether removing a covering layer, merely break a few peptide bonds, which might allow some rearranging of the surface molecules, or remove some steric barrier to proper binding of the lectin: we were unable to detect release of any significant amount of surface components after the mild trypsin treatment which rendered cells agglutinable (Burger, 1970b). Phillips and Morrison (1973), however, found that treating erythrocytes with a mild dose of trypsin did still remove about 20% of the major surface glycoprotein in the form of glycopeptides, but that the remaining glycoprotein had become ten times more amenable to lactoperoxidase iodination. It should be mentioned that Phillips and Morrison treated their cells with 25 μg of trypsin per milliliter for 15 minutes, whereas we found a 5-minute treatment with similar (or even smaller) amounts of trypsin to be sufficient to uncover the agglutinin sites of BHK cells or 3T3 mouse fibroblasts. In any case, it is clear that trypsin revealed previously unavailable glycoprotein sites, and one can imagine that transformation might also produce a similar type of unmasking of sites (see also Section IV,A,5).

The other mechanisms which we shall now examine take into account the fact that normal cells also bind lectins, and they attempt to explain increased agglutinability by a different arrangement of receptor sites on the cell surface, rather than by the emergence of new sites on this surface.

2. *Concentration of Sites Due to Surface Shrinkage*

In 1971 the group of Sachs (Ben-Bassat *et al.*, 1971) suggested that since transformation of certain cells is accompanied by a decrease in their size, such a shrinkage might bring about a higher site density. They indicated, however, that this model was only valid for certain cells, and they suggested as other models the cryptic site hypothesis which we have just discussed and the following model.

3. *Clustering of Sites*

Increased agglutination may be brought about, in some cases, not by an increase in available sites, but by regrouping of already exposed sites: if cells have areas of high receptor density and if they meet at such areas in the presence of agglutinin, this may increase the likelihood of the formation of stable aggregates. To investigate the possibility of such regroupings, one has to map these receptor sites, and show that their location is changed as a result of transformation or of protease treatment.

Several types of markers have been used for this purpose: binding of fluorescent agglutinins can be observed directly under the microscope, whereas the electron microscope must be employed to locate hemocyanin-

or ferritin-conjugated agglutinins. Another ingenious method consists in incubating cells with a peroxidase–lectin complex which after reaction with diaminobenzidine forms a precipitate visible in the electron microscope (Bernhard and Avrameas, 1971; François *et al.*, 1972; Huet and Garrido, 1972; Stobo and Rosenthal, 1972; Torpier and Montagnier, 1973). Another possibility is to use radioactive marked lectins and to locate them by autoradiography. Examples of these techniques will be seen in this and in the next sections. As was the case for binding studies, the techniques and the exact conditions used in each case should be examined carefully and critically, as rearrangement of sites has been shown in many cases to be an artifact of preparation or fixation of the cells.

a. Studies Indicating a Difference in Topography of Sites between Normal and Transformed Cells. Ben-Bassat *et al.* (1971) were the first to propose that in some cases receptor sites might be concentrated in certain areas of the transformed cells, although they had not actually visualized such a case. Later they showed a higher degree of fluorescence when treating transformed cells with fluorescein-Con A as compared with normal cells (Shoham and Sachs, 1972). They suggested as one possibility that clustering of several Con A binding sites might result in a generally better detectable fluorescence than if the sites were scattered.

Mallucci (1971), who also used fluorescent Con A, but in larger amounts than did Shoham and Sachs, observed that fluorescence of transformed cells often occurred in large, localized areas. Similar conclusions were reached by De Salle and collaborators (De Salle *et al.*, 1972), but they were based, like those of Ben-Bassat, on measurement of Con A binding per cell and per unit surface area, not on visual examination of sites.

In 1971 Nicolson labeled normal and virally transformed 3T3 fibroblasts with ferritin-Con A, and he observed in the electron microscope that the labeled lectin was distributed randomly on the surface of normal cells, whereas on transformed or trypsinized cells the sites were grouped in clusters. He and Singer then proposed a model (Nicolson, 1971; Singer and Nicolson, 1972) according to which trypsinization or malignant transformation would cause a redistribution of sites in the plane of the membrane in the form of patches or clusters; and they further proposed that such a grouping of agglutinin sites would be more favorable for cell agglutination than would be dispersed sites. This is a very attractive theory, but it should be noted that ferritin-Con A was absorbed to cells at room temperature, and that the cells were previously fixed with only 0.1% formaldehyde and then lysed at an air–water interface to produce flattened membranes suitable for electron microscopy; while on the one hand

0.1% formaldehyde did not necessarily fix molecular movement prior to addition of the lectin, one must also consider, on the other hand, that the lysis procedure may have introduced unwanted alterations.

Martinez-Palomo *et al.* (1972) and Bretton *et al.* (1972) incubated *live* cells with Con A, then peroxidase (at room temperature), and only then did they fix the cells with glutaraldehyde and treat them with diaminobenzidine to reveal the Con A binding sites. These authors found, in agreement with Nicolson's model, that Con A receptors were randomly dispersed on normal fibroblasts but usually clustered on cells transformed by polyoma or SV40 virus. Torpier and Montagnier (1973) using this technique observed a rather variable distribution of Con A sites: often but not always patchy in transformed cells, and also patchy in some types of normal, nonagglutinable cells. They concluded that there was no clear correlation between the transformed state of cells and a special distribution of Con A receptor sites.

b. Studies Showing No Difference in Topography of Sites on Normal and Transformed Cells. At the same time that several investigators were describing the lectin receptor sites of transformed cells to be more clustered than those of normal cells, others could see no difference in the topography of these sites. François *et al.* (1972) labeled normal and RSV-transformed embryo fibroblasts with complexes of Con A or WGA with peroxidase. WGA could be bound only to transformed cells, where it formed a discontinuous pattern, whereas both normal and transformed cells were labeled in a continuous fashion by Con A.

Smith and Revel (1972) labeled living cells fixed on coverslips with Con A and hemocyanin, and subsequently fixed them for electron microscopy with glutaraldehyde and osmium tetroxide. Interestingly, they found that on normal and transformed fibroblasts, as well as on polymorphonuclear leukocytes, the distribution of sites depended not on transformation, but on the temperature: at 4°C all cells showed a uniform distribution of the lectin, whereas the sites, on normal as well as on transformed cells, were clustered when the lectin incubation was performed at 37°C. These observations were very important, for they showed that methods used for incubation and preparation of the cells have a definite influence on the final appearance of sites, so that it may be difficult to distinguish between inherent properties of cells and modifications brought to them as artifacts of preparation. Furthermore, the difference in distribution of sites observed at 4° and 37°C lent support to the concept of the fluidity of membrane components, which was just then beginning to take hold (see Frye and Edidin, 1970; Singer and Nicolson, 1972; and see Section III,C).

4. *Other Rearrangements*

Before leaving this section on changes brought to the surface membrane by the transformation process, we should consider two cases where neoplastic transformation, instead of uncovering lectin receptor sites, seems to have buried them deeper. We had mentioned in Section II,A the case of normal liver cells which are agglutinated by lentil agglutinin whereas rat hepatoma cells were not, and we had proposed that in that case transformation had resulted in burial of sites, rather than bringing them to the surface (Borek *et al.*, 1973). An analogous but not identical case is that of polyoma-transformed hamster embryo cells, which Sela *et al.* (1970) found to be just as poorly agglutinable by soybean agglutinin (SBA) as were the untransformed parent cells. Trypsinization nevertheless revealed the presence of SBA receptor sites in both types of cells; these sites may, however, have been less available (buried deeper?) in the transformed than in the normal cells, as it took a longer trypsin treatment to render the transformed cells agglutinable than it did the normal cells.

C. Altered Mobility of Membrane Components

1. *The Fluid Mosaic Membrane Model*

a. Membrane Structure, in General. The structure of plasma membranes had been the object of considerable study in the last few years, and since these studies have been well documented and extensively reviewed (Bangham, 1972; Chapman, 1968; Fox and Keith, 1972; Guidotti, 1972; Korn, 1966, 1969; Manson, 1971; Oseroff *et al.*, 1973; Rothfield, 1971; Wallach, 1972), we shall limit ourselves here to a few general remarks.

Although the model proposed by Danielli and Davson in 1935 and its modification by Robertson in 1960 have since undergone considerable further modification, they taught us that basically all membranes consist of a phospholipid bilayer, with the hydrophobic fatty acid chains directed inside and polar heads lining the inner and outer surfaces. Later studies showed that protein molecules, instead of being placed outside the lipid bilayer as originally proposed by Robertson, are probably intercalated between lipid molecules. Glycoproteins and lipoproteins, being amphipathic, have their polar extremities emerging from the inner or outer surface of the membrane, while their hydrophobic parts are inserted within the lipid bileaflet. The resulting structure is a sort of mosaic of interspersed lipid and protein subunits. Such a view of membrane structure has resulted from biophysical studies by optical rotatory dispersion

and circular dichroism on membrane protein conformation (Lenard and Singer, 1966; Wallach and Zahler, 1966), X-ray diffraction and calorimetric measurements on natural as well as model lipid membranes (Chapman and Wallach, 1968; Levine and Wilkins, 1971; Luzzati, 1968; Wilkins *et al.*, 1971), and electron microscopic observations, in certain cases after freeze-etching (Pinto da Silva and Branton, 1970; Tillack and Marchesi, 1970), to name but a few.

b. The Fluid Mosaic Membrane Model. The general membrane model described above is the one currently favored, but it has within the last few years received a very important qualification: under physiological conditions the structure of membranes is not rigidly fixed, but exists in a fluid and dynamic state. In 1972 Singer and Nicolson proposed their "fluid mosaic model" of cell membrane structure, in which they indicated that since lipids are in a fluid state at physiological temperatures, this allows for lateral diffusion of the proteins embedded within the membrane. Singer (1973) has recently graphically described his model membrane as consisting of protein icebergs floating in a sea of lipid. Two important consequences of such a fluid membrane structure are that the membrane will form a heterogeneous mosaic and that the subunits of this mosaic will be liable to redistribution, depending upon physiological conditions operating either on the whole membrane or locally on certain portions of it. Before discussing implications of this fluid model for neoplastic transformation and for the agglutinin reaction, let us first review some of the evidence which supports this new membrane model.

i. *Nuclear-resonance spectra.* Motion of molecules within phospholipid bilayers was demonstrated by Kornberg and McConnell (1971), who prepared vesicle membranes from spin-labeled phospholipids. Nuclear resonance spectra in such artificial membranes showed that lateral diffusion of phospholipids within the bilayer was very rapid; exchange of molecules from one monolayer of the bileaflet to the other ("flip-flop") was possible, but very much slower.

ii. *X-Ray diffraction studies.* Diffraction patterns of artificial lipid mixtures as well as of membranes give support to the fluid mosaic membrane model: phospholipids were shown to form bilayers whose orientation and packing depended on the type of lipid, the cholesterol content, and the temperature. The lipids were in a fluid state above their phase transition temperature, and raising the temperature increased the degree of fluidity and the "disorder" of the membrane (Levine and Wilkins, 1971; Wilkins *et al.*, 1971). The measured thickness of the membrane was incompatible with an external layer of protein, whereas diffraction patterns were compatible with proteins inserted into the phospholipid bilayer (Blaurock, 1972, 1973). It should be recalled that in 1962 already

Luzzati, as a result of X-ray diffraction studies, emphasized the fluidity of lipids and the conformation changes and the disorder caused by increases in temperature (Gulik-Krzywicki *et al.*, 1969; Luzzati and Husson, 1962). Blasie and collaborators (Blasie and Worthington, 1969; Blasie *et al.*, 1969) found that the X-ray diffraction patterns for rhodopsin molecules within retinal receptor disk membranes were consistent with a planar liquidlike arrangement rather than a crystalline lattice of these molecules. Interestingly, they found that binding antirhodopsin molecules to the membranes changed the pattern somewhat, indicating that these extraneously added molecules caused a certain rearrangement of the proteins within the membrane; this would be impossible if the membrane had a rigid structure.

iii. *Other studies on rhodopsin.* An elegant demonstration of the mobility of rhodopsin in the visual receptor membrane was made by Cone (1972) and by Brown (1972), by measuring photoinduced dichroism in frog retinas. They found that a retina fixed with the bifunctional and cross-linking agent glutaraldehyde (but not with formaldehyde, which is mainly monofunctional) became highly dichroic when partially bleached with polarized light. In a nonfixed retina, however, dichroism was transient, lasting only for the very brief time (20 μsec) needed for the rhodopsin molecules to reorient themselves parallel to the light beam.

iv. *Electron microscopic observations.* Freeze-etching studies on lymphocytes by Scott and Marchesi (1972) and on red cell membranes by Tillack and Marchesi (1970) and by Pinto da Silva and Branton (1970) and radioactive labeling of the inner and outer surface of erythrocyte membranes by Bretscher (1971) indicated that the majority of the proteins do not float on the outer side of the membrane but that their span is from the outer surface through the membrane leaflet to the inner cytoplasmatic side.

Singer and Nicolson (1972) labeled erythrocytes with ferritin-conjugated antibodies and found that H-2 antigens and Rh antigens were distributed in a random, aperiodic fashion on the cell membrane, an observation which they presented in support of their model of an irregular mosaic membrane structure, and they suggested that such irregular distributions would be more likely to occur in a fluid than in a rigidly fixed membrane.

v. *Membrane fluidity demonstrated by fluorescent labeling of antibodies.* Fundamental to the development of the fluid mosaic membrane model were the elegant experiments of Frye and Edidin (1970), who fused mouse and human cells and thereafter labeled them with fluorescein- and rhodamine-conjugated antibody. The heterokaryons originally presented one green and one red hemisphere, but mixing of the antigens

could be observed subsequently; within 40 minutes at 37°C, 90% of the hybrid cells showed green and red speckled mosaics. This mixing was independent of protein synthesis, but it did not take place at low temperatures. Frye and Edidin consequently proposed that molecules are not rigidly fixed in membranes but are free to "float in a liquid-like environment" and "re-orient relative to one another."

In further work from Edidin's laboratory (Edidin and Fambrough, 1973), the spread of fluorescent antibody bound to the surface of cultured myotubes was observed, as were the patching and capping of fluorescent antigen–antibody complexes on the surfaces of lymphocytes and fibroblasts (Edidin and Weiss, 1972); and Sundqvist (1972) observed similar reactions in various cell types. In all cases antigen movement was temperature-dependent; and Edidin and Fambrough (1973) showed that it was abolished by previous fixing of the cells with 5% glutaraldehyde.

vi. *Lymphocyte triggering: patching and capping due to antibodies.* In 1970 Raff *et al.* demonstrated the presence of Ig (immunoglobulin) receptors on the surface of lymphocytes, by use of fluorescein-labeled or radioactive anti-Ig antibodies. Subsequent experiments showed (de Petris and Raff, 1972; Taylor *et al.*, 1971) that at 0° the label was diffusely spread over the cell, but that raising the temperature to 24–37°C produced capping of the antibodies over the pole of the cell containing the Golgi apparatus and other organelles. Capping, which was inhibited by azide and dinitrophenol, was rapidly followed by pinocytosis, and the authors postulated that the internalization of antigen in an organelle-rich part of the cell might be instrumental for triggering lymphocyte transformation. Particularly significant was the observation that monovalent Fab antibody fragments could bind to the cells, but resulted neither in capping, nor in triggering of the lymphocytes; in other words, cross-linking by *divalent* antibody was necessary for antigen redistribution and for triggering. It might be added that disappearance of surface antigens was due not only to pinocytosis, but to a process of continuous shedding of Ig receptors from the surface, followed by regeneration (Loor *et al.*, 1972; Wilson *et al.*, 1972).

An important group of experiments by de Petris and Raff (1972, 1973; Raff and de Petris, 1973) and by Loor and collaborators (1972) lends further support to the fluid mosaic membrane model, and the results agree with and extend the observations of Edidin and Weiss (1972) described above. They showed that clustering of receptors was a passive process, induced by cross-linking with divalent or polyvalent antibody and which was inhibited by cold, but not by metabolic inhibitors. This clustering was a prerequisite to capping, but the role of these different processes and of pinocytosis in lymphocyte activation is still open to ques-

tion, especially in view of the very recent observation by Elson *et al.* (1973) that antibody-induced cap formation in lymphocytes was not necessarily followed by DNA synthesis and mitosis.

A significant recent observation was that different antigens can move past each other and independently of each other in the surface membrane, as shown by Ashman and Raff (1973) and by Kourilsky *et al.* (1972).

vii. *Lymphocyte triggering as a result of lectin binding.* Lymphocytes can be stimulated to blast formation and to mitosis, not only by antibodies and polymeric antigens, but also by certain lectins. It must be emphasized, however, that not all lectins are mitogenic: Con A and PHA, for instance, are mitogenic, whereas WGA and SBA are not.

As was seen before, lectins interact with specific carbohydrate receptors on the cell surface, and Karnovsky and Unanue and their coworkers (Karnovsky and Unanue, 1973; Karnovsky *et al.*, 1972; Unanue *et al.*, 1972) have shown that, similarly to anti-Ig, fluorescent or radioactive Con A was randomly distributed on the lymphocyte surface at 4°C, but moved into patches and then caps after warming to 37°C. Interestingly, anti-H-2 antibody was seen to bind to surface H-2 antigens, but capping could be produced only after further cross-linkage in an indirect, or sandwich, reaction, and the authors observed by electron microscopy of antibody-coupling that H-2 antigens seem to be situated far apart from each other in the membrane, whereas both Ig and Con A receptors were situated close together, hence the ease with which they could be cross-linked and could then move into clusters and caps (the authors were aware of the possible artifactual redistribution of the antibody-linked H-2 antigens). Comoglio and Guglielmone (1972) observed that binding of fluorescent Con A to both fibroblasts and lymphocytes produced temperature-dependent patching, which was followed by capping. In their hands, azide inhibited both patching and capping. When cells were treated with glutaraldehyde prior to addition of Con A, the fluorescent lectin remained uniformly distributed, movement of molecules being inhibited by the cross-linking reagent. Conversely, Yahara and Edelman (1972) observed that clustering and capping of Ig–anti-Ig complexes on lymphocytes was inhibited if the cells had previously been incubated with Con A, and these investigators suggested that lectin binding on the membrane might somehow affect the mobility of other receptors.

More recent experiments from Edelman's laboratory (Edelman *et al.*, 1973; Gunther *et al.*, 1973) showed that caps were formed when fluorescent Con A was bound to lymphocytes at 0° and the cells were then brought to 37°C. When Con A, which normally exists at neutral pH as a tetramer, was treated with succinic anhydride, it was apparently present in dimer form, i.e., prevented from forming tetramers. This succinyl-

Con A could bind to lymphocytes but, contrary to tetrameric Con A, it did not inhibit cap formation induced by anti-Ig, and did not itself form caps; moreover, this succinylated Con A had lost much of its agglutinating activity. All these effects were explained by considering the dimeric lectin to be a less effective cross-linking agent than the tetramer. Surprisingly, succinyl Con A was as fully mitogenic as the native lectin, though much less toxic; this would indicate that, with this lectin anyway, extensive cross-linkage and capping are not required for lymphocyte activation. Based on experiments with colchicin, Edelman *et al.* (1973) proposed that interaction of Con A receptors with a microtubule type of protein might be involved in events leading to mitogenesis; with high concentrations of Con A extensive interaction and cross-linking would eventually lead to cell death, but this would not be the case with succinyl Con A. To what degree some cross-linking can still occur, enough, for example, to trigger the "wound up" and highly sensitive lymphocytes into transformation, will be an important subject for further investigation.

After his experiments on the distribution and movement of Ig receptors (see above), Loor (1973) studied the effect of PHA on lymphocytes. By using mitogenic doses of rhodamine-PHA, he could observe formation of fluorescent caps when the cells were incubated at 0° then warmed to 37°. He then stopped the capping process by cooling the cells to 0° when 30–40% of them were capped, and he processed them for freeze-fracture electron microscopy. This enabled him to observe in a number of cases a gradient of membrane particles, with some areas practically devoid of them, while particles seemd to be increasingly concentrated in other areas; in contrast, membrane particles were always homogeneously distributed or grouped in small clusters on cells not treated with PHA. Loor suggested that the lectin receptors are associated with the membrane particles [as had been demonstrated by Marchesi *et al.* (1972) for PHA receptors on the major glycoprotein of the erythrocyte membrane], and that the changed distribution of these particles after PHA treatment of the cells corresponded to the capping observed by fluorescence microscopy. Loor believes that this redistribution of membrane particles is relevant to the mechanism of lymphocyte triggering; the observations from Edelman's laboratory on succinyl Con A would, however, make this suggestion questionable, if the interpretations of the Edelman group are correct.

viii. *Conclusions.* The experiments on lymphocyte triggering by antibodies and by lectins leave a number of questions unanswered. They certainly do not tell us yet by which molecular mechanism the stimulation is effected. They do indicate, however, that membrane phenomena are an important initial part of this process, and they lead to the following

important conclusion: Surface membranes are in a fluid and dynamic state and membrane components therefore do not have rigidly fixed positions but are on the contrary free to move in the plane of the membrane. Any statements as to inherent distribution of membrane components must therefore be interpreted with great caution, since distribution can be radically changed by a variety of agents and conditions, and probably not only by cross-linking agents or by changes of temperature.

2. *Role of Membrane Mobility for Cell Agglutination*

Normal as well as transformed cells have been shown to possess lectin receptor sites (see Section II,B); and since these receptors are probably more or less mobile within the plane of the membrane, it is improbable that their distribution in native tumor cells should be very different from what it is in native normal cells. One is therefore again confronted with the question of the basis for the difference in agglutinability between normal and transformed cells, a question that must now be investigated in the light of the newer concepts on membrane structure.

One possible difference might be that membranes of agglutinable cells (i.e., transformed cells, or normal cells during mitosis or after protease treatment) are somehow more fluid, so that receptor sites could more easily be gathered together into some conformation favorable for agglutination. This was the conclusion reached, among others to be discussed below, by Rosenblith *et al.* (1973) from their electron microscope observations on the interaction of hemocyanin-Con A with 3T3 mouse fibroblasts, and with 3T3 cells treated with trypsin or transformed with SV40. A dispersed and random distribution of receptor sites was observed for all three cell types at 4°, but after warming to 37°, the lectin remained dispersed in 3T3 but was gathered in clusters on SV 3T3 and on trypsinized 3T3; there was, however, no clustering if the cells were previously fixed with 1% formaldehyde, thus preventing the lectin-induced receptor movement.

When they presented their fluid mosaic model for membrane structure in 1972, Singer and Nicolson suggested that both the transformation process as well as trypsinization might alter membrane glycoproteins and glycolipids in such a way that, taking advantage of membrane fluidity, the lectin-binding sites would redistribute themselves into aggregates prior to the addition of lectin. In support of this view, Nicolson (1972) showed by electron microscopy that ferritin-Con A was clustered on trypsinized 3T3 but randomly distributed on untreated cells, and one of his pictures shows agglutinated cells (trypsinized 3T3) with a large amount of ferritin-Con A, presumably forming cross-bridges, on the area where the two cells were in contact. Nicolson also observed that

trypsinized erythrocytes incubated at 37°C agglutinated better with *Ricinus communis* agglutinin and had a more clustered distribution of sites than cells kept at 0°, the clustering and the consequent increase in agglutinability being explained by increased mobility of sites at the higher temperature. According to this view, then, transformation or trypsinization would effect a first clustering of sites, and this would be enhanced by warming of the cells. A direct effect of the lectin on the redistribution was ruled out by Nicolson since 0.1% formaldehyde apparently did not prevent the clustered appearance if the lectin was added subsequently. It should also be noted that a very mild trypsin treatment is sufficient to cause agglutination, whereas rearrangements of the type mentioned by Nicolson would require massive doses of trypsin (Speth *et al.*, 1972).

More recently, however, Nicolson observed (1973) a uniform distribution of fluorescent Con A on SV40-transformed 3T3 cells if the cells were fixed in 2% formaldehyde prior to labeling. This observation could then only lead to the conclusion that patching did not preexist in transformed cells, but depended on cross-linking by the lectin and on molecular movement made possible at higher temperatures, a view that is now the prevalent one.

Starting from the observation that in many cases agglutination is temperature dependent, investigators in the laboratory of Sachs have been studying physiological aspects of the agglutination process. They first proposed (Inbar *et al.*, 1971, 1972a; Vlodavsky *et al.*, 1972) a two-site hypothesis according to which lectins like Con A and PHA would have two kinds of sites on cells: a binding site, present in both normal and transformed cells, and a second site, which would be present in active form only in transformed cells; this second site would also be present, but inactive, in normal cells, where it could be activated by trypsin. This special site would, however, be fully active only at 37°C, owing to some metabolic activity. Lectins with which agglutination is not temperature dependent (WGA and SBA) would apparently not need such a second site.

This theory may seem somewhat complicated in view of current concepts about temperature-dependent mobility of membrane components, but it certainly cannot be dismissed offhand, and it ultimately led to further interesting findings.

Surprisingly, in contrast to what one might expect if a metabolic activity were involved, agglutinable cells (virally transformed fibroblasts) were found (Vlodavsky *et al.*, 1973) to have a much lower ATP content than nonagglutinable cells. Furthermore, nontransformed cells when grown to high densities could be made agglutinable by Con A after treatment with such metabolic inhibitors as dinitrophenol (DNP),

fluoride, or azide which depleted their ATP, whereas transformed cells grown in the presence of glucose acquired a higher ATP content and lost their agglutinability by Con A. The authors suggested that ATP might stabilize the cell membrane in such a way as to inhibit the clustering of sites necessary for agglutination. According to this view, transformed cells would again be postulated to have more mobile membrane sites, but in this case this increased mobility would be attributed to a lowered ATP content. In contrast to the above findings are those of Kaneko *et al.* (1973), who found that agglutination of rat ascites cells by Con A or *Ricinus communis* agglutinin was inhibited not only by low temperatures, but by DNP or azide, and they postulated the involvement of a metabolic activity requiring ATP; no measurements of ATP were reported in this preliminary communication, however, and the two groups worked with different cells.

In a study on aggutinability of fibroblasts and lymphocytes by Con A, Inbar, Shinitzky, and Sachs (Inbar and Sachs, 1973; Inbar *et al.*, 1973c; Shinitzky *et al.*, 1973) came to conclusions similar to those reached by others at this time: clustering of sites is necessary for agglutination; it is induced by cross-linking lectins, maybe dependent on membrane fluidity, and is inhibited at low temperatures. Furthermore, by measuring rotational relaxation times of fluorescent lectins bound to cells, they deduced increasing degrees of rotational mobilities for membranes of normal fibroblasts, membranes of transformed or trypsinized fibroblasts or of lymphoma cells, and finally lymphocytes. Although the relaxation times measured seem to be high for what would be expected for a membrane receptor, these data correlated with the degree to which fluorescein-labeled lectin redistributed first into small clusters only, and then into confluent clusters called caps. This scheme for correlation between mobility and clustering of sites and agglutinability is not a universally applicable one: with WGA and SBA, agglutination is not temperature-dependent (Inbar *et al.*, 1971), and Vlodavsky and co-workers found (1973) that agglutination with these lectins is not affected, as it is for Con A, by the ATP content of the cells. These lectins are also set in a class apart by the fact that they are nonmitogenic (Inbar *et al.*, 1973a); they do not induce cap formation in lymphocytes, and the mobility of their receptor sites on normal lymphocytes is about half that of Con A sites on these cells, and comparable to the mobility of Con A sites on normal fibroblasts (Inbar *et al.*, 1973c; Shinitzky *et al.*, 1973). It seems that agglutination by WGA and SBA might not require the degree of site mobility needed for agglutination by Con A; but, according to the results just quoted, a high degree of mobility and the ability to form caps might be a prerequisite for lymphocyte triggering.

Recent work in our laboratory leads us to retain our original "cryptic

site hypothesis" (Burger, 1970b; and see Section II,B), but in a form modified in the light of newer findings from this and other laboratories, chief among which would be those demonstrating the fluidity of membrane components, which we feel plays a crucial role in cell agglutination.

a. Many Transformed Cells Do Have More Lectin Binding Sites Than Do Normal Cells. As shown in Section II,B, lectin binding studies must be done under conditions that minimize nonspecific binding and endocytosis, both of which effects can be drastically reduced at 0°. We have already mentioned in Section II,B the observation by R. S. Turner (unpublished, 1973) that transformed cells consistently bind somewhat more fluorescent Con A than do normal cells. More quantitative binding studies were undertaken with radioactively marked Con A, and Noonan and Burger (1973b) found that virally transformed fibroblasts bound 2.5–5 times more Con A per unit surface area (or taking cell size into account by any other way) than did the untransformed parent cells. Interestingly, cells from a rat minimal deviation hepatoma bound 2.5 times more Con A than did normal rat liver cells, and one can speculate that these cells which have a rather low degree of malignancy might have surface membranes with properties intermediate between those of normal and of fully malignant cells.

Temperature-sensitive SV40-transformed 3T3 mutants, which were recently isolated by Renger and Basilico (1972), express the transformed phenotype at 32°C but a normal phenotype at 39°C. We found (Noonan *et al.*, 1973b) that the cells grown at 32°C bound 4–5 times more Con A-^{3}H and were much more agglutinable than were the same cells cultivated at 39°C, which incidentally were of the same size.

b. Experiments Reflecting the Need for Mobility of Membrane Components. When the binding of Con A-^{3}H is measured as a function of increasing temperature, a sharp break in the curve occurs at 15°C, and there is a similar sharp increase in the agglutination curve at that temperature (Noonan and Burger, 1973b,c). That such an increased availability of binding sites at higher temperatures is, however, not relevant for the temperature-dependent increase in agglutinability was shown in the following experiment. Cells were incubated with Con A at 0° then, after the unbound lectin was washed off at 0°, the temperature was raised to 22°C and agglutination assayed after 15 minutes at that temperature (Noonan and Burger, 1973c): these cells were as fully agglutinable (95%) as a parallel sample which had been incubated with Con A at 22°C, whereas an aliquot kept continuously at 0° was not agglutinated to more than 15%. In other words, sufficient Con A was bound at 0° for full agglutination, but the agglutination could not take

place at that temperature. That the transition point in both binding and agglutination curves should occur at 15°C seems significant in view of the fact that this is close to the temperature at which many membrane lipids shift from a semicrystalline to a fluid phase (Reinert and Steim, 1970).

In agreement with others (see above) we have found that cells treated with glutaraldehyde are no longer agglutinable by Con A; and since binding of the lectin was found not to be decreased by this treatment, the effect may be on immobilization of membrane proteins by cross-linking. We have also found that Con A agglutination is inhibited by neither azide, cyanide, nor fluoride, which may be taken as an argument against direct involvement of a metabolic process for agglutination (but see experiments of Vlodavsky *et al.*, 1973, described above, in connection with ATP content and metabolic inhibitors) (Noonan and Burger, 1973c).

We also have indications that lipids may be directly involved in the agglutination process, for treatment of transformed or trypsinized cells with phospholipase C prevented their agglutination by Con A. If 3T3 cells were treated with this lipase prior to trypsinization, agglutination was not affected, indicating that a lipase-sensitive site on the membrane is also cryptic and only becomes available after trypsin treatment or transformation (Noonan and Burger, unpublished). The role of the lipid in this case is still not clear, but it might favor a secondary binding of Con A, or a stabilization of clumped receptors needed for agglutination.

We have shown several cases where transformed cells do bind more lectin than nontransformed cells. Although the temperature dependence for agglutinability by Con A may indicate the necessity for mobility of the receptor sites in the course of agglutination, we do not know whether increased mobility of the receptor sites is the prime reason for increased agglutinability. Increased cluster formation—if it is indeed relevant for agglutination—should clearly be favored by a general increase in number of sites. Like Inbar *et al.* (1973a), we are aware that agglutination by the non-temperature-dependent lectins like WGA probably takes place by a completely different mechanism (preliminary experiments indicate, however, that glutaraldehyde inhibits agglutination by WGA as well as by Con A: Inbar *et al.*, 1973b; A. M. C. Rapin and M. M. Burger, unpublished, 1973). It seems very likely that movement of receptor sites is induced by the cross-linking lectin; this movement becomes significant only at temperatures above the freezing point of the membrane lipids (around 15°C), and it is increased as the temperature is raised.

Recent work indicates the possible involvement of microtubules in bringing about a redistribution of membrane receptors favorable for agglutination. Berlin has shown (Berlin and Ukena, 1972; Yin *et al.*,

1972) that the agglutination of fibroblasts and of polymorphonuclear leukocytes is inhibited by the microtubular-disrupting alkaloids colchicine and vinblastine. It will also be recalled that Edelman *et al.* (1973) suggested that Con A-induced mitogenesis depended on interaction of the lectin receptors with colchicine-binding proteins (see Section III,C,1,b,vii). They also proposed (Edelman *et al.*, 1973) that failure of Con A and PHA to agglutinate cells at low temperature might partly be due to the dissociation of microtubular proteins in the cold (see Tilney and Porter, 1967). A role for microfilaments in receptor site movement has also been proposed (Kaneko *et al.*, 1973; Nicolson, 1974), but solid evidence supporting this proposal is still lacking.

It is quite possible that membrane components of transformed cells are more mobile than those of normal cells, which would explain the difference in agglutinability of these cells. Inbar *et al.* (1973c) have reached this conclusion after measuring the rotational relaxation time of cell-bound fluorescent Con A. The suitability of such a procedure for determining the mobility of membrane components still requires further critical examination, however.

D. Other Possible Mechanisms

We have stated the current views—and controversies—concerning the increased agglutinability of transformed cells, but one must be aware that this does not exhaust all the possibilities. We considered some other possibilities earlier (Burger, 1973) and briefly list them here; see also Figs. 1–4. These factors need not be mutually exclusive, and indeed they might well play a role in addition to those mentioned above under Sections III,B and C.

1. *Difference in Surface Charge between Normal and Transformed Cells*

The negative surface charge of cells is due in part to their surface sialic acid. Reports on the sialic acid content of normal and of transformed cells are contradictory, and there are no doubt variations between different cell types; however, much of the more recent work suggests that transformed cells contain less sialic acid (Grimes, 1970; Ohta *et al.*, 1968; for discussion on this subject, see Burger, 1971a, and see Section VI). If transformed cells do indeed have a reduced electronegative charge, they might be more easily agglutinated owing to reduced repulsive forces.

On the other hand, we showed (Burger, 1968a; Burger and Goldberg, 1967) that treatment of transformed cells by sialidase decreases their agglutinability by WGA, although sialic acid is not a hapten inhibitor

of this lectin, and therefore not involved in the binding specificity of this lectin. It might, however, play a role in maintaining the surface in a proper conformation for interaction of the agglutinin with the cell surface, acting through steric or charge effects, or both. Kapeller and Doljanski (1972) also suggested that the negative charge of sialic acid might be necessary for maintaining the proper tertiary structure of the surface glycoproteins. Yamada and Yamada (1973) recently reported that Con A induced a concentration-dependent biphasic change in the electrophoretic mobility of hepatocarcinoma cells. Such a change could be induced in regenerating liver cells only after they had been treated with trypsin. Neuraminidase treatment increased the Con A-induced electrophoretic mobility change, and the authors suggested that rearrangement of the surface glycoproteins subsequent to Con A binding might either expose (at low Con A concentrations) or cover (at higher Con A concentrations) sialic acid molecules, resulting in different electrophoretic mobilities at different Con A concentrations. It is not clear whether these effects, which depend both on surface charge and on membrane site mobility, are relevant for agglutination.

Other surface charge effects (due to cations, for example) may also play a role for the differential agglutinability, but these have not been assessed as yet.

2. *Increased Flexibility of the Membrane*

Evidence given above (Section III,C) indicates that at least some components of the membrane of transformed cells are more mobile than those of normal cells. If the transformed cell membrane is altogether in a more fluid state, this would also render it more flexible, and hence possibly better able to undergo the deformations that occur when cells are tightly agglutinated together.

3. *Lipophilic Interactions*

We have indicated above that lipids seem to play a role in cell agglutination, and that this might be for proper interaction of the lectin with the cell surface. Whether lipophilic interactions between cells might play a role in their agglutination, and whether they would differ in normal and in transformed cells remain open questions.

4. *Availability of Bound Lectins*

One should also consider the possibility that receptor sites on normal cells, although available to lectins, are set in more deeply than on transformed cells. The lectins could then bind, but they would remain some-

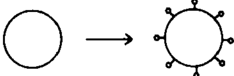
what recessed, so that intercellular lectin bridges, or other interactions between cells mediated by lectins, could not be formed.

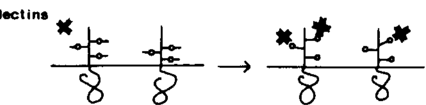
E. How Are Cells Agglutinated?

Various possible differences between normal and transformed cells have been considered that might explain their differences in agglutination, but something should still be said concerning the actual process of agglutination. These remarks will be brief, as we still do not know exactly how lectins bring cells together and hold them tightly clumped, but a few possibilities will be considered here (illustrated in Figs. 1–4); this should also serve as a summary of present views about the steps in the agglutination process.

1. *Binding and Availability*

a. Lectin Does Not Bind to Normal Cells. As prerequisite to agglutination by a given lectin cells must have specific receptors for it on their surface. Figure 1 illustrates three cases where a lectin cannot bind (or only in reduced amounts) to normal cells, because of (a) absence of





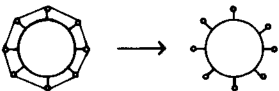


FIG. 1. Lectin Binding. (a) No sites on normal cells. Lectin receptor sites are induced as result of transformation. (b) Sites chemically different. Drawing shows glycoprotein receptors in the cell membrane. Only those on the transformed cell have sites to which the lectin can bind. (c) Sites on normal cells cryptic. Transformation makes the cryptic sites available to the lectin.

sites, (b) chemically different sites, or (c) cryptic sites. Case 1,b refers to any chemical alteration of the receptor site; the particular one illustrated here could in fact also be considered as a special case of cryptic sites, but which would be uncoverable by glycosidases rather than proteases, and the transformed cells in this case would be those deficient in certain glycosyl transferases (see Section VI,E) or having increased glycosidases. Lectin sites are apparently cryptic (Fig. 1,c) because covered over by protease-labile molecules; transformed cells might lack this surface cover, or their glycoprotein receptors might be able to penetrate it and become exteriorized, owing to increased endogenous protease activity, or to increased membrane fluidity (see Sections III,C and IV,A,5). Sites can, however, be cryptic without being actually covered over by a protein layer: they can be otherwise trapped in a conformational position making their interaction with the lectin impossible. A protease, by breaking just a few bonds in the membrane protein, and not necessarily in the immediate proximity of the receptor site, could induce general membrane rearrangements which would ultimately result in exposure of the cryptic site.

b. Lectin Binds to Normal Cells, but It Is Not Available to Neighboring Cells. In Fig. 2 are illustrated cases where a lectin can be bound to normal cells as much as to transformed cells, but where it would remain unavailable (or less available) to neighboring cells, either because the receptor sites are recessed in the normal cell so that the lectin

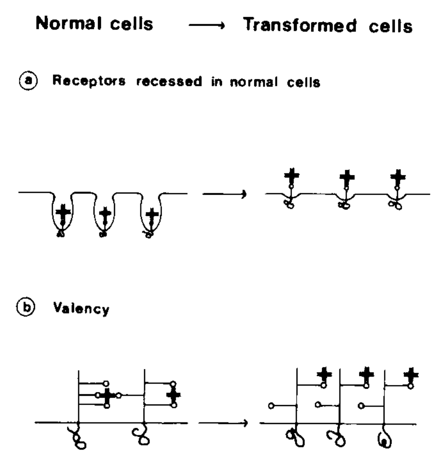


FIG. 2. Availability. Lectin can bind to normal cells, but remains unavailable to neighboring cells. (a) Sites in normal cells are recessed, can become exteriorized as result of transformation. (b) Valency: In normal cells all lectin valencies are used on the same cell; none are available to interact with another cell. In transformed cells, some lectin valencies remain available to another cell.

remains partly buried (Fig. 2,a), or because all the valencies of the lectin are used on the same cell (because of smaller distances between receptor sites on the normal cells for instance), so that no valencies remain for building bridges to another cell (Fig. 2,b); it is not yet proved, however, that such bridges are an absolute requirement for agglutination.

2. *Initial Stages of Agglutination*

a. Increased Likelihood of Aggregation through Increased Site Density. A fairly dense distribution of lectin sites, on the whole cell surface or in certain areas, is presumably favorable for agglutination: electron micrographs by both Nicolson (1972) and de Petris *et al.* (1973) show agglutinated cells with large amounts of ferritin-Con A in the area where the two cells are juxtaposed. Unambiguous proof that agglutinins do form bridges between cells is, however, missing, and it is also possible that cells are held together by noncovalent bonds between the lectins, or between the cells themselves, in areas of high lectin density (see below, hydrophobicity).

Figure 3 illustrates several ways in which a high lectin density on the transformed cell surface could be achieved:

i. *Shrinkage of cells as result of transformation (Fig. 3,a).* In cases where transformation produces smaller cells but no change in total number of receptors, these will then obviously be more densely located on the transformed cells, and they could then be more easily cross-linked by the lectin (Ben-Bassat *et al.*, 1971; and see Section III,B,2).

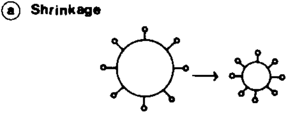
ii. *Clustering of sites induced by transformation, and present prior to lectin addition (Fig. 3,b).* Although present evidence speaks against it, the possibility that sites are more clustered in some transformed cells than in normal cells (Singer and Nicolson, 1972) still cannot be altogether ruled out at this point (see Section III,C,2).

iii. *Clustering of sites on microvilli (Fig. 3,d).* Transformed cells are covered with numerous microvilli, and Porter *et al.* (1973) have suggested that these might be bearers of lectin receptor sites, a location that would certainly be favorable for agglutination as the receptors would thus be highly available to neighboring cells (see also Section V,E).

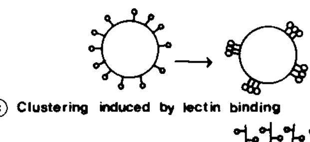
iv. *Clustering of sites induced by lectin binding (Fig. 3,c).* Recent evidence indicates that clustering of sites is brought about by lectin cross-linkage and translational movement of these linked sites in the membrane, which may be more fluid in transformed than in normal cells (see Section III,C). The relevance of site clustering to cell agglutination is still questionable, especially in view of the recent observation by de Petris *et al.* (1973) that nonagglutinable 3T3 cells showed the same clustering of labeled Con A as did agglutinable Py3T3 or trypsinized 3T3 cells.

Normal cells ⟶ Transformed cells

(a) Shrinkage



(b) Clustering induced by transformation



(c) Clustering induced by lectin binding

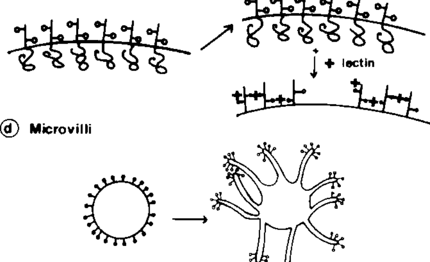


FIG. 3. Increased likelihood of aggregation through increased site density. (a) Shrinkage of cells due to transformation results in higher density of sites per unit cell surface area. (b) Clustering of receptor sites could result from transformation. This view is at present not held as likely. (c) Clustering of receptor sites as a result of lectin binding. Sites are not clustered in transformed cell prior to lectin addition. Lectin cross-linking and membrane fluidity bring about clustering. (d) Microvilli appear on transformed cells. As discussed in text, they can play a physical role in cell–cell association: tangled microvilli can hold cells together. Additionally, microvilli may bear lectin receptor sites at their tips, which are thus easily available to neighboring cells.

b. Increased Likelihood of Aggregation Due to Physical Alterations.

i. *Decreased electronegativity* (*Fig. 4,a*). If the surface of transformed cells is less electronegative than that of normal cells (see Section III,D,1), repulsive forces between cells will be diminished, an obvious advantage for formation of the initial contacts which can subsequently lead to agglutination. Bound lectins (irrespective of their cross-linking or bridge-building functions) can also play a role in decreasing the cell surface charge by partly masking charged groups.

ii. *Cells held together by microvilli* (*Fig. 3,d*). Aside from being possible bearers of receptor sites (see above), the microvilli of transformed cells may play a role in holding cells together physically. The tangling of microvilli from neighboring cells could hold the cells associated, either as a prelude to, or a reinforcement of, other intercellular bonds.

3. *Late Stages of Agglutination*

Also to be considered in the process of agglutination are physical factors that might increase the likelihood of cells remaining stably agglutinated (see also Section III,D).

a. Hydrophobicity (Fig. 4,b). It is not inconceivable that transformation might bring about a certain increase in hydrophobicity of the membrane. This could be a general phenomenon or might occur in topographical zones. Areas of increased hydrophobicity could result for instance from site clustering made possible by the increased fluidity of the transformed cell membrane. Cells could then adhere closely to each other in these more lipophilic areas, even without the building of lectin bridges. In addition, one will also have to consider that the binding of a lectin to the cell surface might be promoted by this increase in hydrophobicity.

b. Flexibility of the Membrane (Fig. 4,c). Agglutinated cells are packed close together, which results in a certain deformation of the cell

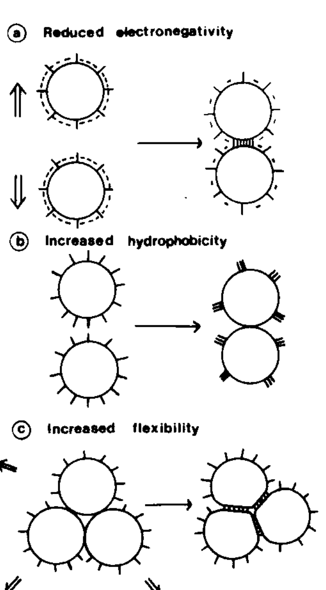


FIG. 4. Physicochemical factors that can play a role in agglutination. (a) Reduced electronegativity of transformed cell surface reduces repulsive forces between them. (b) Increased hydrophobicity, local or general, of transformed cell membrane would favor cell–cell contacts; noncovalent linkages between cells become possible. (c) Increased flexibility of transformed cell membrane allows deformations enlarging the zone of interaction and facilitating tight packing of cells.

surface. An increased fluidity of the transformed cell membrane may increase its flexibility, and thus enable it to withstand such deformations, which would be favorable for increasing the area of contact between cells.

Finally we should consider that, aside from their role to cluster receptor sites and to counteract surface charges, lectins may effect other membrane changes which might favor intercellular adhesions. Several cases have been found where binding an agglutinin apparently increases the number of receptor sites available for binding another lectin or the agglutinability by other lectins (Majerus and Brodie, 1972; Metz, 1973). Similarly Ahmann and Sage (1972) observed that the nonmitogenic lectin from *Agaricus bisporus,* when added together with a suboptimal dose of the mitogenic lectin from *Lens culinaris,* stimulated lymphocytes, suggesting that some alteration of the membrane or of membrane functions had occurred as a result of lectin binding. These cases have been cited to illustrate the multiplicity of effects which lectins can have on the cell membrane. In conclusion then we have to realize that we do not know how cells are effectively held together after their interaction with agglutinins, and this question awaits further investigations.

IV. Relevance of Cell Surface Alterations in Growth Control

A. Influence of Surface Changes on Growth Control

1. *The Concepts of Contact Inhibition and Density-Dependent Inhibition of Growth*

Malignant cells are by definition invasive: they continue to divide under conditions where normal cells cannot, and their growth is little hampered by the presence of other cells or tissues. In tissue culture conditions normal cells do not go much beyond the monolayer stage: they maintain a certain saturation density, characteristic for a given cell type; transformed cells on the contrary can easily grow over each other to form multiple layers, which leads to a much higher saturation density. Abercombie and Heaysman (1954) coined the expression *contact inhibition* after observing that the forward movement of fibroblasts was stopped when cells collided. These authors (Abercombie *et al.,* 1957) later observed that malignant cells did not display this behavior of contact inhibition of movement, and Abercombie (Abercombie and Ambrose, 1962) proposed that a change in cell surface must play a key role in malignant transformation; but he left open the question of whether membrane changes might also be involved in release from

mitotic inactivity. The concept of the reduced contact inhibition of transformed cells has lately been questioned again, particularly by the observation from the group of Gelfand and Vasiliev (Guelstein *et al.*, 1973) that contact inhibition of movement can be an attribute of transformed as well as normal cells.

The term of *density-dependent inhibition* of growth (referred to as DDI) was proposed by Stoker and Rubin (1967) in order to emphasize that inability of normal cells to grow beyond a relatively low density might be due to a number of factors in their environment, and not merely to cell–cell contact. And Martz and Steinberg (1973) have lately insisted that "post-confluence inhibition of cell division" was not necessarily equivalent to contact inhibition. It seems nevertheless clear that the membrane must somehow be involved, whether causally or not, in growth control, since the response of cells to their environment (be it physical contact, or differential ability to use certain nutrients or to respond to changes of pH or to toxic agents) must necessarily be mediated through the cell membrane. Aaronson and Todaro (1968) clearly demonstrated the relationship between tumorigenicity and the loss of DDI of division for a series of well defined mouse fibroblast cells by selecting cells that were increasingly insensitive to density inhibition of growth: they observed that such cells displayed an increasing ability to produce tumors.

When a cell culture that has reached its saturation density and stopped growing is "wounded" by cutting across it with a razor blade, cells at the edge of the wound will move into this free space, and they will begin to divide. Vasiliev *et al.* (1969) explain both migration and mitosis as release of the cells at the edge of the wound from contact inhibition. Castor (1969) suggested that cells moving into a wound would be more flattened, and thus better able to take up nutrients necessary for growth and mitosis, and he described the cell membrane as being a "transducer" in the mechanism of growth regulation, i.e., it could sense the conditions in the environment (crowded or not) and transmit this observation to an effector system. Dulbecco (1970) coined the term *topoinhibition* to describe the inhibition of DNA synthesis due to extensive cell–cell contacts, and he stated that the transformed phenotype resulted chiefly from great decrease or absence of this property, which would be one of the important mechanisms for growth regulation *in vivo* as well as *in vitro*.

Considerable effort has been devoted in the last few years to the search for a messenger that could relay information from membrane to nucleus. Some of this work will be discussed in Section IV,B, but we shall now first describe some of the factors, other than cell contact, that can influence cell growth and may play a role in its regulation.

2. *Release from Growth Control by Serum*

Serum is an indispensable component of culture medium for survival and growth of a majority of cells, and it was therefore the obvious place to look for some growth-promoting factor.

Todaro, Lazar, and Green observed in 1965 that a change of medium induced cell division, and that this induction was dependent on the concentration of serum used. The authors suggested that some factor in serum might act directly at the cell surface to modify the interaction between cells in contact; or that it could act intracellularly on a process leading to DNA synthesis, and probably at the level of RNA synthesis, since RNA was observed to increase very rapidly after serum addition.

One of the characteristics of transformed cells is that they have little or no serum requirement for growth. Bürk (1966) found evidence for a growth inhibitor which was apparently produced by normal cells and whose effect was counteracted by serum; transformed cells would lack this inhibitor. On the other hand, since growth of normal cells in the presence of fresh serum can go well beyond confluency, and since the saturation density of cells is proportional to the concentration of serum added, Holley and Kiernan (1968) suggested that a factor (or factors) in serum, probably protein in nature, must be necessary for growth of normal, but not of transformed cells.

Castor (1969, 1971) developed a system for cultivating cells with perfused medium and using simultaneous cinematographic observation. In this way depletion of the medium was avoided, and inhibitors which might have been produced by the cells were removed. Nontransformed cells were again found to cease to divide unless the serum was elevated, while transformed cells were not as sensitive to crowding, suggesting to Castor that they must produce endogenously a factor that the non-transformed cells get from the serum. Westermark (1971) studied growth of glia-like cells under steady-state medium conditions, and he came to the same conclusion as Castor about inhibition of growth due to cell contacts, and release of the inhibition by serum. Humphreys (Baker and Humphreys, 1971; Humphreys, 1972) also considered cell–cell contacts of prime importance for regulation of growth control. He believes that serum added to confluent cultures separated cells from each other, so that they were able to move again, to divide and pile up; the serum independence of transformed cells could then be simply explained by their lack of adhesiveness (see Section I; and Coman, 1944). Edwards *et al.* (1971) and Gail and Boone (1971) likewise correlated increased motility of transformed cells with their decreased mutual adhesivity.

Serum independence need not be an exclusive property of trans-

formed cells, for "flat" variants have been obtained from transformed cells which had lost most of their transformed phenotype: they were morphologically similar to nontransformed cells, were poorly agglutinable, and they were contact inhibited and grew to a low saturation density; also, they were less tumorigenic. These variant cells, however, were able to grow in medium depleted of serum (Dulbecco, 1970; see also Inbar *et al.*, 1969; Pollack and Burger, 1969; Pollak *et al.*, 1968; H. S. Smith *et al.*, 1971); i.e., some of the properties generally associated with the transformed phenotype can be dissociated from each other.

It is generally agreed that serum promotes cell division, yet it is totally unknown by what means. Serum activation is certainly a complex process since a number of serum growth factors have been isolated, but none have been fully characterized. Investigations in the laboratory of Holley (Holley and Kiernan, 1968, 1971; Lipton *et al.*, 1971; Paul *et al.*, 1971) have resulted in the isolation of several factors, some of which must act concurrently, since they act on "survival" (Paul *et al.*, 1971), growth, or migration only (Lipton *et al.*, 1971). The fact that a factor might act on migration, i.e., breaking contact only, indicates that increased mobility is not alone sufficient to induce cell division (cf. Baker and Humphreys, 1971, quoted above).

Although proteases have been shown to induce cell growth (see Section IV,A,4), most serum factors tested so far have no common protease or esterase activity (Pierson and Temin, 1972). Activities on specific protein substrates, such as surface glycoproteins, will yet have to be ruled out. Frank *et al.* (1970, 1972) obtained from serum a protein factor which could trigger resting rat embryo cells (in the G_1 phase) to DNA synthesis and then mitosis, and which was not required for growth of transformed cells; and hybrids of normal cells with X-rayed transformed cells were much less serum-dependent than normal cells. These experiments would speak against production of a growth inhibitor by normal cells, and for the possible production of an activating substance by transformed cells.

Serum has also been reported to stimulate membrane transport, and Cunningham and Pardee (1969) observed that it increased the uptake of uridine and of phosphate by confluent 3T3 cells, while having little or no effect on uptake in growing 3T3 cells or in Py-transformed 3T3 cells. They isolated from serum a transport-stimulating factor that did not have DNA synthesis stimulating activity. Sefton and Rubin (1971) showed that addition of serum to contact-inhibited cells immediately increased their rate of uptake of 2-deoxyglucose, but this was interpreted as a secondary rather than a primary effect of serum, as the transport stimulation was blocked by inhibitors of protein synthesis.

The literature quoted points to the diversity and complexity of the

effects of serum. The salient question is still whether serum acts as a nutrient, either directly, or indirectly, for instance by permitting other molecules to enter, or whether it acts in a yet other undefined manner by triggering growth through disturbing the microenvironment of a cell, or finally by attaching to the surface membrane and setting in motion unknown processes.

3. *Insulin*

Insulin was already reported in 1924 (Gey and Thalhimer, 1924) to have a growth-stimulating effect on cells in culture, but although, in contrast to serum, insulin is a well-defined entity, its effect on growth is not yet better understood.

Although Temin (1967) could show that addition of insulin enabled chicken embryo fibroblasts to grow in medium depleted of serum factors, a growth-stimulating factor which he isolated from serum was different from insulin, since it could not be inactivated with anti-insulin antiserum (Pierson and Temin, 1972). Griffiths (1970), on the other hand, suggested that stimulation of growth by insulin was not a primary effect, but was due rather to the general increase in transport and metabolic functions produced by the hormone. It should be pointed out in this context that insulin can exert its effects from the cell surface, without entering the cell (Cuatrecasas, 1969). This again shows involvement of the membrane in growth-controlling functions, and it is quite conceivable that the very binding of insulin to its membrane receptor produces a conformation change that would be sufficient to start up the activating process.

We might also mention here briefly an example of the morphotropic effect of insulin on cells: Piatigorsky *et al.* (1973) recently reported that insulin could replace serum in promoting differentiation of lens epithelium in tissue culture, the primary effect being an elongation of the cells, probably brought about by microtubules, which were seen to assemble and orient longitudinally. The effect was not as long-lasting as that of serum, possibly because only a small stimulation of protein synthesis was obtained.

4. *Proteases and Other Enzymes*

We had briefly mentioned in Sections II,A and III,B, that a very mild treatment of normal cells with trypsin rendered them agglutinable (Burger, 1969; Inbar and Sachs, 1969), thus apparently making their membranes similar to those of transformed cells. Cells treated with trypsin become analogous to transformed cells in yet another way: they are released from DDI of growth, begin to divide or grow faster than control cells (Burger, 1970a; Sefton and Rubin, 1970). We found

(Burger, 1970a, 1971b) that not only trypsin but a number of other proteases (ficin, papain, chymotrypsin, Pronase) were effective in releasing 3T3 fibroblasts from growth control. As low a concentration of trypsin as 10 μg/ml was sufficient, and it need not be applied for more than 5 minutes; once the signal was given, the chain of events was started, and the stimulus was no longer needed. The signal seems to be initiated at the membrane level, as trypsin attached to beads was equally active (Burger, 1971b, 1973). Cells treated with trypsin did not become permanently transformed: after one round of division, they returned to the nonagglutinable, static state, having repaired the trypsin-caused lesion; the treatment could be repeated on the same cells, resulting in a new burst of growth (Burger, 1971b). Sefton and Rubin (1970) found independently that addition of trypsin (3 μg/ml) to chicken embryo fibroblasts resulted in a burst of DNA synthesis after 6–7 hours, followed 6–10 hours later by one round of mitosis. Weber (1973) treated a number of cell types (both normal and transformed) with trypsin (1.25 μg/ml) and observed that nonagglutinable or poorly agglutinable cells became agglutinable by Con A or WGA, whereas the agglutinability of transformed cells was not appreciably changed. He found a positive correlation between the agglutinability of a given cell line after trypsin treatment and the saturation density that it then reached.

Effects of trypsin on the cell membrane were shown by Mallucci *et al.* (1972) and by Day and Maddy (1968). The latter authors showed that as little as 0.1 μg/ml trypsin reduced the resistance of fibroblasts to mechanical deformation, but in their conditions the surface recovered its original stiffness only after 4–5 days. This increase in surface flexibility after trypsin treatment may be relevant for the agglutinability of the cells, as we have pointed out earlier (Burger, 1973; see also Section III,D). Mallucci and co-workers (1972) on the other hand observed that a dose of trypsin (1–5 μg/ml) which induced mitosis of normal cells caused an increase in the thickness and overall mass of both normal and transformed cells, but this response was blocked when protein synthesis (which was shown to peak 30 minutes after addition of trypsin) was blocked by cycloheximide. The authors suggested that these morphological changes might correspond to protein syntheses at the cell surface, related to stimulation of DNA synthesis.

We mentioned earlier the stimulating effect of serum on differentiation of lens epithelium. Attardi *et al.* (1967), however, reported that a protein extracted from mouse salivary gland, and which had esterase and peptidase activity, caused both growth and loss of differentiation of cultured embryonic muscle cells. This is a case where enzymatic action again mimics neoplastic transformation, since the latter phenomenon also brings about a certain degree of dedifferentiation.

Vasiliev *et al.* (1970) found that a variety of agents—hyaluronidase, digitonin and ribonuclease—could bring about the same stimulation of DNA synthesis and subsequent mitosis as did a change of medium, and they suggested that these agents might act through some effect on the cell surface; this would be quite logical for digitonin and hyaluronidase, and perhaps less evident for ribonuclease, unless RNA is present at the cell surface as suggested by Weiss (1970).

Neuraminidase has been reported by Vaheri *et al.* (1972) to release chick embryo fibroblasts from growth control: this enzyme had the same effect as insulin and trypsin in stimulating DNA synthesis and cell division; moreover, these agents all increased uptake of 2-deoxyglucose and glucosamine. In view of the inhibitory effect of neuraminidase on agglutination by WGA of L1210 leukemia cells (Burger and Goldberg, 1967) and of transformed chick embryo fibroblasts (Kapeller and Doljanski, 1972; and see Section III,D), it would be interesting to know whether cells stimulated to grow by neuraminidase would be agglutinable by this lectin. One might also mention here two cases where neuraminidase was found to have an antitumor effect. Codington *et al.* (1970) and Hughes *et al.* (1972) found that, after treatment with neuraminidase, ascites cells were less transplantable—i.e., they were rejected by their hosts; this was ascribed to their elimination by a cytotoxic factor which had no effect on the nontreated cells. Simmons and Rios (1971) likewise observed the immunospecific rejection by mice of neuraminidase-treated fibrosarcoma cells, and they suggested that the cells, possibly because of decrease in negative surface charge, might more easily interact with immunocompetent cells, or might be more easily phagocytized, or lysed by complement. Since the negative charge on the cell surface is to some degree due to neuraminic acid, and since this sugar covers a large portion of the cell surface and is part of its antigenic makeup, it is not surprising that its removal should have varied and complex effects on the cell.

5. *Evidence for Increased Cell Surface Proteases in Transformed Cells*

Since proteases render normal cells in several respects analogous to transformed cells, we considered the possibility that proteases on the surface of tumor cells might be important for the maintenance of the transformed state (Burger, 1969). Much evidence accumulated in the last few years points in that direction. In 1957, Sylvén (Sylvén and Malmgren, 1957) already had observed that rapidly growing cells had higher catheptic activity than those growing more slowly, and in 1965 (Sylvén and Bois-Svensson, 1965) he reported high levels of peptidases and cathepsins in interstitial fluids from several tumors: he then suggested

that these degradative enzymes might play an important role in the invasive and destructive role of the tumors.

Rubin (1970) isolated from the culture medium of Rous sarcoma cells a factor which stimulated overgrowth of confluent and static chicken embryo cells, and it was suggested that the nondialyzable and virus-free material might act as a protease or peptidase. Bosmann (1969, 1972b) found elevated levels of several glycosidases, as well as both acid (cathepsinlike) and neutral (trypsinlike) protease activities in extracts from several fibroblast lines transformed by RNA viruses; Schnebli (1972) demonstrated a higher protein-degrading activity by an intact culture of PY3T3 cells than by a culture of normal 3T3 cells.

Fibrinolytic activity was found by Reich and his collaborators (Ossowski *et al.*, 1973; Unkeless *et al.*, 1973) in a variety of cells transformed by either DNA or RNA viruses. The material was present close to the cell surface, and could be released into the medium under special conditions. Interestingly, these workers found a fibrinolysin inhibitor in the serum of tumor-bearing animals; this would then be a defense against the degradative effects of the tumor cells.

Other hydrolytic enzymes found at increased levels in transformed cells were collagenase in an ascitic carcinoma (Harris *et al.*, 1972) and neuraminidase in transformed fibroblasts (Schengrund *et al.*, 1973). In the latter case both normal and transformed cells had the same amount of total sialic acid, and both had sialidase activity directed against endogenous substrate; however, after hydrolysis and removal of this material, exogenously added disialo- and trisialogangliosides were hydrolyzed by the transformed, but not by the normal, cells.

If transformed cells owe their ability to grow to high saturation density to a higher sensitivity to or to higher amounts of proteases present in their membranes, it should be possible to restrain their growth with protease inhibitors. And indeed we found that TLCK, TPCK, TAME, ovomucoid, and trasylol at nontoxic doses inhibited the growth of polyoma-transformed 3T3 or BHK21 cells, yet had less of an effect on the corresponding nontransformed cells. Since the inhibitors used have quite different modes of action, it does seem most probable that their effect results from protease inhibition. It should be mentioned here that results of studies with the irreversible protease inhibitors, such as TLCK and TPCK, i.e., chloromethylketones, can be held suspect of being artifacts due to the reactivity of the compounds with sulfhydryl groups, unless the same results can be achieved (as was the case here) with other types of protease inhibitors, such as TAME or ovomucoid. Interestingly, Py3T3 cells, whose growth was inhibited by TLCK, were much less agglutinable by WGA and Con A than untreated cells (Schnebli and Burger, 1972).

We have also shown that protease inhibitors act directly on the cell membrane with the use of ovomucoid coupled to polyacrylamide beads of Bio-Gel P-10 whose size is comparable to that of Py3T3 cells attached to a culture dish. The coated beads were layered over cultures of 3T3 and Py3T3 cells, and growth of the transformed cells was clearly inhibited after 3–4 days, whereas 3T3 cells remained unaffected. Growth of Py3T3 cells resumed normally, and they reached their characteristic high saturation density 2–3 days after the beads had been washed off the plates. We have been able to show that radioactive ovomucoid, coupled to beads, was not taken up by the cells; also, beads coated with a number of other proteins had no growth inhibitory effect (Talmadge *et al.*, 1974).

An interesting piece of work indicating the surface location of degradating enzymes, presumably also proteases, was carried out on polymorphonuclear leukocytes (Arend and Malchow, 1972). Incubation of these with autologous red blood cells resulted in loss of N_{hu}-receptor activity, without loss of M_{hu}-receptor, or of the receptor for *Vicia graminea* agglutinin; at the same time, however, H-receptor activity appeared (as shown by agglutinability with two different H-specific lectins). These modifications in blood group activity were attributed to some proteolytic activity of the leukocytes, as they could be prevented by a number of protease inhibitors.

Warren and Glick (1968) have shown that confluent cells have a higher membrane turnover than nonconfluent cells, and they should therefore have higher activity of both protein synthesizing and proteolytic enzymes than do growing cells. Baker and Humphreys (1972) found that chicken embryo fibroblasts, when confluent, could be made agglutinable by use of cycloheximide, pactamycin, or emetine, which had no effect on growing cells. In collaboration with Borek of Columbia University (Borek *et al.*, 1973), we recently confirmed and extended these findings to confluent 3T3 fibroblasts or normal rat liver cells treated with cycloheximide which became agglutinable with WGA and Con A. Growing cells, on the other hand, could not be rendered agglutinable with this protein synthesis inhibitor. This was interpreted as the uncoupling of the normal balance between protein breakdown and resynthesis: when resynthesis was blocked by cycloheximide the action of the proteases was not compensated for, and the cells temporarily acquired the surface properties of transformed cells; after removal of the inhibitor the cells returned to their normal, nonagglutinable state. If the protease-inhibitors TAME or TLCK were added together with cycloheximide, the confluent cells remained nonagglutinable.

Protease inhibitors have also been shown to inhibit the growth of tumor cells *in vivo:* Troll *et al.* (1970) found that direct application of the irreversible chloromethylketone inhibitors TLCK and TPCK as well

as the reversible and competitive inhibitor TAME to the skin of mice 1–2 hours after it had been treated with croton oil or phorbol ester greatly reduced the number of tumors and delayed their appearance. This experiment, which is of obvious interest from a clinical point of view, is a confirmation of the *in vitro* experiments pointing to the role of proteases in growth regulation.

Since normal cells do have surface proteases, we have proposed (Burger, 1971b, 1973; Talmadge *et al.*, 1974) that triggering of growth by such various agents as serum factors, insulin, or proteases may start a chain of reactions in the cell surface, where one protease would activate the next one in a cascade system, such as in the systems leading to complement lysis or to blood clotting. It is interesting in this context to recall that Ossowski *et al.* (1973) and Unkeless *et al.* (1973) found fibrinolytic activity in transformed cells (see above). A multiple-step system, which is at this time but a hypothesis, would allow a variety of controls; this would be an advantage for a phenomenon as important and as complex as regulation of growth.

6. *Miscellaneous Other Growth-Stimulating Agents. Conclusions*

The complexity of growth regulation is shown by the number of ways in which it can be modified, and a few other modifying agents will be mentioned here.

Weston and Hendricks (1972) obtained release from DDI of growth of fibroblasts with 0.2 *M* urea, and the cells so treated became agglutinable, showing that release from growth control was accompanied by a change in the cell surface. This change was reversible after removal of urea, but restoration of the normal cell surface was prevented by cycloheximide. This agrees well with the experiments of Baker and Humphreys (1972) and of Borek *et al.* (1973) described above, which also indicated degradation and resynthesis of cell surface proteins, preventing the agglutinable surface configuration. In the case of Weston and Hendricks, cell surface material which was removed by urea could be added back to the cells in the presence of cycloheximide, and normal contact inhibition was restored, showing that enzymes with a fast turnover relevant for transferring material onto the cell surface had not been involved.

Since the chain of events leading from stimulation of the cell surface to the final event of cell division is a long one, it can be modified at various points, as was suggested in the last sections. Vasiliev *et al.* (1971) were able to stimulate DNA synthesis with such "metaphase inhibitors" as colchicine, Colcemid, or vinblastine. These agents interfere with locomotor functions of the cells by causing microtubular disaggregation, thus presumably permitting micromorphologic rearrangements including certainly changes in number and form of the microvilli.

Proper cell growth is very dependent on growth conditions, and some of the differences between normal and transformed cells no doubt reside in their sensitivity to external conditions. Ceccarini and Eagle (1971) showed that different types of cells differ in their optimum pH for growth, and they were able to stimulate overgrowth in a number of contact-inhibited cells by using buffers which maintained a strictly defined pH above neutrality. The usual bicarbonate buffers shift toward acidity during cell growth, and the authors suggested that this is what stops growth of cells at a given level, whereas transformed cells are not so sensitive to pH and can continue growing to higher densities (it might be recalled here that the peptidases found by Sylvén and Bois-Svensson (1965) in tumor cells were essentially of the acid type; see Section IV,A,5).

Pardee suggested in 1964 that alteration of cell membrane permeability for certain critical compounds might be involved in the control of cell growth and division. Along similar lines, Holley (1972) proposed that tumor cells are better adapted for growth because of their altered membranes which allow for better uptake of certain nutrients and growth factors. Growth would then be triggered by the increased concentration of these critical substances in the cells. These hypotheses rest of course on the fact, already discussed earlier (see Section IV,A,2,3), that transformed cells often show increased uptake rates for a number of substrates.

Many agents and conditions affecting cell growth have been discussed here, and their mode of action is still not well understood. It is clear, however, that the cell membrane plays a crucial role in growth control, and we might recall here experiments which we did a few years ago, and which showed that certain membrane sites, in fact those involved in cell agglutination, might well play a role for growth control: trypsinized or chymotrypsinized Con A (which is presumably no longer in the native tetravalent form, but rather in the di- or monovalent form) binds to Py3T3 cells but does not cause them to agglutinate; it is nontoxic, but it did restore DDI of growth in these cells. The inhibition could, however, be reversed when the Con A was removed by its specific hapten α-methylmannose. The inhibition of growth was not obtained with other proteins such as hemoglobin or ovalbumin, nor with two other lectins which could bind to the cells (Burger, 1973; Burger and Noonan, 1970). The mechanism by which this nonagglutinating lectin preparation can influence growth control remains an open question.

In closing this discussion of a number of factors which can be involved in growth control, we would like to mention the unifying hypothesis of Hershko *et al.* (1971). They defined growth-stimulating factors, such as insulin or serum as "pleiotypic activators" and the whole chain of events leading to cell growth which these activators produce would be a "positive pleiotypic response." These activating agents would act, presumably

at the cell membrane level, to activate a pleiotypic mediator which could then start up macromolecular synthesis, increased uptake, and the other responses observed in cells released from growth control. Transformation of cells would act on the mediator, so that it would remain in the activated state. The existence of this "pleiotypic mediator" was invoked to account for the fact that growth can be stimulated by a number of different agents and environmental factors, which start up a number of apparently coordinated reactions in the cells; the mediator would then have regulatory functions through its action as messenger from membrane to cytoplasm and nucleus. Considerable effort has been directed in a number of laboratories toward identifying such a messenger, and elucidating its mode of action, and this will be reviewed in Section IV,B.

B. Cyclic Nucleotides

A great deal of effort has been directed toward looking for a universal mediator initiating the multitude of phenomena instrumental in stimulating cell growth (pleiotypic mediator of Hershko *et al.*, 1971; see Section IV,A,6), but the problem has still not been solved, and one may eventually find that different cells use different chains of events, different mediators, or a combination of messengers. Cyclic AMP (cAMP) is known to be a messenger for a number of metabolic processes (Robison *et al.*, 1968, 1971; Sutherland, 1972); moreover, since it is probably formed on the inner surface of the cell membrane by the membrane-bound adenyl cyclase, it would be a likely candidate for a membrane-to-nucleus messenger (see Burger, 1971b), all the more so as correlations have been found between cAMP levels and the state and growth rate of cells. This is also the conclusion that was recently reached by Tomkins (Kram *et al.*, 1973), who now considers cAMP to be the pleiotypic mediator.

We shall summarize here the evidence accumulated in a number of laboratories which suggest a role for cAMP in growth control. Attention has been so generally focused on that topic in the past few years that this part of the review will primarily be concerned with this nucleotide. But we want to point out already now that a whole series of other factors, which for lack of data cannot yet be described fully, may eventually be found to be at least as important.

1. *Cyclic AMP Levels High in Nongrowing Cells, Low in Growing and in Transformed Cells*

The earliest reports on cellular cAMP levels were made in 1968 by Bürk and by Granner *et al.* (1968). The latter authors found a rat mini-

mum deviation hepatoma to have 10 times less cAMP than normal cells, and little or no adenyl cyclase activity; they considered this to be a particularity of that line, and did not relate it to its transformed character. Bürk, on the other hand, hypothesized that adenyl cyclase, and hence cAMP, might be instrumental in regulating growth; he found greatly reduced levels of cyclase in polyoma-transformed BHK cells as compared with normal cells, and suggested that virally transformed cells might then no longer be regulated by hormones which turn on adenyl cyclase or act through phosphodiesterase. An inverse correlation between growth rate and intracellular levels of cAMP was observed by Heidrick and Ryan (1971) and by Otten *et al.* (1971) in a number of cell lines, and transformed cells were consistently found to have lowered levels of cAMP. Heidrick and Ryan suggested that in normal cells the cell-to-cell contacts formed at confluency might activate the membrane-bound cyclase, whereas such a response could not be made by transformed cells. The relationship between growth rates and cAMP levels was demonstrated in 3T3 cells by Seifert and Paul (1972): cells which had stopped growing had levels of cAMP twice as high as growing cells, whether in sparse (1% serum) or in dense (10% serum) culture, and the authors concluded that cAMP levels were probably regulated by serum factors, not by contact between cells.

It had been observed in 1966 by Butcher *et al.* that the level of cAMP in rat adipose tissue was greatly lowered within 5 minutes after incubation of the tissue in the presence of insulin; and Illiano and Cuatrecasas (1972) demonstrated with isolated cell membranes that insulin lowers the glucagon-, fluoride-, or epinephrine-stimulated cyclase in these membranes. The hypothesis about the role of cAMP in growth control was further strengthened when Sheppard (1972) and Otten *et al.* (1972b) found that not only insulin, but also other growth-stimulating agents, such as serum and trypsin, caused a rapid fall in the cAMP levels of normal cells in culture considerably before onset of DNA synthesis. Conversely, serum deprivation raised the cAMP levels of 3T3 cells, but did not appreciably change the already low levels in SV3T3 cells (Kram *et al.*, 1973). Recently de Asúa *et al.* (1973) showed that BHK fibroblasts stimulated to overgrowth and transformed morphologically by insulin had low levels of cAMP and reduced cyclase activity; the effects of insulin on both growth and morphology were inhibited, thus clearly showing the involvement of cAMP in growth control.

We have also recently shown (Bombik and Burger, 1973; Burger *et al.*, 1972) that serum as well as a number of proteases which produce overgrowth of 3T3 fibroblasts reduced intracellular levels of cAMP within 5 minutes, and that growth stimulation could be inhibited by addition of

diBcAMP (see next section). Other agents which were shown by various other authors to stimulate fibroblasts to overgrowth were also found to reduce cAMP levels; this was the case with sialidase, colchicine, digitonin, ribonuclease, hydrocortisone, hyaluronidase, lysolecithin, and hydroxyurea (B. M. Bombik and M. M. Burger, unpublished data, 1973).

Correlation between lowered cAMP levels and the transformed character could be shown with a temperature-sensitive mutant of Rous sarcoma virus (Anderson *et al.*, 1973; Otten *et al.*, 1972a). Chicken embryo fibroblasts infected at the permissive temperature (37°C) had low levels of cAMP and of adenyl cyclase, whereas these were normal when the cells were cultivated at the nonpermissive temperature (41°C). If cAMP was added to the cultures before a temperature shiftdown, they retained the untransformed phenotype. Further work by this group (Rein *et al.*, 1973) demonstrated that infection of confluent 3T3 fibroblasts with SV40 virus caused a transitory decrease in the cAMP levels of these cells, later followed by DNA synthesis and cell multiplication. Treatment of the cells with UV-inactivated virus, which did not induce DNA synthesis and did not transform the cells, also did not change their cAMP levels. The lowering of cAMP following viral infection and preceding DNA synthesis was compared with that obtained with insulin, serum, or trypsin: the fact that the latter agents caused a more immediate lowering of cAMP (within minutes, as against 2–3 hours for the virus) was ascribed to their direct interaction with the enzymes of cAMP metabolism in the cell membrane, whereas the virus would first have to be uncoated and its genome translated. Similar conclusions were reached by Raska (1973), who observed a drop in cAMP after infection of BHK21 cells with adenovirus 12, and he noted that both adenyl cyclase and phosphodiesterase activities were correspondingly lowered [the level of the cAMP-degrading phosphodiesterase is thought to be regulated by the intracellular concentration of cAMP (d'Armiento *et al.*, 1972)]. In transformed cells having low basal activity of adenyl cyclase, this could usually be increased to a certain extent by fluoride or prostaglandin E_1 (PGE_1); but two transformed 3T3 lines were found where cyclase was activated by fluoride but not by PGE_1, indicating that transformation had here resulted in loss or alteration of PGE_1 receptors (Peery *et al.*, 1971).

Further correlations between cAMP levels and growth control have been found in experiments with exogenously added cAMP, and these will be discussed in Section IV,B,4.

2. *cAMP and Cell Differentiation*

Mouse neuroblastoma cells could be stimulated to differentiate irreversibly and produce axons by addition of diBcAMP, an agent which also

considerably inhibited growth of the cells (Furmanski *et al.*, 1971; Prasad and Hsie, 1971); cAMP was also growth inhibitory, but did not induce differentiation (Prasad and Hsie, 1971). Differentiation could also be induced by PGE_1 and by an inhibitor of phosphodiesterase, and it was accompanied by increased levels of cAMP and higher activity of phosphodiesterase (the latter as a result of the increased concentration of cAMP, and observed also, but later, after stimulation with phosphodiesterase inhibitor). Protein synthesis was required for differentiation and for maintaining high levels of cAMP and its metabolizing enzymes, but the level at which it was necessary was not ascertained (Prasad and Kumar, 1973; Prasad *et al.*, 1973; Sheppard and Prasad, 1973). Lim and Mitsunobu (1972) investigated differentiation of neuroblastoma and astrocytoma cells of tumor origin under the influence of diBcAMP. Morphological differentiation was observed within 1–2 days, at the same time that growth was inhibited; DNA and RNA synthesis were both inhibited, but protein synthesis was increased. cAMP was also found by Hier *et al.* (1972) and by Roisen *et al.* (1972) to stimulate nerve outgrowth from embryonic sensory ganglia.

The reports from these groups indicate a positive correlation, though not necessarily causal, between the differentiated state and higher levels of cAMP in neural cell lines. The opposite was observed in a study on myoblasts by Wahrmann *et al.* (1973). Adenyl cyclase was found to drop as myotubes were formed from cultured myoblasts. In cells from a temperature-sensitive line, the decrease in cyclase was observed at the permissive temperature which allowed differentiation; at the nonpermissive temperature, however, cells grew to a certain level and became static, but without differentiating, and their levels of cyclase increased during that time.

The contrast between these two systems shows that, even if cAMP does play a role in differentiation, this is probably not of a simple and direct nature.

3. *cAMP Levels during the Cell Cycle*

If there is a correlation between the state of the cell surface in normal and transformed cells and the cellular levels of cAMP, one might expect this to be also manifested during mitosis, since normal cells undergoing mitosis have certain surface properties similar to those of transformed cells (see Section V); and this has indeed been shown to be the case.

Sheppard and Prescott (1972) found that levels of cAMP in CHO cells were at a minimum during mitosis and at a maximum in early G_1, and they suggested that this sharp rise during G_1 might be correlated with the cell's "decision" at that time about whether to go on to another

cycle of division or to remain static in G_1. Working with fibroblasts we also observed (Burger *et al.*, 1972) that levels of cAMP were at a minimum during mitosis; an initial drop in cAMP was observed during the late S-phase, and a second drop coincided exactly with the onset of mitosis, reaching a minimum at the height of the mitotic peak, when DNA synthesis was also at a minimum, and after that the cAMP levels rose to a maximum in early G_1.

The fluctuations in cAMP levels during the cell cycle are one more indication that the nucleotide may be involved, as cause or effect, with metabolic changes related to DNA synthesis and cell growth. Willingham *et al.* (1972) attempted to clarify the role of cAMP on DNA synthesis and mitosis by adding the nucleotide to cultures at different stages of the cell cycle. They observed that diBcAMP added at the time that 3T3 cells were plated inhibited their DNA synthesis and stopped them in the G_1 phase; the onset of DNA synthesis was however accelerated when cAMP was added 3–6 hours after plating. When the nucleotide was added at the beginning of DNA synthesis this proceeded normally, but the cells were then arrested in G_2 and did not go on to mitosis. The effects of cAMP appear quite complex, and it is probable that it can act on several different processes. Other studies, to be described in the next section, indicate that cAMP may interact with microtubules, and possibly with microfilaments, and this might be a reason for its inhibitory effect on the cell cycle at various stages.

4. *Effects of cAMP and diBcAMP on Cell Cultures*

The possible role of cAMP on cell growth has been investigated in a number of studies where cAMP or derivatives of the nucleotide were added to cell cultures. DiBcAMP has been chiefly used because it apparently penetrates the cells better (Posternak *et al.*, 1962); also, cAMP has lately been found to be degraded extracellularly (Kaukel *et al.*, 1972; MacManus *et al.*, 1971) so that some effects formerly attributed to it may actually have been caused by its degradation products or metabolites such as 5′-AMP or ADP. DiBcAMP in contrast was resistant to extracellular degradation, and to intracellular hydrolysis by phosphodiesterase (Kaukel and Hilz, 1972). It was converted in the cell to monobutyryl cAMP which, in view of its biological activity, the authors considered to be the true imitator of intracellular cAMP.

Related to the studies on cell differentiation described above are those on CHO cells, which can be considered as dedifferentiated epithelioid cells, which are agglutinable and not contact inhibited. DiBcAMP converted these cells (Hsie and Puck, 1971; Hsie *et al.*, 1971) to monolayered, fibroblastic cells which synthesized collagen and which were only

poorly agglutinated by WGA or Con A. Also noteworthy, in view of the observations of Porter *et al.* (1973; see Section V,E) was the disappearance of the bleblike protuberances characteristic of CHO cells as they elongated to fibroblasts. Microtubules were apparently necessary for the conversion, and they were considered to be the possible site of action of cAMP, as Colcemid and vinblastine prevented the differentiation. The effect of the cyclic nucleotide was potentiated by testosterone and by prostaglandins, but contrary to the differentiation of neuroblastoma cells described above, the differentiation in this case was reversible and lasted through one cell cycle only. Rozengurt and Pardee (1972) also observed the growth inhibition of CHO cells by diBcAMP, and they found that this was accompanied by a decrease in amino acid transport ability and an increased serum requirement, both of which are characteristic of untransformed cells; serum addition reversed the effects of diBcAMP.

CHO cells were found by Roberts *et al.* (1973) to have another characteristic of transformed cells, namely, a surface fucose-containing sialopeptide not found in normal cell membranes (Buck *et al.*, 1971; see Section VI). Conversion of CHO to fibroblastlike cells caused this glycopeptide to disappear, another indication for the conversion of these cells to a "nontransformed" state under the effect of cAMP. Very recently, however, Wright *et al.* (1973) have reported finding the same conversion of CHO to fibroblast-type cells by using phenethyl alcohol instead of diBcAMP and, like that by the cyclic nucleotide, the conversion was inhibited, or could be reversed, by the use of colchicine; phenol or ethanol had no effect on CHO cells. These experiments would indicate, and this was the conclusion reached by the authors, that growth inhibition and cellular differentiation are initiated by a membrane alteration, which can apparently be brought about equally well by phenethyl alcohol as by cAMP.

The earliest report about the effects of exogenous cAMP on cell growth was that of Ryan and Heidrick (1968) who found that cAMP, or better diBcAMP, in the culture medium of HeLa or L cells inhibited their growth. The authors extended their studies (Heidrick and Ryan, 1970) to other cell lines, which were also inhibited; cGMP was also found to have an inhibitory effect on certain cell lines, and in particular on a normal diploid cell line which was refractory to cAMP.

At the basis of many further studies on the effect of cAMP on cell growth were those of Sheppard (1971) who restored contact-inhibited growth to virally and spontaneously transformed 3T3 cells by addition of diBcAMP and theophylline (an inhibitor of phosphodiesterase); rapid growth resumed immediately upon removal of the drugs. The treated cells had a morphology similar to that of the nontransformed parents,

and their agglutinability by WGA was very much decreased; as in nontransformed cells, however, agglutinability could be restored by a mild trypsin treatment. These results were earlier interpreted as evidence for a correlation between growth control and surface structure, but since even 3T3 cells can be inhibited, one will have to be cautious about comparing inhibition of growth of transformed cells with contact inhibition of growth in untransformed cells.

In the last two years a number of reports have appeared, indicating an inhibition of growth of transformed cells by cAMP derivatives. Masui and Garren (1971) obtained the same inhibition of DNA synthesis and growth of adrenal tumor cells by ACTH as by cAMP, diBcAMP and other adenine nucleotides, and the corticotropic hormone was assumed to act through stimulation of cAMP. Zimmerman and Raska (1972) observed that BHK cells in which growth had been induced by adenovirus infection or by serum treatment could again be arrested by addition of diBcAMP to the cultures; interestingly, T antigen, normally induced by the viral genome, was not expressed in the treated cells, indicating that nuclear as well as surface changes induced by the virus had been temporarily obliterated.

Surface changes accompanying cAMP growth inhibition can apparently be more complex than a simple reversion to the normal phenotype, as shown by experiments of Kurth and Bauer (1973). Cells from an RSV-induced tumor, when treated with diBcAMP and theophylline ceased growing and became less agglutinable by Con A; it seems, however, that their expression of embryonic antigens and of the virus-induced tumor-specific surface antigens was still greater than in normal cells, while that of the normal cell antigens was reduced. Unfortunately the antigenic picture of comparable nontransformed cells could apparently not be made, so that the direction and extent of surface rearrangements obtained is difficult to assess. Growth inhibition of a malignant hepatoma cell line, a KB line and an SV3T3 line were reported by three different laboratories (Smets, 1972; Teel and Hall, 1973; van Wijk *et al.*, 1972). Frank (1972) found that, in rat embryo cells stimulated by serum, DNA synthesis was inhibited by diBcAMP. Serum apparently activates phosphodiesterase, hence the decreased cAMP levels in stimulated cells and the antagonistic effects of serum and diBcAMP.

The motility of fibroblasts treated with diBcAMP or PGE_1 was followed by time-lapse cinematography by Johnson *et al.* (1972) and was found to be strongly inhibited, and the authors postulated a possible interaction of cAMP with microfilaments, such as phosphorylation. Johnson and Pastan (1972) also found that along with the decrease in motility, adhesiveness of cells to the substratum was increased after treatment with

diBcAMP or PGE_1, another indication of an effect on surface properties of the cells. This increase in adhesiveness was observed in transformed as well as in normal cells, but was lost within 30 minutes after removal of the drug. The growth-inhibitory effect and induction of morphological differentiation by diBcAMP was shown by Johnson *et al.* (1971) to apply to a number of cell types, but apparently not to epithelioid cells under the conditions used (CHO cells were not tested). As in the studies of Hsie and Puck (1971), colchicine inhibited the formation of cell processes induced by cAMP, so that here also microtubules were believed to be involved.

Results obtained in our laboratory indicated also a causal, and not only a correlative, relation between cAMP and growth control. Growth stimulation of 3T3 cells (stimulated by proteases, 50% serum, or insulin) was prevented if the cells were treated simultaneously for 10 minutes with the growth-promoting agent and with 1 to 5×10^{-5} *M* diBcAMP (Bombik and Burger, 1973; Burger *et al.*, 1972). Growth was not inhibited if cAMP was added later than 3 minutes after removal of Pronase, which would indicate that an apparently irreversible chain of events had been started as a result of the protease-induced membrane changes. We have been able to show that it is not the membrane change itself which is prevented by cAMP, which indicates that the nucleotide must act on some "signal" induced immediately by the membrane alteration. Thus 3T3 cells which were treated simultaneously with Pronase and with 5×10^{-4} *M* diBcAMP were as agglutinable by WGA as cells treated with Pronase alone; likewise, polyoma-transformed 3T3 cells remained agglutinable after treatment with a concentration of diBcAMP (10^{-4} *M*) sufficient to inhibit their growth (Bombik and Burger, 1973). More recently, we have obtained similar results with cells stimulated with sialidase, colchicine, or hydrocortisone (B. M. Bombik and M. M. Burger, unpublished observations, 1973). These results have led us to propose (Bombik and Burger, 1973) that surface alterations (due to transformation, to mitosis, or to agents like proteases) trigger a chain of events that eventually lead to DNA synthesis, protein synthesis, and mitosis; these alterations are normally accompanied by, but are not necessarily the cause of, a decrease in the intracellular level of cAMP. Evidence from a number of laboratories as well as our own indicates that cAMP plays a regulatory role in this chain of events; but we still do not know how or where it acts, and, judging from the experiments we have described above, it is quite possible that it can intervene in a variety of ways, depending on the physiological condition of the cells.

Recently, B. J. Smith *et al.* (1973) reported that transformation by SV40 and by polyoma virus was increased 3–5-fold by diBcAMP. This

does not seem to have been a question of better adsorption or penetration of the virus in a modified membrane, as the effect was greatest when cAMP was added 8 hours after infection and left in for up to 24 hours, apparently the period when the virus stimulates DNA synthesis and integrates into the host genome. DNA and protein synthesis were obviously not inhibited in this system, but a study of them was not given in this short report.

At the end of this list of studies, which are all indicative of an involvement of cAMP in regulating growth control, we should also mention the serious criticism raised by Paul (1972). He found 10^{-4} *M* diBcAMP + 10^{-3} *M* theophylline to be toxic to SV3T3 cells, although the effect could be overcome by serum, and he suggested that the saturation density observed by other workers did not reflect a growth arrest, but an equilibrium between dead and newly formed cells. He observed by autoradiography that diBcAMP-treated SV3T3 cells still synthesized DNA. But since his cells were not synchronized, it is quite possible, in view of the experiments of Willingham *et al.* (1972; see Section IV,B,3) that some of the cells may still have been synthesizing DNA; also, the extent of DNA synthesis cannot be judged as accurately from an autoradiogram as from thymidine-^{3}H incorporation studies, which makes it difficult to evaluate Paul's results. In our hands cAMP and related nucleotides were indeed toxic at concentrations of 4×10^{-3} *M* and above; but we did not find them toxic at the concentration of 10^{-4} *M*, which was sufficient to inhibit DNA synthesis and growth. We also observed that cells are more sensitive to diBcAMP shortly after plating than when the drug is added 24 hours later (Bombik and Burger, 1973). When considering the effects of cAMP on cell cultures, one must certainly take into account the conditions used; it might not seem warranted at the present time to ascribe all growth inhibitory effects of the drugs to toxic effects.

5. *Effects of cAMP on Cells in Vivo*

A few studies on animals indicate that cAMP may also have a growth-inhibitory effect *in vivo*. Gericke and Chandra (1969) reported that mice which had been injected with lymphosarcoma cells and which were then treated daily with cAMP or cIMP had considerably smaller tumors than did untreated controls or mice treated with cUMP; tumor growth, however, resumed as soon as the treatment was interrupted.

Reddi and Constantinides (1972) obtained a partial protection against tumor production by adenovirus-transformed cells by injecting theophylline before or at the same time as the cells; if the drug was given after the tumors had started to develop, they remained smaller. If the transformed cells were treated directly with diBcAMP and theophylline for

24 or 48 hours before injection, tumors failed to develop or appeared after a longer incubation period. This is interesting since the effects of cAMP *in vitro* are reversible after removal of the drug; but apparently in this case a complete reversion to the transformed state was not obtained after injection into the animal.

Protection by theophylline was also obtained by Webb *et al.* (1972), either by pretreatment of the RLV tumor cells, or by injection into the animals. The synthetic nucleotides poly(A,U) and poly(I,C), especially the latter, were also effective; and since the favorable effects were also observed in irradiated mice, they were considered to be due, not to an enhanced immune response of the animals, but to stimulation of endogenous cAMP. The authors did not have any further data to either support or invalidate their hypothesis, but one can be sure that they, and investigators in other laboratories, have since been actively looking for an antitumor effect of cAMP.

6. *Conclusions*

a. Possible Role of cGMP. The experiments described in this section lend support to the hypothesis that cAMP is the "pleiotypic mediator" (Kram *et al.*, 1973), one of the important functions of which would be to regulate growth control. Its mode of action, which is no doubt complex, is still not properly understood.

Recently a few investigators have directed their attention toward the only other naturally occurring cyclic nucleotide, cGMP. Sutherland (1972) has referred to it as a nucleotide in search of a function, but he and others have nonetheless found a few functions for it. One important observation was that physiological concentrations of cGMP stimulate the hydrolysis of cAMP by liver phosphodiesterase (Beavo *et al.*, 1971), and a few recent experiments point to the possible importance of a balance between cAMP and cGMP. For a recent review about the properties and biological functions of cGMP, see Goldberg *et al.* (1973).

Experiments with perfused rat hearts indicated that cholinergic agents, which decreased heart rate and beating amplitude, elevated cGMP levels but had no effect on cAMP, except a possible slight decrease; a combination of theophylline and isoproterenol, on the other hand, increased the cAMP level considerably and raised heart rate and amplitude, but did not appreciably change the cGMP level (George *et al.*, 1970). With lymphocytes, cholinergic agents were shown by Strom *et al.* (1972) to increase their cytotoxicity toward sensitized cells, whereas agents such as PGE_1 and theophylline, which increased levels of cAMP, inhibited the cytotoxic effect, and it was suggested that the cytotoxic action of lymphocytes was regulated by a balance between the two nucleotides.

The effect of cGMP on cAMP phosphodiesterase from thymic lym-

phocytes was found by Franks and MacManus (1971) to be complex: this phosphodiesterase apparently has two K_m's, and the enzyme with the lower K_m was inhibited by cGMP, while that with the higher K_m was stimulated; this would mean that the levels of cAMP would be controlled by the concentration of cGMP. Further reports from that laboratory (MacManus and Whitfield, 1969, 1972) indicate opposite effects of cAMP and cGMP on lymphocytes: at low concentrations (10^{-8} to 10^{-6} M) cAMP was mitogenic for lymphocytes, while cGMP had no effect. Very low ($\leq 10^{-10}$ M) or considerably higher (1 to 5×10^{-6} M) concentrations of cGMP however stimulated cAMP and, probably through it, stimulated DNA synthesis and mitosis (Whitfield *et al.*, 1971). Hadden *et al.* (1972) also found opposite effects of the two cyclic nucleotides on growth regulation: induction of mitogenesis by Con A or PHA was accompanied by a rise in intracellular cGMP, but no change in cAMP, and the authors concluded that cGMP would be the messenger delivering mitogenic signals, whereas cAMP at high concentrations would on the contrary be responsible for inhibiting cellular division and maintaining cells in the steady state.

The only possible conclusion at this time is that if these two cyclic nucleotides play a role in growth control, and indications are that they do, then this growth regulation must depend on a very delicate balance between them. It is obvious that more cell types will now have to be tested. Considerably more work needs to be done in order to understand what affects this balance between cAMP and cGMP, and mostly, to try to ascertain what relevance these observations may have to *in vivo* systems.

One recent clinical observation indicates that the relative amounts of cAMP and cGMP may indeed be of importance for regulation of cell division in the organism: Vorhees *et al.* (1973a,b) found that in psoriasis the affected epithelial cells had very low levels of cAMP, but normal or high levels of cGMP, and he suggested that in this case hydrolysis of cAMP by phosphodiesterase was activated by cGMP. And in fact, an unguent containing the phosphodiesterase inhibitor papaverin was found to be effective in raising the cellular levels of cAMP and succeeded in moderating the inordinate cell growth. If similar effects can be obtained in other systems, they would be indications in favor of a regulating role for cGMP, possibly acting through cAMP, but too few data are at present available to allow such conclusions. And even if these cyclic nucleotides are unequivocally found to be implicated in the regulation of cell growth, the question of their site and mode of action will still have to be answered.

b. Further Considerations about Growth-Stimulating Agents and about Intercellular Contacts. The concluding remarks of this section will

have to remain inconclusive, since we have by no means a precise view of the way in which growth is regulated at a molecular level.

While we are waiting for a pleiotypic mediator or a triggering agent to be found and its mode of action to be elucidated, it might not be amiss to consider again some of the effects of the growth-stimulating agents that were discussed in Section IV,A. These agents all seem to act, directly or indirectly, on the cell surface, which they can modify in a number of ways, and these alterations in cell surface properties certainly play an important role, not only for cell-to-cell contacts and interrelationships, but for the internal economy of each cell. Stimulated cells, as well as transformed cells, were shown to have an increased capacity for transport of certain nutrients and ions, and of the RNA precursors adenine and uridine. This is probably more than a trivial correlation, for the ability to take up certain substances and to maintain them at a critical concentration may well be determinant for a cell's ability to undergo division. Pardee had already in 1964 stressed that changes in uptake capacity might be important in the regulation of cell growth, and a similar conclusion was reached again by Holley (1972), who considered that cell growth was regulated by critical internal concentrations of nutrients. And we might also recall that Castor (1969: see Section IV,A,1) considered the membrane as a transducer in the mechanism of growth regulation, with the double function of sensing conditions in the environment and initiating the proper metabolic response in the cell, and of controlling the uptake of mitosis-promoting substances. It should be clear from what has been said earlier in this review (see in particular Section III) that cell surface membranes are not fixed and rigid entities, but that they undergo considerable variations in structure and properties as a result of a number of agents or of changes in their environment, and this plasticity of the membrane enables it to exert very fine controls over the cell's metabolism.

The earlier studies on growth control stressed contact inhibition as a primary factor, until it was recognized that density-dependent inhibition was a more appropriate term (see Section IV,A,1). The question of contacts between cells should, however, not be altogether dismissed, for it is quite possible that "messages" may be transferred between cells through intercellular contacts; and the fact that some neoplastically transformed cells, owing to their altered surface membranes, no longer have normal contacts with each other or with their substratum or surrounding tissues may also be a factor in their aberrant behavior. The improved resolution recently brought by scanning electron microscopy has allowed us to see that cells can be covered with impressive appendages (filipodia and microvilli in particular; see Section V,E), and these may well be found to play very important roles for nutrient uptake and

for intercellular communication. Porter *et al.* (1973) have found that microvilli were particularly numerous in rounded cells, and Wallach and collaborators (Lin *et al.*, 1973) have observed microvilli in lymphocytes, i.e., in suspension cells, and these processes may be the means for cells which are no longer in close contact to communicate.

These questions of cell permeability and of cellular contacts may seem simplistic as compared with molecular models and intracellular mediators; and indeed, we do not deny the importance of searching for a proper mediator, but we also feel that some of the basic factors which we have just mentioned should not be overlooked.

V. Surface Changes in the Cell Cycle

We already briefly mentioned in Section II,A that normal cells bind more lectin during the time that they are in mitosis than they do at other times (Fox *et al.*, 1971; Shoham and Sachs, 1972). This is indicative of a transitory change occurring in the membrane of these cells, and it is of obvious interest to see how this is brought about, and to what extent it is related to the permanent change caused by neoplastic transformation. Some of the other changes occurring in the membrane during the cell cycle will be described first.

A. Antigens

Blood group H specificity in HeLa cells was found by Kuhns and Bramson (1968) to increase considerably at mitosis, as shown by specific agglutinations. Thomas (1971), using immunofluorescence, observed cyclic variations in the expression of B and H blood group specificities on mastocytoma cells, and on lymphocytes stimulated by PHA: Contact-inhibited cells and cells in early G_1 were B^- and H^+, but commitment to division, such as after PHA stimulation, resulted in appearance of B specificity, and at mitosis cells were labeled with both B and H antibodies (after going through a transitory period, during DNA synthesis and G_2, where the H specificity was not expressed). Just how this antigen modulation took place is not clear, but the appearance of B positivity did seem to correlate with initiation of events leading to mitosis, and in transformed cells B antigen was permanently expressed. Thomas also commented on the fact that at least in several cell types H specificity and H-2 alloantigens (see below) seemed to vary concomitantly, whereas B specificity varied inversely, except at mitosis.

Cikes and Friberg (Cikes, 1970; Cikes and Friberg, 1971) used fluorescent antibodies to study the expression of surface antigens at different times during the cell cycle of MLV-induced mouse lymphoma cells. They found cyclic variations in fluorescence, with maximal expres-

sion of both cellular and viral antigens during the early part of the G_1 period; and the coordinated expression of host and virally determined antigens indicated their regulation by some common, but as yet not determined mechanism.

Cyclic variations of the histocompatibility H-2 antigen were also observed by Pasternak *et al.* (1971) with a cytotoxicity test. In accord with Cikes they found that in mouse mastocytoma cells H-2 antigens were maximally expressed in the early G_1 period, then decreased during the period of DNA synthesis, to increase again as the cells went through G_2 and approached mitosis.

B. Changes in Membrane Glycoproteins and Sialic Acid

Growing cells have been shown (Warren and Glick, 1968) to synthesize new cell surface material but to have relatively little turnover, whereas nongrowing cells have a high rate of turnover, with synthesis and degradation of membrane glycoproteins balancing each other. In KB cells synchronized by a double thymidine block Gerner *et al.* (1970) found that lipids, carbohydrates, and proteins were synthesized throughout the cell cycle, but that there was an increased rate of membrane synthesis in the early G_1 phase, just after division, then after a few hours rates returned to what they were before division. Onodera and Sheinin (1970) pulse-labeled synchronized cells with ^{14}C-labeled glucosamine and analyzed a surface component which was released when the cells were treated for 10 minutes with 0.1% trypsin (which left them fully viable). The composition of this surface material, which was partially purified by DEAE-cellulose chromatography, was different in 3T3 and in SV40-transformed 3T3 cells. Synthesis of this material was high just after the cells had been subcultured with trypsin and replated, and again, as in the work cited above, just after mitosis, i.e., as cells entered the G_1 phase. It is interesting to note that fucose and sialic acid-containing glycopeptides which are characteristic of transformed cells, although also found in small amounts in normal cells (Buck *et al.*, 1971; and see Section VI,A), were transiently present in larger amounts in normal cells while they were in mitosis (Glick and Buck, 1973).

In 1966 Mayhew reported an increase in the electrophoretic mobility of osteosarcoma cells at mitosis; this was thought to be due to the appearance of neuraminic acid on the cell surface, since the transient increase was abolished by sialidase treatment. Kraemer (1967), however, noted that in CHO cells the onset of mitosis was accompanied by a decrease in cell size; when sialic acid (assayed colorimetrically) was measured as a function of cell surface area, it increased gradually and only slightly as the cells proceeded through the cycle, and with no abrupt

change at mitosis. He concluded that the electrophoretic mobility increase observed by Mayhew at mitosis might be due to conformational rearrangements on the cell surface during the cycle. Quite different are the conclusions of Rosenberg and Einstein (1972), who measured in human lymphoid cells a definite increase in sialidase-removable neuraminic acid just prior to mitosis, and a decrease thereafter. They ascribed this increase to a synthesis of membrane sialic acid during the late G_2 period of the cycle (or possibly a reduced degradation of sialoproteins at that time). Rosenberg and Einstein followed the sialic acid content of Raji lymphoid cells through the cell cycle, but they also found that sialic acid levels could vary as much as 5 times between different lymphoid cell lines. Kraemer used CHO cells, also grown in suspension cultures, but which may have a different metabolism of cell surface glycoproteins. More cells will have to be studied before the discrepancy between the results from these two laboratories can be simply ascribed to the difference in cell type studied.

C. Transport

The involvement of transport alterations in cellular growth control was proposed by Pardee (1964) several years ago. In 1969 he could show that transport activity was at a minimum in nondividing cells, and that it increased rapidly after stimulation by serum (Cunningham and Pardee, 1969; see also Section IV,A,2). In synchronized CHO cells Sander and Pardee (1972) observed that rates of transport of aminoisobutyric acid, thymidine, and uridine were low at mitosis and in early G_1, but then increased as the cells progressed through the cycle, and they proposed that this increased transport might be correlated with the commitment of the cell to divide: i.e., this would be yet another way in which the membrane could be involved in growth. It is interesting to note that this is one case where mitotic cells differ from transformed cells, since in the latter transport rates are usually higher.

Cation content and flux has also been observed to change during the cell cycle. Jung and Rothstein (1967) observed that in synchronized lymphoma cells the net potassium content decreased during G_1 and the early S phase, then returned to normal. The Na^+ fluxes were more complex, and particularly remarkable was an abrupt drop in Na^+ at mitosis. The fluxes of Na^+ were partially opposite to those of K^+, but a net deficit of cations was observed during the early S period, and the authors suggested that this must be compensated for by other osmotically active substances, since no cyclic changes in cell volume were seen. Orr *et al.* (1972) observed that growth of BHK cells was inhibited when they were exposed to a high internal concentration of potassium (114 m*M*):

DNA synthesis was considerably inhibited, whereas the effect on RNA synthesis was much smaller, and the cells were essentially blocked in the mid-G_1 phase. Interestingly, there was no appreciable change in intercellular K^+, or in ATP, content of cells maintained in the high K^+ medium, which indicates that the inhibition of DNA synthesis must have been effected through some changes in the cell membrane, whose potential would then play a role in growth control. It might be added that transformed cells were affected in the same way by the high K^+ medium, and that for both types of cells the effect was reversible after return to normal medium.

D. Cyclic AMP

Despite the fact that low levels of cAMP were observed during mitosis of fibroblasts (Section IV,B,3) the enzymatic activity of both basal and epinephrine-stimulated adenylate cyclase was found to be greatly increased during mitosis (Makman and Klein, 1972). The enzymatic fluctuation was interpreted as a variation in the availability of cyclase membrane receptors which would be uncovered (or possibly synthesized) at mitosis; such considerations, however, are still very hypothetical.

E. Morphological Changes

A freeze-cleavage electron microscope study of CHO and L cells by Scott *et al.* (1971) showed changes in the intramembranous protein particles during the cell cycle. Particularly notable was a sharp decrease in the density of these particles in late telophase and early G_1, but which was immediately followed by an increase during mid and late G_1, so that the level was back to normal before onset of DNA synthesis. These morphological observations are interesting in that they tie in quite well with the data of Gerner *et al.* (1970; see Section V,B), who found an increased rate of synthesis of membrane components in the early G_1 phase.

Follett and Goldman (1970) observed, by an electron microscope replica technique, that mitotic fibroblasts, as well as cells treated by trypsin, were covered by an abundance of microvilli, absent from non-dividing and flattened cells. They attributed the appearance of these structures to the rounding process, but there may also be significance in their presence in states of the cells where they are similar to transformed cells. Recently extensive studies of Porter *et al.* (1973) and of Rubin and Everhart (1973) by scanning electron microscopy revealed many details of surface structure which cannot be observed by ordinary procedures. They followed changes in cell shape and processes occurring

as CHO cells progressed through the cycle. Interphase cells in the S phase were flat and had few processes, except for ruffles, whereas cells in G_1 and G_2 were characterized by numerous microvilli and blebs, as well as ruffles; and mitotic cells, which are rounded, were covered with microvilli and were attached to the supporting substrate by long, slender filipodia. These observations were all made on cells growing at fairly high densities, and the change in the various processes must have been involved in intercellular contacts, as CHO cells growing at low density did not show these remarkable cyclic changes, but remained fairly rounded and blebbed. In contrast with the CHO cells, normal, nontransformed cells were flat and devoid of processes. The authors suggested that the microvilli might not only be involved in intercellular contacts, but also be related to the increased transport seen in transformed cells during the G_1 period (see Section V,C), and might possibly also bear agglutinin receptors in a conformation or density particularly favorable for agglutination (cf. their absence in nontransformed cells and their prominence in mitotic cells and, as shown by Follett and Goldman, on trypsinized, agglutinable fibroblasts). It will certainly be interesting to follow these studies and to find out what the role of the protuberances are for intercellular contacts, and to what extent they may be involved in agglutination.

F. Lectin Binding during Mitosis

Our initial observations that normal cells in mitosis could be labeled by fluorescent WGA while transformed cells could be labeled at all times (Fox *et al.*, 1971) were extended by a careful study of fluorescent Con A binding. When 3T3 or Py3T3 cells were incubated with subagglutinating doses of fluorescein- or rhodamine-Con A at 4°C, only mitotic cells were labeled, but fluorescent cells were observed during all stages of mitosis. This increased binding was shown to be indeed due to mitosis, not trivially to a possible concentration of sites due to rounding up of the cells occurring during mitosis, because interphase cells rounded by treatment with EDTA were not labeled by the fluorescent agglutinin (Turner and Burger, 1974). We emphasize again that binding assays must be performed under carefully controlled conditions: when the binding was done at 37°C, or with higher concentrations of Con A, all cells were unspecifically labeled (R. S. Turner, unpublished observations, 1973). Shoham and Sachs (1972) confirmed an increased binding of fluorescent Con A (also using very small amounts of lectin) to mitotic normal cells and to interphase transformed cells. On the other hand, they observed considerably less binding to mitotic than to interphase transformed cells after 1 minute of labeling; they were, however, equally

labeled after 5 minutes. The difference between these results may well be due to the different cells used (hamster embryo fibroblasts by Shoham and Sachs; mouse 3T3 fibroblasts by us), or it may reflect a difference in the binding techniques (Shoham and Sachs incubated at 37°C and measured the increase of labeled cells with time, from 0.5 to 20 minutes, whereas we incubated at 4°C and counted the number of labeled cells after 15 minutes). Glick and Buck (1973) have recently reported agglutination by Con A of BHK cells blocked in metaphase, in agreement with agglutinability during mitosis found for normal 3T3 cells (K. D. Noonan, unpublished, 1973) and hamster embryo fibroblasts (Shoham and Sachs, 1972).

We undertook a quantitative study of radioactive Con A binding to 3T3 fibroblasts during the cell cycle (Noonan and Burger, 1973b; Noonan *et al.*, 1973a). Mitotic cells were found to bind at least 3 times more lectin than cells in other phases of the cycle; however, if cells were blocked in metaphase by colchicine, the same transient increase in Con A binding was observed as in nontreated cells, showing that after the proper time the surface had regained its normal, interphase properties, even though the nucleus was blocked in metaphase. Lectin binding was not inhibited by cycloheximide added at that time, but protein synthesis was required during the late stage of DNA synthesis (late S phase) for increased binding at mitosis to take place. Interestingly, in transformed cells (SV-3T3) protein synthesis was needed in the early, rather than the late, S phase for the increase in binding, thus indicating that transformation results in a change in the order of synthesis of at least certain proteins, as proposed in the model by LeVine and Burger (1972). Since increased binding does not require protein synthesis during mitosis, and since the cell surface can return to its nonmitotic structure while the nucleus is blocked in mitosis, membrane alterations during mitosis are apparently not controlled by nuclear events occurring simultaneously; our experiments would indicate, however, that the alterations would be predetermined during the S phase.

VI. Survey of Chemical Differences Found in the Surfaces of Transformed Cells

This review has dealt with several biological differences between the surfaces of normal and tumor cells; neoplastic transformation was shown to be accompanied by alterations in antigenicity, in adhesiveness, in surface charge, in transport properties, and in a broad sense in intercellular contacts. Basic to these alterations must be differences in the chemical makeup of the cell surface, a subject which has been considerably investigated, yet still leaving many controversies unresolved. A major dif-

ficulty for the study of membrane components is the isolation of pure membranes. It is also necessary to distinguish between properties specifically due to transformation and those due to growth and cell density. A complete discussion on the chemistry of normal and transformed cell surfaces would be beyond the scope of this paper, and since this has been reviewed recently, we shall limit ourselves to listing some of the main differences between membrane components of normal and of transformed cells. For further details the reader is referred to previous reviews (Burger, 1971a; Emmelot, 1973; Herschman, 1972; Hughes, 1973; Meyer, 1971), and to the original papers reviewed here.

We shall confine ourselves to the cell surface membrane, as that has been most extensively studied. One should nevertheless be aware that the fuzzy coat may also play a role in cellular interactions *in vivo,* since it is external to the cytoplasmic membrane; it is difficult, however, to obtain it undamaged in cells harvested from culture plates. A preliminary study by Chiarugi and Urbano (1973) indicates that material released from cells with EDTA was made up of glycoproteins and mucopolysaccharides, possibly associated with phospholipids; little difference in composition was found between this cell coat material from BHK cells and polyoma- or RSV-transformed BHK. Onodera and Sheinin (1970) did, however, find differences in the glycopeptides isolated from a trypsin-labile surface component, probably membrane-associated, obtained from 3T3 or SV-3T3 cells.

A. Glycoproteins

A number of differences in membrane glycoproteins have been found, not only between normal and transformed cells, but among various transformants of a given cell line (Buck *et al.*, 1971; Sheinin and Onodera, 1972). Several cases have been reported where membrane glycoproteins of transformed cells were less glycosylated than those of normal cells (Chiarugi and Urbano, 1972; Makita and Seyama, 1971; Meezan *et al.*, 1969; Wu *et al.*, 1969). However, transformed BHK cells have been reported to excrete into the medium a more glycosylated glycopeptide than did normal cells (Chiarugi and Urbano, 1972), an indication of the malfunctioning of glycosyltransferases in transformed cells, as also suggested by Meezan and Wu, and by other investigators (see Section VI,E). Sakiyama and Burge (1972) found little difference in glycopeptide patterns between normal and transformed 3T3 cells, in spite of decreased carbohydrate levels, indicating a reduced density of glycoproteins on the membrane of transformed cells.

Warren, Glick, and their associates have probably come closest to finding a "tumor characteristic" glycoprotein, as in Sephadex chromatography of membrane glycopeptides they have consistently found those

from transformed cells to contain some earlier eluting material present only in traces in normal cells (Buck *et al.*, 1971; Warren *et al.*, 1973). This material was present in cells transformed by both DNA and RNA viruses, and, as seen in Section V, its levels in normal cells were elevated during mitosis (Glick and Buck, 1973). It was characterized by its content of fucose and sialic acid, and transformed cells had elevated levels of a specific sialyl transferase which transferred neuraminic acid from CMP-neuraminic acid to a fucose-containing acceptor on the cell surface (Glick and Buck, 1973; Warren *et al.*, 1973).

Evidence for alterations in glycosyltransferase activity in transformed cells has come from several other laboratories, this will be discussed in Section VI,E. The decreased complexity of the glycoproteins of transformed cells can also be partly accounted for by the elevated levels of certain glycosidases (Bosmann, 1969, 1972b; Sela *et al.*, 1970) and of membrane proteases observed in transformed cells (see Section IV,A,5).

B. Sialic Acid

In spite of the elevated amounts of the sialic acid-containing glycopeptide of Warren, the total amount of sialic acid (which includes that present in both glycolipids and glycoproteins) has usually been found to be lower in transformed cells (Grimes, 1970, 1973; McClelland and Bridges, 1973; Makita and Seyama, 1971; Meezan *et al.*, 1969; Ohta *et al.*, 1968; Wu *et al.*, 1969). The levels of neuraminidase-labile sialic acid were found to decrease with the progression and increase in tumorigenicity of a rat ascites hepatoma (Smith and Walborg, 1972). "Flat" variants derived from transformed cells showed partial recovery of sialic acid content (Grimes, 1973), and a direct relationship was found between the degree of contact inhibition of cells and their sialic acid content (Culp *et al.*, 1971).

C. Glycolipids

In 1968 Hakomori and Murakami reported that transformed cells had decreased levels of the higher sphingolipid hematoside, but increased levels of less glycosylated precursors. After observing several cases where the glycolipid pattern of growing normal cells was similar to that of transformed cells, i.e., reduction or absence of higher gangliosides and accumulation of their precursors, Hakomori (1970) suggested that transformed cells lacked the "glycosyl extension response" by which normal cells at confluence added sialic acid or galactose residues to their less glycosylated glycolipids (see also Hakomori, 1971; Kijimoto and Hakomori, 1971, 1972). Interestingly, Hakomori and his co-workers (1968) found that a mild trypsin treatment of normal cells uncovered cryptic glycolipids as well as cryptic lectin sites (cf. Sections II,B and III,B,

and see Forssman antigen, below). Reduction in higher gangliosides and accumulation of precursors was also observed by Mora *et al.* (1971) in virally transformed 3T3 cells, whereas "flat" variants had recovered to a great degree the higher ganglioside content.

Sakiyama and Robbins (1973) and Sakiyama *et al.* (1972) found that NIL hamster fibroblasts at confluency synthesized larger amounts of trihexosyl-ceramide and gangliosides than did growing cells. Hematoside (*N*-acetylneuraminyl-galactosyl-glucosyl-ceramide) was however present in both normal and transformed cells, and its level was not correlated with cell density. No general correlation was found between tumorigenicity and the complexity of the glycolipid pattern; however, inability of cells at confluency to synthesize increased amounts of glycolipids was correlated with tumorigenicity (Sakiyama and Robbins, 1973). Critchley *et al.* (1973) have recently reported that these "density-dependent" glycolipids, which are virtually absent from transformed cells, are present at the cell surface, but are not exclusively confined to it.

A glycolipid which has received particular attention is the Forssman antigen, whose structure was established by Siddiqui and Hakomori (1971) and confirmed by Sakiyama *et al.* (1972). Forssman antigenicity was shown (Fogel and Sachs, 1962; Makita and Seyama, 1971; O'Neill, 1968) to appear as a result of transformation of BHK cells, but Fogel and Sachs (1962, 1964) observed that it could appear also in normal cells as a result of *in vitro* culture. We observed (Burger, 1971c; Noonan and Burger, 1971) that, as in the case of lectin receptors, normal BHK cells did have Forssman antigen reactivity, but in cryptic form that could be revealed by mild trypsin treatment. A similar observation was made by Makita and Seyama (1971). In NIL hamster fibroblasts, however, Forssman antigen was present in the nontransformed cells only (Hakomori and Kijimoto, 1972; Kijimoto and Hakomori, 1972; Sakiyama *et al.*, 1972), and this glycolipid was among the "density-dependent" glycolipids which were found to increase at confluency. Hakomori and Kijimoto (1972) observed, however, that, in spite of an increased net synthesis of the antigen at confluency, the Forssman reactivity (as measured with specific antiserum) decreased at that time, and it was postulated that the antigen probably became masked or otherwise blocked at that time.

Few data are available on the neutral lipid composition of tumor cell membranes, and there seems to be considerable variation between different species and different types of tumors, and no typical alteration related to neoplastic transformation. Van Hoeven and Emmelot (1972) found elevated levels of cholesterol and variable content of other lipids in hepatoma membranes; little overall difference in phospholipid com-

position of these membranes was detected by Bergelson *et al.* (1970). A trend toward unsaturation of phospholipids and an elevated oleate: stearate ratio was observed in various tumor cell membranes by both Veerkamp *et al.* (1962) and Selkirk *et al.* (1971). Since membrane lipids play such an important role in the flexibility of the membrane and the mobility of the membrane components, it would be of obvious interest to have more comparative studies on the lipid composition and in particular on the degree of unsaturation of fatty acids of normal and transformed cells from the same origin.

D. Embryonic Antigens or Oncofetal Antigens

Tumor tissues often express embryonal antigens which are absent from normal adult tissues (Abelev, 1971; Abelev *et al.*, 1963; Alpert *et al.*, 1968; Gold and Freedman, 1965a,b; for review, see Alexander, 1972). The carcinoembryonic antigen of the colon discovered by Gold and Freedman (1965a,b) and the α-fetoprotein found by Abelev in hepatomas (see Abelev, 1971, for review) are probably the best characterized chemically, but several other oncofetal antigens have been detected immunologically in other tissues. Recently Ting *et al.* (1972) found several fetal antigenic specificities in cells transformed *in vitro* by SV40 or polyoma virus; embryonic antigen expression has also been found in chemically induced rat tumors (Baldwin *et al.*, 1972a,b; Iype *et al.*, 1973). It should also be mentioned in this context that embryonic cells are agglutinable by Con A (Moscona, 1971; Noonan and Burger, 1971; Steinberg and Gepner, 1973; Weiser, 1972). The presence of oncofetal antigens on tumor cells has been considered to be an example of their dedifferentiated character; it is assumed that synthesis of these antigens is repressed in adult tissues, but it is not known whether normal adult cells might possess certain cryptic oncofetal antigens. The finding by Gonano *et al.* (1973) that phenylalanine-tRNA from fetal rat liver and from a rat hepatoma were similar, but different from that of adult rat liver, may be of interest in this context, if this proves to be more than an isolated correlation.

E. Transferases

The changes in glycolipids and glycoproteins described in the previous sections can be attributed at least partly to alterations in glycosyltransferases. A transferase defect was suggested to Wu *et al.* (1969) and Meezan *et al.* (1969) when they found that in spite of a decreased carbohydrate content of membrane glycoproteins and glycolipids in transformed cells the levels of nucleotide sugars were normal. Some of the first measurements of several glycosyltransferase activities in transformed cells came

from Roseman's group when Den *et al.* (1971) found reduced levels of sialyl-, galactosyl-, and glucosaminyltransferases in homogenates from polyoma-transformed BHK cells. Grimes (1970, 1973) measured transfer of sialic acid and of fucose and galactose from their respective sugar nucleotides to the proper glycoprotein acceptors; sialyltransferase was consistently lower in transformed cells, while fucose and galactose transfer were either lower or normal. Glycosyltransferase defects have also been reported by Mora *et al.* (1971) and by Hakomori's group (see above, glycolipids). In contrast, elevated glycosyltransferase activity has been reported by Bosmann (1972a) in cells from confluent cultures of several virally transformed 3T3 lines (transferase levels in sparse cultures were the same, however, in normal and in transformed cells). The reason for the discrepancy between the results from these different groups is not clear, but it might be because Bosmann measured surface enzyme activities since he used whole cells, whereas Grimes and Mora used cell-free homogenates. As noted above, Warren's group (Buck *et al.*, 1971; Warren *et al.*, 1973) found in transformed cells increased sialic acid transfer to a fucose-containing glycopeptide, but this was considered to be a specific transferase with an acceptor specificity different from that of other sialyltransferases.

Roseman proposed in 1970 that glycosyltransferases might play an important role in intercellular adhesions; according to his model, adhesions would result from intercellular glycosylation, where glycosyltransferases on the surface of one cell would bind to and glycosylate an acceptor on another cell. One piece of evidence in support of this model was the finding by Roth and White (1972) that whole cells could incorporate galactose from UDP-galactose into their surface components; the nucleotide sugar was not taken up into the cells, and radioactive galactose became attached to components on the cell periphery, indicating that both enzyme and substrate were on the cell surface. Evidence for intercellular glycosylation was deduced from the fact that the rate and extent of the reaction were greatest when it took place in a cell suspension, under conditions allowing for maximal contact between cells. According to Roseman's theory, normal cells which have the proper complement of transferases and acceptors can adhere strongly to each other, whereas transformed cells cannot bind to each other strongly because of their defects in certain transferases. Roth and White (1972) also indicated that, in contrast with normal cells where glycosylations are essentially intercellular, glycosyltransferases on tumor cells would also be able to glycosylate substrates on the same cell, presumably because enzyme and substrate are closer to each other on tumor than on normal cells. This proximity of molecules might be explained by their

increased mobility in the more fluid membrane of transformed cells (see Section III,C), and the ability not to depend on intercellular glycosylations would be of obvious advantage to transformed cells that do not have normal intercellular contacts. Roseman believes that such intercellular glycosylations can explain many biological phenomena involving cellular interactions. It is, however, not possible to subscribe fully to this theory, nor to make predictions concerning cellular interactions *in vivo* before we have more precise knowledge about the structure of the cell membrane and the pathways for its biosynthesis.

VII. Conclusions

The last few years have led to new insights into the structure of the cell surface. Particularly valuable is the concept of the membrane as a dynamic and fluid entity, as its plasticity makes it more amenable to alterations such as those occurring during neoplastic transformation. Too little is yet known, however, about the nature and the mechanism of these alterations; there is still a great need for careful chemical and biochemical studies on the membranes of homologous normal and transformed cells before meaningful conclusions can be drawn about their alterations. Some evidence points among others to the revealing of cryptic components (antigens, glycolipids, lectin receptors) during transformation, which leads to the following questions: By which process are the components uncovered? What determines and regulates this uncovering? Normal, nongrowing cells were seen to have a high rate of membrane turnover (Warren and Glick, 1968), where synthesis and degradation of surface components are in balance. Transformed cells do not exhibit this balance; we have shown that they have elevated levels of surface proteases, and we have suggested (Borek *et al.*, 1973; Talmadge *et al.*, 1974) that transformation might initiate a chain of reactions involving proteases and possibly other membrane components; in such a system the membrane would be both site of action and effector, since its modification in the course of this cascade reaction would ultimately lead to triggering of DNA synthesis and cell division.

Agglutinins enable us to monitor some of these cell surface alterations, and the discovery (Fox *et al.*, 1971; Shoham and Sachs, 1972) that the surface of normal cells during mitosis is in several ways analogous to that of transformed cells is enlightening, as it suggests the existence in the cell cycle of a critical switching point: transformation can operate like the throwing of a switch which sidetracks the cell from completion of the normal cycle with eventual shutoff in G_1, and keeps it oriented toward continual division (Burger, 1973; Burger *et al.*, 1971). We still do not understand how this switch operates, but it is important that

there is a critical point in the cell cycle when any normal cell can either be steered toward quiescence or switched over toward malignant growth and transformation. Evidence from the studies we have described in this review indicates that the cell membrane plays a role in this modulation of growth control, but much more research is still needed before it can definitively be decided whether the membrane alterations observed are only correlates or whether they do have a causal role.

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PRINCIPLES OF IMMUNOLOGICAL TOLERANCE AND IMMUNOCYTE RECEPTOR BLOCKADE

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I. Introduction

The original definition of immunological tolerance as a specific, central failure of the immune response to an antigen, brought about through prior exposure of the lymphoid system to that antigen (Medawar, 1956) still serves to contain most of the different phenomena studied under the heading of tolerance or paralysis by present-day immunologists. Nevertheless, there is a danger in being locked into an overly traditional approach to the tolerance problem. The early thinking (Burnet and Fenner, 1949; Medawar, 1956; Lederberg, 1959) was dominated by the concept that the normal reaction of an immunocyte to an encounter with an antigenic "nonself" molecule was the initiation of a cascading immune response. The paradigm was the appearance, after a short latent period, of exponentially increasing amounts of antibody following a single injection of antigen given in particulate or insolubilized form. If that were, in fact, the norm, then it followed that the failure of the body to form antibodies against autologous components required specialized

mechanisms of an intricate, and perhaps unique, nature. By the same token, the "typical" infections which preoccupied classical immunologists were diseases like smallpox or diphtheria, where the immune system either "won," leaving the patient cured and immune, or "lost," resulting in death of the patient. Relatively little attention was paid, either at the experimental level, to antigens which failed to elicit a convincing immune response in the adult animal or, at the interpretative level, to host–parasite relationships where chronic infection coexisted with modest or minimal immune responses. Few workers stopped to ponder, for example, why the levels of serum antibody to the commensal microorganisms of the gut were uniformly quite low.

We now know that union of a lymphocyte surface receptor with an appropriate antigenic determinant by no means always results in immunocyte activation. This insight immediately prompts the question of what other trigger or signal is needed for the initiation of immunity (Cohn, 1971) and what the effects of a "sterile" encounter might be for the immunocyte concerned (Nossal, 1969). As soon as one admits the possibility that an encounter with antigen may block or even eliminate a lymphocyte, it becomes clear that the "decision" which the immune system encountering an antigen must make between immunity and tolerance is nothing more than the summation of all the individual decisions made, over a period of time, by all its reactive lymphocytes. This effectively demythologizes immunological tolerance and shows it as just one facet of the wide problem of regulation of the immune response, with its myriad of control processes and feedback loops. Nothing has done more to accelerate this system or "network" (Jerne, 1974) approach to immunology than recent developments in tumor immunity. In most cancer induction models, the simplistic notion of an exponentially increasing immune avalanche overwhelming the antigen lets us down. Rather, we have to analyze an ecological puzzle in which the inherent proliferative drive of the tumor is impeded, but only partially and diminishingly, by a strictly limited immune response. For this attempt to offer any hope of success, we must understand the effects of steadily increasing levels of antigen, present in both cellular and soluble form, on the responding lymphoid population, and, in particular, the effects on immunocytes of soluble complexes of antigen and antibody (Sjögren *et al.*, 1971; Feldmann and Diener, 1971; Feldmann and Nossal, 1972). The development of some degree of immunological tolerance is only one of many factors that frequently allow the tumor to "win" over the immune system.

It is clear that growing tumors, releasing chemically undefined and unquantitated antigens, do not provide the ideal model for working out these basic rules. In fact, students of immunological tolerance have

increasingly sought to use pure haptens, proteins, or carbohydrates for their work. Unfortunately, however, the bulk of the monumental body of work on tolerance was done before the influence of the thymus-derived (T) lymphocyte system on antibody production by bone marrow-derived (B) lymphocytes was realized. Therefore, all the classic tolerance experiments are in the midst of a somewhat agonizing reappraisal. There is bewildering profusion of experimental designs, and the casual reader could be excused for being confused by apparently contradictory tolerance findings. Yet, immunological tolerance is important to cancer researchers; it will be the chief purpose of this review to provide a general framework supported by selected experiments. Several excellent recent sources are available for a fuller summary of the literature (Ada and Cooper, 1973; Weigle, 1973; Katz and Benacerraf, 1974; Mitchell, 1974b).

II. Background Concepts about the Mechanism of Action of Antigens

It is helpful to set down the preconceptions about the immune system that underlie the analysis of tolerance which will follow. While many of the "dogmas" remain without rigorous proof, most of them have passed sufficiently into the folklore of modern immunology not to require detailed documentation (e.g., Nossal and Ada, 1971) in this brief paper.

1. The induction of an immune response depends on macromolecular recognition. An antigen, possibly after complex processing steps, encounters receptors of one or more chemical types on the surface of a lymphocyte. These receptors belong to a family or families of molecules endowed with the property of uniting with the whole universe of molecules termed antigens. The genetic capacity to synthesize the receptor family is inherent in the lymphocyte population, and, though large, is not infinite. The finite population of receptors is charged with the responsibility of recognizing the larger population of antigens present in nature or synthesized in the laboratory.

2. The act of receptor-antigen recognition is not an all-or-none phenomenon. Rather, the association constants of reactions which clearly belong into the category of immune recognition vary over a range of at least one millionfold. A given recognition site may recognize any one of a large number of antigenic determinants, and a given antigenic determinant may be recognized by any one of a large number of receptor molecules, but with varying affinities of binding. Failure to recognize this single, simple concept has caused much of the confusion in the tolerance literature.

3. Whether a given antigenic determinant is immunogenic *vis à vis*

a given lymphocyte receptor, in the sense of triggering that lymphocyte to undergo the various metabolic changes involved in immune activation, depends not only on the value of the K_a, which is indeed an important variable, but on a variety of operational circumstances, which include the valency and molar concentration of the antigen; the concentration in the local microenvironment of the lymphocyte-antigen interaction of certain poorly understood cofactors in activation, including products of already activated lymphocytes or macrophages; and, less certainly, the differentiation state or maturity of that cell.

4. While the introduction of an antigen has many immediate effects, largely related to the existence in the body of specific recognition molecules either on cell surfaces or in the serum, the induction of an immune response as usually defined embraces a longer time dimension involving days and even weeks. The lymphocyte activated by antigen embarks on a series of division and differentiation steps which result in the production of a clone of specialized effector cells that mediate the immune response. Remarkably little is known about the details of these proliferation processes, but the strong likelihood exists that antigen plays a continuing inductive role during the clonal expansion, rather than acting solely as an initiator.

5. The lymphocytes which initiate immune responses fall into a number of functional categories, which are just beginning to be defined with precision. The most important subdivision is into thymus-derived or T lymphocytes, versus bone marrow-derived, "bursal equivalent," or B lymphocytes. The latter are the precursors of antibody-forming cells. The former initiate cell-mediated immune phenomena, such as delayed hypersensitivity, graft rejection, graft-versus-host reactions and cytotoxic killing of antigenic cells independent of added complement or antibody. T cells also act as "helper" cells in the initiation and amplification of B cell responses to many antigens.

6. B cell receptors for antigen are immunoglobulin (Ig) molecules. The operative portions in antigen recognition are combining sites the specificity of which depend on the amino acid sequences of the variable (V) portions of Ig light and heavy chains. These in turn are coded for by V_κ, V_λ, and V_H genes possessed by the receptor-bearing cells. While a given B cell may carry more than one class of heavy chain on its surface, the evidence is strong, as recently summarized (Nossal, 1974), that each B cell only expresses one unique type of combining specificity, i.e., a single pair of Ig V regions, one from either κ or λ light chains, and one coded for by the heavy-chain V gene, common to all heavy chains. Activation of a B cell results in the secretion at high rate by its clonal

progeny of antibody molecules bearing the combining specificity of the original receptor. There is some evidence that the primordial receptor in most species is IgM and that certain lymphocytes undergo an IgM to IgG transition after activation (Nossal *et al.*, 1964, 1971). Whether that is so or not, it is clear that the production of IgG antibody is intimately dependent on T cell activation under most experimental circumstances.

7. T cells do not secrete large quantities of antibody. Their defense function against antigenic microorganisms or cells depends on close contact between antigen and lymphocyte. It is generally believed that such close contact is mediated by an interaction between T lymphocyte receptors and antigenic determinants. Various pharmacologically active substances, collectively termed lymphokines, are released on such contact, and these are profoundly important in cellular immunity. They promote chemotaxis, cause macrophage activation, and potentiate B cell responses to antigen. The molecular mechanisms by which T lymphocytes kill antigenic cells and collaborate with B cells are not fully elucidated.

8. The chemical nature of T cell receptors for antigen, both those responsible for the initial activation of the cell and those (perhaps identical) receptors permitting close contact between activated T lymphocytes and their target antigens, is the subject of much current debate. One line of experimentation (Marchalonis and Cone, 1973; Feldmann and Basten, 1972b; Warner, 1974; Roelants *et al.*, 1973) suggests that the receptor is an IgM-like molecule, perhaps consisting of standard κ or λ light chains, and a μ-chain-like heavy chain which might be coded for by a fourth set of *V* genes linked to separate *c* genes, i.e., a fourth "translocon" (Gally and Edelman, 1972). The second line of experimentation (Crone *et al.*, 1972; Benacerraf, 1974) argues for a family of recognition molecules that are not Ig at all. The suggestion is that the T cell receptor is coded for by a set of immune response (*Ir*) genes that are closely linked to the major histocompatibility genes of the species. A compromise suggestion recently proposed was that there are two kinds of T cells, one working through IgM-like receptors and the other through *Ir* gene products, which collaborate in many T cell responses (Wagner and Nossal, 1973). In the light of this uncertainty, it is not possible to be dogmatic about the question of whether a given T cell displays only a single receptor specificity, but fair arguments in favor of this view can be mustered (Nossal, 1974). Many immunologists assume that T lymphocytes undergo a somatic diversification process comparable with that of B lymphocytes, with the production of mature T cells, each with a single receptor specificity.

9. Both T and B lymphocytes are generated continuously throughout life, but particularly in immature animals, from receptor-free precursor cells. This lymphoneogenesis is antigen-independent, occurring in the thymus for T cells and probably chiefly in the bone marrow for B cells. Such "virgin" T and B cells are exported from these so-called primary lymphoid organs and reach lymph nodes, spleen, Peyer's patches, and other lymphoid collections which constitute the secondary lymphoid organs. Little is known about the life-span of virgin T and B cells that fail to encounter an antigen capable of triggering them, but this may be short, e.g., a few days. When antigen activates T or B cells, progeny result that are termed memory cells. These exhibit the same specificity as the original cell, but probably differ in a number of other respects, e.g., smaller median cell volume, higher density, lesser tendency to adhere to glass, and longer life-span. Memory cells recirculate extensively, and may live for many months in the G_0 state of the mitotic cycle if not triggered again by antigen. It is probable that most mitotic division of lymphocytes in secondary lymphoid organs is driven by antigen, though potentiated by lymphokines and macrophage factors. In a mature animal, memory cells constitute the great majority of the lymphon, as evidenced by the small total number of peripheral lymphocytes present in an adult germfree animal. Thus the response of an animal to an antigen is influenced profoundly by its previous history of antigenic exposure (Fazekas de St. Groth, 1967).

10. Genetic factors influence immune responses at least as profoundly as prior antigenic load. Three main streams of investigation document this. First, outbred individuals can be selectively mated to produce lines of animals that are either good or poor producers of antibody to a wide variety of antigens. This can be shown to depend on a multiplicity of genes, which influence, *inter alia,* macrophage performance and B cell mitotic rate (Biozzi *et al.*, 1974). Second, the histocompatability-linked *Ir* genes determine how well the T cells of an individual can respond to a given antigen. This may determine how much antibody will be produced by the B cells, which require T cell help under many circumstances (McDevitt and Landy, 1972). Third, the immunoglobulin structural genes present in the germ line of an animal may determine the efficiency of antibody production to particular antigens (Cohn, 1973). These genetic influences may not be evident in many cases where animals are given powerful antigens like bacterial or viral vaccines, where a multiplicity of different antigenic determinants contributes to the overall response as usually titrated. They may be most important in responses to tumor antigens, where the critical period of immune defense involves low concentrations of poorly immunogenic molecules.

III. Possible Levels for Immunity-Tolerance Signal Discrimination

Tolerance does not embrace all the mechanisms of immune hyporeactivity. Unresponsiveness due to drugs, ionizing radiation, reticuloendothelial system blockade, malnutrition and cachexia, and genetic or acquired diseases of the lymphoid system will not concern us further; and phenomena such as antigenic competition or desensitization by antigen with exhaustion of pharmacologic mediators are only marginally relevant. We must certainly consider hyporeactivity to antigen caused by enhancing antibody and perhaps IgG-mediated negative feedback under the broad heading of tolerance, as these are specific with respect to antigen. Our main concern, however, must be for the properties of lymphoid cell populations which respond suboptimally to antigen even when placed in environments where such inhibitors are inoperative.

There appear to be four basic ways in which a state of tolerance might be achieved. These we can term clonal abortion, clonal deletion, receptor blockade, and activation blockade.

A. Clonal Abortion

Clonal abortion (Nossal and Pike, 1973) implies that immunocompetent cells of a given specificity are eliminated before they reach a sufficient degree of maturity to allow activation by antigen. This theory was first proposed in 1959 (Lederberg, 1959; Burnet, 1959) but, despite some tentative experimental support (Scott and Waksman, 1969; Nossal and Pike, 1974), it remains largely speculative. The main point in favor of the view is its elegance from a teleologic viewpoint. The most important "goal" of tolerance is to allow discrimination between "self" and "non-self" molecules. One key operational difference between these two is that "self" molecules are ever present, right throughout the differentiation process which forms lymphocytes from more primitive precursors, whereas foreign antigens are pulsed in unexpectedly. If the maturing T or B cell passed through a transient phase during which any contact with antigen capable of interacting with its receptors killed it, every self-reactive cell would be eliminated as it was being produced by the generator of diversity. If a foreign molecule entered the system, it might encounter a few cells in this phase, which would be killed, but many more that had passed through the transition period, which would initiate the immune response and rapidly eliminate the antigen. It has been difficult to construct really precise experiments to test the clonal abortion theory, because not enough is known about lymphocyte differentiation to allow isolation and study of the different postulated maturation stages. The kinetics of tolerance induction in secondary lymphoid organs, which

presumably contain chiefly cells that have already passed through the critical phase, can sometimes be so rapid as to make it most unlikely that clonal abortion is the sole mechanism of tolerogenesis.

B. Clonal-Deletion and Two-Signal Theories

According to this mechanism, the tolerant state is due to actual deletion of cells on contact with antigen. It is postulated that contacts with antigen can be either stimulatory or tolerogenic. A stimulatory interaction causes clonal proliferation and differentiation; a tolerogenic interaction is followed by death of the cell within a reasonably brief period. An elaboration of this view, which has been helpful in encouraging more precise formulation of the problem, has been the "two-signal" theory of immunity-tolerance signal discrimination (Bretscher and Cohn, 1970; Cohn, 1971; Schrader, 1973). This hypothesis states that the triggering of a lymphocyte depends on two different "signals" reaching the cell simultaneously. Signal 1 involves the surface receptor uniting with an antigenic determinant. Signal 2 involves some chemical transmitter substance coming from either an activated macrophage or an activated T cell. If the cell receives *only* signal 1, it is believed to be turned off, and perhaps destroyed. Certain chemicals with known adjuvant properties, such as bacterial lipopolysaccharides, may substitute for the physiological 2 substance. Some models of immunological tolerance are readily reversed when the tolerant cell population is placed in an antigen-free environment. In many cases, the speed of such reversal is too rapid to make clonal deletion a likely mechanism. There are other models in which radioautographic studies show a reduction in the number of lymphocytes capable of binding the tolerogenic antigen (Louis *et al.*, 1973) and, in these, clonal deletion may well be at work.

C. Receptor Blockade

If interaction between antigen and receptor is, in itself, insufficient to activate an immunocyte, one could envisage a simple saturation of surface receptors acting as an efficient mechanism to block immune induction. Let us spell out one example of this concept. Suppose union of B cell surface Ig and soluble, monomeric protein antigen is insufficient to trigger a cell, but that an encounter with the same antigen bound to a macrophage surface is effective in triggering. Consider a B cell which emerges from the "generator of diversity" bearing receptors for the animals own serum albumin. As soon as they were expressed at the cell surface, these receptors would be occupied by albumin present in the extracellular fluid, and the cell would be incapable of responding to heat-aggregated albumin injected by an investigator. Tissue culture experi-

ments have shown that such a mechanism is a real possibility in some experimental situations (Feldmann, 1972b). Nonimmunogenic hapten-protein conjugates can bind to B cell receptors and effectively inhibit the activity of immunogenic conjugates. They can do so reversibly, although the possibility exists that the process decomes irreversible within 24 hours or so (J. W. Schrader, unpublished). It is doubtful that such simple receptor blockade is the sole way in which self-tolerance actually works. A system based on this principle might be unduly sensitive to autoimmunization under conditions, e.g., gram-negative bacterial septicemia, where molecules with "signal 2" reactivity could then trigger all the many self-reactive cells already in receipt of signal 1 because of their bound antigen. It should be noted, however, that if the life-span of untriggered, virgin lymphocytes is indeed very short, the mechanisms of clonal deletion and receptor blockade tend to merge into each other.

We shall be devoting special attention to a particular type of receptor blockade induced by multivalent antigens. This is covered in Sections V–VII.

D. Activation Blockade

Under this heading we can place all those mechanisms of nonreactivity that do not depend on some basic property of the immediate precursors of immunological effector cells. For example, one may be considering tolerance as measured by the amount of antibody formed after a challenge injection of antigen. The B cells in the responding lymphoid population may be perfectly normal, but T cells may be required to initiate antibody formation. If the T cell population is tolerant, the operational end result may masquerade as B cell tolerance, revealing its true nature only when the antigen concerned is presented on some other carrier. Another emerging concept is that of suppressor T cells (reviewed in Mitchell, 1974b), i.e., an apparently deficient reaction of B cells to antigen which can be shown to be due to the presence of T cells that exert some inhibitory influence. The term also covers more complex regulatory defects which one can postulate, such as a failure of correct activation due to an excess of the stimulatory signal. Other examples of apparent nonreactivity such as the postulated balanced coexistence of activated lymphocytes and serum blocking factors (Hellström and Hellström, 1969) do not fit comfortably into this framework, and must be considered separately (Sections VI and IX).

In any discussion of possible mechanisms of tolerogenesis, it is difficult to avoid using language that implies an absolute degree of reactivity between antigen and cell. In fact, as we have discussed, this is not true. We know that in the case of B cells and a hapten molecule, 1% or more

(reviewed in Ada, 1970) of the lymphocyte population may possess receptors capable of specifically binding the hapten, yet it is probable that in a typical antihapten immune response only a small proportion of those cells actually become activated. Serious mistakes could result from a failure to appreciate this point. For example, an investigator may so treat an animal with a tolerogenic antigen as to lower its response to a challenge injection of antigen 100-fold. This response must be measured by some methodology, such as a plaque technique, for the enumeration of antibody-forming cells. The investigator, seeking to test the clonal deletion theory of tolerance, looks for antigen-binding B cells in the animal's spleen by a sensitive autoradiographic technique. He finds a normal or even an increased number, and concludes that clonal deletion is not operating. Let us suppose, however, that his radiolabeling method is capable of marking all B cells which possess Ig with K_a of 10^4 moles or greater; and his plaque method reveals only antibodies with a K_a of 10^6 moles or greater. The small number of B cells with Ig of the high association constant may well have been reduced drastically, but the less avid B cells shown by the labeling test may not have been tolerized at all; they may, in fact, have been immunized by the putatively tolerogenic pretreatment. The investigator, though possessed of tests that can reflect the avidity of lymphocyte receptors or antibody formed by single cells in an approximate way (Andersson, 1970), has no technique for measuring the K_a really accurately in the two situations, so, in short, cannot test the hypothesis formally with the model chosen. It may well be that normal animals possess cells that would be classified as self-reactive on certain laboratory criteria. If, operationally, the avidity of such cells for the self antigen were too low to allow for triggering under most conceivable circumstances; or, if the antibody formed after triggering were of too low a binding affinity to alter the physiology of the self constituent, none of the fears of Ehrlich's "horror autotoxicus" would be realized.

It is unwise to regard the four basic mechanisms described above as being in any sense alternatives. I have myself been guilty of engaging in debates about *the* mechanism of tolerance, where "reversibly tolerant cells" have been regarded as antithetical to clonal deletion (e.g., Nossal, 1969). The sharply different behavior on adoptive transfer of lymphocytes from animals rendered tolerant to different antigens (e.g., Howard, 1972) shows clearly that no unique mechanism exists. There is a temptation to hope that self-tolerance may depend chiefly on one mechanism, which would then become "the main" mechanism, but even this seems unlikely when one considers the wide range of concentrations, molecular forms, and arrangements of self constitutents. For the cancer worker as

for the basic immunologist, the problem must be particularized to be described or understood.

IV. Tolerance Induced in T and B Lymphocytes by Soluble, Oligovalent Antigens

Ever since the early experiments of Smith and Bridges (1958) in the rabbit, and of Dresser (1961) in the mouse, heterologous serum proteins have been favorite tools for the quantitative assessment of tolerance induction. These substances are typical of a group of molecules which share certain characteristics that appear to promote tolerogenesis. These include wide tissue distribution (Nossal and Ada, 1971), relatively slow rates of removal from the extracellular fluids and the absence of multiple repeating identical antigenic determinants. If care is taken to remove small aggregates, such antigens cause tolerance even in adult animals. We now know that these antigens require the helper effect of T cells in order to elicit a maximal response. The challenge injections given to test the state of tolerance usually consist of aggregated, alum adsorbed or emulsified antigen, and the assay procedures used usually concentrate on IgG antibody production. In other words, when the great importance of T cells in humoral immunity was fully realized, it became clear that either T or B cell tolerance could have accounted for most of the results in the literature. The intriguing observation had been made by Mitchison (1964) that two zones of antigen dosage existed which could cause tolerance in adult mice, intermediate dosage levels causing immunization. This also had to be fitted into the new framework of T-B cooperation.

We owe to the group of Weigle (Chiller *et al.*, 1970, 1971; Chiller and Weigle, 1973; Weigle *et al.*, 1972) the first frontal attack on the question of whether T or B cells were predominantly tolerized by such soluble protein antigens. They used mouse thymus as a source of T cells and bone marrow as a source of B cells. Donor mice were given deaggregated human gamma globulin (HGG) as a tolerogen. The success of tolerance induction for either the whole animal or for component tissues was tested by challenge with aggregated HGG, in the latter case after transfer of cells to lethally irradiated recipients. The experimental design allowed various mixtures to be made, i.e., normal thymus plus tolerant marrow, tolerant thymus plus normal marrow, and so forth. Final readout was by a test for anti-HGG antibody-forming cells (AFC) as measured by a hemolytic plaque test. Two injections of antigen were given, the first at the same time as cell transfer and the second 10 days later. Animals were killed 15 days after cell transfer, and host spleens were tested for AFC content.

The results showed that tolerance was achieved rapidly in thymus cells, reaching maximal levels 2 days after infusion of HGG into mice, and with 100 μg or less of antigen. Tolerance of bone marrow populations was achieved more slowly, reaching maximal effectiveness only 21 days after antigen, and required 2.5 mg of antigen. The conclusion was reached that T cells are rendered tolerant much more readily than B cells, particularly with low antigen doses. These pioneering studies have gained wide general acceptance, and have indeed been valuable guides to future work, but the conclusions may not be equally applicable to all experimental situations. Both of the organ sources studied, thymus and bone marrow, contain a high proportion of *precursors* of mature (T and B) lymphocytes, and there is no guarantee that these respond to antigen similarly to mature, peripheral T and B cells. The kinetic aspects of the study may well reflect the speed of maturation of cohorts of precursor cells and concomitant export of more mature cells, rather than tolerance-inducing phenomena at the single cell level. Indeed, the authors themselves present data to suggest that peripheral B cells are rendered tolerant at a different, faster rate than bone marrow itself. The problem is particularly complex in the case of bone marrow. This organ contains multipotent hematogenous stem cells, lacking Ig receptors, which clearly cannot be affected by tolerogens, and which may differentiate into T and B cells in adoptive hosts. It contains more differentiated B cell precursors, which can be termed pre-B cells (Lafleur *et al.*, 1972) and which may acquire their Ig receptor coat during a nonmitotic maturation phase (Osmond and Nossal, 1974). Its content of functional B cells as tested by adoptive transfer and challenge with a T cell independent antigen is actually quite low (Stocker *et al.*, 1974), and thus the kinetics and cell dose-response relationships of AFC appearance in recipients of marrow require careful analysis. A delayed injection of antigen allows significant improvement in the adoptive transfer performance of marrow cells, presumably because of B cell neogenesis in the host from receptor-free precursors (Lafleur *et al.*, 1972; Stocker *et al.*, 1974). The receptor-free pre-B or stem cells presumably cannot be altered by exposure to antigen. When bone marrow cells from a tolerant donor are transferred, there is some danger that antibody production by descendants of such cells might mask adequate tolerance induction in more mature B cells (Kaplan and Cinader, 1973).

Fortunately, a variety of other studies suggest that T cells are more readily tolerized than B cells. Mitchison (1971), investigating the cellular basis of low and high zone tolerance induced in mice by multiple injections of bovine serum albumin, concluded that low zone tolerance affected T cells, and high zone tolerance affected B cells. Again, the

experimental protocol was complex and some details are open to different interpretations. Rajewsky (1971) also noted the difficulty of rendering B cells tolerant and showed that successful tolerogenesis affects only those B cells the progeny of which make high affinity antibody (Rajewsky and Pohlit, 1971). Miller *et al.* (1971), using fowl gamma globulin as antigen, failed to produce tolerance in B cells with a single injection of deaggregated antigen, but readily tolerized T cells. Their assay system might have missed tolerogenesis in a subset of high affinity B cells. Experiments from our own laboratory (reviewed in Feldman and Nossal, 1972) compared the relative capacity of fowl gamma globulin to tolerize T and B cells *in vitro* within 16 hours, and, noted that T cells were rendered unresponsive readily with 1 μg/ml of antigen, whereas B cells were unaffected by 100 times this dose. We now know that 16 hours is a suboptimal time for B cell tolerogenesis *in vitro* (Schrader, 1974b).

A convenient technique for studying tolerogenesis of B cells is to couple haptens onto autologous proteins (Havas, 1969; Golan and Borel, 1971) or nonimmunogenic carriers, such as deaggregated heterologous globulin (Taussig, 1973; Nossal and Pike, 1973; Stocker and Nossal, 1974). In most cases, these experiments do not fit the heading of this section, in that the hapten substitution was >1 per carrier molecule, but significant hapten-specific B cell tolerance can be achieved in the mouse with 0.5–2-mg doses of conjugates with a mean of <1 hapten group per molecule. In all, then, the evidence that soluble, monomeric proteins can tolerize B cells, but at a higher dosage than is needed to tolerize T cells, is quite impressive.

How is such an effect achieved? Perhaps the simplest suggestion would be that the tolerogen acts just by occupying the receptors and preventing immunogenic forms of antigen from reaching them. In fact, such receptor blockade can occur readily *in vitro* (Feldmann, 1972b), although multivalent antigens are more effective blockaders than oligovalent ones. Several considerations argue against receptor blockade being the mechanism operative in most *in vivo* models. First, the kinetics of tolerance induction, where carefully investigated, are characterized by a lag of one day or longer. Equilibration of intravenously injected serum proteins between extra- and intravascular fluid compartments is much more rapid than that, and so is equilibrium binding of antigen and receptor at 37°C. Second, tolerance is usually considered unproved until the cell population has been transferred to a tolerogen-free environment (adoptive host or tissue culture) and shown to be hyporeactive to challenge with antigen in immunogenic form. Simple blockade of receptors is rapidly reversed under these circumstances (e.g., Katz *et al.*, 1972), perhaps chiefly because of metabolic turnover of receptors (Wilson *et al.*,

1972). Third, B cells coated with a soluble antigen such as deaggregated fowl gamma globulin (Schrader, 1973) can be triggered into antibody production by exposing them to molecules such as bacterial endotoxin or polymerized flagellin, believed to have "signal 2" properties (see Section III). When tolerogenesis of B cells is induced *in vivo* in congenitally athymic ("nude") mice, it appears that cells go through a progression from a stage where they can form antibody on injection of these substances, to one where they cannot (Schrader, 1974a,b). The postulate is that tolerogenesis is set in motion by the attachment of soluble antigen to B cell receptors, but that cells can be "rescued," during a period of approximately 24 hours, by the addition of the "second signal." After 24 hours, responsiveness is lost, implying that some true metabolic change or conceivably even clonal deletion has taken place in the B cell population. The prior studies of Chiller and Weigle (1973), though not as detailed kinetically, support this viewpoint. Schrader also finds that tolerogenesis can be induced *in vitro* with deaggregated fowl gamma globulin, or $DNP_{4.5}$ HGG, at a concentration and rate broadly similar to that obtaining *in vivo*. If this finding can be confirmed, it will help to resolve a major paradox in the literature. There has previously been no indication that the soluble, monomeric molecules, which are such good tolerogens *in vivo*, can switch off B cells *in vitro*. The absence of the complication of T cells may be one factor in the nude mouse model, but Schrader suspects timing is another very important variable. He finds a much bigger effect after 24 hours of tolerogenesis than after 16 hours.

In Section V, we shall be considering at length the use of multivalent conjugates as tolerogens, and, in view of the great current interest in receptor rearrangement, "patching" and "capping" (see Section V) as signaling mechanisms, it is cogent to ask whether there is any possibility that antigens injected into animals or added to tissue cultures as monomers may in fact exert their effects on lymphocytes as aggregates, polymers, or membrane-bound matrices. This possibility certainly cannot be excluded. *In vivo*, both natural and rapidly formed acquired antibody could cause the formation of soluble complexes. The surfaces of macrophages, dendritic follicle cells (Nossal and Ada, 1971) and, perhaps most importantly, of lymphocytes themselves, present opportunities for the concentration or "focusing" of antigen. The situation is changed only marginally *in vitro*. The use of autologous carrier molecules for tolerization of hapten-specific B cells lessens but certainly cannot eliminate the probability of aggregate or matrix formation. The best test of the capacity of monovalent antigenic determinants to signal tolerance changes to a cell would be to use haptens in a nonreactive molecular form, e.g., DNP-lysine. Most studies of this type have been negative.

Reactive haptens can be injected *in vivo* and can cause B cell tolerance (Fidler and Golub, 1973), but it is likely that this action is preceded by their conjugation with autologous proteins. Nonreactive haptens are excreted very rapidly, and, though they may cause temporary receptor blockade, they have not been reported to cause nonreactivity which survives adoptive transfer. In the Fidler and Golub system, it is probable that the hapten–protein conjugates formed include molecules with multivalent antigenic determinants. However, univalence alone may not be the chief reason why nonreactive haptens appear to be nontolerogenic. It is quite plausible to suggest that tolerogens must have a certain minimal molecular weight to be effective. Even if one accepts the notion that tolerance results from "signal 1" only, nothing is known of the postulated allosteric change in the lymphocyte receptor resulting from antigen binding, and therefore nothing can be said about the influence of ligand size on the change. It is of interest to note that Haustein *et al.* (1974), investigating conformational changes in purified bovine antibody on union with hapten by relaxation studies using the temperature-jump method, found evidence of such changes with 19 S IgM, but not IgG, antibody. The B cell receptor is believed to be IgM, at least for "virgin" B cells in the mouse, though of 7 S rather than 19 S nature. Clearly, the question of whether receptor conformational changes alone can be "read" by the lymphocyte remains unresolved. Nor is it clear that the tolerance signal is necessarily an event involving only the antigen-binding cell. Particularly with *in vivo* tolerogenesis, it could be that the cell which has bound large amounts of monomeric antigen is altered in its migratory behavior, or is rendered more palatable to phagocytic cells.

In view of the above uncertainties, much further work needs to be done on tolerogenesis of T and B cells by soluble, oligovalent molecules. Our recent laboratory experience (Stocker *et al.*, 1974; Stocker and Nossal, 1974; Schrader, 1974a) indicates that unexpected operational complexities remain to be conquered. There is no guarantee that mechanisms of tolerogenesis will be the same for T and B cells, or for "virgin" versus "memory" cells of each category. It is already clear that the testing of the tolerant state by (a) challenge of the whole animal, (b) tissue culture with immunogenic antigen, and (c) adoptive transfer, may yield very different results. In particular, inadequate consideration has been given to the vagaries of the adoptive transfer system, e.g., to the complex cell dose-response relationships and kinetics (Celada, 1967; Stocker *et al.*, 1974). Speculations in the face of these uncertainties may be of limited value, but we are attracted to the view that monomeric antigens can directly tolerize T and B cells, and that the mechanism of this effect differs fundamentally from that considered in the next section.

V. Tolerance Induced in T and B Lymphocytes by Antigens Possessing Multiple Repeating Determinants

The use of antigens with multiple repeating determinants as agents capable of causing nonreactivity antedates even the use of homologous serum proteins (reviewed by Howard, 1972). Felton (1949) coined the word paralysis to describe the failure of animals to respond following the use of supraimmunogenic doses of pneumococcal polysaccharide. A great number of studies have flowed from this seminal discovery, and it now appears that the rules governing tolerogenesis by this class of antigens differ sufficiently from those considered in Section IV to warrant separate consideration. The following characteristics appear to link a certain group of antigens, of which pneumococcal polysaccharides and bacterial endotoxins are typical examples: (1) relatively high molecular weight; (2) repeating antigenic determinants on a single molecule; (3) capacity to trigger B cells *in vivo* or *in vitro* at low dose, without use of adjuvants and without participation of T cells; (4) capacity to cause nonreactivity at tissue culture or whole animal level at doses in excess of those which cause immunity; (5) preferential or exclusive induction of IgM antibody formation; (6) failure to induce "priming" in the sense of leading to a predominantly IgG response on secondary challenge; (7) low or absent capacity to stimulate T cells to become carrier-reactive "helper" cells; (8) low or absent ability to induce delayed hypersensitivity; (9) capacity to activate into proliferative and protein-synthetic activity lymphocytes, particularly B lymphocytes, other than those with specific Ig receptors for the repeating antigenic determinants; (10) ability to activate the third component of complement and thus to initiate the "bypass" system of complement-dependent lysis. Antigens which share some, but not all of these characteristics include polyvinylpyrrolidone, polyfructose or levan, polymerized bacterial flagellin, synthetic copolymers of D-amino acids, serum or other proteins heavily substituted with haptens, aggregated proteins such as hemocyanins of high molecular weight, and intact heterologous erythrocytes.

It will be evident that these characteristics differ sharply from those of the tolerogens which were considered in Section IV. Broadly speaking it could be said that those tolerogens are poor immunogens, and the paradoxical thing about the present set of so-called "T-independent" antigens is that they are both good immunogens and good tolerogens. The confusion is increased by the fact that some of the antigens in the group that have been most extensively studied do not *quite* fit the pattern. Polymerized flagellin (POL), which has been extensively promoted by our group, possesses characteristics 1–5 above, but not 6–8. In this section,

we will seek to sort out what mechanisms may be responsible for the effects of these antigens.

A key development influencing the approach to this problem has been the realization that spatial rearrangement of cell surface macromolecules is possible and may profoundly affect cell behavior. Both the protein and the lipid components of the cell membrane are, in some cells at least, freely mobile in the plane of the membrane (Frey and Edidin, 1970), individual protein molecules or small aggregates being seen as floating in a sea of lipid (Singer and Nicholson, 1972). Moreover, there may be connections of an, as yet, ill-defined nature between membrane proteins and an underlying network of microfibrils and microtubules. When antiglobulin antibodies (Taylor *et al.*, 1971; Loor *et al.*, 1972) or multivalent antigens (Diener and Paetkau, 1972; Raff *et al.*, 1973) are bound to the Ig receptors on the surface of B lymphocytes, the receptors redistribute in a characteristic way. First, they aggregate into "patches" of progressively increasing size by a process that bears some similarities to ordinary serologic agglutination reactions. This "patching" is independent of cell metabolism, but is very slow at temperatures that render the lipid phase of the membrane viscous. Then, by a process which is metabolism-dependent and can occur within minutes at 37°C, the receptors are gathered into a caplike or polar area of the cell membrane, and are subsequently pinocytosed. If the cells are washed and incubated in the absence of the ligand, the receptor coat may reappear, possibly with an increased density of receptors. Simultaneously, the cell may manifest some of the signs of having been induced into immune activation, e.g., it may enlarge, increase its RNA and protein-synthetic rate, and commence DNA synthesis. So far, the link between receptor aggregation and the event of triggering is too tenuous to indicate cause and effect relationship, but interesting speculations about this possibility have begun to appear (Yahara and Edelman, 1972, 1973). Agents which interfere with microtubular function, and thus perhaps with receptor rearrangement, can inhibit lymphocyte triggering (Yahara and Edelman, 1973). A given lymphocyte can undergo cycles of receptor patching, capping, pinocytosis, regeneration, further patching, etc., if reexposed to the appropriate ligand. The situation with a multivalent ligand continuously present in the medium surrounding lymphocytes must be highly dynamic. It is easy to imagine that a given concentration of antigen might initiate an optimal degree of patch formation, but that a higher concentration could so perturb the membrane as to have a deleterious effect on the cell. In this regard, the findings of Diener and Paetkau (1972) are of interest. Working with ^{3}H-labeled POL and mouse spleen lymphocytes (probably B lymphocytes), they found that immunogenic concentrations

of antigen cause patch and cap formation which persists for several hours in the continued presence of antigen. Higher concentrations, known to be capable of inducing nonreactivity in tissue cultures, initially cause capping but after several hours lead to a state of random distribution of receptors which the authors liken to a "frozen" state of the membrane. It is tempting to speculate that what links all the antigens of this group, and distinguishes them from the antigens considered in Section IV, is their capacity to cause receptor rearrangement in specific B cells. At low antigen concentrations, triggering may be favored, and at high, inhibition. Apart from concentration, a variable of great importance may be the exact spacing of antigenic determinants (Feldmann, 1972a).

This perspective, not yet formulated in sufficient detail to be called a theory, is by no means the only one enjoying currency. For example, Dukor and Hartmann (1973) have drawn attention to the capacity of these multideterminant antigens to cleave the third component of complement. Noting the presence of a C'3 receptor on many B lymphocytes, they speculate that the conjoint presence of antigen and C'3 on the cell surface may initiate triggering. Mitchell, in an intriguing series of papers (Mitchell *et al.*, 1972; Mitchell, 1974a) has proposed that B cells of high affinity for a multivalent antigen will tend to be blocked by that antigen. He ascribes to T cells a deblocking and protective function. On this hypothesis, T cells effect antigen digestion or removal at the B cell surface, either directly through secreted enzymes, or via activated macrophages. The reduction in antigen binding is followed by receptor redistribution, which is the actual signal for triggering. Any antigen which fails to activate T cells fails to stimulate this vital deblocking step. Thus such antigens will tend to tolerize high affinity B cells, e.g., those capable of later IgG production. This speculation retains the concept that too much multivalent antigen bound to a cell will result in switching off rather than activation.

In most current debate on this group of multivalent antigens, the lack of capacity to stimulate T cells and the ready tolerogenesis of B cells with excess antigen are considered as linked properties of the antigen. Nevertheless, there are cases that contravene this association. POL, for example, tolerizes well in high dose *in vitro* or *in vivo*, but can also cause good T cell stimulation as manifested by helper effects and delayed hypersensitivity (Nossal and Ada, 1971).

The *in vitro* induction of tolerance in B cells by POL has been carefully documented (Diener and Armstrong, 1969; Diener and Feldmann, 1972). It requires supraimmunogenic concentrations. As with tolerance discussed in Section IV, two stages can be identified, a reversible stage and an irreversible one. In this case, as the multivalent antigen is so firmly

bound, a trypsinization step must be introduced to remove it from the B cell surface. If cells are trypsinized 6 or 16 hours after having bound tolerogenic amounts of POL, they regain their capacity to respond, but if they are left for 3 days, the response capacity can no longer be rescued by trypsinization.

Despite exceptions like POL, the lack of apparent T cell immunization with certain antigens in this group is of interest. It is difficult to believe that the population of T cell receptors cannot recognize and bind this category of antigens. It seems more likely that the defect is one of regulation. Could it be that the "gateway" between no effect at all and tolerizing concentrations is very narrow with respect to T cells and antigens such as pneumoccal polysaccharides? It would then require very sensitive methodologies and careful experiments to document *some* T cell immunization among the predominant tolerance effects. The most careful studies (e.g., Baker *et al.*, 1971) have focused attention on antibody production, and quantitative measurement of T cell activation is still fraught with uncertainties. Analysis of interaction between antigens and T cells will undoubtedly be aided by *in vitro* studies, where at the moment the most convenient parameter to monitor is the induction of helper cell activity (Feldmann and Nossal, 1972; Feldmann, 1974; Ada, 1974). Very preliminary experiments in our laboratory are consistent with the view that T cells can be tolerized with relatively low doses of highly multivalent antigens, but this work needs extension. If it is correct, the ready tolerogenesis in T cells would explain why many of these antigens induce only IgM antibody formation. T cell activation is a prerequisite for IgG antibody synthesis under most circumstances. Possibly the reason why POL can go ahead and stimulate T cells and IgG synthesis relates to its ready degradation to smaller fragments, perhaps not so prone to cause T cell paralysis. In fact, work with synthetic polypeptides lends support to the notion that lack of degradation favors continued IgM production and the other hallmarks of "T cell independence" (Sela *et al.*, 1972).

The effects of multivalent antigens on T lymphocytes can also be studied by activating T cells *in vitro* with subcellular membrane fragments from an allogeneic source (Manson and Simmons, 1969). While this approach suffers from the disadvantage of lack of purity and definition of the antigen, it has the advantage that a highly reproducible, quantitative assay exists for the measurement of T cell activation. This is the ^{51}Cr-release assay of Brunner and Cerottini (1971). We have compared the *in vitro* immunogenicity of crude subcellular membrane fragments with that of soluble H-2 antigens obtained by papain digestion (reviewed in Wagner and Nossal, 1973). The crude membrane frag-

ments caused the generation of cytotoxic ("killer") lymphocytes when incubated with a T cell suspension derived from cortisone-resistant thymocytes. The optimal effect, produced with a concentration of added protein of 15 μg/ml, was weak in comparison with that produced by living, mitomycin-treated allogeneic cells as stimulators. Higher concentrations caused much lower T cell immunization, and the effect was immunologically specific and thus not due to toxicity. In contrast, soluble H-2 antigens could immunize but not cause unresponsiveness at high dose. Thus the physical form of the antigen appears to modulate the effect, and the ready paralysis of T cells by multideterminant, particulate antigens is again documented. Similar conclusions have been reached by Brent and Kilshaw (1970) from *in vivo* work.

If T cells are indeed readily shut off by multivalent antigens, it is possible that the mechanism may resemble the "excessive" series of patch-cap-pinocytosis cycles postulated above for B cells. It has recently been shown that T cell receptors for antigen can also redistribute into caps (Roelants *et al.*, 1973). It would be unwise to conclude, however, that all the phenomena of nonreactivity engendered by multivalent antigens are identical in nature. Reverting to B cell immunization and paralysis, it has been noted that some doses of pneumococcal polysaccharide cause "antibody formation on a treadmill" (Howard *et al.*, 1970), with many antibody-forming cells being generated but with the synthesized antibody being continuously eliminated by depots of nondegradable antigen. As we shall see in Section VII, the apparent tolerance induced in some situations may not be tolerance at all, but a blockade of the effector cells by adherent antigen. Doses of pneumococcal polysaccharide higher than those causing treadmill neutralization do cause central suppression of B cells, though after a prior, transient phase of immunization (Howard, 1972). It is not clear at the moment how much of this effect is really due to effector cell blockade (see Section VII), but a noteworthy feature is a rapid loss of paralysis when cells are transferred to irradiated recipients. In contrast, the polyvalent antigen levan, a fructose polymer with a molecular weight of 23×10^6, causes a nonreactivity with very different features (Miranda, 1972; Miranda *et al.*, 1972). A single injection causes an exclusively IgM response, independent of T cells, over a wide dose range (100 pg to 100 μg). A higher dose, e.g., 1 mg, causes tolerance, apparently without prior immunization or treadmill neutralization. Transfer of spleen cells to lethally irradiated recipients is not followed by rapid loss of tolerance. Therefore, while one could ascribe the pneumococcal polysaccharide nonreactivity to antigen holding cells in a blocked state, the levan nonreactivity appears to rest on a different basis, perhaps reflecting irreversible inactivation of the B cells concerned.

High molecular weight is not an absolute prerequisite for tolerogenesis by multivalent antigens. Depolymerized fractions of levan, molecular weight <10,000, can still cause tolerance (Miranda *et al.*, 1972). Both *in vitro* and *in vivo* studies have demonstrated that the 2,4-dinitrophenyl (DNP) hapten coupled at a high substitution ratio to a copolymer of D-glutamic acid and D-lysine (D-GL) is a powerful inducer of unresponsiveness in hapten-specific B cells (Katz *et al.*, 1972; Nossal *et al.*, 1973). This copolymer has a mean molecular weight of only 50,000. The operative factor seems to be a dense clustering of identical antigenic determinants, allowing extensive cross-linkage of cell surface receptors.

In view of our total ignorance of the intracellular molecular events which follow the binding of multivalent tolerogens, our description of this form of nonresponsiveness as opposed to that caused by monomeric antigens must needs be incomplete. We have some sympathy with the views of Howard (1972), who states that Felton's original term, paralysis, seems highly appropriate to the former group. It is difficult to conceive that the dominant phenomena in the acquisition of self-tolerance depend on the formation of highly clustered antigen aggregates, and thus tolerization by soluble, monovalent antigens may be the model with greater relevance to the self-recognition problem. Even that cannot be regarded as certain. We do not know, for example, whether an animal acquires tolerance to its own histocompatibility antigens via soluble molecules shed from the cell surfaces, or through contact between the potential self-reactive lymphocyte with another cell or cell fragment bearing a matrix of antigen. Much the same is true of the nonreactivity which occurs in late stages of cancer, so it would be wise for the cancer researcher to keep a careful watching brief on both of these major types of nonreactivity as the mechanisms unfold over the next few years.

VI. Tolerance in T and B Lymphocytes Produced by Antigen–Antibody Complexes

It has long been known that antibody administered passively to an animal can hamper an active immune response, and the importance of understanding in detail the role of antibody and antigen–antibody complexes in immunocyte regulation has been sharply underlined by the finding of serum factors which can block cytotoxic killing of tumor cells (Hellström and Hellström, 1969; Sjögren *et al.*, 1971). The detailed cellular mechanisms are difficult to sort out *in vivo*, and therefore our group has spent much effort in defining the effects of antibody *in vitro*. This work has recently been reviewed (Diener and Feldmann, 1972; Feldmann and Nossal, 1972) and so only a brief summary will be presented here.

Two broad categories of antibody-mediated suppression may be dis-

tinguished. First, there is the conceptually simple peripheral suppression (Uhr and Möller, 1968), where the high concentration of serum antibody competes successfully against cell surface receptor antibody for the limited amounts of antigen. This does not involve any changes in the lymphoid cells. More interestingly, we have found (Feldmann and Diener, 1970, 1971) that a central suppression, or true inhibition, of immune competence can occur when lymphocytes are exposed to a mixture of antigen and antibody, with the antigen in slight excess. While documentation is most complete for the effects on B cells, it has been shown that mixtures of fowl gamma globulin (FγG) and anti-FγG can switch off the helper function of FγG-primed T cells (Feldmann and Nossal, 1972). What appears to occur is that soluble complexes of antigen and antibody form, and if sufficient antigenic valencies remain unoccupied by antibody, a multideterminant antigen with many of the characteristics of the tolerogens discussed in Section V is generated. Extraordinarily low concentrations of antigen and antibody suffice for a profound effect. Thus, with the antigen monomeric flagellin (MON) a significant degree of partial tolerance in B cells of spleen cultures could be achieved using 2 pg (2×10^{-12} gm) of MON per milliliter and a 1×10^{-7}% concentration of a hyperimmune antiserum. The onset of paralysis is rapid, significant suppression occurring within 15 minutes, whereas maximal effects are achieved in 4–6 hours. The effect survives transfer of cells to a lethally irradiated animal.

It is interesting to speculate whether this remarkably powerful suppressive mechanism has any *in vivo* significance. Certainly it is not confined to any one group of antigens. It works for all the variants of the flagellin molecule, for serum protein antigens, and for soluble sheep erythrocyte antigens (Diener and Feldmann, 1972). It could provide an effective negative feedback loop in immune regulation, and provides a satisfactory explanation for those forms of tolerance engendered by the repeated injection of extremely small amounts of antigen (e.g., Shellam and Nossal, 1968), where a slight degree of antibody formation frequently precedes the tolerant state. It has even been suggested by Diener that self-tolerance is maintained by the concomitant presence of low concentrations of autoantibodies and autoantigens, the concentration of the former being higher, presumably, in the immediate vicinity of the potential autoreactive B cell which is constantly shedding and regenerating its receptors. Similarly, antibody-mediated tolerance may be a regulatory factor keeping the levels of serum antibody against chronically harbored microorganisms rather low.

The question next arises whether antibody-mediated tolerance bears any relationship to the blocking effects which have been noted in cancer

(Hellström and Hellström, 1969). It appears clear that the cytotoxic effects of activated antitumor lymphocytes can be inhibited by serum factors. These may be antigen–antibody complexes, or tumor antigen, and coating of the tumor cell by antibody alone does not appear to be an effective blocking mechanism (Sjögren *et al.*, 1971; Baldwin *et al.*, 1972, 1973). The very fact that killer cells can arise and inhibit colony formation when incubated with tumor cells in the *absence* of host serum indicates that the inhibitory phenomenon, if it exists *in vivo,* is rather readily reversed when cells are incubated in the absence of the immune complexes. This is reminiscent of the reversible pneumococcal polysaccharide paralysis discussed in Section V for B cells, though it is probable that the cytotoxic killing in the colony inhibition assay is due to T cells, at least partially. In this respect, the blocking phenomenon differs from antibody-mediated tolerance as studied by us *in vitro,* because this persists after the cells have been washed. In fact, blocking of killer cells may be more akin to the phenomena to be considered in the next section.

Even though there are these formal differences between blocking of killer cells and antibody-mediated tolerance, it seems highly likely that the latter phenomenon plays a role in tumor immunity. It could well be that in the absence of circulating soluble complexes of tumor antigen and antibody the quantitative level of killer cells might have been higher than in their presence. In other words, true central suppression of some cells could be occurring *pari passu* with peripheral effector cell blockade of other cells. The two effects together can be disastrous for the cancer patient. For example, in Burkitt's lymphoma the levels of membrane-reactive (anti-MA) antibodies tend to stay at a plateau level in patients in remission. When a sudden fall in anti-MA antibody level occurs, a clinical recurrence frequently follows within a few months. It is likely that the fall in antibody levels is due to complexing with antigen, and that these complexes, by either or both mechanisms, damage the immune response which holds the tumor cells in check (Klein, 1974).

While antibody-mediated tolerance is akin to tolerance caused by multivalent antigens, and may rest on a similar mechanism, it may be wise to keep it in a separate conceptual box for the time being. Operationally, it stands out because of the minute concentrations of reactants that are effective, and because of its potentially universal applicability. Much remains to be learned about the physical chemistry of the tolerogenic encounter between soluble complex and T or B cells. Direct study is rendered difficult by the low molecular concentrations involved, the reversibility of antigen-antibody union, the probable heterogeneity in size and shape, and the strong probability of further complex formation as the affected cell releases some of its own receptors. In the meantime, the

search for obligatorily tolerogenic stable polymers active at low molarity goes on.

VII. Effector Cell Blockade by Multivalent Antigens

Adaptive immune responses are executed by effector cells that are generated through the action of antigen on lymphocytes. For all the functions that have been studied, such as antibody formation, T cell helper activity, or cytotoxic killing of target cells, the effector cells must develop specific metabolic properties that differentiate them from the B or T lymphocytes which preceded them. Most discussion of immune induction and tolerance tacitly assumes that the chief regulatory function of antigen is exerted at the level of initial transformation of a small lymphocyte into an activated cell, preparing for division. In fact, it is probable that antigen plays a continuing role throughout the whole complex series of changes involved in clonal expansion. Nevertheless, until quite recently, few would have challenged the view that the cells representing the end stage of the antigen-initiated differentiation process, e.g., mature, nondividing antibody-forming plasma cells, are autonomous entities, not subject to further regulation by antigen. "Tolerant cells," in most workers' conception, would have represented lymphocytes rendered incapable of reacting by clonal expansion to appropriate antigenic challenge, and *not* activated effector cells somehow held in a blocked state by antigen.

Recent work from our laboratory has shown that hyporeactivity can be induced in B cells even at the latest stage of differentiation, when a cell is engaged in maximal antibody production (Schrader and Nossal, 1974). In other words, the antibody-secreting rate of a single cell can be sharply reduced by attaching multivalent antigen to it. This occurs with concentrations of antigen in the same range as those which induce tolerance, and moreover can be demonstrated both *in vivo* and *in vitro*. The phenomenon appears to involve a true reduction in secretion, not just a temporary adsorption of secreted antibody to cell-attached antigen. We have called this new and surprising effect of antigen effector cell blockade.

A typical example of an antigen capable of causing effector cell blockade is dinitrophenylated *Salmonella adelaide* flagella (DNP-FLA). This antigen consists of small particles made up largely of flagellin polymerized into strands, with the DNP hapten present as multiple determinants on the protein backbone. High molecular weight is not obligatory, however, as DNP-D-GL (see Section V) is also effective. In contrast, lightly substituted serum proteins or free haptens are ineffective. The phenomenon is illustrated in the following way. Hapten-specific AFC

are generated in any standard fashion, either *in vivo* or *in vitro,* and of either IgM- or IgG-producing class. For example, mice can be immunized with a small dose of 0.1 μg of DNP-FLA and the spleen harvested 4 days later. Then, a spleen cell suspension containing AFC is held at 37°C for 30 minutes either in tissue culture medium alone (or with free hapten or an irrelevant antigen), or in medium containing 100 μg/ml of DNP/FLA. Then the cells are carefully washed and assayed for antibody production by standard plaque procedures capable of detecting DNP-specific AFC. It is found that the number of plaques in the suspension that had been held with DNP-FLA is considerably reduced, down to 20–50% of control values depending on exact experimental conditions. More careful examination shows that this is due to a reduction in the antibody secretion rate by each single AFC. Thus, the experimental cell suspension yields plaques that are smaller, and which appear later after incubation. The end result is that cells which would have made small or turbid plaques if left untreated, fail altogether to make plaques after DNP-FLA blockade. The kinetics of blockade can be worked out by binding DNP-FLA to the surface of cells at 0°, washing them, and then incubating at 37°C in the absence of further added antigen. If the cells that have bound DNP-FLA in the cold are immediately placed into a plaque-revealing erythrocyte monolayer, no blockade is observed. After 20 minutes at 37°C between antigen-binding and plaque revelation, a specific AFC loss is seen, and the effect increases in magnitude over the next hour. Clearly some active process is induced by the antigen that has attached in the cold, which progressively reduces antibody secretion rate. No effect is seen on AFC present in the same suspension but directed toward an irrelevant antigen, showing that it is not a nonspecific toxic phenomenon.

A simple method of demonstrating effector cell blockade is to take a group of animals at the height of an immune response and to inject half of them with DNP-FLA. If the spleens are harvested 4 hours later, the blockaded group of animals yield fewer and smaller plaques. The effect is far too rapid for it to be due to any effect at the level of antigen-reactive B lymphocytes.

The most direct proof of the nature of effector cell blockade has come from our single-cell studies. In these, spleens were harvested from DNP-FLA immunized animals, and single AFC were removed from the center of hemolytic plaques by micromanipulation. They were then placed for 30 minutes at 37°C into coded microdrops containing either 100 μg/ml DNP/FLA or medium alone. The cells were then washed carefully by micromanipulation and placed, one by one, into plaque-revealing microdrops, and the rate of plaque growth was measured by

repeated readings with an eye-piece vernier. A 3- to 4-fold reduction in rate of plaque growth as a result of blockade was noted.

Micromanipulation has also resolved the question of whether the blockade is due solely to antibody being held up or trapped by cell-associated antigen. Single AFC that had been subjected to blockade were micromanipulated into droplets containing ^{125}I-labeled antiglobulin, held there for 30 minutes at 0°, and then manipulated on to glass slides, dried, and subjected to autoradiography. It was shown that the blockaded cells demonstrated no more antibody on their surface than did control AFC. This finding demonstrates in a direct way something that could have been inferred from the bulk experiments in which cells were first held with blockading antigen in the cold and then incubated at 37° without antigen. Those cells that were immediately placed into plaque-revealing monolayers performed normally. Had blockade been due simply to secreted antibody being held up at the cell surface by the multivalent, attached antigen, they should have demonstrated full inhibition immediately.

The experiments summarized in Section V showed that certain highly multivalent antigens could directly tolerize B cells, and probably T cells. Now we see that antigens of the same type can slow down antibody secretion by AFC. One must obviously ask whether the two phenomena depend on a similar mechanism. It is even pertinent to wonder whether some examples previously believed to represent tolerance in the sense of inhibition of B cell conversion to AFC may not represent AFC blockade. The rapid reversibility of pneumococcal polysaccharide paralysis suggests that at least part of the effect could be due to blockade. We know little about the regulation of secretion rate by single cells. It is not too fanciful to suppose the following sequence. Most AFC retain surface Ig (Nossal and Lewis, 1972) as an integral part of their membrane. Though direct evidence is not yet available, it seems likely that the attachment of multivalent antigen to the surface of the single AFC could initiate the formation of patches and caps, as in nonsecreting B lymphocytes, and finally result in pinocytosis of immune complexes. This receptor rearrangement and endocytotic activity could interfere with the regulation of secretion at many levels. For example, in B lymphocytes, endocytosed Ig-anti-Ig complexes reach the Golgi apparatus (Santer, 1973), which is believed to be important in presecretion packaging. One can only speculate as to the influences which the surface receptor rearrangements could have on microfibrils and microtubules, but they may well be of a subtle and profound nature (Yahara and Edelman, 1974). The developing molecular biology of membrane function may well reveal important similarities between B cell tolerogenesis and effector cell blockade.

Tumor biologists will no doubt be most interested in whether effector cell blockade can occur at the level of the T cell, and in particular, whether the cytotoxic properties of "killer" T lymphocytes can be abrogated. Our work in this area is not so far advanced, but there is reason to believe that the phenomenon can work at the T cell level. When T cells are induced *in vitro* by alloantigens to become "killer" cells, the efficiency of killing is diminished by exposing the killer cells to crude H-2 antigen containing membrane fragments (Wagner and Boyle, 1972). It is too early to decide whether this is simple receptor saturation or a true metabolic blockade of the cell. The blocking action of antigen–antibody complexes on the cytotoxic properties of antitumor lymphocytes also deserves analysis from this viewpoint. It is our hope that the concept of effector cell blockade will stimulate a large body of research in a variety of model systems, as it appears to us to offer yet another level at which immune responses can be controlled.

VIII. Suppressor T Cells

The field of tolerance was revolutionized by the realization that co-operation between T and B cells is necessary for many immune responses (Miller, 1972). It now seems probable that we are in the early stages of another revolution, as evidence is accumulating to show that T cells cannot only *help* B cells to produce antibody, but can significantly *suppress* their capacity to do so. In fact, in some models of immunological tolerance, the chief lesion is neither a defect in B cells, nor a lack of T cells able to collaborate, but the presence of a T cell population capable of actively interfering with the immunological functions of normal T and B cells. The present review is unfortunately timed for an analysis of the role of suppressor T cells, because, despite a spate of very extensive activity, no clear picture of the relationship between suppressor T cells and helper T cells, on the one hand; and of the physiological role of suppressor T cells on the other, has yet emerged. Nevertheless, one senses a worldwide excitement on the subject, evidenced by the fact that a recent review by Droege (1973) cites 81 references bearing on it. As with any new concept, there has been a certain amount of overreaction, such that some authors wish to force the whole world of tolerance into the new framework, a position rendered impossible by the ready induction of tolerance in congenitally athymic ("nude") mice. Furthermore, it seems probable that the phrase "suppressor T cells" embraces a variety of different phenomena, as indeed does the phrase "immunologic tolerance."

What are the key facts available at present? Important early observations were the finding that procedures such as treatment with anti-lymphocyte serum or thymectomy could *increase* antibody production

against antigens not dependent on T cell help for their action (Baum *et al.*, 1969; Baker *et al.*, 1970a; Kerbel and Eidinger, 1971). The converse observation, namely that the level of antibody produced in response to antigenic challenge could sometimes be reduced by the injection of T cells has also been amply documented (Baker *et al.*, 1970b; Okumara and Tada, 1971; Droege, 1971). The capacity of T cells to produce substances capable of lowering antibody production in tissue culture has been noted (reviewed in Feldmann and Nossal, 1972). A particularly fascinating example of suppressor T cell action is their role in attacking cells with specific receptors, in the phenomenon of allotype suppression (Herzenberg *et al.*, 1971).

The concept of T cell suppression in immunologic tolerance first came into focus when it was noted that cells from tolerant mice, in some systems, could be mixed with normal cells and could cause these to fail to respond adequately (McCullagh, 1970, 1972; Gershon and Kondo, 1971). Two recent studies give interesting insight into this so-called "infectious" tolerance. Ada and Cooper (1973) injected hemocyanin into mice in repeated, large doses beginning at birth, and documented that such mice could not form antihemocyanin antibody. They would not mount a delayed hypersensitivity response. They had normal numbers of cells in their spleens capable of binding ^{125}I-labeled hemocyanin. This suggested a T cell lesion, with retention of normal B cell function (Cooper and Ada, 1972). When lymphoid cells from tolerant mice were mixed with cells from normal mice, unresponsiveness was transferred. Destruction of T cells from tolerant mice by anti-θ treatment, followed by the addition of activated T cells, restored an antibody response. Basten *et al.* (1974) have investigated a system similar in some respects. They injected adult CBA mice with a single, large dose of freshly deaggregated FγG, and 6–12 days later, studies of the whole animal showed a tolerance to challenge with alum-precipitated FγG given together with killed pertussis organisms. The tolerance was predominantly in the IgG phase of the response, which delineates the phenomenon from the work with FγG discussed in Section IV, carried out in "nude" mice. This dealt largely with tolerization of B cell precursors of IgM-forming cells. The tolerance induced by Basten *et al.* (1974) was stable on adoptive transfer to irradiated recipients, was not due to antigen carry-over, and could "infect" both normal spleen cells cotransferred with the tolerant cells, and even the lymphon of a normal animal. This suppressor activity of spleen cells from tolerant mice could be abrogated by anti-θ treatment, confirming the T cell nature of the effect. Most interestingly, it could be eliminated by treatment of the tolerant spleen cells with ^{125}I-FγG for 18 hours at 0° (the so-called radioactive antigen suicide technique). This

substantiated that a specific subset of cells bearing anti-FγG receptors was responsible for the suppressor effect. In retrospect, other tolerance models (e.g., Asherson *et al.*, 1971) are also best explained on the basis of suppressor T cells.

Prior to these key studies, we had speculated on the possible tolerogenic role of T cells in certain *in vitro* systems (Feldmann and Nossal, 1972; Feldmann and Basten, 1972a,b; Feldmann, 1973). We were struck by the observation that an excess of T helper cells caused a profound lowering of antibody production. Our group has reviewed elsewhere (Feldmann and Nossal, 1972; Marchalonis and Cone, 1973) the evidence that T-B collaboration, at least in certain *in vitro* systems, involves the production by activated T cells of small amounts of an IgM-like antibody (IgT) which has a special cytophilia for the macrophage surface (Cone *et al.*, 1974). In our view, this IgT, complexed to the carrier portion of antigen, helps to hold antigen in concentrated form at the macrophage surface. B cells, "addressed" to this macrophage because of free, noncarrier determinants on the antigen, are triggered into antibody formation, aided perhaps by factors synthesized by the macrophage (Schrader, 1974a). It then seemed possible that, if an excessive amount of IgT were produced, such that macrophage sites for IgT became saturated, then complexes of IgT and antigen could form and could reach the B cell directly. These might cause antibody-mediated tolerance akin to that discussed in Section VI, thereby explaining "infectious tolerance." Direct testing of this theory *in vitro* (Feldmann, 1973) showed that T cells could cause shutting off of B cells provided macrophages could be removed or circumvented, and the suppressive factor was (a) antigen specific; (b) inhibitable by antisera directed against light and μ heavy chains of the mouse; and (c) inhibitable by antisera against the B cell-stimulating antigen. It thus had all the characteristics of an IgT-antigen complex.

It seems that these *in vitro* studies fit well with the Ada and Cooper (1973) model, where one could envisage that the constant barrage of antigenic stimulation by repeated hemocyanin injections produces large numbers of antihemocyanin T cells, perhaps sufficient to embarrass the animal's reticuloendothelial system. It is a little less easy to see how the single injection of a deaggregated serum protein in the experiments of Basten *et al.* (1974) could cause a sufficient degree of T cell activation to make the Feldmann (1973) mechanism plausible. It may be wise not to judge all the suppressor T cell effects to be due to any one mechanism. For example, it is possible, as Droege (1973) has suggested, that there are two classes of T cells involved in interaction with B cells, one helper and one suppressor. Furthermore, not all experiments have had specificity

controls as convincing as those of the studies listed above, and some suppressor T cell effects could be due to an excess of lymphokine production. Finally, small differences in experimental design can lead to different conclusions. Thus Scott (1973), using rat lymph node cells and deaggregated sheep immunoglobulins as antigen, could find no evidence for suppressor cells or blocking factors during the induction of tolerance. This section, therefore, ends on the same note of caution with which it began. Suppressor T cells are here to stay, they do play a dominant role in some tolerance models, and there are reasonable ways to study their effects. They do not explain all forms of experimental or autotolerance, and much remains to be done before their place in immunology as a whole can be reasonably assessed.

IX. Relevance of Tolerance and Effector Cell Blockade to Cancer

No regular reader of these "Advances" needs to be reminded that the immune attack against tumor tissues is a complex problem. Even when the investigator can dictate the number and kind of tumor cell inoculum that elicits tumor immunity, and that which tests the resulting level of immunity, he or she is faced with the fact that T cells, B cells, macrophages, antibody and complement can all be shown to play a part in the rejection process, depending on the details of the experimental model. How much more involved is the struggle to assess the role of these cells and molecules in the "real life" situation, where a tumor emerges spontaneously, grows, and metastasizes in the face of whatever immune attack the host mounts, and finally kills the patient, who at this late stage, tends to exhibit immunologic anergy not only to his own tumor but of a generalized nature. As with the field of antibody formation to defined antigens, the problem will yield its secrets only through a patient reductionist approach. In that case, we should not ask whether T and B cell tolerization and effector cell blockade are relevant to tumor immunity. Rather, we should ask at what stages in which cancers these phenomena may occur, and what may their respective roles be.

The debate on this topic has been heavily influenced by the exciting findings of the Hellström group (Hellström and Hellström, 1969, 1973; Sjögren *et al.*, 1971; Hellström *et al.*, 1969, 1971; Wegmann *et al.*, 1971). Their thesis is that many situations where antigenic entities survive, thrive, and even grow in immunologically competent hosts, the immune status of the host may be one *not* of immunologic tolerance, but of a balanced coexistence of activated lymphocytes with toxic potential, and serum factors including antigen–antibody complexes that can block this destructive potential *in vitro*. Thus, they claim the possibility of a common mechanism underlying organ transplant survival, tumor progression,

normal fetal growth, and health of allophenic or chimaeric mice. As a great admirer of both this monumental body of work and of the close and cautious reasoning in the discussion sections of the Hellströms' papers, I am concerned to note that some authors have overinterpreted the data. In fact, a "tolerance is dead" school has emerged, which bases its position largely on the Hellström group's work and on the extraordinary efficacy of passive enhancement in the rat renal allograft model. Two definite conclusions which should have emerged from the present review are: (1) that tolerance and enhancement are *not* antithetical or mutually exclusive concepts; and (2) that blocking factors have the potential to cause *both* true tolerance (Section VI) *and* peripheral effector cell blockade (Section VII), which again are in no sense antithetical. Let us examine these propositions in a tumor setting.

It is axiomatic that, while a tumor grows, the lymphoid system is exposed to a progressively increasing level of its various tumor-associated transplantation antigens (TATA). These must reach the lymphoid system in various molecular forms. First, TATA molecules will be shed from the surface of the living tumor cell, which is metabolically active and continually renews its membrane proteins. Second, fragments of tumor cell membranes will be released as tumor cells are destroyed, either by immunologic attack or shortage of blood supply. Third, lymphocytes may also encounter living tumor cells as they wander around the tissues, providing a transient contact between their receptors and a source of multivalent antigen. In each of the three cases, the end result of a meeting between antigen and lymphocyte must involve principles similar to the ones we have considered in this article. The soluble TATA molecule has the potential of causing tolerance, as in Section IV, except if an activated T cell-macrophage system delivers "signal 2," when immunization will result. The particulate or multivalent antigens, as described in Section V, will have the capacity to immunize or tolerize, dependent largely on dose, and on the presence of antibody, which will cause complex formation and will enhance the possibility of tolerization (Section VI). Broadly speaking, for most tumors some immunization should result in the earlier stages of growth. An exception could be a tumor which exhibits as TATA molecules with only a single determinant foreign to the host, and turning over rapidly in the tumor cell membrane. Such an antigen would reach the extracellular fluids in soluble form and, failing to elicit helper effects, might tolerize first T and then B cells. In the other cases, either helper phenomena or antigen multivalency or both will ensure T and B cell immunity.

As effector cells are formed, various feedback loops will come into operation. Among these will be antibody formation and its eventual

complexing with tumor antigen. Some experimental protocols may highlight the enhancing properties of that antibody. Others may well show that the complexes cause T and B cell tolerance. Others may neglect the effects occurring in antigen-reactive T and B cells and throw the spotlight on effector cell blockade. Another feedback loop may well involve antigen. As immune processes destroy tumor cells, pulses of TATA will be released, which could be immunogenic or tolerogenic. If the inherent growth capacity of the tumor is sufficient to withstand the balance of immune activities engendered, and the tumor progresses, the odds in favor of immunological nonreactivity increase. First, the continued absorption of antibody by the tumor cells (as in treadmill neutralization, Section V) will lower antibody levels. If antibody was originally in excess, it may fall to levels where tolerogenic complexes form. In this regard, it is important to realize that, for antibody-mediated tolerance, the antigen–antibody ratio is critical. Thus, either antigen or antibody could act as "unblocking" factors under appropriate circumstances. Students of unblocking are most particularly urged to consult Feldmann and Diener's (1971) original paper on this point. Second, the increasingly high concentrations of TATA may tolerize T and B cells directly.

Conversely, if the tumor load is suddenly reduced, as after surgery or effective chemotherapy, this alone may tilt the immunologic balance in favor of the host. TATA levels in extracellular fluids will fall, and, as after transfer of paralyzed cells to an antigen-free environment in some systems (Section V), immunocytes may escape from tolerance or effector cell blockade. Tolerogenic complexes will be catabolized, and, as at least some antibody-forming cells are long-lived, free serum antibody may reappear even without further antigenic stimulation. Presumably unblocked killer T cells may similarly appear. The immediate postsurgery or postchemotherapy period may therefore be of critical importance to the cancer patient. He may depend on his immunocytes to remove residual tumor tissue, and therefore this period is also the ideal one for active tumor immunotherapy. If cure is not achieved then, the immune activation may decay for lack of antigen, and the whole cycle may start again later. No doubt, for different tumors the relative importance of direct cytotoxic killing by T cells, antibody-coated target cell killing by lymphocytes (Perlmann and Holm, 1969), and macrophage cytotoxicity (Evans and Alexander, 1972) will vary. However, in a significant number of model situations, these variables are already being patiently sorted out (Baldwin, 1973; Skurzak *et al.*, 1972; Sjögren *et al.*, 1971; Klein, 1973; Lewis *et al.*, 1969; Morton *et al.*, 1970; Rouse *et al.*, 1973; Wagner and Nossal, 1973).

Many of the concepts outlined in this section have already been

validated in one system or another. The principles developed from the fundamental immunologists' simpler systems should continue to provide leads to help the cancer researcher study the basis and kinetics of tumor immunity. Whether this fundamental approach will help to develop therapeutically useful immunization protocols, or whether these will emerge by empiric clinical experimentation, remains to be seen. For the immediate future, it seems particularly important for tumor immunologists to develop assay procedures for TATA and antibody levels that are more quantitative than those commonly employed; and, in their assessment of the cellular immune state, to become more attuned to differentiate the immune *potential* of lymphoid populations, i.e., the number and properties of anti-tumor T and B cells, from the immune *performance* as measured by the levels and activities of antitumor effector cells. As with the field of organ transplantation, a continued discourse between basic immunologists and clinical oncologists cannot help but be mutually fruitful.

X. Summary

The problem of immunologic tolerance has been examined from two perspectives. First, from the viewpoint of the susceptible T or B lymphoid cell, four possible ways of achieving an insusceptibility to appropriate activation signals have been outlined. These are clonal abortion, clonal deletion, receptor blockade, and activation blockade. Clonal abortion was defined as an elimination by antigen of lymphocytes with specificity for a given antigen through contact with that antigen at some postulated early maturation phase, when receptors have just appeared at the cell surface. Clonal deletion implies a destruction of fully developed and competent immunocytes as an end result of the attachment of certain forms and amounts of antigen to their surface. Receptor blockade is the saturation of antigen receptors with antigenic molecules that are, for some reason, nonimmunogenic, and which prevent the cell from being stimulated by immunogenic antigen. Activation blockade is a more general concept relating to any factor or influence which may alter the lymphocyte's reactivity to antigen. A plea is made for *not* regarding these four mechanisms as alternatives, but rather as complementary concepts. It is possible that each comes into play in some of the various phenomena which exist under the broad umbrella of tolerance.

The second approach has been to divide tolerance phenomena on the basis of the structural nature of the antigen causing them. A case is made for differentiating between those tolerogens that are soluble, oligovalent and poorly immunogenic; and those that are polymeric, multivalent, and highly immunogenic in low dose but paralyzing in high dose. The former group require T cell help for antibody formation to occur, and low

antigen concentrations may tolerize T cells selectively, higher doses affecting also B cells. The latter group can immunize B cells without T cell intervention, and the view that tolerance with such antigens represents essentially supraoptimal quantitative stimulation is proposed. A special example of multivalent antigen is that of soluble antigen–antibody complexes in slight antigen excess. These are profoundly tolerogenic for both B and T cells.

A new phenomenon is briefly described. This is the reduction in antibody secretion rate caused by the attachment of highly multivalent antigen to the surface of an antibody-forming cell. As we believe it is possible that cytotoxic cells of the T lineage can be similarly affected, we propose the broad term effector cell blockade. It is suggested (a) that some examples previously considered to be due to tolerance really represent effector cell blockade; and (b) that soluble immune complexes may cause *both* true tolerance at the level of antigen-reactive T or B cell, and effector cell blockade at the level of the terminal activated immunocyte.

Consideration is given to the interrelationships between T and B lymphocytes and their influence in tolerance. Failure of antibody production by whole animals may sometimes be due either to a lack of competent T lymphocytes required for a helper effect, or to the presence of antigen-activated T lymphocytes that exert some suppressive influence on B cells. One speculative mechanism for suppressor T cell action is presented.

The likely scenario of immunologic events during tumor progression is outlined from a basic immunologist's viewpoint. The reasons for a relentless switch from immunity to tolerance and blockade during tumor progression are reviewed. The immunologic significance of the immediate post-surgery or post chemotherapy period is discussed. The conclusion is reached that there will be much two-way traffic between basic immunologist and clinical or experimental oncologist in the years ahead.

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THE ROLE OF MACROPHAGES IN DEFENSE AGAINST NEOPLASTIC DISEASE[1]

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I. Introduction

Recent successes in immunochemotherapy of neoplastic disease have focused interest and stimulated research on the mechanisms of natural and adjuvant-induced host antitumor activity. For many years the major concern of both research and reviews on tumor immunology has been the antitumor activity of antibodies and lymphocytes. More recently, macrophages have been found to play a significant role in both immune and nonimmune antineoplastic host activities. In this review, we have attempted to extract from a mass of heterogeneous and sometimes contradictory material the established and speculated roles for macrophages in the host defense against neoplastic disease.

The macrophage has long been known to be an integral part of the host's complex defense system. The full potential of the macrophage and the reticuloendothelial system (RES) to which it belongs has been the

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subject of many articles and several recent books (van Furth, 1970; Vernon-Roberts, 1972; Pearsall and Weiser, 1970; Nelson, 1969) and is well beyond the scope of this review. However, a brief summary of the macrophage's recognized activities is presented below in order to provide a base from which one can discuss the role of macrophages in the defense against neoplastic disease. Where specific periodical references are not given, the reader is referred to the texts mentioned above.

A. Definition of Macrophage

The term "macrophage" refers to a group of cells given many different names (e.g., Kupffer cells, dust cells, microglial cells, histiocytes), residing in many different organs (e.g., liver, lung, brain, spleen), as well as in almost all tissues and serosal cavities of the body. Macrophages constitute the cellular component of the RES responsible for particle clearance, storage, and degradation. RES function, in general, is affected by alterations in macrophage activity, but may be unaffected when such alterations occur at a local level. In addition, both macrophage and RES activities are affected by factors such as opsonins and lymphokines elaborated by accessory lymphoid cells. Such complex interactions should be kept in mind when considering discordant data regarding the role of the RES or of macrophages in host defenses against neoplastic disease.

The macrophage is a large cell possessing a single, eccentrically located, indented nucleus, frequently containing peripheral chromatin and one or two nucleoli. The cytoplasm is often foamy or granular, containing many vesicles including lipid droplets, lysozomes, phagosomes, and phagolysosomes. The enzymatic content of such vesicles may be highlighted by stains specific for peroxidase, esterase, or acid phosphatase. The cytoplasmic membrane is usually active, appearing ruffled and often engaged in forming small endocytic vacuoles. The cytoplasmic membrane has also been shown to contain receptor sites for opsonins and for the Fc portion of immunoglobulins (LoBuglio *et al.*, 1967; Arend and Mannik, 1973). Two of the most striking features of macrophages are their phagocytic activity and their trypsin-resistant adherence to glass surfaces (Evans, 1970, 1973a). Macrophages can move across glass surfaces and are sensitive to both chemotactic and migration inhibitory factors (David, 1971; Nathan *et al.*, 1971). A number of biological and chemical agents have been demonstrated to activate resting macrophages, transforming them into cells that are metabolically and morphologically hyperactive and display accelerated attachment and spreading onto glass surfaces and enhanced pinocytic, phagocytic, bactericidal, and cytopathic activities (Blanden, 1968; Mackaness, 1970; McLaughlin *et al.*, 1972).

B. Participation of Macrophages in Immune and Nonimmune Host Responses

The macrophage originates from radiosensitive precursor cells in the bone marrow, which, upon maturity, become relatively radioresistant (Chen and Schooley, 1970) and participate in many immune and nonimmune functions. As either blood monocytes or wandering or tissue-fixed macrophages, these mononuclear phagocytes take up and process particulate antigens, thereby initiating a sequence of cellular reactions leading to the primary (Panijel and Cayeux, 1968; Gallily and Feldman, 1967; Cruchaud and Unanue, 1971) as well as, in some cases, the secondary (Panijel and Cayeux, 1968; Feldman and Palmer, 1971) antibody response to these antigens. Macrophages can also facilitate 'T' cell–'B' cell interaction in this antibody-forming process and can aid lymphocytes in reversing specific immunosuppression caused by minute doses of passive antibody (Haughton, 1971). Beyond this participation in the afferent limb of the immune response, macrophages can act as nonspecific, yet necessary, amplifying or effector cells in delayed hypersensitivity reactions (Volkman and Collins, 1968, 1971; Salvin *et al.*, 1971). Within this system, macrophages have been shown to be necessary for optimal blast transformation and lymphotoxin release by sensitized lymphocytes upon exposure to their specific antigen (Hersh and Harris, 1968; Levis and Robbins, 1970b; Seeger and Oppenheim, 1970). Additional studies indicate that macrophages play an important activating or regulating role in mixed leukocyte reactions (Blaese *et al.*, 1972; Twomey and Sharkey, 1972), *in vitro* graft reactions (Lonai and Feldman, 1971), and the blastogenic and interferon responses of lymphocytes to nonspecific mitogens such as phytohemagglutinin, concanavalin A, and lipopolysaccharide (Levis and Robbins, 1970a; Epstein *et al.*, 1971; Gery *et al.*, 1972; Gery and Waksman, 1972). Within these cellular reactions, macrophages somehow facilitate contact between the stimulus and the lymphocyte or between lymphocyte and lymphocyte, but they do not appear to account for significant immune specificity, radioactive label incorporation, or release of lymphotoxin and interferon. Activated macrophages have been shown to release a lymphocyte-activating factor (LAF) that may play a role in the above reactions (Gery *et al.*, 1972; Gery and Waksman, 1972; Mitchell *et al.*, 1973a).

Macrophages and the entire RES constitute a barrier to pathogenic infection of the host. Aided by specific cytophilic antibodies, antigen–antibody complexes (LoBuglio *et al.*, 1967; Shin *et al.*, 1972; Fakhri *et al.*, 1973; Uhr, 1965; Hoy and Nelson, 1969a; Cruse *et al.*, 1973; Liew and

Parish, 1972), or nonspecific opsonins (Pisano *et al.*, 1970a; Kampschmidt and Pulliam, 1972), macrophages exert their own brand of cellular immunity to invading microorganisms (Mims, 1964; Silverstein, 1970; Ruskin *et al.*, 1969). Finally, macrophages have many additional nonimmune functions, some of which were thought originally to be the only functions possessed by these cells (Pearsall and Weiser, 1970; Vernon-Roberts, 1972). Macrophages have been shown to scavenge tissue debris and effete cells, aid in wound healing, detoxify certain chemical substances, inactivate thromboplastin, and participate in the metabolism and disposition of iron, lipids, and proteins. Macrophages are also involved in the synthesis of various biologically important products, such as transferrin, complement, interferon, pyrogen, colony-stimulating factor (CSF) (Chervenick and LoBuglio, 1972), lymphocyte-activating factor (LAF) (Gery *et al.*, 1972; Gery and Waksman, 1972), and several cytotoxic factors (Sintek and Pincus, 1970; Heise and Weiser, 1969; Kramer and Granger, 1972).

II. RES and Tumor Resistance

A. Historical Perspective

The importance of the RES in the defense against neoplastic disease was stressed by Stern (1941), who noted previous reports of depressed RES function in cancer patients and was able to correlate such depressed function with both the severity of disease and the lack of clinical response to therapy. Conversely, a similar correlation was found between survival of cancer patients and the presence of marked lymph node sinus histiocytosis (Black *et al.*, 1953). In early animal studies, high-cancer-incidence inbred strains of mice were shown to have depressed RES activity when compared to low-cancer strains (Stern, 1948). In addition, agents which depressed RES activity increased susceptibility to tumors (Baillif, 1956; Ghose, 1957), whereas agents which stimulated RES activity increased the resistance to tumor transplants and oncogenic viruses (Old *et al.*, 1961; Bradner *et al.*, 1958; Diller *et al.*, 1963; Lemperle, 1966). Inbred strains of mice known to have a high spontaneous tumor incidence were less responsive to RES stimulation, again indicating an inherent weakness in the ability of their RES to combat neoplastic disease (Old *et al.*, 1961). Finally, reports of enhanced RES activity observed in certain tumor-bearing mice were interpreted as further evidence that the RES was participating in the host defense against neoplastic disease (Old *et al.*, 1960, 1961).

B. Depression of RES Activity

1. *RES Depression and Increased Susceptibility to Neoplastic Disease*

Based upon the observations mentioned above and the well documented increased incidence of cancer in patients with defined primary and secondary immunodeficiency states (Gatti and Good, 1971; Penn, 1970), many studies into the influence of primary and secondary RES depression on the incidence of neoplastic disease have been performed. Depressed RES activity has been observed in aged animals associated with impaired immunocompetence and increased susceptibility to neoplastic disease (Teller *et al.*, 1964; Stjernswärd, 1966; Wigzell and Stjernswärd, 1966; Aoki *et al.*, 1965; Hanna *et al.*, 1971). Altered localization of antigen and decreased responsiveness to sheep red blood cells, skin grafts, as well as to a known RES stimulant, zymosan, paralleled an increased incidence of spontaneous tumors and susceptibility to chemically induced or transplanted tumors in these aged animals.

Depression of RES function may be induced exogenously by various biological and chemical agents. The administration of a wide variety of chemicals which enhance tumor growth (Baillif, 1956) and metastasis (Ghose, 1957) have been shown to blockade transiently the RES and depress humoral immunity (Sabet *et al.*, 1968). Treatment of neoplastic disease patients with cytotoxic drugs and ionizing radiation is often followed by depressed RES function (Chen and Schooley, 1970; Zscheische, 1972; Volkman and Collins, 1968, 1971), an effect that may compromise the therapeutic efficacy of these procedures (Magarey and Baum, 1970; Sheagren *et al.*, 1967; Donovan, 1967; Al-Sarraf *et al.*, 1970). Such depression of RES function might also account for some of the increased incidence of neoplasia in transplant recipients receiving immunosuppressive, cytotoxic drugs (Gatti and Good, 1971; Penn, 1970).

2. *RES Depression in Neoplastic Disease*

The neoplastic process itself has been shown to have varying effects on RES activity, although in general, heightened RES activity has been correlated with a more favorable, and depressed RES activity with a less favorable, clinical course (Magarey and Baum, 1970; Sheagren *et al.*, 1967; Al-Sarraf *et al.*, 1970; Old *et al.*, 1961; Groch *et al.*, 1965; Donovan, 1967). Significant RES depression has been observed in spontaneous tumor-bearing mice when compared to their tumor-free littermates (Stern *et al.*, 1967). Similarly, metastasis in tumor-bearing mice has been shown to cause a marked reduction in RES clearance activities (Franchi *et al.*,

1972). In some instances, the RES depression observed in tumor-bearing animals and human cancer patients has been attributed to a depletion of nonspecific opsonins (Pisano *et al.*, 1970a,b, 1973; Di Luzio *et al.*, 1972). A 90% reduction of an essential serum opsonin, called human recognition factor (HRF), has been observed in patients with untreated carcinoma and leukemia (Pisano *et al.*, 1970a,b). A significant decrease in opsonin levels was also observed in mice following the intravenous administration of leukemic, but not in normal, leukocytes (Di Luzio *et al.*, 1972), indicating that the RES depression observed in these animals was directly caused by the neoplastic process. The restoration of HRF to near normal levels following successful antineoplastic therapy further suggests that HRF can be of prognostic, and even of therapeutic, value (Pisano *et al.*, 1970b).

C. Stimulation of RES Activity

1. *RES Stimulation and Increased Resistance to Neoplastic Disease*

Additional evidence supporting the role of the RES in the defense against neoplastic disease comes from animals treated with various chemical and biological RES stimulants. Restim (Lemperle, 1966; Bliznakov, 1968), glucan (Lemperle, 1966; Di Luzio *et al.*, 1970; Diller *et al.*, 1963), zymosan (Diller *et al.*, 1963; Kampschmidt and Upchurch, 1968; Bradner *et al.*, 1958), triolein (Kampschmidt and Upchurch, 1968), lipopolysaccharide (Lemperle, 1966), stilbesterol (Magarey and Baum, 1970, 1971; Kampschmidt and Upchurch, 1968), pyran (Kapila *et al.*, 1971; Remington and Merigan, 1970; Hirsch *et al.*, 1972), Triton WR 1339 (Franchi *et al.*, 1971, 1973), and tilorone (Munson *et al.*, 1972) have all been shown to enhance RES activity. After such stimulation, experimental animals have shown increased rejection of tumor grafts, resistance to oncogenic viruses, reduction of metastases, and regression of established tumors, with or without resultant long-term immunity. In addition, the antineoplastic effects of biological agents such as *Bordetella pertussis* (Malkiel and Hargis, 1961; Guyer and Crowther, 1969), *Corynebacterium parvum* (Woodruff and Boak, 1966; Currie and Bagshawe, 1970; Fisher *et al.*, 1970), Bacille Calmette-Guerin (BCG) (Larson *et al.*, 1971; Davignon *et al.*, 1970; Old *et al.*, 1961), and a methanol extraction residue (MER) of BCG (D. W. Weiss *et al.*, 1966; Weiss, 1972) have been attributed to their RES stimulatory activities. The exact mechanism by which these agents increase resistance to tumors is uncertain since they have been shown to be immune adjuvants as well as RES stimulants. Regardless of the mechanism, the macrophage, an integral component of the RES, must be involved in the increased tumor resistance produced

by these agents since, as will be presented in Section III, plays a crucial role in both immune adjuvant reactions and antineoplastic host defenses.

2. *RES Stimulation in Neoplastic Disease*

As mentioned in Section II,B,2, the neoplastic process has been shown in some instances to have a stimulatory effect on the RES. Studies using different animal hosts and tumor types have yielded contradictory data. One possible source of discrepancy may be that enhanced liver activity accounts for the majority of RES stimulation by tumor or exogenous agents (Kampschmidt and Pulliam, 1972; Di Luzio *et al.*, 1970; Warr and Sljivic, 1973; Sljivic and Warr, 1973). Since the liver functions mainly in clearance and degradation of antigen (Frei *et al.*, 1965; Franzl, 1972), RES stimulation, although aiding in reduction of tumor load, may be of limited benefit in the establishment of long-term immunity to tumor and may therefore not be an adequate defense against those neoplastic diseases that require both immune and nonimmune clearance. Nevertheless, as a rule, patients responding to neoplastic disease with enhanced RES activity do seem to follow a better clinical course (Magarey and Baum, 1970; Sheagren *et al.*, 1967; Donovan, 1967; Black and Leis, 1971), again implying that the RES does contribute to the defense against neoplastic disease.

III. Macrophages and Tumor Resistance

A. Historical Perspective

The apparent involvement of the RES in the host defense against neoplastic disease has stimulated research into the antineoplastic activities of macrophages. Several transplanted animal tumors were found to elicit a marked histiocytic response not only in draining lymph nodes, but also at the transplant site (Gorer, 1956; Amos, 1960, 1962; Weaver, 1958; Gershon *et al.*, 1967). Macrophages from tumor-immunized animals caused specific, contact-mediated destruction of tumor cells *in vitro* (Granger and Weiser, 1964; Bennett, 1965; Bennett *et al.*, 1964). Hepatic and splenic macrophages isolated from immunized guinea pigs were shown to be capable of phagocytizing tumor cells and pinocytizing solubilized tumor antigens (Draz *et al.*, 1971). Immune peritoneal macrophages were also shown to be capable of transferring adoptive immunity to ascitic tumor transplants (Amos, 1962; Tsoi and Weiser, 1968a; Bennett *et al.*, 1964; Bennett, 1965; Baker *et al.*, 1962). Such immunity was equal to or greater than that conferred by transfer of immune peritoneal lymphocytes.

In the remainder of this review, we attempt to cover the research,

conducted mainly in the past 5 years, on the participation and possible mechanisms of macrophage activity in the host response to neoplastic disease.

B. Depressed Macrophage Function

1. *Depressed Macrophage Function and Increased Susceptibility to Neoplastic Disease*

The age of dependence of RES activity has been found to apply to macrophage function as well. The RES depression and increased incidence of tumors in aged mice have been attributed to defective macrophage function (Hanna *et al.*, 1971). In addition, Argyris (1968) has shown that the immunologic immaturity of newborn mice is due, at least in part, to the lack of competent macrophages. Macrophage maturation has also been shown to play an important role in the development of resistance to infection by various pathogenic viruses (Allison, 1970; Johnson, 1964). Accordingly, several animal species are susceptible to certain oncogenic viruses or to tumor cell transplants for only a brief period following birth (Gross, 1970). Finally, macrophage function has been impaired by infection with a number of nononcogenic viruses (Gledhill *et al.*, 1965) and by the oncogenic Friend leukemia virus (Wheelock *et al.*, 1974). Impairment of macrophage function may lead to acceleration of the neoplastic process through reduction of direct macrophage antitumor activity and of macrophage-dependent immune responses to tumor antigens. One may further speculate that the failure of several alkylating agents to produce remission in patients with neoplastic disease may be due, in part, to the depression of macrophage function caused by these agents (Zscheische, 1972). In like fashion, the decrease in number of mononuclear phagocytes in both peripheral circulation and inflammatory exudates, caused by the administration of glucocorticosteroids (Thompson and van Furth, 1970) may contribute to the increased incidence of neoplasia in patients on steroids for immunosuppressive purposes (Penn, 1970).

Depression of macrophage function may also be achieved through the *in vivo* or *in vitro* administration of amantin-albumin conjugates (Barbanti-Brodano and Fiume, 1973), carrageenan (Catanzaro *et al.*, 1971), silica (Kessel *et al.*, 1963), or specific antimacrophage serum (AMS) (Loewi *et al.*, 1969). Amantin–albumin conjugates have been shown to selectively kill macrophages in mixed leukocyte cultures (Barbanti-Brodano and Fiume, 1973). Carrageenan has been shown to impair *in vitro* macrophage dependent, antigen-induced lymphocyte transformation and *in vivo* anti-sheep red blood cell antibody formation (Lake *et al.*,

1971; Bice *et al.*, 1972), although one recent report has indicated that these immunodepressive effects may be unrelated to malfunction of the macrophages (Aschheim and Raffel, 1972). To date, there are no reports on the effects of amantin–albumin conjugates or carrageenan on tumor growth. Silica, on the other hand, has been shown to increase host susceptibility to infection by certain pathogenic viruses, including the oncogenic Friend leukemia virus (Allison, 1970; Larson *et al.*, 1972; Wheelock *et al.*, 1974). In addition, silica pretreatment has been shown to interfere with the establishment of active (Pearsall and Weiser, 1968a; Wheelock *et al.*, 1974; Erb *et al.*, 1972) and adoptive (Zarling and Tevethia, 1973) antitumor immunity, and has been shown to actually induce lymphomatous tumors in Wistar rats (Wagner and Wagner, 1972). Silicosis has been correlated with an increased incidence of tuberculosis (Mayers, 1969), a disease in which macrophages play a major role in host defenses. In addition, silicosis has been related causally to an increased incidence of neoplastic disease (Bryson and Bischoff, 1967). Whether this oncogenic relationship is due to a direct carcinogenic effect of silica or to its subversion of macrophage-mediated antitumor activity has not yet been determined.

The administration of AMS to animals has been shown to impair carbon clearance, antibody production, delayed hypersensitivity, and host resistance to certain pathogenic viruses (Panijel and Cayeux, 1968; Loewi *et al.*, 1969; Smith and Pont, 1972). In addition, injection of AMS into Ehrlich tumor-bearing mice produced significant acceleration of tumor growth (Yamashiro, 1972). Accordingly, the antimacrophage activity found in various antilymphocyte serum (ALS) preparations (Clarke and Boak, 1970; Patterson *et al.*, 1970; Sheagren *et al.*, 1969; Musher *et al.*, 1972), has been suggested to be responsible, at least in part, for the neoplasia-enhancing effects of ALS (Gatti and Good, 1971; Penn, 1970; Larson *et al.*, 1972).

Of added interest are recent reports of the toxic effect of cigarette smoke and asbestos fibers on macrophages. In contrast to macrophages of nonsmokers, macrophages from cigarette smokers failed to show any migration inhibition in response to MIF or to specific antigens to which *in vivo* delayed hypersensitivity skin reactions had been demonstrated (Warr and Martin, 1973). In addition, the *in vitro* exposure of lung explant cultures containing macrophages and epithelioid cells to filtered cigarette smoke resulted in decreased phagocytic function and death in the macrophage population, followed by a loss of contact inhibition of epithelioid cells (Leuchtenberger and Leuchtenberger, 1971). This sequence of events suggested both particle-clearing and growth-regulating functions for alveolar macrophages.

Treatment of macrophage cultures with asbestos resulted in increased cell membrane permeability and phagosome membrane damage, leading to release of lysosomal enzymes into supernatant fluids. This enzyme release was paralleled by a decrease in macrophage total lipid content and an increase in lysolecithin levels (Miller and Harington, 1972; Beck *et al.*, 1972). The correlation of asbestosis and increased incidence of neoplasia has been well documented (Newhouse *et al.*, 1972; Enterline *et al.*, 1973). In addition, asbestos has been shown to induce tumors in experimental animals (Shin and Firminger, 1973; Stanton and Wrench, 1972). Furthermore, prior to the induction of tumors, intraperitoneal injection of asbestos produced an acute peritonitis in which death of both polymorphonuclear leukocytes and macrophages in contact with asbestos fibers was observed. Finally, epidemiological studies have suggested that asbestos could also act as a cocarcinogen for cigarette smoke (Kannerstein and Churg, 1972). Whether the oncogenicity of these agents is due to their direct carcinogenic effects or their macrophage-toxic effects remains to be determined.

2. *Depressed Macrophage Function in Neoplastic Disease*

Twomey and Sharkey (1972) have found that the monocyte-macrophages in patients with leukemia and asplastic anemia were reduced in number but were functionally competent when tested in a mixed leukocyte reaction. Blaese *et al.* (1972) obtained similar results with Hodgkins disease and Wiskott-Aldrich syndrome patients, but they cautioned that the methods used to collect and test for macrophage function selected for healthy macrophages and thus may not have detected functionally defective cells in the macrophage population of the patients studied. Using a skin-window technique, Ghosh *et al.* (1973) have been able to demonstrate decreased cytoplasmic inclusions and depressed phagocytic activity in macrophages from patients with Hodgkins disease and lymphoreticulosarcoma. Murine leukemic monoblasts and their mature macrophage progeny have been found to have subnormal phagocytic capacity as well as quantitatively and qualitatively abnormal IgG receptors (Cline and Metcalf, 1972). More recently, Cline (1973) has shown that human mononuclear phagocytes from myelomonocytic leukemia and lymphoma patients display defective bactericidal activity that not only could account for the high incidence of infections in cancer patients (Klastersky *et al.*, 1972), but also might reflect a diminished antineoplasia response of these patients. In addition, it has been suggested that the depletion of HRF levels observed in the serum of cancer patients may depress RES activity by interfering with the ability of host macrophages to recognize foreign elements, such as neoplastic cells (Pisano *et al.*, 1970a,b). It has

also been suggested that serum blocking factors in cancer patients might similarly prevent macrophages from recognizing, processing, or destroying tumor cells (Allison, 1972; Mitchell and Mokyr, 1972). Finally, Keller (1973a) has demonstrated that serum from rats infected with *Nippostrongylus brasiliensis* not only can block the antitumor activity of activated macrophages, but also can return these cells to an inactivated state.

C. Stimulated Macrophage Function

1. *Stimulated Macrophage Function and Increased Resistance to Tumor*

Similar to RES stimulants, agents that enhance macrophage activity have been shown to increase host resistance to neoplastic disease. Examples of such agents are Withaferin A (Shohat and Joshua, 1971), peptone (Keller and Hess, 1972), wheat straw hemicellulose B, lichen polysaccharide GE-3, and lentinan (Tokuzen, 1971), glycoprotein pituitary hormone (Yamada *et al.*, 1969); pyran (Hirsch *et al.*, 1972), endotoxin, double-stranded RNA, and lipid A (Parr *et al.*, 1973; Evans, 1973b), statolon (Wheelock *et al.*, 1974), *Bordetella pertussis* (Guyer and Crowther, 1969), *Corynebacterium parvum* (Woodruff and Boak, 1966; Currie and Bagshawe, 1970), BCG alone (Zbar *et al.*, 1971; Hanna *et al.*, 1972; Hibbs, 1973; Hoy and Nelson, 1969a; Gutterman *et al.*, 1973), BCG in complete Freund's adjuvant (Hibbs *et al.*, 1972b; Lemperle, 1966), a methanol extraction residue of BCG (Weiss, 1972), *Listeria monocytogenes* (Hibbs *et al.*, 1972a), *Nippostrongylus brasiliensis* (Keller and Jones, 1971; Keller *et al.*, 1971), *Toxoplasma gondii* and *Besnoitia jellisoni* (Hibbs *et al.*, 1971b). In addition, macrophage from animals treated with PPD (Holterman *et al.*, 1972), endotoxin (Alexander and Evans, 1971), or peptone (Keller, 1973b) or normal macrophages treated *in vitro* with double-stranded RNA, endotoxin, or lipid A (Alexander and Evans, 1971; Hibbs, 1973) have been shown to be nonspecifically cytotoxic to tumor cells *in vitro*.

2. *Stimulated Macrophage Function in Neoplastic Disease*

As mentioned in Section III,A, a marked histiocytic infiltration is frequently a part of the host response to neoplastic disease. A massive proliferation of activated histiocytes has been observed in draining lymph nodes and tissues surrounding the implantation of a nonmetastasizing lymphoma (NML); no such reaction occurred in recipients of its metastasizing lymphoma (ML) counterpart (Gershon *et al.*, 1967). Activated macrophages were also found at the rejection site of a secon-

dary NML implant in NML-bearing animals. The concomitant immunity responsible for the rejection of these secondary NML implants was considered to be the underlying cause for macrophage activation in primary NML-bearing animals and was not found in ML-bearing animals (Birbeck and Carter, 1972). Activated macrophages have also been observed in the peritoneal cavities of mice during the course of rejection of Ehrlich ascites tumor cells to which they had been previously immunized (Sakashita, 1971). Similarly, an early invasion of activated macrophages has been observed in chemically induced lymphomas undergoing spontaneous regression in Swiss and NZW mice (Rice, 1972). Finally, increased *in vitro* macrophage colony formation by the bone marrow cells of tumor-bearing animals has been observed in marrow samples harvested between 4 days and 2 weeks after tumor implantation (Baum and Fisher, 1972).

It is difficult to discern whether macrophage proliferation and activation is induced by tumor or by the host's immune response to tumor. However, such activation by tumor, by the host immune response, or by exogenous stimulants appears to be beneficial to the host and will be considered in greater detail in Sections III,D and E.

D. Afferent and Efferent Macrophage Activity in Immunotherapy of Neoplastic Disease

The demonstrated involvement of macrophages in cell-mediated immune reactions (Volkman and Collins, 1971; Vernon-Roberts, 1972; Pearsall and Weiser, 1970) and the presence of large numbers of macrophages in various untreated animal tumors (Evans, 1972, 1973b) suggest that these cells play a vital role in tumor rejection produced by the establishment of local delayed hypersensitivity. Topical application of dinitrochlorobenzene (DNCB) and/or 2,3,5-trisethyleneiminobenzoquinone (TEIB) induces delayed hypersensitivity and leads to both the resolution of established neoplasms and the eradication of lesions that were clinically undetectable at the time of therapy (Klein, 1969; Klein and Holtermann, 1972). Administration of BCG by scarification has also produced positive therapeutic effects in the treatment of human melanomas (Gutterman *et al.*, 1973). In addition, intratumor inoculation with BCG, transplantation of tumor cells mixed with BCG, or the transplantation of tumor cells into the site of an ongoing delayed hypersensitivity reaction to an antigenically different tumor resulted in tumor regression or rejection, as well as elimination of lymph node metastases. These effects occurred only where an ongoing delayed hypersensitivity reaction could be observed (Zbar *et al.*, 1970a,b, 1971, 1972; Bartlett *et al.*, 1972; Hanna *et al.*, 1972; Hoy and Nelson, 1969a). In some cases,

such treatment led to systemic tumor immunity (Zbar *et al.*, 1972), presumably due in part to increased antigen processing by activated macrophages. However, this specific tumor immunity did not appear to be crucial to tumor rejection (Bartlett *et al.*, 1972).

Zbar *et al.* (1972) have suggested that BCG-mediated tumor cell death occurs in three steps: (1) the host produces lymphocytes which recognize antigens of BCG; (2) specifically sensitized lymphocytes react with BCG leading to the production of soluble mediators of cellular immunity including MIF which promote local accumulation of macrophages; (3) the macrophages may then directly destroy the tumor cells or may process tumor antigens leading to the formation of tumor-specific "killer" lymphocytes. Osteen and Churchill (1972) and Holtermann *et al.* (1972) have demonstrated a similar acquisition of antitumor activity by macrophages mixed with sensitized lymphocytes which have been exposed to their specific antigens.

In agreement with the above findings, two lymphocyte-produced mediators of delayed hypersensitivity, migration inhibitory factor (MIF) and interferon, have also displayed antitumor activity (Bernstein *et al.*, 1971; Gresser *et al.*, 1970). MIF has been shown to enhance macrophage membrane activity, glucose oxidation via the hexose monophosphate shunt, adherence to glass, and phagocytosis (Nathan *et al.*, 1971). Intradermal injection of MIF led to a reaction similar in appearance to delayed cutaneous hypersensitivity and resulted in the suppression of growth of syngeneic tumor grafts at the reaction site (Bernstein *et al.*, 1971). Interferon has also been shown to enhance macrophage phagocytosis (Huang *et al.*, 1971). The administration of interferon or interferon inducers has been shown to confer nonspecific resistance to bacterial and protozoan infections (Remington and Merigan, 1970; Regelson and Munson, 1970; Jahiel *et al.*, 1968; Weinstein *et al.*, 1970; Remington and Merigan, 1968). In addition, animals treated with interferon or interferon inducers showed enhanced resistance to infection with oncogenic viruses and to intraperitoneal transplantation of tumor cells (Kapila *et al.*, 1971; Hirsch *et al.*, 1972; Gresser *et al.*, 1970; Wheelock *et al.*, 1973). Such activities go well beyond interferon's previously known role in the inhibition of virus replication at the molecular level (Vilcek, 1969) and have been attributed to macrophage activation.

Macrophages have been shown to participate in the adjuvant effects of several other biological agents employed in the immunochemotherapy of neoplastic disease (Yashphe, 1971). Spitznagel and Allison (1970a,b) have shown that the adjuvant effect of lipopolysaccharide on the antibody response of mice to bovine serum albumin was correlated with macrophage activation and lysosome labilization. Bovine serum albumin

taken up by lipopolysaccharide-treated macrophages was more immunogenic than that taken up by normal macrophages even though adjuvant-treated macrophages showed no increase in uptake or degradation of this antigen. It appeared that adjuvant-treated macrophages somehow stimulated the multiplication of immunocompetent cells and promoted antibody production rather than tolerance induction. These effects were thought to be caused by macrophage-bound antigen, as well as by the delivery of the macrophage-contained adjuvant to lymphoid cells. Similarly, Unanue *et al.* (1969), found that the adjuvant effects of *Bordetella pertussis* or berillium sulfate on the antibody response of mice to hemocyanin was seen only after the uptake of these adjuvants by the macrophage. Adjuvant-treated macrophages but not adjuvant-treated lymphocytes showed morphologic abnormalities and increased acid phosphatase staining indicating a general state of activation. Again, these researchers suggested that either the adjuvants contained within these activated macrophages or the activated state itself somehow stimulated the proliferation of surrounding antigen-sensitive lymphoid cells. Both BCG and lipopolysaccharide have been shown to induce in macrophages the production of a lymphocyte-activating factor (LAF), which could account for such a proliferation of lymphoid cells (Gery *et al.*, 1972; Gery and Waksman, 1972; Mitchell *et al.*, 1973a). Finally, an increase in cell size and in the number of cellular immunoglobulin receptor sites, accompanied by increased binding of soluble immune complexes was observed in alveolar macrophages isolated from rabbits given multiple intravenous injections of complete Freund's adjuvant (Arend and Mannik, 1973). This enhanced binding capacity of macrophages could play an important role in the clearance of such complexes which may act as blocking factors of tumor immunity. In addition, binding of antigen–antibody complexes or cytophilic antibody to macrophages may lead to specific cytotoxicity against tumor cells (see Section III,E,3).

Macrophages may also participate in the antineoplastic effects of concanavalin A (Con A) and *Vibrio cholera* neuraminidase (VCN). Con A has been shown to enhance tumor cell agglutination and adhesion to macrophages (Inoue *et al.*, 1972), which could lead to increased phagocytosis and antigen processing, if not outright tumor cell destruction. In addition, Con A-coated, irradiated, leukemic cells were significantly more immunogenic than noncoated irradiated cells (Martin and Wunderlich, 1972). Similarly, the interaction of Con A with Friend leukemia virus has been shown to block the immunosuppressive effect of this virus complex (Kately and Friedman, 1973). Also, infection with Con A-Friend leukemia virus caused significantly less splenomegaly and mortality (Dent, 1973), an effect accounted for by agglutination of the virus. Such

agglutination could possibly lead to some direct inactivation of virus but might also enhance virus phagocytosis and processing by macrophages. Intraperitoneal injection of Con A alone has been shown to increase the numbers and enhance the glass spreading and phagocytic activities of peritoneal macrophages (Smith and Goldman, 1972). In addition, *in vitro* treatment of purified macrophages with Con A has been shown to inhibit their migration (Kumagai and Arai, 1973) and enhance their lysis of syngeneic and allogeneic erythrocytes (Melsom and Seljelid, 1973). Whether Con A bound to cells or virus has the same activity as free Con A has not yet been reported.

Vibrio cholera neuraminidase (VCN) has been shown to produce an immunospecific regression of human and animal tumors (Simmons *et al.*, 1971; Weiss, 1973; Rios and Simmons, 1972). Treatment of tumor cells with VCN enhances their immunogenicity apparently by exposing previously masked tumor-specific membrane antigens (Weiss, 1973). In addition, VCN treatment alters cell surface charge and deformability, thereby promoting increased contact with antigen-processing macrophages (Weiss, 1965; L. Weiss *et al.*, 1966). Treatment of various cell types with VCN has been shown to enhance their phagocytosis by macrophages (L. Weiss *et al.*, 1966; Lee, 1968; Evans, 1971a). In addition, the combination of a macrophage stabilizer, poly-2-vinyl pyridine-*n*-oxide (Rios and Simmons, 1972; Holt *et al.*, 1970) or a macrophage activator, BCG (Simmons and Rios, 1971), with VCN therapy have produced additive beneficial effects. Surface alterations have also been proposed as an explanation of the macrophage uptake and processing of previously nonphagocytizable tumor cells after heat killing or irradiation of these cells (Sezzi *et al.*, 1972). The immunogenicity of irradiated cells has been further enhanced by VCN treatment prior to vaccination (Oxley and Griffen, 1972). Finally, intimate contact between cell membranes of macrophages and cancer cells has been observed following X-ray treatment of human basal cell carcinomas, suggesting to these authors that macrophages were participating in the therapeutic effects of X-irradiation (Vorbrodt *et al.*, 1972).

E. Antineoplastic Activities of Macrophages

1. *Nonspecific Antitumor Effects of Activated Macrophages*

Macrophage activation per se has been found to be the basis for the resistance of mice and mouse macrophage monolayers to phylogenetically diverse, intracellular organisms observed upon immunization and mixed challenge using *Salmonella typhimurium, Listeria monocytogenes, Mycobacterium tuberculosis, Pasteurella tularensis, Besnoitia jellisoni,* and

Toxoplasma gondii (Blanden, 1968; Ruskin *et al.*, 1969). In addition, chronic infection of mice with *Toxoplasma gondii* or *Besnoitia jellisoni* produced resistance to transplantable and autochthonous tumors as well as to challenge with Friend leukemia virus (Hibbs *et al.*, 1971a,b). The increased resistance to Friend leukemia virus infection, sarcoma 180 and leukemia L1210 transplantation, and to the development of spontaneous mammary tumors in C_3H and leukemia in AKR mice, paralleled an enhanced nonspecific resistance to challenge with *Listeria monocytogenes* (Hibbs *et al.*, 1971b). Since the macrophage appeared to be the effector arm in nonspecific bacterial resistance (Ruskin *et al.*, 1969), its role in tumor resistance was considered to be highly likely. To this end, purified macrophage cultures from *Toxoplasma*-infected mice were shown to have the capacity, via a nonphagocytic mechanism, to nonspecifically destroy target L cells, as well as syngeneic KHT sarcoma and allogeneic MET-6 mammary adenocarcinoma cells (Hibbs *et al.*, 1972a). Neither peritoneal or spleen lymphocytes from stimulated mice nor macrophages from nonstimulated mice showed any such antitumor cytopathic effect.

Macrophages activated *in vivo* by intraperitoneal injection of peptone, double-stranded RNA, or endotoxin or by *in vitro* exposure to double-stranded RNA, poly(I)·poly(C), endotoxin, or Lipid A have also been shown to be cytotoxic to tumor cells *in vitro* (Alexander and Evans, 1971; Keller, 1973b; Hibbs, 1973). Exposure to such nonspecifically activated macrophages resulted in growth inhibition of tumor cells as measured by their reduced capacity to divide when transferred to a permissive culture environment (Alexander and Evans, 1971) or by their markedly reduced RNA and DNA synthesis (Keller, 1973b). Such cytostatic effects were not observed if silica was added to the macrophage/tumor cell mixture, or if tumors were exposed only to supernatants from either macrophage or macrophage/tumor cell cultures (Keller, 1973b).

Further studies have shown that animals treated with complete Freund's adjuvant (CFA), but not with saline or incomplete Freund's adjuvant were protected from autochthonous and transplanted tumors as well as infection by Friend leukemia virus (Hibbs *et al.*, 1972c). Macrophages from CFA-treated and *Toxoplasma gondii*-infected mice displayed selective but nonimmunologic cytotoxicity to cells with abnormal growth characteristics, such as syngeneic and allogeneic tumor cell cultures and established cell lines, but did not affect embryonic cell cultures or fibroblastic cell strains (Hibbs *et al.*, 1972b,d). The loss of contact inhibition and its concomitant modification of target cell surface, high *in vitro* cell density, agglutinability by plant lectins, and tumorigenicity, was shown to evoke a cytotoxic response from macrophages previously activated by *in vivo* infection with *Toxoplasma gonadii* or BCG, as well as by *in*

vitro exposure to endotoxin (Hibbs, 1973). It was suggested that the destruction of cells with abnormal growth characteristics, but not of normal cells by activated macrophages might constitute a primitive, yet highly effective, antitumor surveillance mechanism.

2. *Cooperation of Macrophages and Lymphocytes*

In addition to the nonspecific cooperation between macrophages and lymphocytes in host antitumor activities mentioned in Section III,D, immune lymphocytes have been shown to recruit and immunospecifically activate host macrophages (Evans and Alexander, 1970). Purified syngeneic peritoneal macrophages from mice immunized against lymphoma cells were shown to be cytotoxic to lymphoma cells *in vitro*. In addition, normal syngeneic macrophages could be rendered cytotoxic to lymphoma cells after *in vitro* incubation with immune spleen cells, but not with immune serum. Macrophage-tumor cell toxicity, as measured by the failure of macrophage-exposed lymphoma cells to proliferate in culture, was immunologically specific and required cell–cell contact.

Additional studies showed that macrophages from the peritoneal cavity of hyperimmunized mice or normal macrophages exposed *in vitro* for 24 hours to either hyperimmune spleen cells or to the supernatant from immune lymphocytes incubated with cells to which they had been sensitized became specifically cytotoxic to those sensitizing cells (Evans and Alexander, 1972a). Such macrophages, referred to as "armed macrophages," could be rendered nonspecifically cytotoxic to all target cells following activation by exposure to their specific arming antigens. This system was operative not only when the target cells served as both immunizing and test antigens, but also in a BCG–PPD, sensitization-specific reaction system. It was also noted that macrophages removed earlier than 10 days after hyperimmunization with BCG or tumor cells were nonspecifically cytotoxic, most likely owing to interaction of armed macrophages and activating antigen persisting within the peritoneal cavity. After 10 days, macrophages were still specifically armed but were no longer nonspecifically cytotoxic, presumably owing to the disappearance of antigen from the peritoneal cavity. It is therefore likely that the nonspecific macrophage activation observed by Hibbs *et al.* (1971a,b) after chronic infection with *Toxoplasma gondii* as well as that seen in varied BCG systems (Bartlett *et al.*, 1972; Hibbs, 1973) could be due to initial arming and consequent activation by persistent intracellular parasite antigens.

Specific macrophage antitumor arming in a mouse allogeneic system was found to be destroyed in trypsin treatment of immune macrophages but unaffected by rabbit antimouse gamma globulin (Den Otter *et al.*,

1972). Lymphocytes were not needed for tumor cell cytotoxicity but were thought to play a role in the arming of the macrophages. Isolated trypsinized macrophages were unable to recover their cytotoxic potential, indicating that they were not capable of synthesizing the active component of the arming reaction. Previously, Denham *et al.* (1970) had observed two separate classes of lymphocytes, early lymphoblasts and late memory cells, both cytotoxic to allogeneic tumor cells. More recent studies have confirmed Denham's observations and demonstrated the existence of two similar classes of cytotoxic thymocytes (Grant *et al.*, 1972; Alexander *et al.*, 1972). In addition, these studies revealed the presence of a third class of lymphoid cells, one that upon exposure to sensitizing antigens could render monocytes and/or macrophages specifically toxic to target cells but displayed no such cytotoxicity itself. This class of lymphoid cells produced a specific macrophage arming factor (SMAF) that was thought to be related to macrophage migration inhibitory factor (MIF) in its ability to activate macrophages (Nathan *et al.*, 1971) and recognize specific antigens (Amos and Lachmann, 1970).

Further investigation into the properties of SMAF in a syngeneic tumor system (Evans *et al.*, 1972) showed that SMAF did not affect the growth of specific target cells in the absence of macrophages. The arming activity of SMAF could be totally absorbed out by specific target cells, but not by unrelated cells. Upon gel filtration, SMAF activity was found in two major peaks, one containing material of molecular weight greater than 300,000 and another between 50,000 and 60,000. Both fractions could bind specifically to target cells and nonspecifically to mouse as well as rat macrophages. In addition, it was found that SMAF production could be eliminated by the exposure of immune cells to anti-theta serum and complement as well as by whole-body irradiation and thymectomy prior to immunization. Again, in this syngeneic system as in the allogeneic system (Alexander *et al.*, 1972), SMAF-producing cells were not directly cytotoxic to tumor cells. Additional studies showed that, similar to the BCG–PPD system previously reported (Evans and Alexander, 1972a), immunization with sheep red blood cells (SRBC) could result in SMAF arming and SRBC antigen activation of normal macrophages (Evans *et al.*, 1973). Although having the binding and specificity properties of cytophilic antibodies, SMAF did not seem to belong to one of the established classes of immunoglobulins since high activity was found in a fraction with a molecular weight of 50,000 to 60,000. It was suggested that SMAF arming and antigen activation of macrophages bore a formal similarity to the IgE coating and specific antigen-induced degranulation of mast cells (Stanworth, 1970; Ishizaka *et al.*, 1971). Again, however, molecular weight analysis dictated that

SMAF was not a classical immunoglobulin, although the possibility that it contained subunits of known immunoglobulins has not yet been excluded (Evans *et al.*, 1973).

In another series of experiments no difference in the degree of macrophage arming was observed using SMAF derived from lymph node as compared to spleen cells (Evans and Alexander, 1972b). In addition, SMAF was shown to arm both allogeneic and syngeneic macrophages but not fibroblasts. Macrophages could be armed *in vivo* by a single injection of hyperimmune spleen cells. *In vitro,* physical contact was necessary between lymphoid cells from hyperimmune mice and normal macrophages for successful arming. In contrast, lymphoid cells from singly immunized mice released significant filterable SMAF when exposed to their sensitizing antigen *in vitro.*

In continued investigations, it was found that coating of target lymphoma cells with specific antiserum blocked the cytotoxic effects of immune macrophages on these cells, but conferred antitumor activity to nonimmune macrophages (Evans and Alexander, 1972c). Treatment with a 1% solution of trypsin, but not antimouse gamma globulin, depressed the tumor cell-growth inhibitory effects of immune macrophages. Significant levels of immunoglobulin were deposited on macrophages after contact with spleen cells from both hyperimmunized and singly immunized mice. However, only those macrophages exposed to hyperimmune spleen cells were rendered cytotoxic. There was no increase in the level of membrane-bound immunoglobulin after incubation of macrophages with SMAF. Thus, again it seemed that SMAF and cytophilic gamma globulin were not identical. Similar studies have been performed in a tumor allograft system (Evans and Grant, 1972) and have yielded essentially the same results as described above.

Finally, Evans (1973b) has shown that, in tumor-bearing animals, growth-inhibitory macrophages can be found not only in the peritoneal cavity, but also within the growing tumor itself. An inadequate ratio of cytotoxic macrophages to tumor cells or the presence of blocking factors were suggested as possible reasons for the failure of these macrophages to bring about tumor rejection. It was further speculated that these macrophages might play a significant role in the rejection of tumors induced by various macrophage activating agents (see Section III,E,1).

3. *Cooperation of Macrophages and Antibody*

The importance of cytophilic antibody in macrophage-mediated antitumor activity, suggested in early studies (Baker *et al.*, 1962; Bennett *et al.*, 1964), has been pursued by Granger and Weiser and their as-

sociates. They observed that immune peritoneal macrophages could cause acute allogeneic disease in mice and suggested that this reaction might be mediated by cell-bound antibodies on the surface of the immune macrophages (Weiser *et al.*, 1965). Immune macrophages could also destroy allogeneic sarcoma and syngeneic fibroblast monolayers *in vitro* via a nonphagocytic form of cell contact (Granger and Weiser, 1964, 1966). Immune macrophages specifically adhered to monolayers of cells syngeneic to those used for immunization resulting in the mutual destruction of both macrophage and target cells; macrophages from various nonspecifically immunized mice showed no toxicity to the target cells. Heat eluates from immune macrophage monolayer cells were capable of activating complement and caused specific 2-mercaptoethanol sensitive, agglutination of red blood cells bearing the same histocompatibility antigens as the immunizing and target cells (Granger and Weiser, 1964). While no evidence was obtained for the participation of complement in macrophage-mediated target cell destruction, the necessity for intact, general biosynthetic activities of the macrophage was demonstrated (Granger and Weiser, 1966). Cytotoxicity but not specific adherence was blocked by sodium azide, 2,4-dinitrophenol, sodium fluoride, actinomycin D, chloramphenicol, or puromycin. It was suggested that passive adherence might lead to sufficiently intimate contact of cell membranes to promote exchange of cytoplasmic materials which could secondarily produce cell injury. Treatment of sarcoma cells *in vitro* with immune serum did not enhance macrophage phagocytosis even though intraperitoneal injection of such sera plus rabbit complement significantly increased injury to tumor cells present in the peritoneal cavity (Holmes and Weiser, 1966). Nevertheless, a predominance of macrophages with marked affinity for tumor cells was seen in the ascitic fluid samples from mice actively rejecting tumor transplants with the aid of injected sera and complement.

Further studies showed that mild trypsinization of immune macrophages destroyed their capacity to transfer immunity suggesting that such activity was due to cytophilic antibody, apparently acquired following its production by immune lymphocytes (Pearsall and Weiser, 1968b). Passive transfer studies were then extended into irradiated hosts (Tsoi and Weiser, 1968a); the degree of observed suppression of tumor growth was directly related to the number of immune macrophages added to the tumor inoculum. The necessity of cell-cell contact for the expression of macrophage tumor-suppressive activity was indicated by the relative inefficiency of macrophage-mediated protection against tumors transplanted to peritoneal as opposed to subcutaneous sites as well as by the lack of protection when tumor cells and macrophages were

injected into different sites. In the same system, it was also observed that tumor growth was inhibited when the tumor cells were mixed with immune serum and injected intraperitoneally (Tsoi and Weiser, 1968b). The effective dose range of immune sera in these experiments was quite low, suggesting the participation of accessory inhibitory cells in the peritoneal cavity. To this end, the addition of immune peritoneal macrophages to tumor cell inocula significantly increased the host rejection of both intraperitoneal and subcutaneous transplants (Tsoi and Weiser, 1968c). Moreover the addition of immune serum to this mixed cell inoculum as well as the combination of immune serum with nonimmune macrophages and tumor cells produced striking immunity in recipient mice. The high degree of this protection implied a synergistic rather than a merely additive effect of such a combination. Further studies concerning the role of cytophilic antibodies in macrophage-mediated immunity showed that immune macrophages allowed to spread onto glass surfaces lost their immune capacities within a matter of hours (Weiser *et al.*, 1969). Macrophages disarmed by trypsin treatment could be rearmed by incubating them with cytophilic antibody in either immune serum or heat eluate from immune macrophages. In addition, migration of nonsensitized macrophages armed with BCG-specific antibody was inhibited by PPD in the absence of MIF-producing lymphocytes.

The importance of antibody for macrophage activity has been observed by several other investigators. Receptor sites for monomeric IgM have recently been found on guinea pig splenic macrophages (Rhodes, 1973). In addition, LoBuglio *et al.* (1967) and Temple *et al.* (1973) have shown that cytophilic antibody was responsible for specific macrophage binding and destruction of target erythrocytes. Uhr (1965) has shown that normal macrophages exposed to antigen–antibody complexes can specifically adhere to cells coated with the sensitizing antigen. Hoy and Nelson (1969b) have found cytophilic antibody receptors with different susceptibility to trypsin on both immune and nonimmune mouse macrophages. Antitumor antibodies cytophilic for macrophages have been found in IgG, IgM, albumin and fast alpha globulin fractions of serum (Hoy and Nelson, 1969b; Evans, 1971b). Such antibodies were capable of arming normal macrophages and were considered responsible for their specific attachment to sarcoma cells (Granger and Weiser, 1966) and enhanced phagocytosis of lymphoma cells (Evans, 1971a,b). More recently, Liew and Parish (1972) have shown that the attachment of various antigens to macrophages via cytophilic antibody has a significant effect on determining whether the immunized animal will mount a cell-mediated or humoral antibody response to the challenge antigen. In light of the complication of enhancing antibody in the host response to neoplasia

(Kaplan *et al.*, 1971; Allison, 1972), such alteration of antigen form in favor of cell-mediated immunity would be advantageous to the host and might be yet another mechanism by which macrophage-activating adjuvants may exert their therapeutic effects. In addition, Mitchell and Mokyr (1972) have shown that hyperimmune isoantibody to leukemic L1210 cells irreversibly damaged macrophage surface receptors specific for cytophilic antibody and thereby inhibited the development of anti-leukemic, macrophage-mediated immunity. In another system, normal peritoneal macrophages mixed with antitumor antibody were shown to be the only cell type capable of restoring tumor-suppressive capacity to a whole body-irradiated host (Shin *et al.*, 1972). Conversely, in an *in vitro* system, antitumor IgG_2 was shown to block the antitumor cytotoxicity of normal macrophages while it enhanced that of immune macrophages (Cruse *et al.*, 1973). This dichotomous effect on primary versus secondary alloimmune responsiveness was thought to be due to cytophilic activity of antitumor antibodies and an antigen-driven selection for an increased number of cytophilic antibody receptor sites on immune macrophages. Nonimmune macrophages apparently could not bind adequate cytophilic antibody to overcome the blocking activity also present in the IgG_2 antibody fraction. Fakhri *et al.* (1973) and Fakhri and Hobbs (1973) have shown that 7 S antibody bound to injected plasmacytoma cells can attract macrophages with subsequent death of the target cells, resulting in prolonged host survival and short-term resistance to tumor rechallenge. The attachment of antibody to target cell antigens apparently activated the antibody's Fc fragment leading to macrophage binding and destruction of the target cells. This reaction appeared to be similar to the triggering of immediate hypersensitivity by the activation of the Fc in IgE (Stanworth, 1970; Ishizaka *et al.*, 1971). It was also suggested that excess free circulating tumor antigen bound to antibody could produce small immune complexes with activated Fc fragments. Such complexes could act as blocking factors by diverting macrophages and nonimmune lymphocytes away from actual target cells. The increased number of Fc receptor sites on adjuvant-treated macrophages (Arend and Mannik, 1973) could enhance RES clearance of such blocking factors and abrogate their tumor-enhancing effects, suggesting yet another role for activated macrophages in aiding the host in the rejection of neoplastic disease. Finally, Mitchell *et al.* (1973b), have recently detected antibodies cytophilic for macrophages in the serum of human leukemia patients. These antibodies were directed against leukemia-associated antigens common to a histological class of leukemia and were able to mediate the attachment of immunologically naive mouse macrophages to human leukemic cells.

F. Mechanism of Macrophage antitumor Activity

The exact mechanism of macrophage antitumor activity has yet to be defined. As already mentioned (Section III,E) specificity, where it exists, appears to be mediated by factors cytophilic for macrophages or by membrane determinants on target cells with abnormal growth properties. Macrophage-mediated tumor cell death in autologous and syngeneic systems seems to be a cytostatic, growth inhibitory event (Lejeune and Evans, 1972; Evans and Alexander, 1972c; Keller, 1973b; Evans, 1973b), whereas significant tumor cell lysis has been reported in allogeneic systems (Den Otter *et al.*, 1972; Cruse *et al.*, 1973). Intimate contact between target cells and biologically active macrophages appears to be universally requisite for macrophage-mediated cytotoxicity (LoBuglio *et al.*, 1967; Melsom and Seljelid, 1973; Evans and Alexander, 1970; Keller, 1973b; Hibbs *et al.*, 1972a; Granger and Weiser, 1966; Cruse *et al.*, 1973). Despite some reports to the contrary (Chambers and Weiser, 1972; Bennett *et al.*, 1964; Amos, 1960), phagocytosis does not seem to play a primary role in the cytotoxic effects of macrophages on target cells (Melsom and Seljelid, 1973; Temple *et al.*, 1973; LoBuglio *et al.*, 1967; Kramer and Granger, 1972; Keller, 1973b; Hibbs *et al.*, 1972a; Baker *et al.*, 1962; Evans and Alexander, 1972a; Granger and Weiser, 1964).

Early electron microscope (EM) studies revealed a sequence of invagination, ingestion, and consequent digestion of tumor cells by macrophages (Journey and Amos, 1962). This classical phagocytosis, however, accounted for only a small portion of tumor cell death. The majority of tumor cell destruction appeared to be the result of fusion between macrophages and tumor cells leading to possible osmotic instability and/or permitting the transfer of toxic cytoplasmic factors into the tumor cell from the aggressor macrophages. More recent EM studies of target cell destruction by cytophilic antibody-coated macrophages, demonstrated a "piecemeal cytophagocytosis" consisting of the pinching off of target cell protuberances extending into macrophage invaginations (Chambers and Weiser, 1969, 1971). Degenerative changes, such as cytoplasmic patching, plasma membrane "bleb" formation, and cellular ballooning, were visible within 3 hours after the initiation of target cell contact with macrophages. Similar alterations have been reported in antibody-assisted, macrophage-mediated destruction of erthyrocytes (LoBuglio *et al.*, 1967; Melsom and Seljelid, 1973; Temple *et al.*, 1973).

Several studies have suggested that lysosomes may be involved in macrophage-mediated, target cell toxicity (Chambers and Weiser, 1969; Shohat and Joshua, 1971; Temple *et al.*, 1973). EM and cytochemical

studies of the interaction of syngeneic immune macrophages and lymphoma cells demonstrated that immune peritoneal macrophages were larger, contained more lysosomes, and displayed greater acid phosphatase and β-glucuronidase activity than nonimmune macrophages (Lejeune and Evans, 1972). Within 2 hours after the initiation of macrophage–tumor cell interaction, there was a dramatic increase in the number of acid phosphatase-positive, small lysosomes within both normal and immune macrophages. At the cell periphery of immune, but not nonimmune, macrophages, these small lysosomes fused together to form several large lysosomes. The exact significance of the altered enzyme levels was not established in this study since no ultrastructural changes were observed in the growth-inhibited lymphoma cells as late as 24 hours after initial contact with immune macrophages. However, amorphous material considered to be of lysosomal origin has been observed within degenerating tumor cells in several other macrophage-tumor cell cytotoxicity studies (Chambers and Weiser, 1969, 1971, 1972). In addition, phagocytic cells surrounding a *Cryptococcus neoformans* cell have been shown to release detectable hydrolytic enzymes into the encircled yeast (Kalina *et al.*, 1971). The release of enzymes responsible for the nonphagocytic death of the yeast required intimate contact between the macrophages and the organism. Similar enzyme-mediated toxicity has been suggested to be responsible for the lysis of chicken erythrocytes by guinea pig macrophages (Temple *et al.*, 1973).

Alternatively, macrophage-mediated target cell death has been attributed to macrophage secretion of soluble cytotoxic factors. Possibly stimulated by the "piecemeal cytophagocytosis" mentioned above, a growth-inhibitory factor (GIF) has been observed in the media removed from cultures of cytophilic antibody-coated macrophages exposed to their specific target antigen (Weiser *et al.*, 1969). GIF secretion was dependent upon intact macrophage membranes, and peak titers were reached 2 hours after the beginning of cell interaction. GIF suppressed "L" cell protein synthesis within a 2-hour period and was heat labile and specific for "L"-cells, suggesting to these authors that it might be a complex composed of antigen, antibody, and complement. Further studies revealed a different macrophage-produced cytotoxin that could not be distinguished from identically induced lymphocyte cytotoxins by gel filtration, antibody neutralization, heat sensitivity, or biologic assays (Heise and Weiser, 1969; Kramer and Granger, 1972). Unlike GIF, this cytotoxin was nonspecifically toxic for several target cell types and for the macrophages themselves. Finally, Sintek and Pincus (1970) have described a peritoneal cell cytotoxic factor (CTF) which unlike lymphotoxin, appeared to be a phospholipid.

Macrophage-produced cytotoxic factors have not been uniformly detected (Den Otter *et al.*, 1972; Evans and Alexander, 1970; Keller, 1973b; Cruse *et al.*, 1973; Hibbs *et al.*, 1972a). Similarly, mutual destruction of macrophages and target cells has been seen only in a few cases (Journey and Amos, 1962; Hoy and Nelson, 1969b; Holmes and Weiser, 1966). In addition, although *in vitro* immune macrophage–target cell interactions have appeared to be complement independent (Melsom and Seljelid, 1973; LoBuglio *et al.*, 1967; Evans and Alexander, 1970; Granger and Weiser, 1966; Fakhri *et al.*, 1973), the possibility that macrophages could produce some components of complement (Vernon-Roberts, 1972; Pearsall and Weiser, 1970) or substances that could act like complement has not been adequately eliminated. Such substances, in combination with macrophage-bound antibody and target cell antigen, could lead to target cell death via membrane damage as seen in immune hemolysis (Borsos *et al.*, 1964; Rosse *et al.*, 1966).

IV. Conclusion

Concordant with the recent increased use of immunochemotherapeutic agents in the treatment of neoplastic disease, it is important to assess the relative significance of macrophage activities within these systems. Although the extensive studies presented in this review indicate that macrophages play a crucial role in defense against neoplastic disease, there are reports to the contrary. Many studies of cell-mediated immune regression of animal tumors have denied on essential role for macrophages (Denham *et al.*, 1970; Alexander *et al.*, 1966; Berke *et al.*, 1972a,b; Alexander, 1971). Similarly, scalene lymph node sinus histiocytosis has recently been reported to be of no value as a diagnostic or prognostic indicator for human neoplastic disease (Silverberg *et al.*, 1973). In addition, several researchers doubt the significance or even the existence of depressed macrophage function in animal neoplasia-associated immunodepression (Biano *et al.*, 1971; Dracott *et al.*, 1972; Bendinelli, 1968). Certain intact carcinoma cells themselves have been shown to be capable of phagocytosis and digestion of dying tumor cells (Kerr and Searle, 1972), integral disposal activities thought to be the province of macrophages. The therapeutic efficacy of both immune (Dullens and Den Otter, 1973) and nonspecifically activated (Zbar *et al.*, 1972) macrophages in animals has been shown to be exquisitely sensitive to tumor load. In addition, several macrophage-activating, immunostimulatory agents have been shown to enhance tumor growth in both humans and animals (Schoenberg and Moore, 1961; Yashphe, 1971; Weiss, 1972; Gazdar, 1972). Moreover, RES stimulation, per se, appears to play an important role in the development of animal and human lymphomatous disorders,

including Burkitt lymphoma (Isliker *et al.*, 1973; Burkitt, 1969). Finally, macrophages themselves actually have been shown to protect animal tumor cells from antibody and lymphocyte mediated cytotoxicity (Hersey and MacLennan, 1973; Hersey, 1973).

Despite these exceptions and limitations, in many systems macrophages do exert both natural and adjuvant-stimulated, afferent and efferent antineoplastic activities. Macrophages appear to be essential for the uptake and processing of tumor antigens preceding the initiation of an effective immune response. In addition, macrophage alteration of antigen may promote successful immunization as opposed to induction of tolerance or production of enhancing or blocking factors. The macrophage might further enhance the immune response by stimulating the proliferation of immunocompetent cells through the production of LAF or the delivery of macrophage-contained adjuvant. Aided by cytophilic antibody, SMAF, MIF, interferon, nonspecific opsonins, or nonspecific activation, macrophages, either alone or in concert with other immune cells, can exert both immune and nonimmune cytotoxicity toward neoplastic cells. This antitumor activity is most likely mediated through a nonphagocytic, contact-dependent mechanism, associated in only a few systems with the release of soluble toxic substances. Such direct macrophage-mediated antitumor activity, as well as macrophage-mediated induction and amplification of antitumor immune responses, appear to contribute significantly to host survival and deserve careful consideration in both the experimental and clinical study of animal and human neoplastic disease.

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EPOXIDES IN POLYCYCLIC AROMATIC HYDROCARBON METABOLISM AND CARCINOGENESIS

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I. Introduction

One of the most intriguing problems in cancer research concerns the mechanism by which the relatively inert polycyclic aromatic hydrocarbons initiate tumors. Since the isolation and characterization of polycyclic hydrocarbons as the first pure chemical carcinogens (Kennaway, 1930; Cook *et al.*, 1933), the original idea that the hydrocarbons are carcinogenic per se has slowly given way to the idea that metabolism may precede biological activity. Thus, although correlations had been drawn between the electronic structures and the chemical carcinogenicities of a wide range of polycyclic hydrocarbons (Pullman and Pullman, 1955a,b), it was still thought at one stage that the physicochemical binding of hydrocarbons to nucleic acids might be sufficient to account for their biological effects (Boyland and Green, 1962; Liquori *et al.*, 1962). In other laboratories, particularly in that of the Millers and their colleagues, considerable progress was being made in studies on the metabolic activation of the carcinogenic aromatic amines and azo dyes (reviewed by Miller and Miller, 1969). These compounds were found to be metabolized to derivatives that gave rise to reactive electrophiles, which it was suspected could cause permanent alterations in cell phenotype by reacting with genetic material.

Indirect evidence that metabolic activation of the polycyclic hydrocarbons occurs in cells was obtained when the hydrocarbons were first prepared labeled with either ^{14}C or ^{3}H. In experiments using these radioactive compounds, the hydrocarbons became covalently bound to the macromolecular constituents of mouse skin (Heidelberger and Davenport, 1961; Brookes and Lawley, 1964; Goshman and Heidelberger, 1967) and of mammalian cells in culture (Diamond *et al.*, 1967)—two situations in which hydrocarbon metabolism occurs (Wattenberg and Leong, 1962; Andrianov *et al.*, 1967).

Polycyclic hydrocarbons are present in tobacco smoke (Wynder and Hoffman, 1959), are common contaminants of the urban environment (Kennaway and Lindsey, 1958), and are suspected of contributing to the increasing incidence of cancer of the respiratory tract in man (Doll, 1955); interest in their mechanism of action has therefore intensified.

Although their metabolism had been studied in detail in several systems (see, for example, Boyland and Weigert, 1947; Conney *et al.*, 1957; Sims, 1970b), their metabolic conversions had not been directly linked to hydrocarbon carcinogenesis, although carcinogenesis by polycyclic hydrocarbons is often inhibited by compounds that alter the levels of metabolizing enzymes (Huggins *et al.*, 1964; Wattenberg and Leong, 1968, 1970a; Wheatley, 1968; Diamond *et al.*, 1972).

The first clear evidence that metabolism might be involved in the covalent binding of polycyclic hydrocarbons to cellular constituents was obtained using an *in vitro* system, in which radioactively labeled hydrocarbons were metabolized by rat liver microsomal preparations in the presence of DNA or of protein (Grover and Sims, 1968). The results showed that the hydrocarbons became covalently bound to the macromolecules in the presence, but not in the absence, of the cofactors necessary for the function of the microsomal oxygenases, enzymes that catalyze the addition of oxygen across aromatic double bonds. Using similar systems, this metabolic activation of polycyclic hydrocarbons has been confirmed by other workers, including Gelboin (1969). Wang *et al.* (1971), Hey-Ferguson and Bresnick (1971), Bogdan and Chmielewicz (1973), and Meunier and Chauveau (1973), and the general principle has been applied in studies with other chemical carcinogens (Meunier and Chauveau, 1970; Garner *et al.*, 1972; Garner, 1973; Ames *et al.*, 1973b; Rocchi *et al.*, 1973; Swenson *et al.*, 1973).

The microsomal metabolites of the hydrocarbons that reacted with DNA and with protein probably included epoxides, the formation of which, as metabolites, was first suggested by Boyland (1950). These epoxides are now known to arise as metabolites of polycyclic hydrocarbons, and a certain amount of evidence has been obtained that indicates that they may be involved in hydrocarbon carcinogenesis. The aim of this review is to assemble and to present the information that exists on the epoxides and to discuss the relationship of this information both to the metabolism and to the biological activities of the polycyclic aromatic hydrocarbons.

II. Metabolism of Polycyclic Aromatic Hydrocarbons

A. General Aspects

It is now believed that the first step in the metabolism of polycyclic hydrocarbons is carried out by the "mixed-function oxidases" (or "oxygenases") present on the endoplasmic reticulum of cells. These enzymes are NADPH-dependent, and they catalyze the incorporation of

molecular oxygen into the substrate molecules. Evidence for this was provided by the work of Holtzman *et al.* (1967a,b), which showed that in the formation of *trans*-1,2-dihydro-1,2-dihydroxynaphthalene from naphthalene in a hepatic microsomal system, one atom of ^{18}O was incorporated into each dihydrodiol molecule when the incubations were carried out in the presence of $^{18}O_2$, the second oxygen atom being derived from water. The modes of action of the oxygenases are not yet completely understood, but the enzymes appear to consist of electron-transporting systems together with the terminal cytochromes P-448 and P-450. The overall reactions can perhaps be represented by Eq. (1).

$$\text{NADPH} + O_2 \longrightarrow \text{NADP}^+ + OH^- + \ddot{\underset{..}{O}}: \quad (1)$$

where $\ddot{\underset{..}{O}}$: is the so-called oxene radical (Ullrich and Staudinger, 1971). Although there is no direct evidence for its formation, either free or in association with cytochrome P-450, the reactions of a radical of this type with polycyclic hydrocarbons can account for the formation of most, if not all, of the primary metabolic products of aromatic hydrocarbons so far identified. Jerina *et al.* (1970a) have pointed out that the carbene radical, which is isoelectronic with the oxene radical, has properties analogous to those expected of the oxene radical, including the ability to add to double bonds and to insert between carbon and hydrogen. The oxygenases are involved in the metabolism of most foreign compounds (or xenobiotics) including drugs and insecticides as well as in the metabolism of many steroids. The mechanisms involved have been reviewed (e.g., Estabrook, 1971).

Detailed studies on the metabolism of polycyclic hydrocarbons in whole animals have been restricted mainly to the simpler compounds

such as naphthalene (I) (Bourne and Young, 1934; Young, 1947; Booth and Boyland, 1949; Corner *et al.*, 1954; Corner and Young, 1954; Boyland and Sims, 1958; Sims, 1959), anthracene (II) (Boyland and Levi, 1935, 1936a,b; Sims, 1964), phenanthrene (III) (Boyland and Wolf, 1950;

Sims, 1962; Boyland and Sims, 1962a,b), pyrene (IV) (Harper, 1957, 1958a; Boyland and Sims, 1964a), and benz[*a*]anthracene (V) (Harper, 1959a,b; Boyland and Sims, 1964b) although a limited amount of work has been carried out with the more complex compounds, such as benzo-[*a*]pyrene (VI) (Berenblum and Schoental, 1943; Weigert and Mottram,

(V) (VI)

1946; Harper, 1958b,c; Falk *et al.*, 1962; Raha, 1972), dibenz[*a,h*]anthracene (VII) (Dobriner *et al.*, 1939; Boyland *et al.*, 1941; LaBudde and Heidelberger, 1958), and 3-methylcholanthrene (VIII) (Harper, 1959a;

CH_3

(VII) (VIII)

Takahashi and Yashuhira, 1972). The earlier work on the metabolism of polycyclic aromatic hydrocarbons has been reviewed (Boyland and Weigert, 1947; Young, 1950). With the simpler hydrocarbons, phenols, dihydrodiols, and mercapturic acids were excreted, whereas with the more complex compounds only phenols were isolated. These products are discussed in more detail in Section II,B.

Because of the toxicity of the larger hydrocarbons and the complexity of their metabolism, most of the work on the metabolism of these compounds has been carried out using hepatic homogenates or microsomal preparations, together with an NADPH-generating system containing glucose 6-phosphate and glucose-6-phosphate dehydrogenase. However, comparisons of the metabolites excreted by animals treated with a hydrocarbon with those formed when the same hydrocarbon is incubated with hepatic homogenates or microsomal fractions showed that the same types of products are formed (Boyland *et al.*, 1964); naphthalene, for example, is converted into 1-naphthol and *trans*-1,2-dihydro-1,2-dihydronaphthalene both in whole animals and by tissue preparations (see Fig. 1). The use of isolated tissue preparations in metabolic studies therefore seems justified.

In recent years, the metabolism of some hydrocarbons by cells in tissue culture, in particular in mouse and hamster embryo cells, has also been investigated. In general, although many of the nonpolar metabolites

formed are, at least in their chromatographic properties, similar to those formed by hepatic homogenates and microsomal fractions, the water-soluble products differ (Sims, 1970a; Sims *et al.,* 1973). Attempts to identify the water-soluble products have not been successful (Diamond *et al.,* 1968; Sims, 1970a). In other metabolic studies with rodent cell cultures, the metabolism of benzo[*a*]pyrene, dibenz[*a*,*h*]anthracene, and

CH_3
CH_3
(IX)

7,12-dimethylbenz[*a*]anthracene (IX) was studied under the conditions necessary for the malignant transformation of the cells (Huberman *et al.,* 1971b). Nonpolar and water-soluble metabolites were estimated, and it was shown that the nonpolar products consisted of dihydrodiols and smaller amounts of phenols. A study has also been carried out on the metabolism of benzo[*a*]pyrene in human embryonic fibroblasts and epithelial cells (Huberman and Sachs, 1973), when water- and alkali-soluble products were measured.

In other studies in cells, the extent of metabolism of benzo[*a*]pyrene was measured by the disappearance of substrate in normal and neoplastic fibroblasts (Andrianov *et al.,* 1967). The disappearance of substrate has been estimated in mouse embryo cells treated with benz[*a*]anthracene, benzo[*a*]pyrene, dibenz[*a*,*h*]anthracene, dibenz[*a*,*c*]anthracene (X),

(X)

7-methylbenz[*a*]anthracene, 7,12-dimethylbenz[*a*]anthracene, and 3-methylcholanthrene (Duncan *et al.,* 1969), benzo[*a*]pyrene (Duncan and Brookes, 1970), dibenz[*a*,*c*]anthracene, and dibenz[*a*,*h*]anthracene (Duncan and Brookes, 1972), in fibroblasts derived from various human embryo tissues and in HeLa cells treated with benzo[*a*]pyrene and 7,12-dimethylbenz[*a*]anthracene (Brookes and Duncan, 1971). Estimates of water-soluble metabolites formed from hydrocarbons by a variety of cell cultures of both normal and transformed cells have been made (Diamond *et al.,* 1968; Diamond, 1971). In general, levels of metabolism of hydro-

carbons are higher in normal cells than in malignant cells, and this is correlated in normal cells with a greater toxicity and a higher level of binding to cellular macromolecules. The use of fetal cell cultures as an experimental system for predicting hydrocarbon metabolism in the whole animal has been discussed (Nebert, 1973).

Cultures of alveolar macrophages obtained from the lungs of guinea pigs metabolize benzo[*a*]pyrene to 3-hydroxybenzo[*a*]pyrene (Tomingas *et al.*, 1971a,b), and there is some evidence that the alveolar macrophages of rats also metabolize this hydrocarbon (Dehnen *et al.*, 1970a). However, microsomal fractions from alveolar macrophages obtained from rabbits, even from animals pretreated with the oxygenase inducer 3-methylcholanthrene, were unable to metabolize 7,12-dimethylbenz[*a*]anthracene (Reid *et al.*, 1972).

B. Types of Products Formed

Three types of products, dihydrodiols, glutathione conjugates, and phenols, have been found as metabolites of aromatic hydrocarbons in tissue preparations; these, as will be described below, appear to arise from common types of intermediates. With methylated hydrocarbons, another type of metabolite arising from hydroxylation of methyl groups has been identified in experiments with tissue preparations. The hydroxymethyl compounds thus formed (Boyland and Sims, 1965a, 1967a; Sims, 1967a; Gentil and Sims, 1971) probably arise by a mechanism different from that giving rise to the other types of products, but it is not known whether this mechanism involves either the direct hydroxylation of the methyl groups or the intermediate formation of hydroperoxides of the type detected in the metabolism of tetralin (Chen and Lin, 1968) and fluorene (Chen and Lin, 1969).

In experiments with animals, however, two other types of metabolites of polycyclic hydrocarbons have also been recognized, dihydromonols and products formed by metabolism at positions equivalent to the meso position of anthracene. Products of this type have not been identified with certainty in experiments with tissue preparations.

Differences in metabolism among the limited number of the polycyclic hydrocarbons that have been studied in detail appear to arise (a) from differences in the proportions of the amounts of the various types of metabolites formed and (b) from differences in the proportions of the amounts of one type of metabolite formed at the various sites on a hydrocarbon. It should be borne in mind, however, that estimations of the amounts of metabolites formed at any one site may not be a true estimate of the amount of metabolism occurring at that site, since reactions of metabolic intermediates with cellular constituents may occur, and the

FIG. 1. The metabolism of naphthalene in rats.

metabolites themselves may undergo further enzymatic or nonenzymatic oxidation.

The metabolites normally seen when hydrocarbons are metabolized by tissue preparations may be regarded as the phase I metabolites of Williams (1959, 1971). However, these metabolites can undergo further metabolism, either by oxidation or, in the case of phenols and dihydrodiols, by conjugation with glucuronic acid or sulfuric acid, and these are often the products excreted by animals treated with hydrocarbons. Some of the secondary metabolites, particularly the conjugates, can be regarded as the phase II metabolites of Williams (1959, 1971), but as will be discussed below (Section V,F), secondary metabolism, at least in isolated tissue, can also result in the formation of products that will react with cellular constituents. The metabolic pathways of naphthalene are shown in Fig. 1, and these are typical of the more complex hydrocarbons.

1. *Properties and Metabolism of Dihydrodiols*

a. Configuration. In the few cases where the configuration of the dihydrodiols has been established by their direct comparison with the

(XI)

(XII)

(XIII)

authentic compounds, e.g., 1,2-dihydro-1,2-dihydroxynaphthalene (Fig. 1) (Booth and Boyland, 1949; Booth *et al.*, 1950), 9,10-dihydro-9,10-dihydroxyphenanthrene (XI) (Boyland and Wolf, 1950), 5,6-dihydro-5,6-dihydroxybenz[*a*]anthracene (XII) (Boyland and Sims, 1964b), and 4,5-dihydro-4,5-dihydroxypyrene (XIII) (Boyland and Sims, 1964a), the compounds have the *trans*-configuration. These experiments were carried out in whole animals, but in many experiments with tissue preparations, where the *cis*- and *trans*-isomers can be separated by chromatography, the dihydrodiols formed by metabolism have the mobilities of the *trans*-isomers. Thus, *trans*-5,6-dihydro-5,6-dihydroxydibenz[*a,h*]anthracene (XIV) is a metabolite of dibenz[*a,h*]anthracene (Boyland and Sims,

(XIV) (XV)

1965c); *trans*-10,11-dihydro-10,11-dihydroxy-3-methylcholanthrene (XV) is a metabolite of 3-methylcholanthrene (Sims, 1966); and *trans*-5,6-dihydro-5,6-dihydroxy-7-methylbenz[*a*]anthracene and *trans*-5,6-dihydro-5,6-dihydroxy-12-methylbenz[*a*]anthracene are metabolites of 7- and 12-methylbenz[*a*]anthracene, respectively (Sims, 1967a).

The dihydrodiols possess asymmetric centers and can therefore exist in optically active forms. In whole animals, the simpler hydrocarbons, such as naphthalene (Young, 1947; Booth and Boyland, 1949), anthracene (Boyland and Levi, 1935; Sims, 1964), and phenanthrene (Boyland and Wolf, 1950; Boyland and Sims, 1962b) are converted into mixtures of the (+) and the (−) forms of the dihydrodiols, often with one form predominating, and these mixtures are excreted in the urine. It is difficult to determine the relative proportions of the optical forms of the dihydrodiols as they are first formed, since conjugation reactions and further metabolism also occur in the body. In the metabolism of naphthalene by microsomal fractions from the livers of mice, rats, rabbits, and guinea pigs, mainly (−)-*trans*-1,2-dihydro-1,2-dihydroxynaphthalene is formed (Jerina *et al.*, 1970c), although an earlier report (Holtzman *et al.*, 1967b) suggested that mouse liver microsomal fractions metabolized naphthalene to the (+)-dihydrodiol. The optical activities of dihydrodiols formed from other hydrocarbons by microsomal preparations have not been examined. The absolute configuration of (−)-*trans*-9,10-dihydro-9,10-dihydroxyphenanthrene as 9S,10S and that of (+)-*trans*-1,2-dihydro-1,2-dihydroxynaphthalene as 1S,2S have been reported (Miura *et al.*, 1968).

b. Conjugation. Dihydrodiols are often excreted in conjugation with glucuronic acid (Boyland and Levi, 1935, 1936a; Corner *et al.*, 1954; Corner and Young, 1954). The glucuronic acid conjugates of both (+)- and (−)-*trans*-1,2-dihydro-1,2-dihydroxynaphthalene were isolated from the urine of rabbits dosed with naphthalene (Sims, 1959).

The monosulfuric acid ester of *trans*-9,10-dihydro-9,10-dihydroxyphenanthrene [9,10-dihydro-9-hydroxy-10-phenanthryl sulfate (XVI)] was isolated from the urine of rats dosed with (±)-*trans*-9,10-dihydro-9,10-dihydroxyphenanthrene (Boyland and Sims, 1962c).

O·SO$_3$H OH

XVI

O·SO$_3$H OH

(XVII)

c. Dehydrogenation. In whole animals, many dihydrodiols are dehydrogenated to catechols, which are usually excreted in the urine as sulfuric esters. Thus 2-hydroxy-1-naphthyl sulfate (XVII) is excreted by animals treated with either naphthalene or (±)-*trans*-1,2-dihydro-1,2-dihydroxynaphthalene (Boyland and Sims, 1957; Sims, 1959). Phenanthrene and (±)-*trans*-9,10-dihydro-9,10-dihydroxyphenanthrene are similarly metabolized to 9,10-dihydroxyphenanthryl sulfate (Boyland and Sims, 1962c).

These dehydrogenations are presumably carried out by the soluble dehydrogenases present in liver (Mitoma *et al.*, 1958; Ayengar *et al.*, 1959). The dehydrogenases are stereoselective *in vitro* in their action on (±)-*trans*-1,2-dihydro-1,2-dihydroxynaphthalene and (±)-*trans*-9,10-dihydro-9,10-dihydroxyphenanthrene (Jerina *et al.*, 1970c) in that the (+) forms of the dihydrodiols are preferentially dehydrogenated. In animals treated with (±)-*trans*-9,10-dihydro-9,10-dihydroxyphenanthrene, more than twice the amount of the glucuronic acid conjugate of the (−)-isomer than that of the conjugate of the (+)-isomer was present in the urine (Boyland and Sims, 1962c), an observation that suggests that a similar mechanism is in operation *in vivo*.

d. Formation of Phenols. It is probable that the phenols present in the urine and feces of animals treated with polycyclic aromatic hydrocarbons arise by two distinct routes. The first, through the intermediate formation of epoxides, is discussed in detail in Section V,C. The second route involves the breakdown of conjugates of the dihydrodiols formed during metabolism. The sulfuric esters are the conjugates most likely to undergo this breakdown for, although the ester of (±)-*trans*-9,10-dihydro-9,10-

dihydroxyphenanthrene is stable in the solid state (Boyland and Sims, 1962c), the corresponding ester of (±)-*trans*-1,2-dihydro-1,2-dihydroxynaphthalene rapidly decomposes to 1-naphthyl sulfate and 2-naphthol (Sims, 1959) [Eq. (2)].

(2)

The urine of animals treated with (±)-*trans*-1,2-dihydro-1,2-dihydroxynaphthalene contained 1-naphthyl sulfate and free 2-naphthol (Corner and Young, 1955; Sims, 1959), but little 2-naphthyl sulfate and free 1-naphthol, suggesting that the sulfuric ester of the dihydrodiol is formed in the body but decomposes spontaneously during the processes of excretion. In contrast, the sulfuric ester of (±)-*trans*-9,10-dihydro-9,10-dihydroxyphenanthrene was detected in the urine of rats treated with the dihydrodiol (Boyland and Sims, 1962c), so that the conjugates of "K-region"[1] dihydrodiols may be more stable than those of dihydrodiols formed elsewhere on the hydrocarbon molecules. The phenolic metabolites detected in the early work on hydrocarbon metabolism were not "K-region" products and could well have arisen from the decomposition of conjugates of dihydrodiols. Thus, for example, the benzo[*a*]pyrene metabolite designated F_1 by Weigert and Mottram (1946) and later identified as 9-hydroxybenzo[*a*]pyrene (XVIII) (Sims, 1968), probably

(XVIII) (XIX)

arose by this route. The dihydrodiol precursor of the phenol, 9,10-dihydro-9,10-dihydroxybenzo[*a*]pyrene (XIX), is a metabolite of benzo[*a*]-pyrene in rat liver microsomal systems (Waterfall and Sims, 1972), although in these experiments the phenol itself was not detected as a metabolite. More recently, however, the formation of the phenol by a rat liver microsomal system has been reported (Kinoshita *et al.*, 1973).

The dihydrodiols are themselves decomposed chemically into phenols by hot mineral acid, but it now seems unlikely that they are the sources of phenols formed as metabolites. The gains in resonance energy that

[1] "K-region" products are those formed on the "K-region" (Pullman and Pullman, 1955a) of aromatic hydrocarbons, and non-"K-region" products are those formed on bonds other than those of the "K-region."

should occur when a number of dihydrodiols are dehydrated to phenols have been calculated (Pullman and Pullman, 1955b).

2. *Properties and Metabolism of Glutathione Conjugates*

A typical glutathione conjugate, S-(1,2-dihydro-2-hydroxy-1-naphthyl)glutathione, has the structure (XX).

(XX)

a. Properties. Glutathione conjugates of this type are acid labile and are usually readily decomposed by cold mineral acid. However, some, such as S-(5,6-dihydro-6-hydroxy-7,12-dimethylbenz[*a*]anthracene-5-yl) glutathione (Sims, 1973), require warm acid to effect their decomposition. The nature of the decomposition depends on whether or not the glutathione conjugate formed is a "K-region" conjugate; S-(9,10-dihydro-10-hydroxy-9-phenanthryl)glutathione, for example, yields an S-arylglutathione conjugate together with the parent hydrocarbon and oxidized glutathione (Eq. 3) (Boyland and Sims, 1965b). Conjugates formed on

(3)

bonds other than those of the "K-region" yield these products together with small amounts of phenols, the conjugate (XX), for example, yielding 1- and 2-naphthol (Boyland and Sims, 1958). These decomposition reactions are clearly complex since migration of hydroxyl occurs, but the mechanisms involved have not been investigated.

The glutathione conjugates are converted into the related phenols by Raney nickel (Boyland *et al.*, 1961); these reactions enable the positions of the hydroxyl groups in the conjugates to be established. With the "K-region" glutathione conjugates of 7,12-dimethylbenz[*a*]anthracene, alkaline hydrolysis yielded a mixture of products including the *trans*-dihydrodiol (Booth *et al.*, 1973). Non-"K-region" glutathione conjugates have not been examined, but the related mercapturic acid, *N*-acetyl-S-

FIG. 2. The enzymatic conversion of a glutathione conjugate into a mercapturic acid.

(1,2-dihydro-2-hydroxy-1-naphthyl)cysteine, derived from naphthalene yields 2-naphthol on treatment with alkali (Boyland and Sims, 1958).

b. Metabolism. The glutathione conjugates are converted into the related mercapturic acids by the mechanisms outlined in Fig. 2, in which the metabolism of S-(1,2-dihydro-2-hydroxy-1-naphthyl)glutathione is illustrated. The reactions are enzymatic (Booth *et al.*, 1960a) and involve first the loss of glutamic acid to form the cysteinylglycine conjugates. These are converted into the cysteine conjugates by loss of glycine and acetylation of the amino groups of the cysteine conjugates yields the mercapturic acids, the conjugates normally excreted in the urine of animals treated with aromatic hydrocarbons. All four types of conjugates are present in the bile of rats treated either with naphthalene (Boyland *et al.*, 1961), or with phenanthrene (Boyland and Sims, 1962a). Enzymes that convert the glutathione conjugate into the cysteine derivative are present in rat kidney; those that convert the cysteine derivative into the mercapturic acid are present in both rat kidney and liver (Booth *et al.*, 1960a). Rat liver preparations appear to be unable to convert glutathione conjugates into cysteinylglycine derivatives (Revel and Ball, 1959), and glutathione conjugates are those normally seen in metabolic experiments with aromatic hydrocarbons using rat liver preparations. However, S-(*p*-chlorobenzyl)glutathione is converted into the corresponding cysteine derivative by guinea pig liver slices and guinea pig, rat, and rabbit liver homogenates (Bray *et al.*, 1959). Some of the cysteine derivatives are substrates for the aminoacyl-RNA synthetase present in baker's yeast (Bucovaz *et al.*, 1970).

Acid-labile mercapturic acids of the type described above have been called "premercapturic acids" (Knight and Young, 1958).

3. *Metabolism of Phenols*

Phenols are excreted by animals either free or in conjugation with sulfuric or glucuronic acid. Conjugates of both types were detected when 1- or 2-naphthol was fed to rats (Berenbom and Young, 1951).

With simpler hydrocarbons, there is little evidence that phenols are further metabolized to dihydric phenols; the formation of catechols by dehydrogenation of the dihydrodiols has been discussed above. With the more complex hydrocarbons, however, further hydroxylation does occur. 6-Hydroxybenzo[*a*]pyrene is metabolized in rats to 1,6- and 3,6-dihydroxybenzo[*a*]pyrene (Falk *et al.*, 1962) (Eq. 4) and the dihydroxy-

OH OH → OH OH + OH OH (4)

dibenz[*a,h*]anthracenes are metabolites of dibenz[*a,h*]anthracene (Dobriner *et al.*, 1939; Boyland *et al.*, 1941; LaBudde and Heidelberger, 1958). 1,6- and 1,8-Dihydroxypyrene, formed in the metabolism of pyrene (Harper, 1957), probably arise through the initial formation of monohydroxylated derivatives.

4. *Metabolic Formation of Dihydromonols*

It was observed by Bourne and Young (1934) that, when the urine of rabbits that had been treated with naphthalene was acidified, crystals of the hydrocarbon separated out, the hydrocarbon apparently arising from the decomposition of an acid-labile precursor. Similarly, anthracene was liberated by acid from a compound present in the urine of rats and rabbits that were treated with the hydrocarbon (Boyland and Levi, 1936b). The urine of rats treated with naphthalene, phenanthrene, or anthracene, but not of those treated with benzo[*a*]pyrene, dibenz[*a,h*]-anthracene, or 3-methylcholanthrene contained acid-labile hydrocarbon precursors (Chang and Young, 1943). Glucuronic acid conjugates of hydrocarbon precursors were detected in the urine of rats and rabbits treated with pyrene (Boyland and Sims, 1964a) and benz[*a*]anthracene (Boyland and Sims, 1964b), and of mice treated with 3-methylcholanthrene, dibenz[*a,h*]anthracene, benz[*a*]anthracene, chrysene and anthracene (Harper, 1959a) and pyrene and benzo[*a*]pyrene (Harper, 1958a,c). Although with some hydrocarbons the liberated compounds could have arisen by the decomposition of acid-labile mercapturic acids (Section II,B,4), Boyland and Solomon (1955) showed that compounds

were present in the urines of naphthalene-treated rats and rabbits that appeared to be glucuronic acid conjugates of one or other of the optically active forms of 1,2-dihydro-1-naphthol; these conjugates readily yielded naphthalene on acidification. A possible route to the biosynthesis of the naphthalene dihydromonol in the body is by the reduction of naphthalene 1,2-oxide (Eq. 5).

2H (5)

5. *Metabolism at the Meso Positions of Aromatic Hydrocarbons*

Metabolism of aromatic hydrocarbons normally takes place at the double bonds, but the formation of metabolites at the meso positions (the so-called "L-regions") of some hydrocarbons has also been reported. Thus anthracene is metabolized in rats at the 9- and 10-positions to yield *trans*- (and possibly also *cis*-)9,10-dihydro-9,10-dihydroxyanthracene (Sims, 1964), and although this dihydrodiol was present in the urine only in small amounts, larger amounts of 2-hydroxy-9,10-anthraquinone and conjugates of 9,10-dihydroxy- and 2,9,10-trihydroxyanthracene were present (see Fig. 3). These compounds were also formed when rats were treated with *trans*-9,10-dihydroxyanthracene (Sims, 1964), so that they presumably arise from the parent hydrocarbon through the intermediate formation of the dihydrodiol.

Benz[*a*]anthracene is metabolized in rats, rabbits, and mice to conjugates of an unidentified hydroxy compound that yielded benz[*a*]-anthracene 7,12-quinone (XXI) on hydrolysis (Boyland and Sims, 1964b). It is possible that the hydroxy compound was 7,12-dihydro-7,12-dihydroxy-benz[*a*]anthracene (XXII); this compound is itself excreted by these

FIG. 3. The metabolism at the "meso" position of anthracene.

(XXI)

(XXII) (XXIII)

animals partly in conjugation with sulfuric and glucuronic acid and partly in the form of conjugates of 7-hydroxybenz[*a*]anthracene (XXIII). The monohydroxy compound is readily oxidized by air to the 7,12-quinone.

Dibenz[*a,h*]anthracene is similarly metabolized to dibenz[*a,h*]anthracene 7,14-quinone in animals (Heidelberger *et al.*, 1953) and benzo[*a*]-pyrene is converted into 6-hydroxybenzo[*a*]pyrene in rats (Falk *et al.*, 1962), which is excreted in conjugation with glucuronic acid. The 6-position of benzo[*a*]pyrene may be regarded as a meso position, equivalent to the 7-position of benz[*a*]anthracene since both these positions are the sites of chemical substitution reactions, with, for example, bromine or lead tetraacetate.

Metabolites formed at the meso positions of some aromatic hydrocarbons are found in experiments with animals, but there is as yet no evidence that metabolism of this type can be carried out by hepatic homogenates or microsomal fractions. The formation of quinones from hydrocarbons in these systems has been reported: benz[*a*]anthracene was converted into benz[*a*]anthracene 7,12-quinone (Boyland *et al.*, 1964) and benzo[*a*]pyrene into the 3,6- and sometimes into the 1,6-quinone (XXIV and XXV) (Conney *et al.*, 1957; Sims, 1967b; Borgen

(XXIV) (XXV)

et al., 1973; Kinoshita *et al.*, 1973). However, these quinones were not detected in other experiments with the hydrocarbons (Sims, 1970b; Grover *et al.*, 1974), suggesting that they may arise from chemical oxidations occurring during working-up procedures.

The sites of formation of these products in the body are thus not yet established, and it is possible that metabolism of the hydrocarbons by gut bacteria is involved. In the metabolism of anthracene, either anthracene 9,10-oxide, as shown in Fig. 3, or anthracene 9,10-peroxide (anthracene 9,10-photo-oxide (XXVI) are the likely metabolic intermediates in the formation of 9,10-dihydro-9,10-dihydroxyanthracene. Cyclic peroxides are formed in the microbial oxidation of several benzenoid compounds, the addition of two atoms of oxygen being catalyzed by a

(XXVI) (XXVII)

dioxygenase (Gibson *et al.*, 1970). Naphthalene is metabolized by *Pseudomonas* strains to *cis*-1,2-dihydro-1,2-hydroxynaphthalene, a reaction that suggests that naphthalene 1,2-cyclic peroxide, rather than naphthalene 1,2-oxide, is formed as a metabolic intermediate by bacteria (Jerina *et al.*, 1971; Catterall *et al.*, 1971). The transannular epoxide, naphthalene 1,4-oxide (XXVII), does not appear to be involved in the metabolism of naphthalene in rats (Sims, 1965).

6. *Metabolism of Hydroxymethyl Compounds*

Compounds of this type have been identified as products of hydrocarbon metabolism only in isolated tissue preparations. A typical hydroxymethyl compound is 7-hydroxymethyl-12-methylbenz[*a*]anthracene, which is formed when 7,12-dimethylbenz[*a*]anthracene is metabolized by rat liver homogenates or microsomal fractions (Boyland and Sims, 1965a, 1967a; Jellinck and Goudy, 1966, 1967; Flesher *et al.*, 1967; Sims and Grover, 1968; Sims, 1970b,c). As outlined in Fig. 4, three metabolic

CH_2OH CH_2OH CH_3 CH_2OH CH_3 CO_2H

CH_3 CH_2OH H OH HO · CH_3 CH_2OH HO H OH · CH_3 CH_2OH OH H HO H · CH_3 CH_2OH OH

Fig. 4. The metabolism of 7-hydroxymethyl-12-methylbenz[*a*]anthracene.

pathways are possible with this hydroxymethyl compound involving either (a) further metabolism of the hydroxymethyl groups, (b) metabolism of the second methyl group, or (c) metabolism on the aromatic nucleus.

There is some evidence that metabolism of the hydroxymethyl group of 7-hydroxymethylbenz[*a*]anthracene, 7-hydroxymethyl-12-methylbenz[*a*]anthracene, and 12-hydroxymethyl-7-methylbenz[*a*]anthracene occurs in liver homogenates to yield the related carboxylic acids (Sims, 1967a; Boyland and Sims, 1967a), reactions that presumably involve the intermediate formation of the related aldehydes. Small amounts of 12-methylbenz[*a*]anthracene-7-carboxylic acid have been detected in the urine of rats treated with the parent hydrocarbons (P. Sims, unpublished observation). With 7,12-dimethylbenz[*a*]anthracene derivatives, hydroxylation of the methyl groups also occurs to a small extent to yield 7,12-dihydroxymethylbenz[*a*]anthracene (Boyland and Sims, 1967a). The major routes of metabolism, however, involved metabolism of one or other of the aromatic rings to yield dihydrodiols, glutathione conjugates, and phenols (Sims, 1967a; Boyland and Sims, 1967a; Sims, 1970c; Booth *et al.*, 1973), presumably by the same processes as those involved in the metabolism of the parent hydrocarbons.

3-Methylcholanthrene, the molecule of which possesses a methylene bridge as well as a methyl group, is hydroxylated by rat liver homogenates on all three nonaromatic carbon atoms to yield 1- and 2-hydroxy-3-methylcholanthrene, (XXVIII) and (XXIX), *cis*- and *trans*-1,2-dihydroxy-

(XXVIII) (XXIX)

(XXX) (XXXI)

3-methylcholanthrene (XXX), and 3-hydroxymethylcholanthrene (XXXI) as well as on the aromatic rings to yield the "K-region" dihydrodiol (XV) and unidentified phenols (Sims, 1966). The further metabolism of these compounds by liver preparations has not yet been studied. In mice, 3-methylcholanthrene is metabolized to cholanthrene-3-carboxylic acid

(Harper, 1959a) and to *cis*- and *trans*-1,2-dihydroxy-3-methylcholanthrene and 2-hydroxy- and 2-keto-3-methylcholanthrene (Takahashi and Yasuhira, 1972).

C. Epoxides as Primary Metabolic Products

The metabolic formation of *trans*-dihydrodiols from naphthalene (Booth and Boyland, 1949), anthracene (Boyland and Levi, 1935, 1936a), and phenanthrene (Boyland and Wolf, 1950) led Boyland (1950) to suggest that epoxides were formed as intermediates in the metabolism of polycyclic hydrocarbons, and that these intermediates were involved in the carcinogenic activity shown by many hydrocarbons. Although in the early years evidence to support the suggestion of Boyland was difficult to obtain, more recent work has indicated (1) that epoxides are formed as metabolites of hydrocarbons, at least in model microsomal systems, (2) that these compounds can react with cellular constituents, such as proteins and nucleic acid, and (3) that many of them are biologically active and can induce mutations in mammalian cells, bacteria, and bacteriophage, malignant transformation in rodent cells in culture, and cancer in experimental animals. Figure 5, which shows metabolism

Fig. 5. Pathways involved in the metabolism of benz[*a*]anthracene at the "K-region." From Swaisland *et al.* (1973) with permission of Pergamon Press.

at the 5,6-bond of benz[*a*]anthracene, summarizes the types of reactions that appear to occur within mammalian cells after they are treated with polycyclic aromatic hydrocarbons.

III. Synthesis of Epoxy Derivatives of Polycyclic Aromatic Hydrocarbons

The first attempts to synthesize hydrocarbon epoxides,[2] which were not very successful, involved the oxidation of pyrene and benz[*a*]anthracene with perbenzoic acid (Boyland and Sims, 1964a,b). Although the "K-region" epoxides of both these compounds were present in the reaction mixtures as indicated by the formation of "K-region" dihydrodiols or mercapturic acids when the products present in the reaction mixtures were allowed to react with water or *N*-acetylcysteine, crystalline epoxides could not be isolated. Van Duuren *et al.* (1964) have reported the formation of the "K-region" epoxide in a similar reaction with dibenz[*a,h*]anthracene. In these experiments, the presence of the epoxide in the reaction mixture was demonstrated by the formation of 5-hydroxydibenz[*a,h*]anthracene with acid and by the formation of a product, presumably 5,6-dihydro-5-hydroxydibenz[*a,h*]anthracene with lithium aluminum hydride that yielded the parent hydrocarbon when treated with acid. In the oxidations of benz[*a*]anthracene (Boyland and Sims, 1964b) and dibenz[*a,h*]anthracene (Van Duuren *et al.*, 1964), there was also evidence for the formation of transannular epoxides across the meso positions, the 7,12- and 7,14-bonds, respectively, of the hydrocarbons. Thus the oxidation products of benz[*a*]anthracene contained a compound that yielded 7,12-dihydro-7,12-dihydroxybenz[*a*]anthracene with water, whereas those from dibenz[*a,h*]anthracene contained 7,14-dihydro-7-keto-dibenz[*a,h*]anthracene, a product that was probably formed from the spontaneous rearrangement of the epoxide.

More recently, many epoxide derivatives of polycyclic hydrocarbons have been prepared in crystalline form. They can be conveniently divided into two groups, the "K-region" and the non-"K-region" epoxides.

A. "K-Region" Epoxides

When aromatic hydrocarbons possessing "K-region" type bonds are treated with osmium tetroxide, reaction occurs at these bonds to give complexes that can be decomposed to yield "K-region" *cis*-dihydrodiols

[2] The term epoxide is used in this review in the general sense to denote a compound arising from the addition of oxygen across an aromatic double bond. Jerina *et al.* (1968b) prefer the term arene oxide for compounds of this type. However, in naming the compounds, the arene oxide nomenclature (e.g., benz[*a*]anthracene 5,6-oxide) is more convenient than the alternative epoxydihydroarene nomenclature (e.g., 5,6-epoxy-5,6-dihydrobenz[*a*]anthracene).

(Cook and Schoental, 1948). These compounds are conveniently oxidized to the related dialdehydes with either sodium periodate (Hadler and Kryger, 1960) or, for the dihydrodiols related to pyrene and benzo[*a*]-pyrene, with lead tetraacetate (Goh and Harvey, 1973). In some cases, such as phenanthrene (Bailey and Erickson, 1961), the dialdehydes can be prepared directly from the hydrocarbon by ozonolysis. Newman and Blum (1964) were able to cyclize some of these dialdehydes to the related epoxides using trisdimethylaminophosphine (hexamethylphosphoramide), a reagent first introduced by Mark (1963). The synthesis of benz[*a*]anthracene 5,6-oxide by this route is outlined in Fig. 6.

Since aromatic polycyclic hydrocarbons can be generally labeled with tritium by the exchange process on a 0.5–1 gm scale, this method of synthesis provides a convenient route both to the ^{3}H-labeled epoxides themselves and to ^{3}H-labeled *cis*-dihydrodiols and phenols. These latter compounds have been used in conjunction with the ^{3}H-labeled epoxides and hydrocarbons in comparative experiments designed to study reactions with cellular macromolecules both chemically and in cells in culture (Grover and Sims, 1970; Grover *et al.*, 1971a; Kuroki *et al.*, 1971/1972) (see Sections VI and VII).

Two other routes to "K-region" epoxides have been described. The first, which has so far only been used in the synthesis of phenanthrene 9,10-oxide (Mackintosh, 1972), requires the preparation of the monosulfuric ester of *trans*-9,10-dihydro-9,10-dihydroxyphenanthrene (Boyland and Sims, 1962c), and this on treatment with alkali, yields the epoxide.

The second, introduced by Goh and Harvey (1973), requires the synthesis of the related "K-region" *trans*-dihydrodiols. In the synthesis

FIG. 6. Preparation of a "K-region" epoxide (benz[*a*]anthracene 5,6-oxide). Reprinted from "Clinical Carcinogenesis" (P. O. P. T'so and J. A. DiPaulo, eds.) pp. 237–274, by courtesy of Marcel Dekker, Inc.

TABLE I
SYNTHETIC ROUTES LEADING TO EPOXIDES OF POLYCYCLIC HYDROCARBONS

Epoxide	Formula	Method of synthesis[a]	Reference
Naphthalene 1,2-oxide		D E	Vogel and Klärner, 1968 Yagi and Jerina, 1973
Phenanthrene 1,2-oxide		D	Yagi and Jerina, 1973
Phenanthrene 3,4-oxide		E	Yagi and Jerina, 1973
Phenanthrene 9,10-oxide		A B C	Newman and Blum, 1964 Goh and Harvey, 1973 Mackintosh, 1972
Chrysene 5,6-oxide		A	P. Sims, unpublished

Pyrene 4,5-oxide	A	P. Sims, unpublished
Benz[*a*]anthracene 5,6-oxide	A	Newman and Blum, 1964
Benz[*a*]anthracene 8,9-oxide	D	Sims, 1971
7-Methylbenz[*a*]anthracene 5,6-oxide	A	Newman and Blum, 1964
7-Acetoxymethylbenz[*a*]anthracene 5,6-oxide	A	Sims, 1972a

(*Continued*)

TABLE I (*Continued*)

Epoxide	Formula	Method of synthesis[a]	Reference
7-Hydroxymethylbenz[*a*]anthracene 5,6-oxide		F	Sims, 1972a
7,12-Dimethylbenz[*a*]anthracene 5,6-oxide		A	Goh and Harvey, 1973; Sims, 1973
		B	Goh and Harvey, 1973
7-Acetoxymethyl-12-methylbenz[*a*]-anthracene 5,6-oxide		A	Sims, 1973
7-Hydroxymethyl-12-methylbenz[*a*]-anthracene 5,6-oxide		F	Sims, 1973
3-Methylcholanthrene 11,12-oxide		A	Sims, 1966

Dibenz[a,h]anthracene 5,6-oxide		A	Boyland and Sims, 1965c
Dibenz[a,c]anthracene 10,11-oxide		D	Sims, 1972b
Benzo[a]pyrene 4,5-oxide		A, B	Goh and Harvey, 1973
Benzo[a]pyrene 7,8-oxide		D	Waterfall and Sims, 1972
Benzo[a]pyrene 9,10-oxide		D	Waterfall and Sims, 1972

[a] A: Cyclization of the related dialdehydes with hexamethylphosphorus triamide.
B: Cyclization of the related *trans*-dihydrodiol with the dimethylacetal of dimethylformamide.
C: Cyclization of the monosulfuric ester of the related *trans*-dihydrodiol.
D: Dehydrobromination of the related bromotetrahydro epoxide.
E: Dehydrobromination and cyclization of the related bromotetrahydrobromohydrin.
F: Hydrolysis with alkali of the related acetoxymethyl derivative.

of these isomers, the corresponding "K-region" *cis*-dihydrodiols are oxidized to the related quinones with chromic acid. Although this reaction normally proceeds smoothly, the "K-region" quinone derived from 7,12-dimethylbenz[*a*]anthracene could not be prepared by this method (Hadler and Kryger, 1960; Boyland and Sims, 1967a). Other methods of oxidation have been introduced using dimethyl sulfoxide and acetic anhydride (Newman and Davis, 1967) or dimethyl sulfoxide and sulfur trioxide–pyridine complex (Goh and Harvey, 1973). Reduction of the quinones with lithium aluminum hydride normally leads only to the *trans*-dihydrodiols, but with 7-methyl and 7,12-dimethylbenz[*a*]-anthracene-5,6-dione, mixtures of the *cis*- and *trans*-isomer are obtained (Sims, 1967a; Boyland and Sims, 1967a). These are separated either by preparative thin-layer chromatography (Boyland and Sims, 1967a) or, with mixtures of the isomers derived from 7,12-dimethylbenz[*a*]anthracene, by the conversion of the *cis*-isomer into the acetonide with acetone (Goh and Harvey, 1973), so that the *trans*-isomer can be isolated. The *trans*-dihydrodiols are then cyclized with the dimethylacetal of dimethylformamide to yield the "K-region" epoxides. This method could presumably yield epoxides on other bonds of the hydrocarbon molecules if the related *trans*-dihydrodiols were available.

The "K-region" epoxides that have so far been synthesized are listed in Table I.

B. Non-"K-Region" Epoxides

The simplest non-"K-region" epoxide of a polycyclic hydrocarbon, naphthalene 1,2-oxide, was first synthesized by Vogel and Klärner (1968) and involved the preparation of the hydrogenated epoxide 3,4-dihydronaphthalene 1,2-oxide, which was then brominated in the 4-position with *N*-bromosuccinimide. The bromo compound was dehydrobrominated with a suitable base [usually 1,5-diazabicyclo[4.3.0]non-5-ene] to yield naphthalene 1,2-oxide. This synthesis was later modified by Yagi and Jerina (1973), who prepared the trichloro- or the trifluoroacetate of 3,4-dihydronaphthalene 1,2-bromohydrin and subjected these compounds to the bromination procedure. The 4-bromo derivatives thus obtained were hydrolyzed to the 4-bromo-1,2-bromohydrin, and this compound was converted into naphthalene 1,2-oxide by a double dehydrobromination with sodium methoxide.

Only a small number of non-"K-region" epoxides of the more complex hydrocarbons has been synthesized: these are listed in Table I. In most cases, the route used was similar to that of Vogel and Klärner (1968), except that it was necessary to build up the aromatic nucleus using essentially the classical methods of organic synthesis. A typical synthesis,

FIG. 7. Preparation of a non-"K-region" epoxide (benz[*a*]anthracene 8,9-oxide). Reprinted from "Clinical Carcinogenesis" (P. O. P. T'so and J. A. DiPaulo, eds.) pp. 237–247, by permission of Marcel Dekker, Inc.

that of benz[*a*]anthracene 8,9-oxide, is outlined in Fig. 7. Because the non-"K-region" epoxides are very unstable, they are not always amenable to conventional methods of purification, such as crystallization or chromatography. The immediate precursors of the epoxides, the bromo epoxides, have likewise been difficult to purify, so that, as prepared, some of the non-"K-region" are known to contain small amounts of impurities. The modified synthesis of Yagi and Jerina (1973) appears, at least in some cases, to offer some advantages in this respect.

Naphthalene 1,2-oxide was detected in the reaction of naphthalene and pyridine *N*-oxide under the influence of ultraviolet light (Jerina *et al.*, 1970b). The reaction does not appear to be of preparative value.

IV. Metabolic Formation of Epoxides Derived from Polycyclic Aromatic Hydrocarbons

The formation of epoxides from compounds with olefinic double bonds either in whole animals or in microsomal systems has been recognized for many years. Thus, for example, styrene, cyclohexene, and indene oxide (Leibman and Ortiz, 1970) heptachlor oxide (Davidow and Radomski, 1953; Nakatsugawa *et al.*, 1965), dieldrin (Winteringham and Barnes, 1955), endrin (Wong and Terriere, 1965), oxychlordane (Schwemmer *et al.*, 1970), and some allyl-substituted barbituric acid epoxides (Harvey *et al.*, 1972) have been detected as products of the metabolism of the related olefins. 1,4-Epoxy-1,4-dihydronaphthalene is

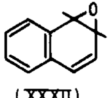
excreted in the urine of rats as 1,4-2,3-diepoxy-1,2,3,4-tetrahydronaphthalene (Sims, 1965). The metabolic formation of epoxides from long-chain olefins such as *n*-4-octene (Maynert *et al.*, 1970) and unsaturated steroids (Kurosawa *et al.*, 1961; Breuer and Knuppen, 1961) have been reported.

The suggestion (Boyland, 1950) that epoxides are formed in the metabolism of aromatic double bonds therefore seems reasonable, and, more recently, evidence, both indirect and direct, that this occurs has been obtained from a number of sources. The indirect evidence arises from (a) comparisons of the structures of hydrocarbon metabolites with those formed from epoxides in the same system, (b) the so-called NIH shift and (c) the effect of inhibitors on the formation of hydrocarbon metabolites that may arise via epoxide intermediates. The direct evidence has come from experiments in which hydrocarbons (usually labeled with ^{3}H) are incubated with microsomal fractions and the presence of epoxides in the reaction mixtures has been detected.

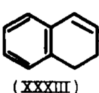
A. Comparison of the Structures of Metabolites Formed from Hydrocarbons with Those Formed from Epoxides

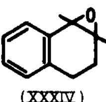
1. *Metabolism in Whole Animals*

The formation of three types of hydrocarbon metabolite, *trans*-dihydrodiols, phenols, and either glutathione or *N*-acetylcysteine conjugates, has been discussed in Section II,B. In suggesting that naphthalene 1,2-oxide was the metabolic intermediate formed from naphthalene, Boyland (1950) predicted that the mercapturic acid excreted by animals dosed with the hydrocarbon should be *N*-acetyl-S-(1,2-dihydro-2-hydroxy-1-naphthyl)cysteine. When this metabolite was isolated from the urine of rabbits dosed with naphthalene (see Fig. 1) (Boyland and Sims, 1958), the formation of naphthalene 1,2-oxide (XXXII) as the primary metabolic



(XXXII)


(XXXIII)



(XXXIV)

product of the hydrocarbon in the body seemed established. 1,2-Dihydronaphthalene (XXXIII) and its oxide (XXXIV), the molecules of which differ from naphthalene and its 1,2-oxide, respectively, only by the presence of two extra hydrogen atoms, are both metabolized by rabbits to the mercapturic acid, (−)-*N*-acetyl-S-(2-hydroxy-1,2,3,4-tetrahydro-1-naphthyl)cysteine (XXXV) (Boyland and Sims, 1960). The formation,

from both compounds, of the same optical isomer of the mercapturic acid suggests that a common metabolic pathway is in operation.

(XXXV) (XXXVI)

The only aromatic hydrocarbon epoxide, the metabolism of which has been examined in animals, is the "K-region" epoxide, phenanthrene 9,10-oxide (XXXVI) (Boyland and Sims, 1965b). A comparison of the metabolites formed from the epoxide with those formed at the 9,10-bond of phenanthrene by rats showed that both compounds yielded *trans*-9,10-dihydro-9,10-dihydroxyphenanthrene, which is excreted both free and in conjugation with glucuronic acid. Both compounds yielded a mercapturic acid, *N*-acetyl-S-(9,10-dihydro-9-hydroxy-10-phenanthryl)cysteine, and a comparison of the optical properties of the methyl esters of the metabolites both from phenanthrene and from its 9,10-oxide, showed that they were the same optical isomer. The metabolism of the two compounds differed in that animals treated with the epoxides excreted 9-phenanthrol, mainly as the sulfate ester, whereas no phenol was formed from the hydrocarbon. This difference exists probably because phenanthrene 9,10-oxide, in common with other polycyclic hydrocarbon epoxides, isomerizes spontaneously to the related phenol. This phenomenon is discussed in more detail in Section VI,A.

2. *Metabolism in Isolated Tissue Preparations*

Glutathione conjugates with structures analogous to those of the mercapturic acids are formed both from naphthalene (Booth *et al.*, 1961) and from 1,2-dihydronaphthalene and its epoxide (Booth *et al.*, 1960b) in hepatic systems containing microsomal and soluble fractions. In the absence of microsomal fractions, a glutathione conjugate is formed only from the epoxide. Thus the enzyme responsible for the activation of the hydrocarbons is located in the microsomal fractions.

Most of the studies in which hydrocarbon and epoxide metabolism was compared were carried out using rat liver homogenates or microsomal fractions, and usually only the less-polar metabolites, those extractable from the reaction mixtures by organic solvents, such as ethyl acetate, were investigated. The metabolism of the epoxides is considered in more detail in Section V, but in general the epoxides are metabolized to dihydrodiols that are the same as those formed on the equivalent bonds of the hydrocarbons, and it is often possible by direct comparison to

show that the metabolites arising from both types of substrates have the *trans* configuration. In experiments with the epoxides, the related phenols are also formed, probably arising from nonenzymatic isomerizations of the epoxides (see Section V,C).

3. *Metabolism in Cells in Culture*

In a direct comparison between the metabolism of hydrocarbons and their related epoxides by hamster embryo cells in culture (Sims *et al.*, 1973), it was shown that benz[*a*]anthracene and benz[*a*]anthracene 5,6-oxide were both converted into *trans*-5,6-dihydro-5,6-dihydroxybenz[*a*]-anthracene.

B. The NIH Shift

In the metabolism of many aromatic compounds specifically labeled with deuterium or tritium in positions where enzymatic hydroxylation is known to occur, this hydroxylation is accompanied by migration of deuterium or tritium into the *ortho*-position, so that these isotopes are retained in the metabolites. This phenomenon has been called the NIH shift (Guroff *et al.*, 1967) and is indicative of the intermediate formation of epoxides, for when naphthalene 1,2-oxide, labeled with deuterium in either the 1- or the 2-position, isomerizes spontaneously under neutral or basic conditions to 1-naphthol, deuterium retentions of about 80% are observed (Boyd *et al.*, 1972). When either of the ^{2}H-labeled epoxides were incubated with rabbit liver microsomal fractions, the observed deuterium retentions in the 1-naphthol thus formed were 72–75% ($\pm 2\%$) and in the metabolic conversion of 1-^{2}H- and 2-^{2}H-labeled naphthalene to 1-naphthol by these fractions, retentions of 64% ($\pm 8\%$) were observed. The mechanism of the metabolic conversion of naphthalene into 1-naphthol was therefore interpreted as involving the formation of naphthalene 1,2-oxide as the rate-limiting step. Since most of the work so far carried out on the NIH shift has been on relatively simple aromatic compounds, the topic will not be discussed further, but a recent review is available (Daly *et al.*, 1972).

C. Effect of Epoxide Hydrase Inhibitors on Hydrocarbon Metabolism

Epoxides are converted into *trans*-dihydrodiols by the microsomal enzyme, "epoxide hydrase" (see Section V,A). Inhibitors of this enzyme are known (Oesch *et al.*, 1971c), so that the incorporation of such an inhibitor into a microsomal system in which a polycyclic hydrocarbon is used as substrate, should, if epoxides are intermediates in dihydrodiol formation, result in reductions in the amounts of the dihydrodiols formed as compared with those formed in experiments carried out in

the absence of inhibitor. A number of experiments in which this occurs have been reported.

Naphthalene is converted into 1-naphthol and *trans*-1,2-dihydro-1,2-dihydroxynaphthalene by reconstituted rat liver microsomal enzyme systems containing either cytochrome P-448 or P-450 preparations (both of which contained epoxide hydrase), together with reductase and lipid fractions (Oesch *et al.*, 1972). The addition to the systems of the epoxide hydrase inhibitor, 3,3,3-trichloropropene oxide blocked the formation of the dihydrodiol and led to increases in the amounts of 1-naphthol formed. The incorporation of the epoxide hydrase inhibitor, cyclohexene oxide, into hamster liver microsomal systems containing benzo[*a*]pyrene reduced the amounts of *trans*-4,5-dihydro-4,5-dihydroxybenzo[*a*]pyrene formed, as compared with those formed in control experiments (Wang *et al.*, 1972). The amounts of 8,9-dihydro-8,9-dihydroxy- and 10,11-dihydro-10,11-dihydroxy-7-hydroxymethyl-12-methylbenz[*a*]anthracene formed when 7-hydroxymethyl-12-methylbenz[*a*]anthracene was incubated with a rat liver microsomal system (see Fig. 4) were reduced in the presence of cyclohexene oxide (Booth *et al.*, 1974). In these experiments the presence of the inhibitor also caused increases in the amounts of water-soluble metabolites formed.

D. Detection of Epoxides as Microsomal Metabolites of Polycyclic Aromatic Hydrocarbons

The first direct evidence for the formation of an epoxide of an aromatic hydrocarbon was provided by the work of Jerina *et al.* (1968a, 1970a), who showed, both by a radiotracer trapping technique and by isolation using countercurrent distribution, that naphthalene is converted into naphthalene 1,2-oxide by rat liver microsomal systems. Reconstituted rat liver microsomal systems containing either cytochrome P-448 or P-450 also converted naphthalene into naphthalene 1,2-oxide (Oesch *et al.*, 1972).

In a more extensive series of investigations (Sims *et al.*, 1971; Grover *et al.*, 1971b, 1972; Keysell *et al.*, 1972, 1973), the formation of "K-region" epoxides from members of a series of aromatic hydrocarbons by rat liver microsomal fractions was demonstrated. In these experiments, livers from rats that had been pretreated with 3-methylcholanthrene were incubated with ^{3}H-labeled hydrocarbons in the presence of either styrene oxide, 3,4-dihydronaphthalene 1,2-oxide, or cyclohexene oxide, all of which act as epoxide hydrase inhibitors. The ethyl acetate extracts of the reaction mixtures were chromatographed on alumina, often in the presence of the related unlabeled carrier "K-region" epoxide, using solvent systems similar to those shown in Fig. 8. The presence of a ^{3}H-labeled "K-region" epoxide in the appropriate fractions was demonstrated (1) by its conversion into

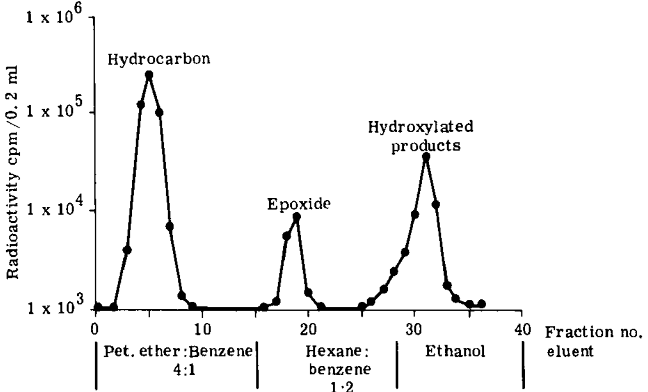


FIG. 8. Alumina column chromatography of ^{3}H-labeled benzo[*a*]pyrene metabolites. The concentrated ether extract from a rat liver microsomal incubation was applied to a column of activated alumina (100–200 mesh, type H), which was eluted with solvent as shown. Fractions (100-drop) were collected, and the radioactivity was measured. From Grover *et al.* (1972) with permission of Pergamon Press.

the related "K-region" phenol with acid, (2) by its enzymatic conversion into the related "K-region" dihydrodiol by the epoxide hydrase present in rat-liver microsomal fractions, and (3) by its chemical conversion into a glutathione conjugate by allowing the material in the appropriate fractions to react with glutathione. In many cases, a mixture of the "K-region" epoxide formed by metabolism with the unlabeled carrier "K-region" epoxide was recrystallized to constant specific activity.

In other experiments, using the methods outlined above, the metabolic formation of "K-region" epoxides from polycyclic hydrocarbons by microsomal fractions from rat lung (Grover, 1974), human lung (Grover *et al.*, 1973) and human liver (P. L. Grover, A. Hewer, and P. Sims, unpublished observations) has been demonstrated. The "K-region" epoxides detected and identified as microsomal metabolites in these systems are listed in Table II.

The formation of a product in the metabolism of benzo[*a*]pyrene by hamster liver microsomal incubation systems that appears from physical and chemical evidence to be benzo[*a*]pyrene 4,5-oxide has also been reported (Wang *et al.*, 1972).

Apart from naphthalene 1,2-oxide, there is as yet no direct evidence for the formation of non-"K-region" epoxides in microsomal systems. This is undoubtedly due to the instabilities of epoxides of this type as compared with "K-region" epoxides (Sims, 1971, 1972b; Waterfall and Sims, 1972).

An epoxide of unknown structure was obtained using a radiotracer

TABLE II
"K-REGION" EPOXIDES IDENTIFIED AS METABOLITES OF ^{3}H-LABELED POLYCYCLIC AROMATIC HYDROCARBONS

Epoxide	Microsomal fractions from: Species	Tissue	Methods of characterization[a]	Reference
Phenanthrene 9,10-oxide	Rat	Liver	A, B, C, D	Grover *et al.*, 1971b
Benz[*a*]anthracene 5,6-oxide	Rat	Liver	A, B, C, D	Grover *et al.*, 1971b
	Human	Lung	A, B, C	Grover *et al.*, 1973
	Rat	Lung	A, B, C	Grover, 1974
7-Methylbenz[*a*]anthracene 5,6-oxide	Rat	Liver	A, B, C, D, E	Keysell *et al.*, 1973
	Rat	Lung	A, B, C	Grover, 1974
7-Hydroxymethylbenz[*a*]-anthracene 5,6-oxide	Rat	Liver	A, B, C, D, E	Keysell *et al.*, 1973
7,12-Dimethylbenz[*a*]-anthracene 5,6-oxide	Rat	Liver	A, B, C, D, E	Keysell *et al.*, 1973
7-Hydroxymethyl-12-methylbenz[*a*]anthracene 5,6-oxide	Rat	Liver	A, B, C, E	Keysell *et al.*, 1973
Pyrene 4,5-oxide	Rat	Liver	A, B, C, E	Grover *et al.*, 1972
Benzo[*a*]pyrene 4,5-oxide	Rat	Liver	A, B, C, E	Grover *et al.*, 1972
	Rat	Lung	A, B, C	Grover, 1974
3-Methylcholanthrene 11,12 oxide	Rat	Liver	A, B	P. L. Grover, A. Hewer, and P. Sims, unpublished observations

[a] A: Conversion by acid into the "K-region" phenol.
B: Conversion with rat liver microsomal fractions into the "K-region" dihydrodiols.
C: Chemical conversion into the "K-region" glutathione conjugate.
D: Recrystallization to constant specific activity.
E: Reaction with polyguanylic acid.

technique combined with thin-layer chromatography when dibenz[*a,h*]-anthracene was incubated with rat liver microsomal fractions (Selkirk *et al.*, 1971).

E. Positions and Extents of Epoxide Formation on Polycyclic Aromatic Hydrocarbons

In most studies on the metabolism of polycyclic hydrocarbons, only the secondary products of metabolism, the dihydrodiols, glutathione conjugates, and phenols, are detected, but it now seems probable that when dihydrodiols and glutathione conjugates are detected as metabolites, the primary metabolic products formed on the bonds metabolized

TABLE III
SITES OF METABOLISM ON POLYCYCLIC AROMATIC HYDROCARBONS BY TISSUE PREPARATIONS THAT PROBABLY INVOLVE EPOXIDE FORMATION

Hydrocarbon	Formula	Species	Tissue[a]	Bonds at which metabolism gives rise to		Reference
				Dihydrodiols	Phenols[b]	
Naphthalene		Rat[c]	Liver (S)	1,2	1,2 (1)	Boyland and Wiltshire, 1953
		Rat[c]	Liver (M)	1,2	1,2 (1)	Booth and Boyland, 1958
		Rat[c]	Liver (S)			Booth *et al.*, 1960b
		Rat[c]	Liver (M)	1,2	1,2 (1 and 2)	Boyland *et al.*, 1964
		Mouse[c]	Liver (M)	1,2		Holtzman *et al.*, 1967b
		Rat[e]	Liver (M)	1,2	1,2 (1)	Jerina *et al.*, 1970a
		Rat[c]	Liver (M)	1,2		
		Mouse[c]	Liver (M)	1,2		
		Rabbit[c]	Liver (M)	1,2		
		Guinea pig	Liver (M)	1,2		Jerina *et al.*, 1970c
Anthracene		Rat[f]	Liver (M)	1,2		Boyland *et al.*, 1964
Phenanthrene		Rat[c]	Liver (M)	1,2; 3,4; 9,10	1,2; 3,4 (1,2,3 and 4)	Boyland *et al.*, 1964
		Rat[c,d]	Liver (M,H)	1,2; 3,4; 9,10	1,2; 3,4 (1,2,3 and 4)	Sims, 1970b

Chrysene		Rat[c,d]	Liver (M,H)	1,2; 3,4	1,2; 3,4 (1,2,3 and 4)	Sims, 1970b
Pyrene		Rat[c,d]	Liver (M,H)	4,5	1,2 (1)	Sims, 1970b
Benz[a]anthracene		Rat[c]	Liver (M)	1,2; 8,9	1,2 (1); 3,4 (3 and 4)	Boyland *et al.*, 1964
		Rat[c]	Liver (H)	1,2; 5,6; 8,9	3,4 (3 and 4)	Boyland and Sims, 1965c
		Rat[c,d]	Liver (M,H)	5,6; 8,9	3,4 (3 and 4)	Sims, 1970b
		Rat[c]	Liver (H)	8,9		Sims, 1971
		Rat[c]	Liver (M,H)	5,6; 8,9		Grover *et al.*, 1974
		Rat[c]	Lung (M,H)	5,6; 8,9		
		Man	Lung (M)	8,9		P. L. Grover, A. Hewer, and P. Sims, unpublished observations
7-Methylbenz[a]-anthracene		Rat[c]	Liver (H)	5,6; 8,9	3,4 (3 and 4)	Sims, 1967a
		Rat[c,d]	Liver (M,H)	5,6; 8,9	3,4 (3 and 4)	Sims, 1970b,c
		Rat[c,d]	Adrenal (H)	5,6; 8,9	3,4 (3 and 4)	Sims, 1970c
		Rat[c]	Liver (M,H)	3,4; 8,9		
		Rat[c]	Lung (M,H)	3,4; 8,9		Grover *et al.*, 1974

(*Continued*)

TABLE III (*Continued*)

Hydrocarbon	Formula	Species	Tissue[a]	Bonds at which metabolism gives rise to		Reference
				Dihydrodiols	Phenols[b]	
7-Hydroxymethylbenz-[*a*]anthracene		Rat[c]	Liver (H)	8,9	3,4 (3 and 4)	Sims, 1967a
		Rat[c,d]	Liver (H,M)	8,9; 10,11	3,4 (3 and 4)	
		Rat[c,d]	Adrenal (H)	8,9; 10,11	3,4 (3 and 4)	Sims, 1970c
		Rat[d]	Liver (M)	5,6; 8,9; 10,11		Sims, 1972a
12-Methylbenz[*a*]-anthracene		Rat[c]	Liver (H)	5,6; 8,9	3,4 (3 and 4)	Sims, 1967a
12-Hydroxymethyl-benz[*a*]anthracene		Rat[c]	Liver (H)	8,9	3,4 (3 and 4)	Sims, 1967a

7,12-Dimethylbenz[a]-anthracene	Rat[c]	Liver (H)	8,9	3,4 (3 and 4)	Boyland and Sims, 1965a
	Rat[d]	Liver (H)	8,9	3,4 (3 and 4)	Boyland and Sims, 1967a
	Rat[c]	Liver (H)[e]	8,9	3,4 (3 and 4)	
	Rat[c]	Liver (M)	5,6	3,4 (3 and 4)	
	Mouse[c]	Liver (H)[e]	8,9	3,4 (3 and 4)	
	Guinea pig[c]	Liver (H)	8,9	3,4 (3 and 4)	
	Hamster[c]	Liver (H)	8,9	3,4 (3 and 4)	Sims and Grover, 1968
	Rat[c,d]	Liver (H,M)	8,9	3,4 (3 and 4)	Sims, 1970b
	Rat[c,d]	Liver (H,M)	8,9	3,4 (3 and 4)	
	Rat[c,d]	Adrenal (H)	8,9	3,4 (3 and 4)	Sims, 1970c
	Mouse[c,d]	Stomach (H)	8,9	3,4 (3 and 4)	
	Mouse[c,d]	Small intestine (H)	8,9	3,4 (3 and 4)	Gentil and Sims, 1971
	Rat[d]	Liver (H)	8,9	3,4 (3 and 4)	Booth *et al.*, 1973
7-Hydroxymethyl-12-methylbenz[a]-anthracene	Rat[d]	Liver (H)	8,9	3,4 (3 and 4)	Boyland and Sims, 1967a
	Rat[c,d]	Liver (H,M)	8,9; 10,11	3,4 (3 and 4)	
	Rat[c,d]	Adrenal (H)	8,9; 10,11	3,4 (3 and 4)	Sims, 1970b
	Rat[d]	Liver (M)	5,6		Sims, 1973
12-Hydroxymethyl-7-methylbenz[a]-anthracene	Rat[d]	Liver (H)	8,9	3,4 (3 and 4)	Boyland and Sims, 1967a
	Rat[c,d]	Liver (H,M)	8,9	3,4 (3 and 4)	Sims, 1970c
Dibenz[a,h]anthracene	Rat[c,d]	Liver (H)	1,2; 3,4; 5,6	3,4 (3 and 4)	Boyland and Sims, 1965c
	Rat[c,d]	Liver (H,M)	1,2; 3,4; 5,6	3,4 (3 and 4)	Sims, 1970b

(*Continued*)

TABLE III (*Continued*)

Hydrocarbon	Formula	Species	Tissue[a]	Bonds at which metabolism gives rise to: Dihydrodiols	Bonds at which metabolism gives rise to: Phenols[b]	Reference
Dibenz[*a*,*c*]anthracene		Rat[c,d]	Liver (H,M)	10,11		Sims, 1970b, 1972b
3-Methylcholanthrene		Rat[c]	Liver (H)	11,12		Sims, 1966
		Rat[c,d]	Liver (H,M)	11,12		Sims, 1970b
Benzo[*e*]pyrene		Rat[c,d]	Liver (H,M)	4,5	Unidentified	Sims, 1970b

Benzo[*a*]pyrene	Rat[c,d]	Liver (H)		1,2,2,3 (1 and 3)	Conney *et al.*, 1957
	Rat[c,d]	Liver (H)	7,8[f]; 9,10[f]	2,3 (3)	Sims, 1967b
	Rat[c,d]	Liver (H,M)	7,8[f]; 9,10[f]	2,3 (3)	Sims, 1970b
	Rat[d]	Liver (H)	7,8; 9,10	2,3 (3)	Waterfall and Sims, 1972
	Hamster[c]	Liver (M)	4,5; 7,8; 9,10	2,3 (3)	Wang *et al.*, 1972
	Hamster[c]	Liver (M)	4,5; 7,8; 9,10	2,3 (3)	Borgen *et al.*, 1973
	Rat[c]	Liver (M)	4,5; 7,8; 9,10	2,3 (3); 9,10 (9)	Kinoshita *et al.*, 1973
	Rat[c]	Liver (H,M)	4,5; 7,8; 9,10	2,3 (3)	
	Rat[c]	Lung (H,M)	4,5; 7,8; 9,10	2,3 (3)	Grover *et al.*, 1973
Dibenzo[*a,h*]pyrene	Rat[c,d]	Liver (H,M)	Unidentified	Unidentified	
	Mouse[c]	Liver (H,M)	Unidentified	Unidentified	
	Hamster[c]	Liver (H,M)	Unidentified	Unidentified	
	Guinea pig[c]	Liver (H,M)	Unidentified	Unidentified	Waterfall and Sims, 1973
Dibenz[*a,i*]pyrene	Rat[c,d]	Liver (H,M)	Unidentified	Unidentified	
	Mouse[c]	Liver (H,M)	Unidentified	Unidentified	
	Hamster[c]	Liver (H,M)	Unidentified	Unidentified	Waterfall and Sims, 1973
	Guinea pig[c]	Liver (H,M)	Unidentified	Unidentified	

[a] S, slices; M, microsomal fractions; H, homogenates.
[b] Figures in parentheses refer to structures of phenolic metabolites formed.
[c] Normal animals.
[d] Animals pretreated with 3-methylcholanthrene.
[e] Homogenates were prepared from the livers of male and female animals of various ages.
[f] Dihydrodiols were incorrectly identified in original work.

are epoxides. The situation is less clear when only phenols are formed as metabolites on a particular bond, but the evidence provided by the NIH shift in general (Section IV,B) and that of Dewhurst and Stephens (1974) (see Section IV,F) in particular suggests that they too are formed through the intermediate formation of epoxides on bonds adjacent to the hydroxyl groups. Table III shows the positions on the hydrocarbon molecules at which metabolism occurs that is probably mediated through the formation of epoxides as primary metabolic products.

Metabolic reactions do not appear to occur on every double bond in the hydrocarbon molecules, and the levels of the reactions that do occur, differ according to the nature of the bond. The amounts of the various dihydrodiols and phenols formed from a number of aromatic hydrocarbons and their derivatives by rat liver homogenates and microsomal fractions from normal and 3-methylcholanthrene-treated animals have been measured (Sims, 1970b,c) and, with 7,12-dimethylbenz[*a*]anthracene, estimates of the amounts of metabolites formed by homogenates of the livers of male and female rats and mice of various ages and of the livers of hamsters and guinea pigs have been made (Sims and Grover, 1968). Comparisons have also been made between the amounts of these products formed from hydrocarbons by microsomal fractions obtained from rat liver and lung (Grover *et al.*, 1974).

It is clear, however, that measurements of the amounts of dihydrodiols and phenols formed in metabolism do not give accurate estimates of the amounts of epoxides formed initially, since other reactions, such as those of the epoxides with microsomal protein, can also occur (Waterfall and Sims, 1973; G. R. Keysell, J. Booth, and P. Sims, unpublished observations) (see Section V,F). In rat liver homogenates, enzyme-catalyzed reactions of the epoxides with glutathione also take place; with 7,12-dimethylbenz[*a*]anthracene, for example, the "K-region" glutathione conjugate is formed (Booth *et al.*, 1973).

In their metabolism by rat liver microsomal fractions, the carcinogenic hydrocarbons 7,12-dimethylbenz[*a*]anthracene, benzo[*a*]pyrene, 3-methylcholanthrene, dibenz[*a*,*h*]anthracene, dibenz[*a*,*h*]pyrene, and dibenz[*a*,*i*]-pyrene yield proportionally less "K-region" dihydrodiols than do the less biologically active hydrocarbons, such as phenanthrene and benz[*a*]-anthracene, and the "K-region" products are often absent when rat-liver homogenates are used (Sims, 1970b; Waterfall and Sims, 1973). This phenomenon may be related to the fact that "K-region" epoxides, the metabolic precursors of "K-region" dihydrodiols, are better alkylating agents than their non-"K-region" isomers (see Section VI,B) and are therefore more likely to react with protein or glutathione. In experiments with rat-liver homogenates "K-region," but not non-"K-region," glutathione conjugates are formed from hydrocarbons (see Section V,B).

Chrysene, which is at most a weak carcinogen (Steiner and Falk, 1951), did not yield a "K-region" dihydrodiol when the hydrocarbon was incubated with rat liver preparations (Sims, 1970b). The relative proportions of the dihydrodiols formed may also depend on the species used; with hamster liver microsomal fractions, the major metabolite of benzo[*a*]pyrene was the "K-region" dihydrodiol, 4,5-dihydro-4,5-dihydroxybenzo[*a*]pyrene (Wang *et al.*, 1972).

F. Species and Tissues That Metabolize Polycyclic Aromatic Hydrocarbons

Most of the detailed studies on the metabolism of polycyclic hydrocarbons have been carried out with rodent liver preparations from rats, hamsters, guinea pigs, or mice. Preparations from the livers of animals of all the species convert 7,12-dimethylbenz[*a*]anthracene (Sims and Grover, 1968) and dibenz[*a,h*]pyrene and dibenz[*a,i*]pyrene (Waterfall and Sims, 1973) into phenols and dihydrodiols. Metabolites of these types have also been identified as products of the metabolism of benz[*a*]anthracene, 7-methylbenz[*a*]anthracene, and benzo[*a*]pyrene in rat lung (Grover *et al.*, 1974) and of benz[*a*]anthracene in human lung (P. L. Grover, A. Hewer, and P. Sims, unpublished observations). They are also formed when hydrocarbons such as 7,12-dimethylbenz[*a*]anthracene, 7-methylbenz[*a*]anthracene, benzo[*a*]pyrene, and benzo[*e*]pyrene are metabolized by mouse embryo cells (Sims, 1970a) and when benz[*a*]-anthracene is metabolized by hamster embryo cells (Sims *et al.*, 1973). Phenols and dihydrodiols are formed when 7-methylbenz[*a*]anthracene, 7,12-dimethylbenz[*a*]anthracene, and their hydroxymethyl derivatives are metabolized by rat adrenal preparations (Sims, 1970c). 7,12-Dimethylbenz[*a*]anthracene is metabolized in small amount to a dihydrodiol by homogenates of mouse stomach and small intestine (Gentil and Sims, 1971).

There is as yet no simple method for estimating the enzymatic conversion of aromatic hydrocarbons into epoxides or of detecting their formation in tissues that possess low enzyme activities. Most studies of these types have therefore used the conversion of benzo[*a*]pyrene into 3-hydroxybenzo[*a*]pyrene (Eq. 6), the phenol being a metabolite that

OH (6)

can be readily detected and estimated in small quantities because of its intense fluorescence in ultraviolet light. The enzyme that carries out this conversion has been called benzpyrene hydroxylase (Conney *et al.*, 1957),

aryl hydrocarbon hydroxylase (Nebert and Gelboin, 1969), (EC 1.14.1.1; 1964 recommendation) or benzopyrene 3-monooxygenase (EC 1.14.14.2; EN 1972),[3] but there is little doubt that it is closely related to, if it is not the same as, the enzyme that converts aromatic hydrocarbons into epoxides. Udenfriend (1971) has also pointed out that in view of the formation of epoxides as intermediates in enzymatic aromatic hydroxylation the generic term oxygenase is more appropriate than hydroxylase. In the related hydroxylation of pyrene to 1-hydroxypyrene, Dewhurst and Stephens (1974) have provided evidence of an NIH shift, which suggests that pyrene 1,2-oxide is intermediate in the formation of the 1-hydroxy compound. Similarly, an epoxide intermediate may be involved in the formation of 3-hydroxybenzo[*a*]pyrene, so that measurements of the levels of benzopyrene 3-monooxygenase probably give estimates of the abilities of the tissues under examination to form epoxides.

A method for the estimation of the monooxygenase was first described by Conney *et al.* (1957), who measured the loss of benzo[*a*]pyrene during metabolism using a fluorescence measurement technique. This was later modified by Silverman and Talalay (1967), who used ^{3}H-labeled benzo[*a*]pyrene. A method for estimating the levels of this enzyme by measuring fluorimetrically the amount of 3-hydroxybenzo[*a*]pyrene formed from benzo[*a*]pyrene was introduced by Wattenberg *et al.* (1962), and this has been modified by Nebert and Gelboin (1968a). The method has recently been simplified (Dehnen *et al.*, 1973). A radioassay of the enzyme activity involving the estimation of tritiated water liberated from ^{3}H-labeled benzo[*a*]pyrene has also been described (Hayakawa and Udenfriend, 1973). The levels of the enzyme have also been measured in individual hamster embryo cells using a microfluorimetric technique (Kouri *et al.*, 1972). However, since it is now known that phenols, including 3-hydroxybenzo[*a*]pyrene, are further metabolized by microsomal oxygenases, it may be that the estimations of benzopyrene 3-monooxygenase that rely on measurements of the amounts of 3-hydroxybenzo[*a*]pyrene formed are not completely reliable.

A method for the estimation of 7,12-dimethylbenz[*a*]anthracene metabolites using a fluorimetric technique has been described (Conney and Levin, 1966).

Benzopyrene 3-monooxygenase activity has been detected in tissues from animals of many species. Thus, for example, it is present in the livers, kidneys, adrenals, testes, thyroid, and lungs of rats (Wattenberg and

[3] Enzyme Commission (EC) numbers introduced in 1972 Recommendations are followed by EN1972 ("Enzyme Nomenclature," 1972 edition).

Leong, 1962), in the intestinal tract of rats, mice, guinea pigs, hamsters, rabbits, dogs, baboons, and man (Wattenberg *et al.*, 1962) and in the sebaceous glands of mouse skin (Wattenberg and Leong, 1970b). Other studies have shown that it is present in the livers, lungs, kidneys and small intestines of rats, hamsters, monkeys, and a number of strains of mice (Nebert and Gelboin, 1969). The enzyme is also present in hamster, mouse, rat, and chick embryo cells in culture (Nebert and Gelboin, 1969).

G. Effect of Inducers and Inhibitors on the Metabolism of Polycyclic Aromatic Hydrocarbons

1. *Effect of Inducers*

When animals are treated with any one of a large number of organic compounds, the levels of the microsomal oxygenases in the liver and in many other organs, such as lung, kidney, skin, and small intestine, are raised (see Conney, 1967; Gelboin, 1967; Mannering, 1968; Gelboin *et al.*, 1972). The effects of pretreating animals with enzyme "inducers" on the *in vitro* hepatic metabolism of polycyclic aromatic hydrocarbons was first described by Conney *et al.* (1957), who treated weanling rats either with benzo[*a*]pyrene or with some other hydrocarbon and examined the effects of this treatment on the levels of hepatic benzopyrene 3-monooxygenase. The amounts of the increases and their duration depended on the dose of the inducer; for example, 100 μg of benzo[*a*]pyrene caused 5-fold increases in enzyme levels within 12 hours, and these returned to normal after 6 days. Since then a number of other workers have investigated these effects (see, for example, Conney *et al.*, 1959; Conney and Burns, 1960; Mullen *et al.*, 1966; Silverman and Talalay, 1967; Gnosspelius *et al.*, 1969/1970). Compounds other than polycyclic aromatic hydrocarbons will also induce the oxygenase; they include those with such diverse structures as phenobarbitone (Conney *et al.*, 1960; Cram *et al.*, 1965; Silverman and Talalay, 1967), aminopyrine (Conney *et al.*, 1960; Conney and Burns, 1960), and chlordane (Mullen *et al.*, 1966), and the enzyme is induced by flavones (Wattenberg *et al.*, 1968a) and 2-phenylbenzothiazoles (Wattenberg *et al.*, 1968b). The effect of diet on the enzyme level in the lungs and intestines of rats has been investigated (Wattenberg, 1972).

In rats bearing Morris hepatomas, enzyme levels in the hepatomas as well as in the host livers are raised in pretreated animals (Watanabe *et al.*, 1970), although the induced levels in the hepatomas are always lower than those in the host livers. Benz[*a*]anthracene induces the oxygenase in regenerating liver to a greater extent than in normal liver (Spencer and Fischer, 1971/1972), and benzo[*a*]pyrene induces the

enzyme in fetal rat liver (Welch *et al.,* 1972a). A comparative study in rabbits, mice, guinea pigs, and rats treated with 3-methylcholanthrene showed no significant increases in enzyme levels in rabbits, 2-fold increases in mice and guinea pigs, and 4- to 5-fold increases in rats (Alvares *et al.,* 1970). The oxygenase present in the nuclear membranes of rat liver cells differs from that present in the microsomal membranes in that the former enzyme is not induced in animals pretreated with phenobarbitone (Kasper, 1971; Khandwala and Kasper, 1973).

Using a histochemical technique, Wattenberg and Leong (1962) found that the levels of benzopyrene 3-monooxygenase are raised in the livers, kidney, thyroid, testis, and lung, but not in the adrenals of rats pretreated with 3-methylcholanthrene, and that feeding rats with benz[*a*]anthracene increases the monooxygenase levels throughout the gastrointestinal tract. In other studies, Nebert and Gelboin (1969) showed that the monoxygenase levels are raised in the livers, lungs, kidneys, and small intestine of monkeys and normal, pregnant, adrenalectomized, and hypophysectomized rats pretreated with 3-methylcholanthrene and in the fetuses from rats similarly pretreated. The enzyme is stimulated in the kidney cortex of rats pretreated with benzo[*a*]pyrene (Grundin *et al.,* 1973).

Enzyme levels are raised in the livers, lungs, kidneys, and small intestines from mice of the Swiss, C-57GK/HEN, and A/HEN strains that are pretreated with 3-methylcholanthrene, but the levels in the organs from mice of the AKR/N and DBA strains are not. These observations imply genetic differences between the mice with respect to the inducibility of the monooxygenase, and this aspect has been investigated in great detail both *in vivo* and *in vitro* by Nebert and his colleagues (Nebert and Bausserman, 1970c; Gielen *et al.,* 1972; Nebert *et al.,* 1972b, 1973; Goujon *et al.,* 1972; Benedict *et al.,* 1972, 1973a; Nebert and Kon, 1973; see also Bürki *et al.,* 1973a). Differences in inducibility of the enzyme in various tissues by pretreatment with 3-methylcholanthrene between inbred mice of the high-leukemia strain AKR and the low-leukemia strain Af have been noted (Bürki *et al.,* 1973b); the enzyme is inducible in most tissues from mice of the Af strain, but not in tissues from those of the AKH strain. There is a close correlation between the susceptibility of mice to the production of subcutaneous tumors by 3-methylcholanthrene (but not by benzo[*a*]pyrene or 7,12-dimethylbenz[*a*]anthracene) and the genetically controlled induction of hepatic oxygenase (Kouri *et al.,* 1973a,b). The oxygenase is inducible in the lungs, small intestine, kidneys, and skin of strains of mice in which the hepatic oxygenase is noninducible (Wiebel *et al.,* 1973). The significance of the above observations is not yet clear.

The levels of the oxygenase present in mouse skin are raised either by the topical application (Gelboin *et al.*, 1970; Kinoshita and Gelboin, 1972a,b) or by the intraperitoneal injection (Gelboin *et al.*, 1970) of benz[*a*]anthracene. The enzyme is inducible in mouse skin (Wattenberg and Leong, 1970b) and in cells of human foreskin (Levin *et al.*, 1972) by polycyclic hydrocarbons.

The inductive effect of pretreatment has also been demonstrated in whole animals. Pretreatment of rats with either benzo[*a*]pyrene, 3-methylcholanthrene, or 7,12-dimethylbenz[*a*]anthracene stimulated the disappearance from the blood and the decrease in concentration in various tissues of ^{3}H-labeled benzo[*a*]pyrene that was subsequently administered by intravenous injection (Schlede *et al.*, 1970a). Pretreatment with pyrene, anthracene, or phenobarbitone gave no such effects. Pretreatment of rats with benzo[*a*]pyrene or phenobarbitone led to increased secretions of ^{3}H-labeled benzo[*a*]pyrene metabolites in the bile after the animals were treated with the labeled hydrocarbon (Schlede *et al.*, 1970b). It was suggested that the two inducers operated by different mechanisms, benzo[*a*]pyrene by enhancing hydroxylation and phenobarbitone by enhancing conjugation.

A number of workers have studied the effects of cigarette smoking on benzopyrene 3-monooxygenase levels in the human placenta. Welch *et al.* (1968, 1969) showed that the enzyme was present in the placentas from mothers who smoked cigarettes, but was absent from those from nonsmokers. Later it was reported by Nebert *et al.* (1969) that, although the enzyme was sometimes present in placentas from nonsmokers, significantly higher levels were present in the placentas from smokers. Juchau (1971) and Pelkonen *et al.* (1972) have confirmed these findings, and the latter workers also showed that the maternal smoking habits had no effect on the enzyme levels in the fetal liver. Enzyme levels in the lungs and placenta of pregnant rats are increased when the rats are exposed to cigarette smoke (Welch *et al.*, 1972b). The enzyme levels in the fetal liver are also raised, but to a lesser extent. Enzyme levels are raised also in the lungs of mice (Holt and Keast, 1973) and in human pulmonary alveolar macrophages (Cantrell *et al.*, 1973) by cigarette smoke.

Levels of the oxygenase are raised when hamster embryo cells (Alfred and Gelboin, 1967; Nebert and Gelboin, 1968a,b; Alfred *et al.*, 1969), rat fetal hepatocytes (Gielen and Nebert, 1971b), hybrids from human, mouse, and hamster embryo cells (Wiebel *et al.*, 1972), mouse hamster and mouse human cell hybrids (Benedict *et al.*, 1972) and cell lines from rat or mouse hepatomas or from normal rat liver (Benedict *et al.*, 1973b) are preincubated with enzyme inducers including polycyclic aromatic

hydrocarbons. Benzopyrene 3-monooxygenase is induced in human lymphocytes when they are cultured in the presence of 3-methylcholanthrene and the mitogen, phytohemagglutinin (Whitlock *et al.*, 1972; Busbee *et al.*, 1972) and these inductions appear to be under genetic control (Kellerman *et al.*, 1973a,c). The mechanisms involved in the induction of the enzyme have been studied in hamster embryo cells (Nebert and Bausserman, 1970a,b; Nebert, 1970) and in rat fetal hepatocytes (Gielen and Nebert, 1971a, 1972; Nebert and Gielen, 1971). Enzyme levels are raised in both parenchymal and nonparenchymal liver cells from rats treated with 3-methylcholanthrene (Cantrell and Bresnick, 1972).

Benzopyrene 3-monooxygenase is induced in fetal rat liver explants by 3-methylcholanthrene and some related compounds (Bürki *et al.*, 1971) and by flavins and phenobarbitone (Cutroneo and Bresnick, 1973), in isolated perfused rat livers by benzo[*a*]pyrene (Juchau *et al.*, 1965) and in chick embryo microsomal fractions by aromatic hydrocarbons (Jellinck and Smith, 1973). The formation of water-soluble metabolites from 7,12-dimethylbenz[*a*]anthracene in hepatic microsomal fractions from rats and chick embryos is also increased by pretreatment with hydrocarbons (Jellinck and Smith, 1973).

Comparisons of the relative amounts of phenols and dihydrodiols formed in the metabolism of members of a series of aromatic hydrocarbons and their derivatives by homogenates and microsomal fractions from the livers of normal rats and of rats that were pretreated with 3-methylcholanthrene showed that, with most hydrocarbons, the relative amounts of the metabolites formed from each hydrocarbon are unaffected by treatment, even though the actual amounts of each metabolite is increased (Sims, 1970b,c). Recent studies on the metabolism of benzo[*a*]pyrene by hepatic microsomal fractions from normal and 3-methylcholanthrene-treated rats have, however, shown that the amounts of the non-"K-region" metabolites, the 7,8- and the 9,10-dihydrodiols, are increased to greater extents than are the phenols and the "K-region" dihydrodiol when livers from pretreated animals are used (Kinoshita *et al.*, 1973). With the methylated hydrocarbons, 7-methylbenz[*a*]anthracene and 7,12-dimethylbenz[*a*]anthracene, and their 7-hydroxymethyl derivative there are also disproportionately large increases in the amounts of the non-"K-region" dihydrodiols formed when hepatic fractions from pretreated animals are used (Sims, 1970b,c). Increases in the amounts of the 8,9-dihydrodiol formed from 7,12-dimethylbenz[*a*]anthracene are found in incubations of homogenates of the livers of mice maintained on a diet containing either 7,12-dimethylbenz[*a*]anthracene or its 7-

hydroxymethyl derivative (Chouroulinkov *et al.,* 1973). Larger amounts of 8,9-dihydro-8,9-dihydroxy-7,12-dimethylbenz[*a*]anthracene (XXXVII) are formed in the incubation of 7,12-dimethylbenz[*a*]anthracene with

(XXXVII)

homogenates from the livers of 25- to 35-day-old rats than in incubations with homogenates of the livers of newborn or adult rats; this increase has been attributed to the presence of enzyme-inducing compounds in the diet during the weaning period (Sims and Grover, 1967). The levels of benzopyrene 3-monooxygenase in rat liver reach a maximum value in 20-day-old animals of both sexes (Dehnen *et al.,* 1970b). These increases in the amounts of 8,9-dihydrodiols formed from 7,12-dimethylbenz[*a*]-anthracene and its hydroxymethyl derivative (Boyland and Sims, 1967a) are also brought about by pretreatment with other compounds such as Sudan III (Huggins and Fukunishi, 1964) that also protect the adrenals of rats against the necrosis induced either by 7,12-dimethylbenz[*a*]-anthracene (Huggins and Morii, 1961) or by 7-hydroxymethyl-12-methyl-benz[*a*]anthracene (Boyland *et al.,* 1965a). The relationship between the

(XXXVIII)

action of such compounds as enzyme inducers and their mode of action in protecting against adrenal necrosis has been discussed (Wheatley and Sims, 1969). 7,12-Dimethylbenz[*a*]anthracene 5,6-oxide (XXXVIII) does not cause adrenal necrosis in rats (Keysell *et al.,* 1973), but the possibility that epoxides are involved in the production of adrenal necrosis cannot be excluded since both 7,12-dimethylbenz[*a*]anthracene and its hydroxy-methyl derivative are metabolized to dihydrodiols by rat adrenal homogenates. The amounts of these products formed by rat-adrenal homogenates are not increased when organs from animals pretreated with 3-methylcholanthrene are used (Sims, 1970c). Both 7,12-dimethylbenz-[*a*]anthracene and its 7-hydroxymethyl derivative are teratogenic in rats

(Currie *et al.,* 1970), and compounds that protect against adrenal necrosis also protect against teratogenicity (Bird *et al.,* 1970), presumably by increasing maternal hepatic enzyme levels.

Since the enzyme-inductive effect of polycyclic hydrocarbons on cells is quite rapid, it seems likely that in tests for carcinogenicity the polycyclic hydrocarbons will induce their own activating and inactivating enzymes. What part this plays in the induction of cancer by these compounds is not yet established, but some possible implications have been discussed (Franke, 1973). It is known, however, that preincubation of hamster embryo cells in culture with benz[*a*]anthracene, which is a good enzyme-inducing agent but a poor transforming agent, will prevent 7,12-dimethylbenz[*a*]anthracene-induced toxicity when the cells are subsequently incubated with the methylated hydrocarbon (Alfred *et al.,* 1969).

2. *Effect of Inhibitors*

The compound most widely used as an inhibitor of benzopyrene 3-monooxygenase *in vitro* is α-naphthoflavone (7,8-benzoflavone). It is effective either when applied directly to mouse skin (Kinoshita and Gelboin, 1972a,b), or when added to cells in culture (Diamond *et al.,* 1972) or to hepatic microsomal fractions (Wiebel *et al.,* 1971). The flavone inhibits the formation of water-soluble metabolites from 3-methylcholanthrene by G23 cells (Marquardt and Heidelberger, 1972a) and from 7,12-dimethylbenz[*a*]anthracene by M2 cells (Marquardt *et al.,* 1974). It also inhibits the formation of water-soluble metabolites from both benzo[*a*]pyrene and 7,12-dimethylbenz[*a*]anthracene by hamster embryo cells (Diamond *et al.,* 1972). The formation of dihydrodiols from compounds such as 7-hydroxy-12-methylbenz[*a*]anthracene by rat liver microsomal fractions is inhibited by the flavone (J. Booth and P. Sims, unpublished observations); it presumably acts by inhibiting epoxide formation.

The formation of dihydrodiols from either 7,12-dimethylbenz[*a*]-anthracene or its hydroxymethyl derivatives by rat liver preparations is inhibited by estradiol (Booth *et al.,* 1974). The estrogen also appears to act by inhibiting the formation of epoxides since the amounts of phenols and glutathione conjugates formed are also much reduced. At high concentration, estradiol inhibits the induction of benzopyrene 3-monooxygenase in mouse embryo cells, and it is a competitive inhibitor of the enzyme in mouse liver, lung, kidney, adrenal, and skin preparations (Nebert *et al.,* 1970). A comparison of the oxygenase with estrogen hydroxylase in rat liver microsomal fractions has been made (Spencer, 1972), and a similar inhibitory effect of estradiol on the oxygenase noted. However, the

topical application of corticosteroids to the skin of mice resulted in an induction of the monooxygenase (Briggs and Briggs, 1973).

V. Metabolic Reactions of Polycyclic Aromatic Hydrocarbon Epoxides

A. Metabolic Conversion into Dihydrodiols

Hydrocarbon epoxides are metabolized by the epoxide hydrase (or hydratases) (see Fig. 5) present in the cells of many tissues in the body. Two such enzymes have been classified "epoxide hydratase" (EC 4.2.1.63; EN 1972) and "arene-oxide hydratase" (EC 4.2.1.64; EN 1972). The properties of these enzymes have recently been reviewed (Oesch, 1973). The enzymes appear to be located exclusively on the endoplasmic reticulum of cells (Oesch *et al.*, 1971a) and are therefore found in the microsomal fractions when cellular components are separated by centrifugation. Unlike the microsomal oxygenase, the epoxide hydrases do not require NADPH or, as far as is known, any other cofactor (Oesch and Daly, 1971; Watabe and Kanehira, 1970).

Most of the detailed studies that have been carried out on the properties of the enzymes have used as substrate an epoxide, styrene oxide, derived from an olefinic hydrocarbon and a method for estimating the enzyme that hydrates ^{3}H-labeled styrene oxide to ^{3}H-labeled

$$C_6H_5-CH\overset{O}{—}CH_2 \longrightarrow C_6H_5-\underset{OH}{CH}-\underset{OH}{CH_2} \qquad (7)$$

styrene glycol (Eq. 7) has been described (Oesch *et al.*, 1971a). A more sensitive assay has recently been developed for measuring epoxide hydrase levels in human liver biopsy specimens (Oesch *et al.*, 1974). The epoxide hydrase in guinea pig liver microsomal fractions that hydrates styrene oxide appears to differ from that which hydrates benzene oxide (Oesch *et al.*, 1971b). Although this suggests that the epoxide hydrase involved in the hydration of styrene oxide may not be the same as that involved in the hydration of aromatic hydrocarbon epoxides, the ratio of the specific activation of purified enzyme preparations toward styrene oxide, naphthalene 1,2-oxide, phenanthrene 9,10-oxide, and some other epoxides was virtually unaltered by the purification procedure (Oesch *et al.*, 1971b) suggesting either the presence of a simple epoxide hydrase with broad substrate specificity or the presence of a group of epoxide hydrases that were purified to the same extent. The solubilization and purification of the epoxide hydrases from guinea pig liver led to a 40-fold increase in specific activity (Oesch and Daly, 1971). An alternative solubilization

and purification of the epoxide hydrases present in microsomal fractions from rabbit liver that converts 1-,2-epoxy-*n*-octane into the related glycol has also been described (Watabe and Kanehira, 1970).

There is, at least in guinea pig liver homogenates, evidence for a coupled benzopyrene 3-monooxygenase–epoxide hydrase complex that is inducible by 3-methylcholanthrene (Oesch and Daly, 1972), a system that may prevent the release of epoxides into the cytosol and thus inhibit the production of toxic effects that might be produced by epoxides. Although soluble cytochrome P-450 and P-488 preparations both contain high levels of oxidase and hydrase, neither of the cytochromes was detected in purified epoxide hydrase preparations, thus indicating that the oxygenase and the hydrase are not common to one protein (Oesch *et al.*, 1972).

The epoxide hydrase that hydrates styrene oxide has been measured in hepatic microsomal fractions from a number of species, and were in the order rhesus monkey > man and guinea pig > rabbit > rat > mice, when expressed in terms of activity per milligram of protein (Oesch, 1973). In rats, the enzyme activity is high in liver and low in kidney, and no activity was detected in muscle, spleen, heart, intestine, lung, and brain (Oesch *et al.*, 1971a). There were low levels of epoxide hydrase activity in guinea pig lung and intestine and no activity in the brain, heart, spleen, and muscle (Oesch *et al.*, 1973a). Levels of the hydrase in adult mouse skin were low, and the enzyme could not be detected in fetal mouse skin or liver (Oesch *et al.*, 1973a). The enzyme is present in human liver (Oesch *et al.*, 1974), in cultured human leukocytes (Kellermann *et al.*, 1973b), and in microsomal fractions from house flies (Brooks *et al.*, 1970).

Studies on the mechanism of action of the epoxide hydrase (arene oxide hydratase) that converts hydrocarbon epoxides into dihydrodiols have been confined mainly to investigations on the configuration of the dihydrodiol thus formed. The experiments have usually been carried out with rodent hepatic homogenates or microsomal systems, and whenever it has been possible to identify the geometric forms of the dihydrodiol, the metabolite appears to have the *trans* configuration. The polycyclic hydrocarbon epoxides examined in these experiments are listed in Table IV.

More detailed experiments with naphthalene 1,2-oxide (Jerina *et al.*, 1970c) have shown that the racemic oxide is converted by the hydrase present in rabbit, rat, guinea pig, and mouse hepatic microsomal fractions into (−)-*trans*-1,2-dihydro-1,2-dihydroxynaphthalene with degrees of optical purity of about 28–35%. In these experiments it was found that the (−) form of the *trans*-dihydrodiol, with about the same degree of

optical purity, was also formed when naphthalene was incubated with the complete hepatic microsomal systems obtained from these animals. The samples of the dihydrodiols obtained from both substrates have a predominantly 1*R*,2*R* absolute configuration; the significance of these observations in relation to the enzymes involved has been discussed (Jerina *et al.*, 1970c), and it was concluded that the enzymatic formation of hydrocarbon epoxides is not a particularly stereoselective process. Phenanthrene 9,10-oxide was metabolized by rabbit liver epoxide hydrase mainly to the (–)-*trans*-dihydrodiol with the 9S,10S absolute configuration. When naphthalene 1,2-oxide was incubated with rabbit hepatic microsomal fractions in the presence of $H_2{}^{18}O$, there occurred incorporation of ^{18}O into the 2-position of the dihydrodiol thus formed (Jerina *et al.*, 1970a).

The enzyme that converts phenanthrene 9,10-oxide and dibenz[*a,h*]-anthracene 5,6-oxide into their dihydrodiols is located in the microsomal fraction of rat liver (Pandov and Sims, 1970). A comparison of the rates of hydration of these two epoxides showed that phenanthrene 9,10-oxide, which is derived from a noncarcinogen, is a better substrate for the enzyme than dibenz[*a,h*]anthracene 5,6-oxide, which is derived from a carcinogen. The significance of this is discussed in Section V,D, where the relative stabilities of a series of "K-region" epoxides is described.

Using a method of assay involving the conversion of ^{3}H-labeled benz-[*a*]anthracene 5,6-oxide into the ^{3}H-labeled 5,6-dihydrodiol, it was found that the levels of the epoxide hydrase in microsomal fractions from rat lung (Grover, 1974) and from human lung (P. L. Grover, A. Hewer, and P. Sims, unpublished observations) are low as compared with the levels of the enzyme in rat liver microsomal fractions. However, rat lung microsomal preparations in the presence of cofactors are still able to convert hydrocarbons into dihydrodiols (Grover *et al.*, 1974).

A large number of compounds have been described that will inhibit the hydration of ^{3}H-labeled styrene oxide (Oesch *et al.*, 1971c) *in vitro*. The inhibitors are of three classes, competitive, noncompetitive, and uncompetitive, depending on the nature and stereochemistry of the substituents on the oxirane ring. This aspect has been discussed in detail (Oesch, 1973). Two inhibitors, cyclohexene oxide and 3,4-dihydronaphthalene 1,2-oxide, which are noncompetitive inhibitors of ^{3}H-labeled styrene oxide hydration (Oesch *et al.*, 1971c) have been used in the epoxide-trapping experiments described in Section IV,D). In the detection of an epoxide as a hepatic microsomal metabolite of dibenz[*a,h*]-anthracene, Selkirk *et al.* (1971) used microsomal fractions that had been heated to 50°C, a treatment that enabled Yamamoto and Bloch

TABLE IV
CHEMICAL AND BIOCHEMICAL REACTIONS OF HYDROCARBON EPOXIDES

Epoxide	Product of reaction of epoxide with			Reference
	Water	Glutathione	Acid	
Naphthalene 1,2-oxide	*trans*-1,2-Dihydro-1,2-dihydroxynaphthalene[b]	*S*-(1,2-Dihydro-2-hydroxy-1-naphthyl)-glutathione[d]	1- and 2-Naphthol	Jerina *et al.*, 1970a
Phenanthrene 9,10-oxide	*trans*-9,10-Dihydro-9,10-dihydroxyphenanthrene[a,b]	*S*-(9,10-Dihydro-9-hydroxy-10-phenanthryl)-glutathione[c,d]	9-Phenanthrol	Boyland and Sims, 1965b
Benz[*a*]anthracene 5,6-oxide	*trans*-5,6-Dihydro-5,6-dihydroxybenz[*a*]anthracene[a,b]	*S*-(5,6-Dihydro-6-hydroxybenz[*a*]anthracen-5-yl)glutathione[c,d]	5-Hydroxybenz[*a*]anthracene	Boyland and Sims, 1965c Newman and Blum, 1964
Benz[*a*]anthracene 8,9-oxide	8,9-Dihydro-8,9-dihydroxybenz[*a*]anthracene[a,b]	*S*-(8,9-Dihydro-9-hydroxybenz[*a*]anthracen-5-yl)glutathione[c,d]	8- and 9-Hydroxybenz[*a*]anthracene	Sims, 1971
7-Methylbenz[*a*]anthracene 5,6-oxide	*trans*-5,6-Dihydro-5,6-dihydroxy-7-methylbenz[*a*]anthracene[a,b]	*S*-(5,6-Dihydro-6-hydroxy-7-methylbenz[*a*]anthracen-5-yl)-glutathione[c,d]	5(or 6)-Hydroxy-7-methylbenz[*a*]anthracene	Sims, 1967a
7-Hydroxymethylbenz[*a*]anthracene 5,6-oxide	*trans*-5,6-Dihydro-5,6-dihydroxy-7-hydroxymethylbenz[*a*]anthracene[a,b]	*S*-(5,6-Dihydro-6-hydroxy-7-hydroxymethylbenz[*a*]anthracen-5-yl)glutathione[c,d]	5(or 6)-Hydroxy-7-methylbenz[*a*]anthracene	Sims, 1972a
7,12-Dimethylbenz[*a*]anthracene 5,6-oxide	*trans*-5,6-Dihydro-5,6-dihydroxy-7,12-dimethylbenz[*a*]anthracene[a,b]	*S*-(5,6-Dihydro-7,12-dimethyl-6-hydroxybenz[*a*]anthracen-5-yl)glutathione[c,d]	5(or 6)-Hydroxy-7,12-dimethylbenz[*a*]anthracene	Sims, 1973; Goh and Harvey, 1973

7-Hydroxymethyl-12-methylbenz[a]anthracene 5,6-oxide	*trans*-5,6-Dihydro-5,6-dihydroxy-7-hydroxymethyl-12-methylbenz[a]anthracene[a,b]	*S*-(5,6-Dihydro-6-hydroxy-7-hydroxymethylbenz[a]anthracen-5-yl)glutathione[c,d]	5(or 6)-Hydroxy-7-hydroxymethyl-12-methylbenz[a]anthracene	Sims, 1973
3-Methylcholanthrene 11,12-oxide	*trans*-11,12-Dihydro-11,12-dihydroxy-3-methylcholanthrene[a,b]	*S*-(11,12-Dihydro-12-hydroxy-3-methylcholanthrene-11-yl)-glutathione[c,d]	11(or 12)-Hydroxy-3-methylcholanthrene	Sims, 1966
Dibenz[a,h]anthracene 5,6-oxide	*trans*-5,6-Dihydro-5,6-dihydroxydibenz[a,h]-anthracene[a,b]	*S*-(5,6-Dihydro-6-hydroxydibenz[a,h]-anthracen-5-yl)glutathione[c,d]	5(or 6)-Hydroxydibenz-[a,h]anthracene	Boyland and Sims, 1965c
Dibenz[a,c]anthracene 10,11-oxide	10,11-Dihydro-10,11-dihydroxydibenz[a,c]-anthracene[a,b]	—	10 and 11-Hydroxydibenz[a,c]anthracene	Sims, 1972b
Benzo[a]pyrene 4,5-oxide	*trans*-4,5-Dihydro-4,5-dihydroxybenzo[a]-pyrene[b]	*S*-(4,5-Dihydro-5-hydroxybenzo[a]pyrene-4-yl)glutathione[c]	4(or 5)-Hydroxybenzo-[a]pyrene	Grover *et al.*, 1972
Benzo[a]pyrene 7,8-oxide	7,8-Dihydro-7,8-dihydroxybenzo[a]pyrene[b]	—	7-Hydroxybenzo[a]-pyrene	Waterfall and Sims, 1972
Benzo[a]pyrene 9,10-oxide	9,10-Dihydro-9,10-dihydroxybenzo[a]pyrene[b]	*S*-(9,10-Dihydro-10-hydroxybenzo[a]pyren-9-yl)glutathione[c]	9-Hydroxybenzo[a]-pyrene	Waterfall and Sims, 1972

[a] Formed chemically from epoxide and water.
[b] Formed enzymatically from epoxide by epoxide hydrase.
[c] Formed chemically from epoxide and glutathione.
[d] Formed enzymatically from epoxide by glutathione *S*-epoxide transferase.

(1970) to isolate squalene 2,3-oxide as a metabolite of squalene. The epoxide hydrase present in insects is inhibited by juvenile hormone analogs, microsomal oxygenase inhibitors, nonhormone epoxides, and organophosphorus compounds (Brooks, 1973).

The levels of epoxide hydrase in the livers of mice are not raised when the animals are treated with 3-methylcholanthrene (Nebert *et al.*, 1972a). However, the levels of guinea pig hepatic epoxide hydrase are raised in animals that are pretreated with phenobarbitone and, to a lesser extent, with those pretreated with 3-methylcholanthrene (Oesch *et al.*, 1973a). With both inducers, however, the increases in levels are small as compared with the corresponding increases in the levels of benzpyrene 3-monooxygenase. Metapyrone and 1-(2-isopropylphenyl)imidazole, which are *in vitro* inhibitors of the oxygenase, activate epoxide hydrase *in vitro*, but not *in vivo* (Oesch *et al.*, 1973a). In studies on a series of inbred strains of mice, it was found that hepatic epoxide hydrase is induced by phenobarbitone in the C57BL/6N,C3H/HeN,NZB/BLN and NZw/BLN, but not in the DBA/2N,N:GP(SW) and AL/N strains. The oxygenase is induced in all seven strains (Oesch *et al.*, 1973b).

Since the levels of epoxide hydrase are low in tissues, such as lung and skin, that are susceptible to hydrocarbon carcinogenesis, it seems unlikely that the induction of the enzyme in these tissues by hydrocarbons will play much of a part in the mechanisms by which the hydrocarbons induce cancer. Similarly, it would not be expected that the application to mouse skin of an epoxide hydrase inhibitor at the same time as a polycyclic hydrocarbon would alter the yield of tumors as compared with the yield obtained by the application of the hydrocarbon alone.

No attempts have been made to demonstrate the presence of epoxide hydrase in rodent embryo cells in culture. However, since benz[*a*]anthracene 5,6-oxide and dibenz[*a,h*]anthracene 5,6-oxide are both converted into the related *trans*-dihydrodiols by hamster embryo cells (Sims *et al.*, 1973), the presence of epoxide hydrases in these cells seems likely.

B. Metabolic Conversion into Glutathione Conjugates

The presence of a series of enzymes in the soluble fraction of rat liver preparations that catalyze the reactions of glutathione with organic substrates that possess reactive groups has been recognized for many years (for a general review, see Boyland and Chasseaud, 1969). With aromatic hydrocarbons such as naphthalene, however, the presence of the microsomal fraction together with appropriate cofactors, as well as soluble fraction, is necessary before conjugation with glutathione can occur (Booth *et al.*, 1961). This observation lent support to the theory that

hydrocarbons were metabolized via epoxides, and, as outlined in Section IV,A, the glutathione conjugates formed from hydrocarbons are similar in structure to those formed from the related epoxides.

In the metabolism by rat liver homogenates of benz[*a*]anthracene (J. Booth and P. Sims, unpublished observations) and 7,12-dimethylbenz-[*a*]anthracene (Booth *et al.*, 1973) and its related hydroxymethyl derivatives (J. Booth, G. R. Keysell, and P. Sims, unpublished observations), "K-region" glutathione conjugates were detected, but there was no evidence for the formation of non-"K-region" conjugates. This is in contrast with the types of dihydrodiols formed, for, although benz[*a*]-anthracene yielded both "K-region" and non-"K-region" products, larger amounts of the non-"K-region" products were formed, and 7,12-dimethyl-benz[*a*]anthracene and its hydroxymethyl derivative yielded only non-"K-region" products (Sims, 1970b). When a mixture of the non-"K-region" epoxide, benz[*a*]anthracene 8,9-oxide, and the "K-region" epoxide, benz-[*a*]anthracene 5,6-oxide, was incubated with rat liver microsomal and soluble fractions in the presence of glutathione, the 8,9-oxide was converted mainly into the related 8,9-dihydrodiol, whereas the 5,6-oxide yielded mainly the glutathione conjugate (J. Booth and P. Sims, unpublished observations). These observations are in agreement with the results obtained in the metabolism of the hydrocarbon (Fig. 9).

The non-"K-region" dihydrodiols of 7,12-dimethylbenz[*a*]anthracene and its hydroxymethyl derivative are themselves further metabolized by rat liver homogenates to products that are probably glutathione conjugates and these are more polar than the simpler conjugates discussed

"OXYGENASE"
O
GSH MINOR PATHWAY
O
GSH MAJOR PATHWAY
"GLUTATHIONE TRANSFERASE"
OH SG
SG
OH

FIG. 9. Formation of glutathione conjugates from benz[*a*]anthracene.

above (Booth *et al.*, 1973). The structures of these metabolites have not yet been determined, although they could well arise via the intermediate formation of epoxides produced by metabolism on other double bonds in the molecules.

The metabolism of a number of aromatic hydrocarbon epoxides has been investigated using either whole liver homogenates or liver soluble fractions and glutathione. These investigations are summarized in Table IV. The structures and properties of the glutathione conjugate thus formed have been discussed in Section II,B.

The enzyme catalyzing the addition of glutathione to alkyl epoxides has been called glutathione S-epoxide transferase (Boyland and Williams, 1965), and presumably the same or a similar enzyme is involved in the formation of conjugates from epoxides formed on aromatic double bonds (Boyland *et al.*, 1965b). The distribution of the epoxide transferase has not been studied in detail, although it is known to occur both in rat liver and kidney (Boyland and Williams, 1965). An enzyme present in rat liver that catalyzes the additions of glutathione to alkyl epoxides has been purified (Pabst *et al.*, 1973). Recent experiments, where the enzyme was assayed by the conversion of ^{3}H-labeled-benz[*a*]anthracene 5,6-oxide into the ^{3}H-labeled conjugate, S-(5,6-dihydro-6-hydroxybenzanthracen-5-yl)glutathione, showed that the levels of glutathione S-epoxide transferase were higher in rat lung (Grover, 1974) and human lung (P. L. Grover, A. Hewer, and P. Sims, unpublished observations) than in rat liver. Rat lung is susceptible to the induction of cancer by polycyclic hydrocarbons, and glutathione S-epoxide transferase may play a more important part than the epoxide hydrases in the detoxification of epoxides formed from hydrocarbons in these tissues.

The levels of the enzyme, glutathione S-aryltransferase, which is closely related to the epoxide transferase, have been measured in the livers of animals of many species (Grover and Sims, 1964). All the livers examined contained measurable levels of the enzyme, but wide variations were observed among the tissues from animals of the various species examined.

There is no evidence yet that cells in culture contain glutathione S-epoxide transferase. Although mouse and hamster cells metabolize both hydrocarbons (Diamond *et al.*, 1968; Sims, 1970a; Diamond, 1971; Huberman *et al.*, 1971b; Sims *et al.*, 1973) and hydrocarbon epoxides (Sims *et al.*, 1973) into water-soluble metabolites, these have not been identified either as glutathione conjugates or as products, such as cysteinylglycine or cysteine derivatives (see Fig. 2), that might arise from the further metabolism of the glutathione conjugates.

Sulfhydryl-dependent enzymes, such as glyceraldehyde 3-phosphate

dehydrogenase, are inactivated by phenanthrene 9,10-oxide. This appears to be because the epoxide reacts with the active site region of the enzyme (Hutcheson and Wood, 1973).

C. Isomerization to Phenols

In hepatic homogenates and microsomal systems, hydrocarbon epoxides are converted into phenols, but there is no evidence that these reactions are enzyme-catalyzed. An earlier report suggested the isomerization of benzene oxide to phenol was catalyzed by proteins or small peptides (Jerina *et al.*, 1968b), but it now seems unlikely that this reaction occurs with naphthalene 1,2-oxide (Boyd *et al.*, 1970) or with polycyclic hydrocarbon epoxides (Swaisland *et al.*, 1973). The detection of phenols in experiments on the metabolism of hydrocarbons depends on (a) a spontaneous isomerization of the epoxide when incubated at the pH of the reaction mixture and (b) an isomerization of epoxide remaining at the end of the incubation periods during the working-up procedures. Although large amounts of phenols were seen in earlier experiments with "K-region" epoxides (Boyland and Sims, 1965c; Sims, 1966, 1967a), only small amounts were detected when more refined chromatographic procedures were used; this finding suggests that these phenols arose from unmetabolized epoxides during chromatography. With non-"K-region" epoxides, isomerization into phenols occurs more readily during chromatography, so that relatively larger amounts of phenols are usually seen in experiments with these epoxides.

D. Stabilities in Tissue Preparations

Although there are wide variations in carcinogenic activities among members of the group of polycyclic aromatic hydrocarbons, all the hydrocarbons that have been examined are metabolized by similar routes that clearly involve the formation of epoxides. If these epoxides play any role in the induction of cancer, then their action may depend on their ability to reach a specific site in the cell. Thus it is important to know which, if any, of the epoxides are sufficiently stable to reach this site. The stability *in vivo* of a given epoxide may depend on one or more of a number of factors including (a) its rate of formation, (b) its rate of enzymatic conversion into the related dihydrodiol, (c) its rate of enzymatic conversion into the related glutathione conjugate, (d) its rate of spontaneous isomerization into the related phenol, and (e) the rate at which it will alkylate cellular macromolecules, particularly protein.

An experiment in which a series of "K-region" epoxides was incubated with rat liver microsomal fractions has been described (Swaisland *et al.*, 1973). Such a system enables factors (b), (d), and (e) to be evaluated

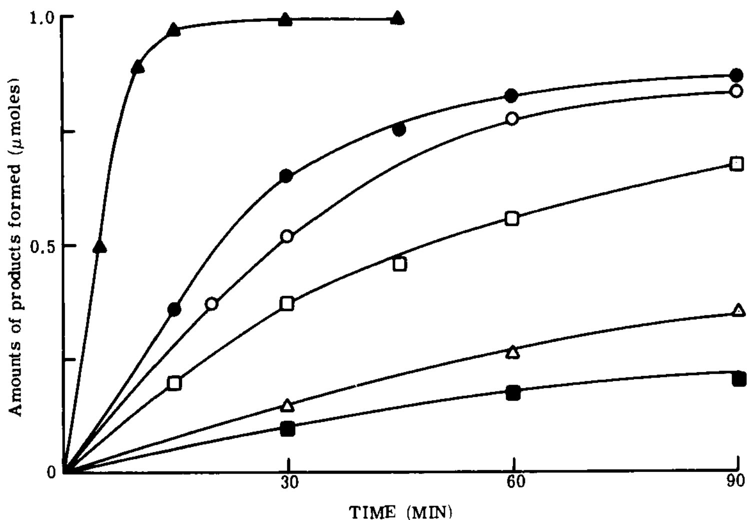


FIG. 10. Rates of formation of phenols and dihydrodiols from epoxides by rat liver microsomal suspensions. Epoxides (1 μmole) in acetone (0.5 ml) were incubated with microsomal fractions (prepared from 2 gm of liver) suspended in pH 7.4 phosphate buffer (10 ml). The mixtures were extracted with ethyl acetate (7 ml), and the amounts of phenols present were estimated fluorimetrically. The samples were acidified with concentrated HCl (0.1 ml), and the amounts of phenols present were again measured. The concentrations of phenols and dihydrodiols in the solutions were calculated. The increases in the concentrations of these products derived from phenanthrene 9,10-oxide (▲—▲), benz[*a*]anthracene 5,6-oxide (●—●), 7,12-dimethylbenz[*a*]anthracene 5,6-oxide (○—○), 7-methylbenz[*a*]anthracene 5,6-oxide (□—□), 3-methylcholanthrene 11,12-oxide (△—△), and dibenz[*a,h*]anthracene 5,6-oxide (■—■) with the times of incubation are shown. From Swaisland *et al.* (1973) with permission of Pergamon Press.

in one experiment. The results shown in Fig. 10 indicate that although there are wide variations in the stabilities of the epoxides examined, there appeared to be no simple relationship between the stability of the epoxide and the carcinogenicity of the hydrocarbon from which the epoxide was derived. Epoxides derived from methylated hydrocarbons appeared to be less stable than those derived from nonmethylated hydrocarbons.

E. METABOLISM BY CELLS IN CULTURE

The metabolism of ^{3}H-labeled benz[*a*]anthracene 5,6-oxide and dibenz[*a,h*]anthracene 5,6-oxide has been studied in hamster embryo cells (Sims *et al.*, 1973). Both epoxides are converted into products with the properties of the related *trans*-dihydrodiols and phenols which are secreted into the media in which the cells are grown. Water-soluble products were also present in the media but were not identified.

F. Further Metabolism of Epoxides and Their Derivatives by Hepatic Preparations

As indicated in Section V,A, the microsomal epoxide hydrase converts hydrocarbon epoxides to dihydrodiols in the absence of NADPH. In the presence of this cofactor, however, other metabolic processes also occur, and with microsomal fractions from the livers of 3-methylcholanthrene-treated rats, benz[*a*]anthracene 5,6-oxide and 7-methylbenz[*a*]anthracene 5,6-oxide are both converted in part into their respective parent hydrocarbons as well as into their related dihydrodiols (A. Hewer and P. Sims, unpublished observations). The mechanism by which these aromatization reactions occur is not known, but it could involve either the direct abstractions of oxygen from the molecules or the reduction of the epoxides to dihydromonols of the type described in Section II,B, that then decompose spontaneously.

"K-region" epoxides react with hepatic microsomal protein in the absence of cofactors (G. R. Keysell, J. Booth, and P. Sims, unpublished observations), but the levels of binding are increased 10-fold when the incubations are carried out in the presence of cofactors. Binding to microsomal protein also occurs when phenols or dihydrodiols (both "K-region" and non-"K-region") derived from 7-methyl or 7,12-dimethylbenz[*a*]-anthracene are incubated with hepatic microsomal fractions.

If the incubations with dihydrodiols and phenols are carried out in the presence of DNA, binding to this macromolecule also occurs. A similar effect has been reported in the benzo[*a*]pyrene series (Borgen *et al.*, 1973), where 7,8-dihydro-7,8-dihydroxybenzo[*a*]pyrene will undergo an enzyme-induced binding to DNA more readily than either the 9,10-dihydrodiol, the 3-hydroxy compound or benzo[*a*]pyrene itself. The reactive species involved in these reactions may well be epoxides and they are possibly related to the species that give rise to the polar glutathione conjugates that are formed during the further metabolism of metabolites of 7,12-dimethylbenz[*a*]anthracene (Booth *et al.*, 1973) (see Section V,B).

VI. Chemical Reactions of Polycyclic Aromatic Hydrocarbon Epoxides

There is the possibility that epoxides derived from polycyclic aromatic hydrocarbons can exist in valence-tautomeric equilibrium with the related oxepin. Although benzene oxide exists in equilibrium with oxepin (Vogel and Günther, 1967), naphthalene 1,2-oxide exists only in the oxide form (Vogel and Klärner, 1968; Boyd *et al.*, 1970). This is presumably because formation of the corresponding oxepin would involve loss of aromaticity

in the benzene ring. On the other hand, naphthalene 2,3-oxide does not appear to exist (Jeffrey and Jerina, 1972), and the corresponding oxepin, 3-benzoxepin is a stable compound. It may be significant that in the metabolism of polycyclic hydrocarbons, metabolites formed on bonds equivalent to the 2,3-bond of naphthalene have never been recognized. If metabolism does occur on these bonds, then the first-formed epoxides would presumably immediately rearrange to the related oxepins. The possible formation of compounds of this type in hydrocarbon metabolism has never been investigated. The synthetic "K-region" and non-"K-region" epoxides described in this review probably do not exist in the oxepin form since to do so would involve loss of aromaticity in one or more of the benzene rings. Their oxide structure is supported by the chemical and biochemical properties of the compounds and by their ultraviolet spectra, which are similar to those of the related dihydrodiols except that in the epoxides the peaks are shifted to slightly longer wavelengths (e.g., Waterfall and Sims, 1972).

A. Aromatization to Phenols

All "K-region" and non-"K-region" epoxides decompose rapidly on treatment with acid. The reactions are energetically favorable because of the gain in resonance energy in going from the nonaromatic to the aromatic state. With naphthalene 1,2-oxide, the isomerization has been studied in some detail (Kasperek and Bruice, 1972; Kasperek *et al.*, 1972), and two types of reaction were distinguished, an acid-catalyzed aromatization at pH 5 and below and a spontaneous aromatization at pH 7 and above. With phenanthrene 9,10-oxide, only the acid-catalyzed aromatization was detected. With many other "K-region" epoxides, however, the spontaneous aromatization does occur and in experiments where a series of "K-region" epoxides was incubated at varying pH, differences in stabilities were found among the various members of the series, those derived from methylated hydrocarbons being the least stable (Swaisland *et al.*, 1973).

The directions in which the oxiran ring opens depends on the nature of the aromatic ring systems and appear to be the same for both the spontaneous and the acid-catalyzed reactions. With most of the "K-region" epoxides, the structures of the resulting phenols have not been unequivocally determined, but in many cases the phenols are chromatographically identical with the phenols produced when the related *cis*-dihydrodiols are decomposed with acid (Cook and Schoental, 1948). The phenol from benz[*a*]anthracene-5,6-oxide has been characterized by a direct comparison of its methyl ether with 5-methoxybenz[*a*]anthracene (Newman and Blum, 1964).

The phenols obtained in the acid-catalyzed isomerization of "K-region" and non-"K-region" epoxides are listed in Table IV. These are also the products that are obtained when the metabolic *trans*- or the synthetic *cis*-dihydrodiols are decomposed with acid.

B. Reactions as Alkylating Agents

1. *With Water*

Hydrocarbon epoxides react slowly with water at room temperature. Prolonged heating under reflux of solutions of the epoxides in aqueous acetone in the presence of sodium bicarbonate yields the corresponding *trans*-dihydrodiols together with the related phenols. The relative amounts of the two types of products depend on the readiness with which the epoxides rearrange to phenols. Thus, for example, relatively large amounts of *trans*-9,10-dihydro-9,10-dihydroxyphenanthrene were obtained from phenanthrene 9,10-oxide (Boyland and Sims, 1965b), whereas only phenols were obtained from benzo[*a*]pyrene 7,8- and 9,10-oxide (Waterfall and Sims, 1972).

2. *With Sulfhydryl Compounds*

Most hydrocarbon epoxides will react with reduced glutathione or *N*-acetylcysteine when mixtures are heated together for some hours under reflux in aqueous acetone in the presence of sodium bicarbonate. The reactions are slow and, as with the reactions of epoxides with water, phenols are also formed in amounts that depend on the stability of the epoxide used. The products formed in the chemical reactions of epoxides with glutathione are identical in their properties with those formed in the enzyme-catalyzed reactions (see Table IV).

3. *With 4-(p-Nitrobenzyl)pyridine*

4-(*p*-Nitrobenzyl)pyridine (XXXIX) is a reagent that has been used in the detection and estimation of alkylating agents (Epstein *et al.*, 1955),

O_2N–C₆H₄–CH_2–C₅H₄N

(XXXIX)

and the mechanisms of these reactions have been reviewed (Bedford and Robinson, 1972). With epoxides such as phenanthrene 9,10-oxide, for example, the reagent probably reacts to give a cation that, in the presence of a base such as trimethylamine, is converted into a violet-colored product with λ_{max} at about 560 nm, that probably has the structure (XL).

(XL)

In experiments in which the alkylating abilities of epoxides were compared, it was assumed that all products of this type have similar molar extinction coefficients; some justification for this is provided by the work of Dipple and Slade (1970), which showed that with another type of 4-(*p*-nitrobenzyl)pyridine derivative, a change in the alkyl moiety from benzyl to 7-methylbenz[*a*]anthracenyl gave rise to a change in molar extinction of less than 1%. The results of one such experiment (Swaisland *et al.*, 1973) carried out on a series of "K-region" epoxides are shown in Fig. 11. The presence of methyl groups close to the "K-region," as in epoxides such as 7,12-dimethylbenz[*a*]anthracene 5,6-oxide and 7-methylbenz[*a*]anthracene 5,6-oxide, is associated with high alkylating abilities

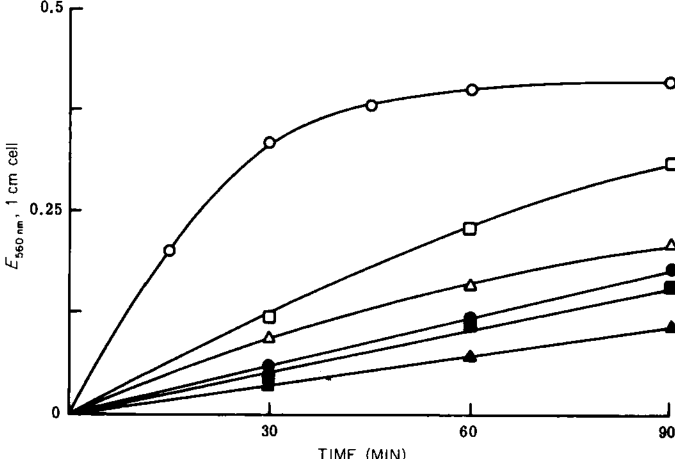


FIG. 11. Alkylation of 4-(*p*-nitrobenzyl)pyridine by epoxides. Epoxides (1.2 μmoles) in acetone (3 ml) were incubated with 4-(*p*-nitrobenzyl)pyridine (0.24 gm) in ethylene glycol (12 ml) and 0.1 *M* Tris·HCl buffer, pH 7.4 (6 ml) at 37°C. Samples (3 ml) were taken at various times and cooled in ice; the extinction at 560 nm was measured immediately after the addition of 50% (v/v) triethylamine and acetone (2.5 ml). The increases in the extinction of the 4-(*p*-nitrobenzyl)pyridine derivatives of 7,12-dimethylbenz[*a*]anthracene 5,6-oxide (○—○), 7-methylbenz[*a*]-anthracene 5,6-oxide (□—□), benz[*a*]anthracene 5,6-oxide (●—●), 3-methylcholanthrene 11,12-oxide (△—△), dibenz[*a,h*]anthracene 5,6-oxide (■—■), and phenanthrene 9,10-oxide (▲—▲) with time of incubation are shown. From Swaisland *et al.* (1973) with permission of Pergamon Press.

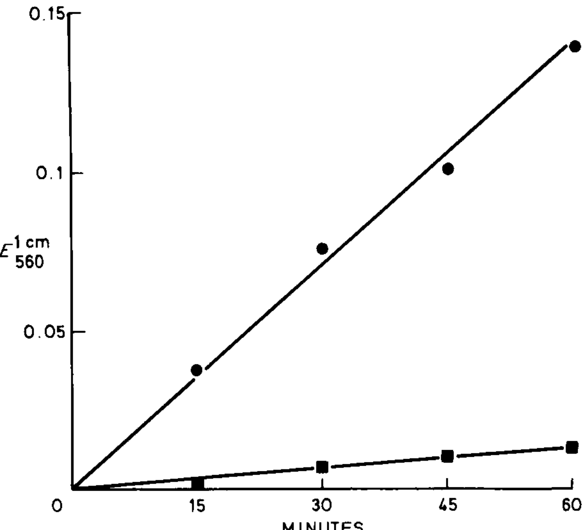


FIG. 12. Alkylation of 4-(*p*-nitrobenzyl)pyridine by the "K-region" epoxide, dibenz[*a,h*]anthracene 5,6-oxide (●—●), and the non-"K-region" epoxide, dibenz-[*a,c*]anthracene 10,11-oxide (■—■). The conditions were similar to those described in the legend to Fig. 11. From Sims (1972b) with permission.

in the epoxides. There is, however, no direct relationship between the ability of an epoxide to alkylate 4-(*p*-nitrobenzyl)pyridine and the carcinogenic activity of its parent hydrocarbon.

In a second type of experiment, the activities toward 4-(*p*-nitrobenzyl) pyridine of "K-region" epoxides were compared with those of non-"K-region" epoxides. In the two cases where this has been done, the non-"K-region" epoxide, benz[*a*]anthracene 8,9-oxide, was less active than the related "K-region" epoxide, benz[*a*]anthracene 5,6-oxide, (Sims, 1971) and, as shown in Fig. 12, dibenz[*a,c*]anthracene 10,11-oxide was less active than dibenz[*a,h*]anthracene 5,6-oxide (Sims, 1972b). Similar differences in activities of the two benz[*a*]anthracene oxides in the enzymatic alkylation of glutathione have been described in Section V,B.

It is not clear whether the results obtained in the experiments with 4-(*p*-nitrobenzyl)pyridine can be related to the observed reactions of the epoxides with macromolecules. Presumably other factors, such as the ease of intercalation of the epoxides with DNA (see Section VIII,C,2) may play some part in their reaction with macromolecules.

4. *Reaction with Polyribonucleotides*

The reactions with members of a series of ^{3}H-labeled "K-region" epoxides with a number of polyribonucleotides have been investigated

TABLE V
REACTIONS OF ^{3}H-LABELED "K-REGION" EPOXIDES OF POLYCYCLIC HYDROCARBONS WITH POLYRIBONUCLEOTIDES[a]

	μMoles/mole P					
"K-Region" epoxide	Poly-(G)	Poly-(A)	Poly-(X)	Poly-(I)	Poly-(U)	Poly-(C)
Phenanthrene 9,10-oxide	280	22	31	42	<9	<9
Benz[*a*]anthracene 5,6-oxide	870	161	86	45	<7	<7
7-Methylbenz[*a*]anthracene 5,6-oxide	1310	566	265	80	27	8
Dibenz[*a,h*]anthracene 5,6-oxide	1860	35	90	51	<5	<5

[a] From Grover and Sims (1973) with permission of Pergamon Press.

(Grover and Sims, 1973). The results are summarized in Table V. In general, the epoxides react with the purine polymers, poly(G), poly(A), poly(X), and poly(I), but not with the pyrimidine polymers, poly(U) and poly(C). Of the reactivities of the epoxides toward the purine polymers, the highest were toward poly(G) and poly(A), suggesting that the amino groups of the purines may be involved in the reactions.

In general, the levels of the reactions of the epoxides with the polynucleotides can be related to the abilities of the epoxides to alkylate 4-(*p*-nitrobenzyl)pyridine (Section VI,B,3), although the reactions of dibenz[*a,h*]anthracene 5,6-oxide are anomalous in this respect. There are presumably factors involved in the reactions with the polyribonucleotides unrelated to the alkylating abilities of the epoxides, since reactions between epoxides and purine or pyrimidine nucleosides or mononucleotides have not been detected (P. L. Grover and P. Sims, unpublished observations).

Reactions with poly(G) were observed with extracts from the incubations of rat liver microsomal fractions with the ^{3}H-labeled hydrocarbons, pyrene and benzo[*a*]pyrene (Grover *et al.*, 1972) and 7-methylbenz[*a*]anthracene, 7,12-dimethylbenz[*a*]anthracene and their 7-hydroxymethyl derivatives (Keysell *et al.*, 1973). These extracts are known to contain "K-region" epoxides (Section IV,D), but the possibility that other reactive intermediates are also present cannot be excluded.

5. *Reactions of Epoxides with DNA and RNA*

^{3}H-Labeled hydrocarbon epoxides react chemically with either DNA or RNA (Grover and Sims, 1970, 1973). The results of a typical experiment are given in Table VI, which shows that although, with some epoxides, extensive reactions with DNA and RNA occur, little if any reaction occurs with apurinic acid. This is not unexpected, since, as out-

TABLE VI
REACTIONS OF ^{3}H-LABELED "K-REGION" EPOXIDES OF POLYCYCLIC HYDROCARBONS WITH DNA, RNA, AND APURINIC ACID[a]

"K-Region" epoxide	μMoles/mole P		
	DNA	RNA	Apurinic acid
Phenanthrene 9,10-oxide	15	<7	<7
Benz[*a*]anthracene 5,6-oxide	680	505	21
7-Methylbenz[*a*]anthracene 5,6-oxide	1630	960	51
Dibenz[*a,h*]anthracene 5,6-oxide	327	417	20

[a] From Grover and Sims (1973) with permission of Pergamon Press.

lined in Section VI,B,4, reactions of epoxides with pyrimidine polymers do not occur readily. The extents of reaction of epoxides with these macromolecules are related to the abilities of these epoxides to alkylate 4-(*p*-nitrobenzyl)pyridine.

The reactions of epoxides with native and with heat-denatured DNA have also been compared (P. L. Grover, A. Hewer, and P. Sims, unpublished observations). Figure 13 shows the G-25 Sephadex column elution profiles obtained after the reaction of ^{3}H-labeled benz[*a*]anthracene 5,6-oxide with double- and with single-stranded DNA. More extensive reactions occur with the double-stranded than with the heat-denatured polymer; this is also true for other "K-region" epoxides. These

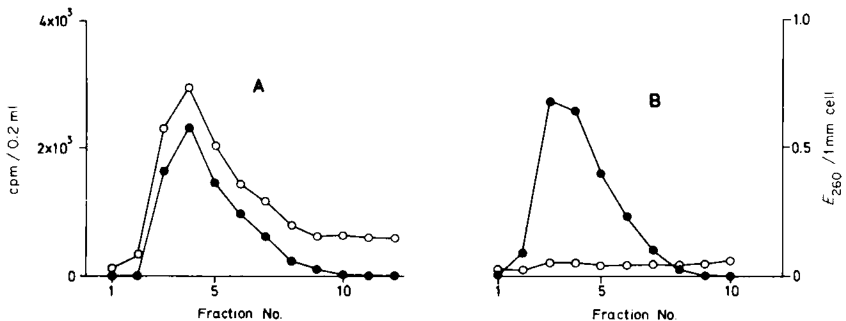


FIG. 13. Reaction of ^{3}H-labeled benz[*a*]anthracene 5,6-oxide with double-stranded or with heat-denatured DNA. The epoxide (0.04 μmole in 1 ml of ethanol) was incubated at 37° for 2 hours either with DNA (≡4 μmoles P) in water (2 ml) or with a similar solution of DNA that had been heated to 100°C for 5 minutes and cooled to 0°C in ice. The solutions were extracted with ether, and the aqueous layers were passed through Sephadex G-25 columns (15 cm). Fractions (12 drops) were collected, and the radioactivity (○—○) and the extinction at 260 nm (●—●) of each fraction were measured. Curves A show results obtained with double-stranded DNA, and curves B those obtained with heat-denatured DNA.

findings appear to support the concept, discussed in Section VIII,C,2, that intercalation of the planar hydrocarbon molecules probably precedes the covalent reaction of the epoxide grouping with an adjacent nucleophilic centre (Ames *et al.*, 1972b) and may facilitate it. These results and those obtained with RNA and with the single-stranded synthetic polynucleotides indicate that reactions of epoxides with single-stranded polynucleotides also occur, but frequently to a lesser extent. The hydrocarbon epoxides may conceivably form two types of physicochemical complexes with nucleic acids, reminiscent of those described for the acridines (Gale *et al.*, 1973): first, an internal intercalated type that is formed with double-stranded macromolecules and, second, an external type that may be formed with both single- or double-stranded macromolecules. The formation of the intercalated type of complex is very probably involved in the action of the polycyclic hydrocarbon epoxides as frameshift mutagens (Section VIII,C,2). If the formation of a physicochemical complex necessarily precedes the covalent reaction of the epoxide group then this may explain the failure, so far, to detect reactions of polycyclic hydrocarbon epoxides with either nucleic acid bases, mononucleosides or nucleotides.

6. *Products of the Reactions with Nucleic Acids and Polynucleotides*

In investigations into the sites of attack and into the nature of the products that are formed when epoxides react with biological macromolecules, it has been necessary to use a range of polynucleotides since the epoxides do not appear to react with monomers. The reaction products, often obtained from reactions employing ^{3}H-labeled epoxides, have been separated or isolated using LH 20 Sephadex columns eluted with increasingly polar solvents (Kreik, 1969/1970; Dipple *et al.*, 1971; Baird and Brookes, 1973; Baird *et al.*, 1973). The products formed in, for example, the reactions of ^{3}H-labeled benz[*a*]anthracene 5,6-oxide with poly(G) and with poly(A) were treated first with alkali and then with alkaline phosphatase, and the ^{3}H-labeled products were separated from the unreacted nucleosides by means of a Sephadex LH-20 column system. The ^{3}H-labeled products were compared with those that were obtained from similar reactions of this epoxide with RNA (A. Swaisland, P. L. Grover, and P. Sims, unpublished observations). The Sephadex LH-20 column elution profiles obtained in such a comparison are shown in Fig. 14, and indicate that, in reactions with RNA, the epoxide yields two principal products, one formed by reaction with guanine and one by reaction with adenine. The radioactive products formed in reactions of ^{3}H-labeled benz[*a*]anthracene 5,6-oxide with poly(A) and with poly(G) are not separable on thin-layer chromatograms from the two main ultra-

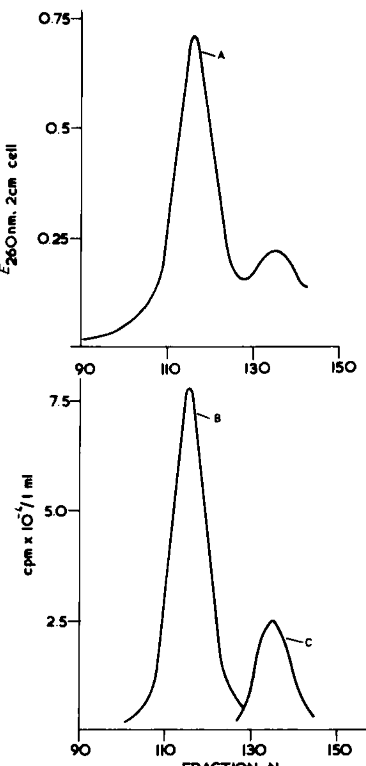


FIG. 14. Reactions of benz[*a*]anthracene 5,6-oxide with RNA poly(G) and poly(A). The alkylated nucleic acid (50 mg) was hydrolyzed in 0.3 *M* KOH (23 ml) at 37°C for 24 hours; the solution was adjusted to pH 7 with HCl and then to pH 9 with 0.1 *M* Tris buffer. The mixture was incubated with alkaline phosphatase (3 ml) at 37°C for 24 hours, evaporated to a small volume, and chromatographed on a Sephadex LH-20 column in a 30–100% water:methanol gradient. Fractions (6 ml) were collected, and the absorption was measured at 260 nm. The alkylated polynucleotides (5 mg) were similarly hydrolyzed, and the products were chromatographed. Curve A shows the products from the hydrolysis of RNA, and curves B and C show the comparable products from poly(G) and poly(A), respectively.

violet (UV)-absorbing products that are formed in reactions of unlabeled epoxide with RNA. Preliminary results show that these RNA products possess, at longer wavelengths, UV spectral properties similar to those of the corresponding "K-region" dihydrodiol, 5,6-dihydro-5,6-dihydroxybenz[*a*]anthracene.

Similar results were obtained in comparisons of the reaction products formed from benz[*a*]anthracene 5,6-oxide and poly(dG), poly(dA), or DNA.

All this evidence confirms that obtained in estimations of the reactions

that occurred between "K-region" epoxides and synthetic polyribonucleotides (Table V) and indicates that the guanine and adenine moieties of nucleic acids are those most likely to be attacked by hydrocarbon epoxides.

Two principal products have also been separated from digests of DNA that had been allowed to react with 7-methylbenz[*a*]anthracene 5,6-oxide (Baird *et al.*, 1973), and two others have been obtained in similar experiments with RNA (W. M. Baird, P. Brookes, P. L. Grover, and P. Sims, unpublished observations); these may also prove to be derivatives of guanine and adenine. At present, however, the sites of attack of polycyclic hydrocarbon epoxides on these nucleic acid bases have not been established.

VII. Reactions of Polycyclic Aromatic Hydrocarbon Epoxides with Constituents of Rodent Cells in Culture

Polycyclic hydrocarbons and epoxide derivatives are active in the *in vitro* systems, developed to study malignant transformation (Berwald and Sachs, 1965; Chen and Heidelberger, 1969a) and mutagenesis (Chu *et al.*, 1969b), that utilize rodent cells grown in tissue culture (Section VIII,B,C). Information concerning the reactivities of these compounds toward the macromolecular constituents of such cells is therefore of interest. While reactions with DNA seem most likely to be responsible for the appearance of permanent alterations in phenotype, reactions of chemical carcinogens with species of RNA (Weinstein *et al.*, 1971) and of protein (Miller and Miller, 1953; Abell and Heidelberger, 1962; Sorof *et al.*, 1963) have also been proposed as possible initiating events in carcinogenesis.

Comparatively few studies of the reactions of polycyclic hydrocarbon epoxides with cellular constituents have been made, probably because such investigations require the radioactively labeled compounds, and have been restricted to "K-region" epoxides since the synthesis of radioactively labeled epoxides on bonds other than that of the "K-region" has not so far been reported. In these investigations, the radioactive hydrocarbon derivatives are usually added in a small volume of solvent to the cell culture medium and the cells are exposed to the compound for the desired period and harvested. The DNA, RNA, and protein is then isolated from the cells by standard fractionation procedures.

A. Reactions with DNA

In one series of experiments (Grover *et al.*, 1971a), ^{3}H-labeled "K-region" epoxides derived from phenanthrene, benz[*a*]anthracene, 7-methylbenz[*a*]anthracene, and dibenz[*a,h*]anthracene were used in

conjunction with confluent cultures of two lines of hamster kidney cells, BHK21 cells and the polyoma virus transformed line of these cells, designated PyY. ^{3}H-Labeled samples of the parent hydrocarbons and of the corresponding "K-region" *cis*-dihydrodiols and phenols were also used with these two cell lines. The epoxides were found to react more extensively than the hydrocarbons, the *cis*-dihydrodiols or the phenols with the DNA of both lines of cells (Table VII).

In a second series of experiments, Kuroki *et al.* (1971/1972) used the ^{3}H-labeled "K-region" epoxide derivatives of benz[*a*]anthracene and dibenz[*a,h*]anthracene, together with the parent hydrocarbons and the corresponding "K-region" *cis*-dihydrodiols and phenols. In these studies, the cells used were exponentially growing cultures of (a) two cell types, secondary hamster embryo cells and a clone of cells (G23) derived from the C3H mouse prostate, the two cell types that form the basis of the two systems most commonly used to investigate malignant transformation *in vitro* (Berwald and Sachs, 1965; Chen and Heidelberger, 1969a) and in which "K-region" epoxides induce transformation (Section VIII,B), (b) the V79 clone of Chinese hamster cells that are extensively used in mutagenesis studies (Chu *et al.*, 1969b) and in which "K-region" epoxides induce mutations (Section VIII,C), and (c) a line of malignant C3H mouse prostate cells (T24) previously transformed by treatment with 3-methylcholanthrene. The results showed (Table VIII) that, as in the studies with BHK21 cells, the "K-region" epoxide of benz[*a*]anthracene reacted to a greater extent with the DNA of the G23 and the hamster embryo cells than either benz[*a*]anthracene itself or the other "K-region" derivatives used. In the V79 cells, the "K-region" phenol also became bound to DNA. However, in the experiments with dibenz[*a,h*]anthracene and its derivatives, the "K-region" phenol became bound to DNA to about the same extent as the corresponding epoxide; the reactions of dibenz-[*a,h*]anthracene itself and the dihydrodiol were lower. In the malignant T24 cells, low levels of binding of benz[*a*]anthracene and the related "K-region" phenol to DNA occurred. The level of reaction of the "K-region" epoxide of benz[*a*]anthracene to the DNA of these chemically transformed cells was also very low. This is in contrast with the results obtained in BHK21 cells where the epoxide reacted more extensively with the DNA of the virus-transformed cells than with that of the untransformed cells (Table VIII). Kuroki *et al.* (1971/1972) also investigated the time-course of the binding of the benz[*a*]anthracene and the dibenz[*a,h*]anthracene series of compounds to the DNA of hamster embryo cells. With most of the compounds, the levels of binding to DNA increased slowly during the first 24 hours after addition of the compound to the culture medium (Fig. 15). The "K-region" epoxide of benz[*a*]anthracene behaved differently;

TABLE VII
THE REACTIONS OF POLYCYCLIC HYDROCARBONS AND THEIR "K-REGION" EPOXIDES, DIHYDRODIOLS AND PHENOLS WITH THE MACROMOLECULAR CONSTITUENTS OF RODENT CELLS IN CULTURE[a,b]

Compound	Cell line					
	BHK21			PyY		
	DNA (μmoles/mole P)	RNA (μmoles/mole P)	Protein (μmoles/400 gm)	DNA (μmoles/mole P)	RNA (μmoles/mole P)	Protein (μmoles/400 gm)
Phenanthrene	0.35	0.18	1.92	0.54	0.07	1.08
Phenanthrene 9,10-oxide	4.10	2.38	112.8	3.31	2.34	156.4
cis-9,10-Dihydro-9,10-dihydroxyphenanthrene	0.25	0.20	4.04	0.22	0.16	3.84
9-Phenanthrol	0.38	0.57	5.40	0.19	0.29	9.25
Benz[*a*]anthracene	0.61	1.18	8.60	0.90	0.79	2.72
Benz[*a*]anthracene 5,6-oxide	26.5	2.67	226.0	67.5	4.95	280.4
cis-5,6-Dihydro-5,6-dihydroxybenz[*a*]anthracene	0.15	0.20	0.60	0.18	0.11	1.40
5-Hydroxybenz[*a*]anthracene	2.62	0.93	26.0	0.39	0.48	15.80
7-Methylbenz[*a*]anthracene	2.21	1.84	6.88	1.24	3.34	8.00
7-Methylbenz[*a*]anthracene 5,6-oxide	17.6	6.34	184.4	36.5	12.1	96.4
cis-5,6-Dihydro-5,6-dihydroxy-7-methylbenz[*a*]anthracene	1.10	1.31	9.64	6.34	1.51	7.88
5-Hydroxy-7-methylbenz[*a*]anthracene	0.49	1.10	14.84	1.10	1.42	8.84
Dibenz[*a,h*]anthracene	0.68	0.35	12.88	0.35	0.26	6.76
Dibenz[*a,h*]anthracene 5,6-oxide	5.63	3.16	86.40	6.31	3.76	87.2
cis-5,6-Dihydro-5,6-dihydroxydibenz[*a,h*]anthracene	0.85	0.45	8.88	0.51	0.29	7.96
5-Hydroxydibenz[*a,h*]anthracene	0.88	0.49	10.96	0.66	0.36	7.12

[a] From Grover *et al.* (1971a) with permission of Microforms International Marketing Corporation.

[b] The ^{3}H-labeled compounds were added in DMSO (1 ml) to the medium (200 ml) in rotating 80-oz bottles containing cell monolayers to give a concentration of 5 μg/ml. After 24 hours the cells were harvested, and the DNA, RNA, and protein were isolated.

TABLE VIII

THE REACTIONS OF BENZ[a]ANTHRACENE AND DIBENZ[a,h]ANTHRACENE AND THEIR "K-REGION" EPOXIDES, DIHYDRODIOLS AND PHENOLS WITH THE MACROMOLECULAR CONSTITUENTS OF EXPONENTIALLY GROWING RODENT CELLS IN CULTURE[a,b]

	Cell line								
	G23			V79			T24		
Compound	DNA (μmoles/mole P)	RNA (μmoles/mole P)	Protein (μmoles/400 gm)	DNA (μmoles/mole P)	RNA (μmoles/mole P)	Protein (μmoles/400 gm)	DNA (μmoles/mole P)	RNA (μmoles/mole P)	Protein (μmoles/400 gm)
Benz[a]anthracene	0.2	—	2.3	0.08	0.08	2.04	0.04	0.04	0.36
Benz[a]anthracene 5,6-oxide	1.4	0.24	5.2	1.6	0.52	24.8	0.08	0.12	4.4
cis-5,6-Dihydro-5,6-dihydroxybenz[a]-anthracene	0.16	0.04	0.12	0.16	0.04	0.72	—	—	—
5-Hydroxybenz[a]-anthracene	0.36	0.24	5.6	1.84	0.28	100	0.12	0.16	5.2
Dibenz[a,h]anthracene	0.2	0.08	0.76	0.28	0.04	0.92	—	—	—
Dibenz[a,h]anthracene 5,6-oxide	0.96	0.84	12.8	0.72	1.44	55.6	—	—	—
cis-5,6-Dihydro-5,6-dihydroxydibenz[a,h]-anthracene	—	—	—	0.48	0.12	13.6	—	—	—
5-Hydroxydibenz[a,h]-anthracene	1.56	0.44	3.84	0.68	0.24	72.4	—	—	—

[a] From Kuroki *et al.* (1971/1972) with permission of Elsevier Publishing Company.

[b] The ^{3}H-labeled compounds were added in DMSO (final concentration 0.5%) to the medium covering exponentially growing cells to give a concentration of 1 μg/ml. After 3 hours, the cells were harvested, and the DNA, RNA, and protein were isolated.

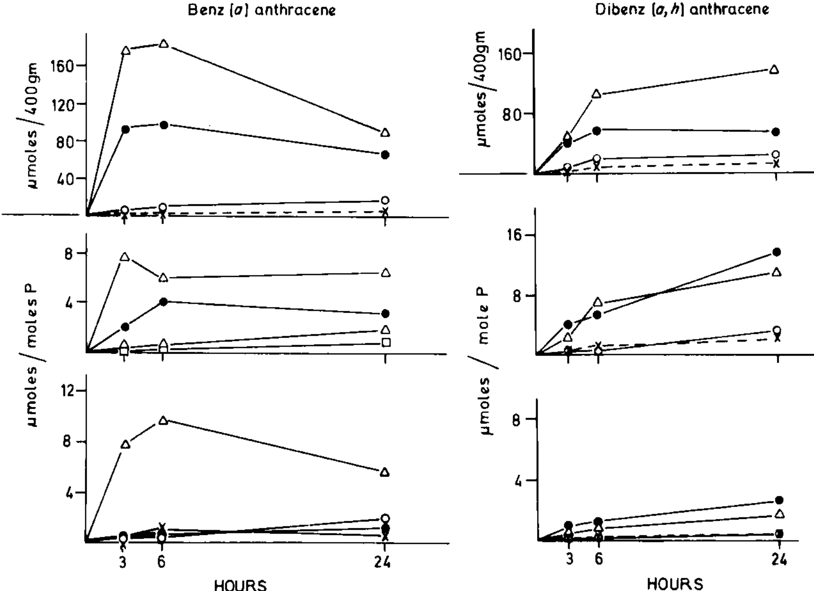


FIG. 15. Time course of the binding of 5 μg/ml of benz[*a*]anthracene, dibenz-[*a,h*]anthracene, and their "K-region" epoxides, dihydrodiols, and phenols to the DNA, RNA, and proteins of exponentially growing hamster embryo cells.

the much more extensive reaction of this epoxide with DNA reached a maximum at between 3 and 6 hours after treatment. Kuroki *et al.* (1971/1972) also used centrifugation in a cesium chloride density gradient to verify that the radioactive benz[*a*]anthracene 5,6-oxide became covalently bound to the DNA of these cells rather than to contaminating protein or RNA isolated with it.

The reaction of polycyclic hydrocarbon epoxides with the DNA of cells in culture is not altogether surprising in view of their chemical reactivity toward nucleic acids (Section VI,B). The mechanism of the reactions of the "K-region" phenols with cellular DNA is not clear, but could be dependent upon their further metabolism; with one exception, 5-hydroxydibenz[*a,h*]anthracene, the "K-region" phenols are all less active biologically than the corresponding epoxides. The results of the experiments on the reactions of ^{3}H-labeled "K-region" derivatives of polycyclic hydrocarbons with the DNA of a variety of rodent cells in culture do not show clear correlations between levels of binding and the biological activities of the compounds in those cells.

The products that are formed when epoxides react with the DNA of cells in culture have been investigated most extensively with 7-methylbenz[*a*]anthracene 5,6-oxide. Baird *et al.* (1973) have isolated and

hydrolyzed the DNA from mouse embryo cells treated with this epoxide and compared the products with those that were obtained when DNA that had been reacted with the epoxide in aqueous solution was similarly hydrolyzed, using the Sephadex LH-20 column separation procedures referred to in Section VI,B,6.

The results showed that similar products are obtained in both cases, indicating that, at least in this example, the reaction of an epoxide with DNA in a biological system is similar to that occurring chemically in solution. More importantly, however, the DNA products formed when mouse embryo cells in culture are treated with the "K-region" epoxides of 7-methylbenz[*a*]anthracene (Baird *et al.*, 1973) and benzo[*a*]pyrene (W. M. Baird, unpublished observations) are not the same as those that are formed in similar cells treated with the parent hydrocarbons. In contrast to these results, the hydrolysis products of the reactions with DNA of metabolites of benz[*a*]anthracene, benzo[*a*]pyrene, or 7,12-dimethylbenz[*a*]anthracene formed by rat liver microsomal fractions in the presence of an epoxide hydrase inhibitor are similar to the hydrolysis products obtained from DNA reacted chemically with the respective "K-region" epoxides (A. J. Swaisland, P. L. Grover, and P. Sims, unpublished observations). Thus the results obtained using cells differ from those obtained using model enzyme systems. These types of investigation, which may yield valuable information concerning the mechanisms of both the metabolic activation of, and chemical carcinogenesis by, the hydrocarbons, should obviously be extended to other hydrocarbons, and such studies are in progress.

The "K-region" epoxide, benz[*a*]anthracene 5,6-oxide, and the related *cis*-5,6-dihydrodiol stimulate DNA synthesis in hamster embryo cells, as measured by the incorporation of ^{3}H-labeled thymidine (Marquardt and Heidelberger, 1972b). Benz[*a*]anthracene itself and the related "K-region" phenol were inactive. The epoxide and the dihydrodiol, are also active in producing malignant transformations in these cells (Grover *et al.*, 1971c).

B. Reactions with RNA

In the two series of experiments that have been described in Section VII,A (Grover *et al.*, 1971a; Kuroki *et al.*, 1971/1972), the levels of reaction of ^{3}H-labeled "K-region" epoxides with the RNA of the cultured rodent cells were also estimated. In some respects these results resemble those obtained when levels of reaction with DNA were measured. In the first series (Grover *et al.*, 1971a), all the four "K-region" epoxides that were tested were more reactive toward the RNA of the BHK and PyY cells than either the respective parent hydrocarbons or their "K-region"

dihydrodiols and phenols although the levels of reaction of the epoxides with RNA were often considerably lower than those found with DNA (Table VII).

In the studies of Kuroki *et al.* (1971/1972) these differences were not always present (Table VIII and Fig. 15); for example, the "K-region" phenol of dibenz[*a*,*h*]anthracene reacted with the RNA of hamster embryo cells to about the same extent as the epoxide. Kuroki *et al.* (1971/1972) also studied the time course of the reaction of benz[*a*]anthracene and of dibenz[*a*,*h*]anthracene and their derivatives with the RNA of hamster embryo cells; the results are shown in Fig. 15, and indicate that both the "K-region" epoxides and the "K-region" phenols derived from these two hydrocarbons became bound more rapidly and more extensively than either the hydrocarbons or the dihydrodiols to the RNA of hamster embryo cells. The method used to isolate RNA in these studies yielded a mixture of 28 S, 18 S, and 4 S RNA (Kuroki and Heidelberger, 1971). Reactions of hydrocarbon epoxides with individual species of RNA in cells have not been investigated but, when ^{3}H-labeled benzo[*a*]pyrene was used, the radioactivity that became bound to RNA was found to be evenly distributed between the 28 S, 18 S, and 4 S components (Kuroki and Heidelberger, 1971).

C. Reactions with Protein

In both the virus-transformed and the untransformed lines of BHK cells, ^{3}H-labeled "K-region" epoxides react extensively with cellular protein (Grover *et al.*, 1971a), and these reactions were consistently greater in extent than those that occurred following the treatment of these cells with either the parent hydrocarbons, the "K-region" dihydrodiols, or the corresponding phenols (Table VII). The extents of reaction of the epoxides with cellular protein were also always higher than the corresponding levels of reaction found with DNA or with RNA. The reactivity of the "K-region" epoxides of benz[*a*]anthracene and of dibenz[*a*,*h*]anthracene toward the proteins of hamster embryo cells (Table VIII) was found by Kuroki *et al.* (1971/1972) to be of a similar order to that reported with BHK21 cells. The time course for the binding of the benz[*a*]anthracene and the dibenz[*a*,*h*]anthracene derivatives to hamster embryo cell protein was also determined by Kuroki *et al.* (1971/1972) (Fig. 15).

In more specific investigations of the reactivity of hydrocarbon epoxides with protein (Kuroki and Heidelberger, 1972), the reactivity of the "K-region" epoxide of dibenz[*a*,*h*]anthracene, dibenz[*a*,*h*]anthracene 5,6-oxide, toward the "h"-protein of rodent cells in culture was measured. This protein, which, it is thought, may play a part in chemical

carcinogenesis in liver (Miller and Miller, 1953), in skin (Abell and Heidelberger, 1962), and in cells in culture (Kuroki and Heidelberger, 1972), is similar to and may be identical with ligandin, a soluble protein present in liver that binds steroids and chemical carcinogens (Litwack *et al.*, 1971). In transformable C3H prostate cells, the ^{3}H-labeled "K-region" epoxide of dibenz[*a,h*]anthracene reacted much more extensively with the "h"-protein fraction than the parent hydrocarbon (Kuroki and Heidelberger, 1972).

The "K-region" has been implicated in the binding of dibenz[*a,h*]-anthracene to protein, since a compound with the chromatographic properties of *S*-(5,6-dihydro-6-hydroxydibenz[*a,h*]anthracen-5-yl)glutathione was detected in the products of the proteolytic enzyme-hydrolysis of the skin of mice that had been treated with the hydrocarbon (Selkirk and Heidelberger, 1972). When benzo[*a*]pyrene was incubated with a liver homogenate and the protein subjected to chemical degradation, chrysene was obtained (Raha *et al.*, 1973), a reaction that implicates one of the "K-regions," the 4,5-bond, of this hydrocarbon in protein binding. Evidence for the binding of benzo[*a*]pyrene through this "K-region" to the proteins of the skin of mice treated with the hydrocarbon has been obtained (Daudel *et al.*, 1962).

The reactivity of polycyclic hydrocarbon epoxides toward cellular proteins is consistent with their action as alkylating agents (Section VI,B), although the products formed have not so far been characterized. The mechanism by which "K-region" phenols become bound to proteins and to the other macromolecules isolated is not clear.

Further studies are obviously required in order to clarify both the reaction mechanism involved and the relevance of these phenol interactions; in most cases the phenolic derivatives do not transform cells (Section VIII,B) and, in situations where further metabolism is unlikely, they do not appear to be mutagens (Section VIII,C).

VIII. Biological Effects Produced by Polycyclic Aromatic Hydrocarbon Epoxides

A. Carcinogenicity in Animals

The route devised by Newman and Blum (1964) for the synthesis of "K-region" epoxides of polycyclic hydrocarbons led to the preparation of a number of compounds of this type (Section III). Carcinogenicity tests on these compounds were carried out in three different laboratories in 1967, and involved both direct tests for carcinogenicity and also tests of the ability of the epoxides to act as tumor-initiators in animals subsequently treated with a tumor-promoting agent. In most experiments

TABLE IX

CARCINOGENICITY TESTS USING POLYCYCLIC HYDROCARBON EPOXIDES ADMINISTERED SUBCUTANEOUSLY TO RODENTS

Compound	Dose (mg)	Species	Strain	Sex	No. of animals	Duration (weeks)	Number with tumors	References
Dibenz[*a,h*]anthracene	10 × 1.0	Mice	C57Bl	M and F	39	80	37	
Dibenz[*a,h*]anthracene 5,6-oxide	10 × 1.0	Mice	C57Bl	M and F	36	80	19	
7-Methylbenz[*a*]anthracene	10 × 1.0	Mice	C57Bl	M and F	20	80	20	
7-Methylbenz[*a*]anthracene 5,6-oxide	10 × 1.0	Mice	C57Bl	M and F	20	80	19	
7-Methylbenz[*a*]anthracene 5,6-oxide	1 × 1.0	Mice	C57Bl	M	10	80	2	Boyland and Sims, 1967b
7-Methylbenz[*a*]anthracene	1 × 1.0	Mice	ICR/HA	F	40	65	25	
7-Methylbenz[*a*]anthracene 5,6-oxide	1 × 1.1	Mice	ICR/HA	F	40	65	7	
7-Methylbenz[*a*]anthracene	1 × 1.0	Rats	CD	M	20	65	15	
7-Methylbenz[*a*]anthracene 5,6-oxide	1 × 2.14	Rats	CD	M	20	65	1	Miller and Miller, 1967
3-Methylcholanthrene	10 × 1.0	Mice	C57Bl	M and F	21	80	20	
3-Methylcholanthrene	3 × 1.0	Mice	C57Bl	M	10	80	10	
3-Methylcholanthrene 11,12-oxide	10 × 1.0	Mice	C57Bl	M and F	20	80	13	
3-Methylcholanthrene 11,12-oxide	3 × 1.0	Mice	C57Bl	M	10	80	2	
3-Methylcholanthrene 11,12-oxide	1 × 1.0	Mice	C57Bl	M	10	80	1	Sims, 1967c
Benz[*a*]anthracene	1 × 1.0	Mice	C57Bl	M	20	80	14	
Benz[*a*]anthracene 5,6-oxide	10 × 1.0	Mice	C57Bl	M and F	21	80	11	
Chrysene	10 × 1.0	Mice	C57Bl	M and F	20	80	2	
Chrysene 5,6-oxide	10 × 1.0	Mice	C57Bl	M	20	80	3	
Phenanthrene 9,10-oxide	10 × 1.0	Mice	C57Bl	M and F	20	80	0	Boyland and Sims, 1967b

the corresponding parent hydrocarbons were also tested in parallel with the epoxides.

Table IX shows the results of the direct carcinogenicity tests performed by Boyland and Sims (1967b), by Miller and Miller (1967), and by Sims (1967c), where the "K-region" epoxides derived from six hydrocarbons were administered by subcutaneous injection to rats or mice. The data clearly show that the "K-region" epoxides of several hydrocarbons are carcinogenic; of the compounds tested by this route only phenanthrene 9,10-oxide gave completely negative results. The experiments also show that the "K-region" epoxides are less active in inducing tumors than equivalent amounts of the corresponding parent hydrocarbons. The sole exception is chrysene, where a low yield of tumors resulted from treatment both with the hydrocarbon and with the 5,6-epoxide.

In two-stage carcinogenicity tests on mouse skin, four "K-region" epoxides have been tested for initiating activity, and the data obtained in the experiments of Miller and Miller (1967) and of Van Duuren *et al.* (1967) are summarized in Table X, which shows that, like the hydrocarbons, the epoxides initiate the formation of tumors in mouse skin and that more tumors arise if the mouse skin is subsequently treated with tumor-promoting fractions of croton resin. Table X also shows that, whereas dibenz[*a,h*]anthracene 5,6-oxide and 7-methylbenz[*a*]anthracene 5,6-oxide are apparently weaker initiating agents than the corresponding hydrocarbons, benz[*a*]anthracene 5,6-oxide was, in the experiments of Miller and Miller (1967), more active than benz[*a*]anthracene itself. Although the administration of phenanthrene 9,10-oxide alone did not induce the formation of either carcinomas or papillomas, this epoxide was weakly active if the mouse skin was subsequently treated with croton resin. Neither the recently synthesized "K-region" epoxides related to the potent hydrocarbons, benzo[*a*]pyrene (Goh and Harvey, 1973) and 7,12-dimethylbenz[*a*]anthracene (Sims, 1973) nor the non-"K-region" epoxides related to benz[*a*]anthracene (Sims, 1971), to dibenz[*a,c*]anthracene (Sims, 1972b), and to benzo[*a*]pyrene (Waterfall and Sims, 1972) have been tested for activity as carcinogens or as initiating agents in whole animals; some data on the activity of these compounds as mutagens and as transforming agents have been reported (Section VIII,B,C).

B. Malignant Transformation of Rodent Cells in Culture

After the discovery of the phenomenon of malignant transformation of rodent cells (reviewed by Macpherson, 1970) that takes place either spontaneously (Gey, 1941) or after the treatment of cultures with a

TABLE X

CARCINOGENIC AND INITIATING ACTIVITIES OF POLYCYCLIC HYDROCARBON EPOXIDES TESTED ON MOUSE SKIN

Compound	Dose (mg)	Croton resin	Mouse strain	Sex	No. of animals	Duration (weeks)	Mice with papillomas	Mice with carcinomas	Reference
Dibenz[*a*,*h*]anthracene	1 × 0.15	+[a]	ICR/HA	F	20	47	18	10	
Dibenz[*a*,*h*]anthracene 5,6-oxide	1 × 0.15	+[a]	ICR/HA	F	20	47	10	2	
Dibenz[*a*,*h*]anthracene	1 × 0.15	−	ICR/HA	F	20	47	3	0	
Dibenz[*a*,*h*]anthracene 5,6-oxide	1 × 0.15	−	ICR/HA	F	20	47	0	0	Van Duuren *et al.*, 1967
7-Methylbenz[*a*]anthracene	1 × 0.3	+[b]	STS	F	26	38	11[c]	9	
7-Methylbenz[*a*]anthracene 5,6-oxide	1 × 0.32	+[b]	STS	F	26	38	10[c]	3	Miller and Miller, 1967
7-Methylbenz[*a*]anthracene 5,6-oxide	1 × 0.3	+[a]	ICR/HA	F	20	47	4	1	Van Duuren *et al.*, 1967
7-Methylbenz[*a*]anthracene	40 × 0.07	−	ICR/HA	F	40	65	4	21	
7-Methylbenz[*a*]anthracene 5,6-oxide	40 × 0.076	−	ICR/HA	F	40	65	0	1	Miller and Miller, 1967
7-Methylbenz[*a*]anthracene 5,6-oxide	1 × 0.3	−	ICR/HA	F	15	47	0	0	Van Duuren *et al.*, 1967
Benz[*a*]anthracene	4 × 0.3	+[b]	STS	F	26	38	6	1	
Benz[*a*]anthracene 5,6-oxide	4 × 0.32	+[b]	STS	F	26	38	15	1	Miller and Miller, 1967
Phenanthrene 9,10-oxide	1 × 1.0	+[a]	ICR/HA	F	20	47	2	1	
Phenanthrene 9,10-oxide	1 × 1.0	−	ICR/HA	F	20	47	0	0	Van Duuren *et al.*, 1967

[a] Croton resin (25 μg/application) was applied 3 times weekly as a 0.025% solution in acetone commencing 2 weeks after initiation.

[b] Croton resin (150 μg/application) was applied twice weekly as a 0.3% solution in acetone commencing 10 days after the last dose of initiator. The croton resin was reduced to 100 μg/application after week 10.

[c] Mice bearing papillomas were recorded at 29 weeks.

chemical carcinogen (Earle, 1943, Earle and Nettleship, 1943), two systems were developed for estimating the frequency at which this transformation occurs. The first system uses primary or secondary cultures of embryonic hamster fibroblasts (Berwald and Sachs, 1963, 1965), and the second system uses cells derived from C3H mouse prostates (Chen and Heidelberger, 1969a,b; Mondal and Heidelberger, 1970). Polycyclic hydrocarbons induce malignant transformations in these two systems (Berwald and Sachs, 1963, 1965; Chen and Heidelberger, 1969b), and both have been used to test the activities of hydrocarbon epoxides: *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, a compound that does not require prior metabolic activation (Lawley and Thatcher, 1970) was often used as a positive control in these tests.

Table XI gives some of the results obtained by Marquardt *et al.* (1972, 1974) using C3H mouse prostate cells. The data show that for some hydrocarbons, the "K-region" epoxides were much more active than the parent hydrocarbons in effecting transformation. For others, notably benz[*a*]anthracene derivatives substituted with methyl or with hydroxymethyl groups in the 7-position, the activities of the "K-region" epoxides were similar to or even slightly lower than those of the related hydrocarbons. The only non-"K-region" epoxide tested, benz[*a*]anthracene 8,9-oxide, was clearly less active than the corresponding "K-region" derivative, benz[*a*]anthracene 5,6-oxide. With phenanthrene and chrysene, neither the hydrocarbons nor their "K-region" epoxides induced transformation.

Results obtained when hydrocarbons and their "K-region" epoxides were tested for their abilities to transform hamster embryo cells (Huberman *et al.*, 1972b) are given in Table XII. Two types of experiments were used. In the first, the compounds were added to the medium in which hamster embryo cells were growing on a feeder layer of irradiated rat cells and left in contact with the cells for 7 days. In the second, the compounds were added to hamster embryo cells growing in conditioned culture medium that, after 4 hours, was replaced by fresh medium. In both types of experiment, the "K-region" epoxides were, in general, more active than the parent hydrocarbons and these differences were often more marked in the experiments that used a treatment time of 4 hours, an observation that is in accordance with the idea that the hydrocarbons themselves require metabolism for activity.

In embryo cells, the non-"K-region" epoxide derived from benz[*a*]-anthracene, benz[*a*]anthracene 8,9-oxide, was again less active than the comparable "K-region" derivative. The main differences between the sets of results obtained with the mouse prostate cells and with the hamster embryo cells concern phenanthrene and chrysene. Both these hydrocarbons were inactive in both cell systems, but the "K-region" epoxides

TABLE XI
MALIGNANT TRANSFORMATION *in Vitro* OF C3H MOUSE PROSTATE CELLS BY POLYCYCLIC HYDROCARBONS AND EPOXIDES

Compound	Concentration (μg/ml)	Plating efficiency (%)	No. of transformed foci/No. of dishes treated
DMSO[a]	(0.5%)	25	0/30
	(0.5%)	24.5	0/12
MNNG[a]	0.2	13	12/13
MNNG[b]	0.2	15.5	32/12
Phenanthrene[a]	1.0	24	0/12
	5.0	20	0/12
	10.0	11	0/12
Phenanthrene 9,10-oxide[a]	1.0	13	0/12
	5.0	1.5	0/15
	10.0	0	—
Chrysene[a]	1.0	22	0/10
	5.0	22	0/12
	10.0	22	0/12
Chrysene 5,6-oxide[a]	1.0	17	0/15
	5.0	1.0	0/18
	10.0	0	—
Benz[*a*]anthracene[a]	1.0	18	0/13
	5.0	15	0/18
Benz[*a*]anthracene 5,6-oxide[a]	0.5	10	9/19
	1.0	3.0	23/25
Benz[*a*]anthracene 8,9-oxide[a]	0.5	17	0/18
	1.0	13	0/14
	5.0	5.0	2/16
	10.0	1.0	0/18
Dibenz[*a,h*]anthracene[a]	1.0	22	0/15
	10.0	20	0/18
Dibenz[*a,h*]anthracene 5,6-oxide[a]	0.5	22	4/20
	1.0	17	9/17
	10.0	6.5	12/15
7-Methylbenz[*a*]anthracene[a]	0.1	21	5/20
	10.0	13	9/17
7-Methylbenz[*a*]anthracene 5,6-oxide[a]	0.01	20	0/10
	0.05	17	4/18
	0.1	14	1/10
	0.2	4.0	0/10
7-Hydroxymethylbenz[*a*]anthracene[b]	0.1	21.5	23/20
	1.0	18.5	37/20
	10.0	10.0	1/12
7-Hydroxymethylbenz[*a*]-anthracene 5,6-oxide[b]	0.05	20.0	8/12
	0.1	20.0	17/20
	0.5	16.5	18/20
	1.0	14.0	9/12

TABLE XI (*Continued*)

Compound	Concentration (μg/ml)	Plating efficiency (%)	No. of transformed foci/No. of dishes treated
7,12-Dimethylbenz[*a*]anthracene[b]	0.1	15.0	20/10
	1.0	11.0	66/12
	10.0	10.0	132/12
7,12-Dimethylbenz[*a*]anthracene 5,6-oxide	0.05	28.5	10/10
	0.1	20.5	12/12
	0.2	11.5	20/12
	0.5	6.0	44/12
	1.0	3.5	16/12
3-Methylcholanthrene[a]	1.5	17	1/13
	10.0	17	10/33
3-Methylcholanthrene 11,12-oxide[a]	0.75	17	38/27
	1.5	9.5	58/26

[a] Tested with the G23 clone of C3H mouse prostate cells (Marquardt *et al.*, 1972).
[b] Tested with the M2 clone of C3H mouse prostate cells (Marquardt *et al.*, 1974).

of phenanthrene, phenanthrene 9,10-oxide, and of chrysene, chrysene 5,6-oxide, appear to be active in embryo cells; the highest frequency of transformation found with hamster embryo cells resulted from treatment with the chrysene epoxide.

Since hydrocarbon epoxides are further metabolized in biological systems to dihydrodiols and can rearrange to the corresponding phenols, the properties of both types of compound were investigated in *in vitro* transformation systems (Grover *et al.*, 1971c; Huberman *et al.*, 1972b; Marquardt *et al.*, 1972). In most cases, these hydroxylated derivatives were inactive as transforming agents. The "K-region" *cis*-dihydrodiols, when active, had been left in contact with cells for 7 days and could therefore have been activated by further metabolism; in experiments with a 4-hour treatment time, essentially negative results were obtained with the *cis*-dihydrodiols (Huberman *et al.*, 1972b). The *trans*-dihydrodiols, which are the isomers likely to be formed in the metabolism of the hydrocarbons, were inactive as transforming agents. The "K-region" phenols were the most cytotoxic compounds tested, but, in general, they did not induce transformation (Grover *et al.*, 1971c; Huberman *et al.*, 1972b; Marquardt *et al.*, 1972). This finding reaffirms the suggestion (Huberman and Sachs, 1966; Chen and Heidelberger, 1969c) that transformation and cytotoxicity are unconnected events.

C. Mutagenicity

Interest in the mutagenic effects of chemical carcinogens has recently increased in parallel with two realizations. The first of these, that very

TABLE XII

MALIGNANT TRANSFORMATION AND TOXICITY OF POLYCYCLIC HYDROCARBONS AND EPOXIDES IN HAMSTER EMBRYO CELLS[a]

Compound	Concentration (μg/ml)	7-Day treatment of cells seeded on feeder layers				4-Hour treatment of cells seeded in condition medium			
		Total No. of colonies	Cloning efficiency (%)	No. of transformed colonies	% Transformation	Total No. of colonies	Cloning efficiency (%)	No. of transformed colonies	% Transformation
Control, acetone	(0.5%)	922	4.6	2	0.2	952	5.3	2	0.2
	(0.5%)	849	15.4	1	0.1	650	13.3	0	0
Phenanthrene	5	—	—	—	—	858	21.4	4	0.5
	10	—	—	—	—	808	20.2	2	0.3
Phenanthrene 9,10-oxide	5[b]	—	—	—	—	757	4.8	8	1.05
	7.5[b]	—	—	—	—	715	4.4	8	1.1
	10	—	—	—	—	475	2.9	23	4.8
Chrysene	5	—	—	—	—	607	19.9	3	0.5
	10	—	—	—	—	789	19.6	3	0.4
	15	—	—	—	—	711	17.8	6	0.8
Chrysene 5,6-oxide	5	—	—	—	—	544	13.6	5	0.9
	10[b]	—	—	—	—	516	3.3	17	3.3
	15[b]	—	—	—	—	158	1.0	28	17.7
Benz[a]anthracene	2.5	875	14.6	2	0.2	663	14.6	1	0.2
	5	851	14.2	1	0.1	621	15.5	0	0
	10	845	14.1	1	0.1	969		1	0.1
Benz[a]anthracene 5,6-oxide	1.2	439	9.7	6	1.4	—	—	—	—
	2.5	946	5.9	38	4.0	631	14.0	5	0.8
	5	0	—	—	—	291	6.5	18	6.2

Benz[*a*]anthracene	2.5	504	11.3	1	0.2	—	—	—	—
	5	544	10.9	7	1.3	473	13.5	0	0
	10	445	8.9	14	3.1	731	15.1	2	0.3
	15	—	—	—	—	279	5.7	4	1.4
Dibenz[*a*,*h*]anthracene	2.5	760	4.2	4	0.5	—	—	—	—
	5	690	3.8	4	0.7	911	5.0	5	0.5
	10	790	4.4	7	0.9	882	4.9	3	0.4
	15	796	4.6	0	0	952	5.4	2	0.2
Dibenz[*a*,*h*]anthracene 5,6-oxide	2.5	598	3.3	3	0.5	722	4.4	8	1.1
	5	601	3.3	12	2.0	740	4.1	14	1.9
	7.5	395	2.5	31	7.8	637	4.0	15	2.4
	10	350	1.9	14	4.0	550	3.4	19	3.5
	15	278	1.5	—	—	—	—	—	—
7-Methylbenz[*a*]anthracene	0.5	351	3.6	9	2.5	657	6.6	2	0.3
	2	412	3.8	7	1.6	569	5.7	1	0.2
	10	458	4.2	5	1.2	557	5.6	0	0
7-Methylbenz[*a*]anthracene 5,6-oxide	0.25	407	4.1	1	0.2	301	4.3	1	0.3
	0.5	197	2.0	1	0.5	246	2.5	5	2.0
	0.75	148	0.7	3	2.0	122	0.6	9	7.4
	1.0	96	0.5	—	—	50	0.3	—	—
3-Methylcholanthrene	2.5	—	—	—	—	404	10.1	9	2.2
	5.0	—	—	—	—	370	9.2	10	2.7
	7.5	—	—	—	—	349	8.7	15	4.3
3-Methylcholanthrene 11,12-oxide	3.5[b]	—	—	—	—	364	2.4	13	3.6
	5[b]	—	—	—	—	245	1.5	8	3.3
	7[b]	—	—	—	—	103	0.7	17	16.5

[a] From Huberman *et al.* (1972b).
[b] 2500 cells/dish.

TABLE XIII
MUTAGENICITY OF POLYCYCLIC HYDROCARBON EPOXIDES

Epoxide	Mutagenesis system					Carcinogenicity of parent Hydrocarbons[f]
	Bacteriophage T_2h^+ [a]	Bacteriophage T_4[b]	*Salmonella typhimurium*[c]	Chinese hamster cells[d]	*Drosophila melanogaster*[e]	
Phenanthrene 9,10-oxide	−		−			−
Chrysene 5,6-oxide	±		−			±
Benz[*a*]anthracene 5,6-oxide	±		+	+	+	±
Benz[*a*]anthracene 8,9-oxide			−		?	±
7-Methylbenz[*a*]anthracene 5,6-oxide			+	+	+	+
7-Hydroxymethylbenz[*a*]anthracene 5,6-oxide			+			+
7,12-Dimethylbenz[*a*]anthracene 5,6-oxide		+	+		+	+
Dibenz[*a*,*h*]anthracene 5,6-oxide	+		+	+		+
3-Methylcholanthrene 11,12-oxide	+		−	+	+	+
Benzo[*a*]pyrene 4,5-oxide		+	+		+	+

[a] Host range mutations (Cookson *et al.*, 1971).
[b] Reversion of frameshift mutations (J. Cresswell, A. Loveless, P. L. Grover, and P. Sims, unpublished data).
[c] Reversion of frameshift mutations in the histidine operon (Ames *et al.*, 1972b; B. N. Ames, E. Yamasaki, P. L. Grover and P. Sims, unpublished data).
[d] Mutation to 8-azaguanine resistance (Huberman *et al.*, 1971a).
[e] X Chromosome mutations (bobbed, minute, recessive lethals, and visibiles) (Fahmy and Fahmy, 1973, and unpublished data).
[f] Hartwell (1951).

many carcinogenic compounds can be shown to be mutagenic if they are tested under the appropriate conditions, has led to the second, which is that the somatic mutation theory of cancer (Boveri, 1914; Tyzzer, 1916) may soon be substantiated. With the polycyclic hydrocarbons, close correlations between carcinogenicity and mutagenicity have not yet been obtained, although some hydrocarbons are mutagenic in some situations (Barratt and Tatum, 1951, 1958; Scheer *et al.*, 1954; Epstein and Shafner, 1968; Fahmy and Fahmy, 1969, 1970, 1973; Chu *et al.*, 1971). As Miller and Miller (1971) have pointed out in a recent review, this is probably because the hydrocarbons have not been tested in a mutagenicity test system that also provides for prior metabolic activation. Work by Ames *et al.* (1973b), in which these criteria are fulfilled, has shown that several carcinogenic hydrocarbons become mutagenic to *Salmonella* as a result of metabolism by a mammalian microsomal oxygenase. Since this enzyme is responsible for the initial oxidation of the hydrocarbons to epoxides, it will be of great interest to see whether further work with this system produces correlations between the carcinogenicity of the hydrocarbons and the mutagenicity of their microsomal metabolites.

Several hydrocarbon epoxides obtained by synthesis have been shown to be active in mutagenicity tests; the existing data are summarized in Table XIII and reviewed in the following subsections.

1. *Mutagenicity in Bacteriophage*

"K-Region" epoxides derived from 7-methylbenz[*a*]anthracene, dibenz-[*a,h*]anthracene, and 3-methylcholanthrene caused host range mutations in bacteriophage T_2h^+ when phage suspensions were incubated with saturated aqueous solutions of the epoxides (Cookson *et al.*, 1971). "K-region" epoxides derived from benz[*a*]anthracene, chrysene, and phenanthrene were less active in these tests. None of the parent hydrocarbons, the related "K-region" dihydrodiols, or the phenols was active when tested under similar conditions (M. J. Cookson, P. L. Grover, and P. Sims, unpublished data).

In more specific tests, the "K-region" epoxides derived from benzo-[*a*]pyrene and from 7,12-dimethylbenz[*a*]anthracene were active in the reversion of a frameshift mutant strain of bacteriophage T4 that was also reverted by the acridine mustard ICR 170 (J. Cresswell, A. Loveless, P. L. Grover, and P. Sims, unpublished data). The parent hydrocarbons, benzo[*a*]pyrene and 7,12-dimethylbenz[*a*]anthracene, and the corresponding "K-region" dihydrodiols and phenols did not revert the frameshift mutant phage. Non-"K-region" epoxides have not been tested for mutagenicity toward bacteriophage. In some respects bacteriophage appears to offer advantages as a test organism for use with reactive com-

pounds such as epoxides, since problems associated with cell-wall permeability, with reactions with constituents of the growth medium, or with further metabolism by the test organism do not arise.

2. *Mutagenicity in Bacteria*

Some of the most interesting data on hydrocarbon epoxide mutagenicity have been obtained using a set of bacterial strains carrying known types of mutations. These *Salmonella typhimurium* test strains, each of which carries one of a variety of mutations in the histidine operon, have been developed by Ames and his colleagues (Ames, 1971; Ames *et al.*, 1973a). They form the basis of a sensitive system for detecting and classifying mutagens, which depends upon reversion from histidine requirement to growth on minimal medium. Several "K-region" epoxides, including those related to benz[*a*]anthracene, 7-methylbenz[*a*]anthracene, dibenz[*a,h*]anthracene, benzo[*a*]pyrene, and 7,12-dimethylbenz[*a*]-anthracene are active in reverting test strains that carried frameshift mutations (Ames *et al.*, 1972b; B. N. Ames, E. Yamasaki, P. L. Grover, and P. Sims, unpublished data). These epoxides did not revert a base-substitution mutant of *S. typhimurium*, and the parent hydrocarbons and the corresponding dihydrodiols and phenols did not revert either base-substitution or frameshift mutant strains of *S. typhimurium*.

Deep rough mutant strains of *S. typhimurium*, which in addition to carrying a frameshift mutation in the histidine operon, also lack most of the lipopolysaccharide component of the outer bacterial membrane, have been isolated (Ames *et al.*, 1972b, 1973a); these strains are frequently more susceptible to reversion by hydrocarbon epoxides, a finding that clearly suggests that bacterial cell walls can act as a barrier to compounds of this type.

The mechanism by which hydrocarbon epoxides act as frameshift mutagens in bacteria and in bacteriophage may involve several stages. The first of these may be an intercalation of the planar hydrocarbon between the base pairs of the DNA, and this is followed by the covalent reaction of the epoxide grouping with an adjacent nucleophilic center (Ames *et al.*, 1972b), probably on a purine (Grover and Sims, 1973). This suggestion stems from earlier studies of the biological effects of other DNA intercalating agents, including several acridine derivatives (Lerman, 1961; Ames and Whitfield, 1966; Creech *et al.*, 1972). The intercalated hydrocarbon or acridine moiety may then lead to a shifted pairing of bases and, during DNA replication, to the addition or deletion of a base pair in the DNA sequence (Brenner *et al.*, 1961; Streisinger *et al.*, 1966; Barnett *et al.*, 1967) with an associated shift in the reading frame

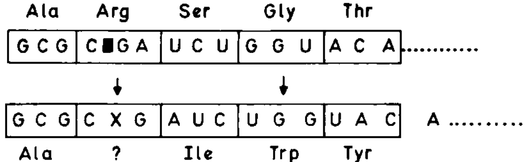


FIG. 16. A diagrammatic illustration of a mechanism involving DNA replication by which an intercalated hydrocarbon epoxide (■) may induce frameshift mutations. × = extra base.

(Fig. 16). Several other types of chemical carcinogens with aromatic ring systems are also metabolized into frameshift mutagens using *Salmonella* as a test organism (Ames *et al.*, 1972a, 1973b); the mechanism of action of these compounds may be similar to that proposed for the acridine mustards and the hydrocarbon epoxides.

3. *Mutagenicity in Mammalian Cells*

The system developed by Chu and Malling (1968) and their colleagues (Chu *et al.*, 1969a,b; Chu, 1971), in which Chinese hamster cell mutants that are resistant to 8-azaguanine are induced, has been used to assess the mutagenicity of some hydrocarbon epoxides (Huberman *et al.*, 1971a). In the experimental procedures, cells in culture are treated with the test compound; then, after an expression time of 48 hours, the treated cells are exposed to a medium containing 8-azaguanine and subsequently scored for colonies that grow in the presence of, and are therefore resistant to, 8-azaguanine. Cytotoxicity estimations are carried out separately on other dishes of cells. The results obtained by Huberman *et al.* (1971a) confirmed the mutagenic activity displayed by epoxides in other biological test systems. The "K-region" epoxides derived from benz[*a*]anthracene, 7-methylbenz[*a*]anthracene, dibenz[*a,h*]anthracene, and 3-methylcholanthrene were actively mutagenic in these mammalian cells; the parent hydrocarbons and the corresponding "K-region" dihydrodiols were inactive; hydrocarbons had earlier been reported to be mutagenic in this system (Chu *et al.*, 1971). The "K-region" phenols derived from benz[*a*]anthracene and dibenz[*a,h*]anthracene did show some mutagenic activity, but it is not clear whether these phenols were active as such or only as a result of further metabolism. Figure 17 shows the results obtained with the benz[*a*]anthracene series of compounds. "K-region" phenols have not been tested in *Drosophila* (see Section VIII,C,4), the other mutagenicity test system in which metabolic activity may be present and that has also been used to test hydrocarbon derivatives.

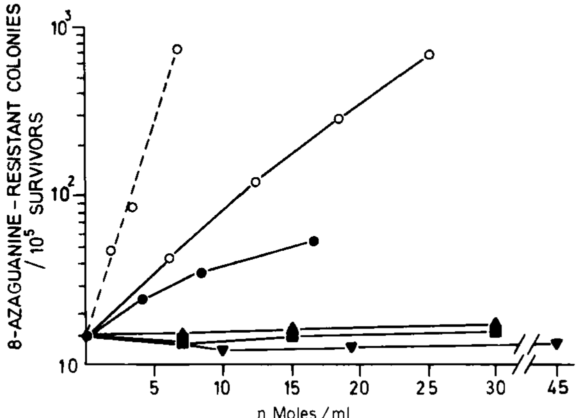


FIG. 17. The mutagenicity in V79 cells of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) (○ - - - ○), benz[*a*]anthracene (■—■), benz[*a*]anthracene 5,6-oxide (○—○), 5-hydroxybenz[*a*]anthracene (●—●), and *cis*- (▲—▲) and *trans*- (▼ — ▼) 5,6-dihydro-5,6-dihydroxybenz[*a*]anthracene.

4. *Mutagenicity in Drosophila*

The mutagenic effects of polycyclic hydrocarbons in *Drosophila* have been described (Fahmy and Fahmy, 1969, 1970, 1973); presumably this species of insect is able, like others, to carry out the metabolic activation of aromatic compounds (Giannotti *et al.*, 1956; Perry *et al.*, 1958; Brooks, 1966).

From the results of their experiments with hydrocarbons and with other classes of chemical carcinogens (Fahmy and Fahmy, 1971, 1972a,b,c), the Fahmy's have suggested that it is the selective induction of mutations at the rRNA and tRNA genes that is relevant to carcinogenesis rather than the other effects on the X chromosome, which give rise to lethal and visible mutations. These authors believe that it is the ratio of the activities of compounds in inducing mutations in the RNA genes of *Drosophila* that give rise to bobbed (*bb*) and to minute (*M*) mutants in comparison to their activities in inducing other mutations on the X chromosome that correlates with the carcinogenicity of the compounds in experimental animals, the carcinogenicities being expressed by the index proposed by Iball (1939).

Recently, epoxides derived from polycyclic hydrocarbons have been tested for mutagenicity in *Drosophila* (Fahmy and Fahmy, 1973, and personal communication); the results are summarized in Table XIV. The Table shows that the frequency with which the epoxides induce RNA gene mutations is, in each case, lower than that produced by equitoxic doses of the corresponding hydrocarbon. In contrast, the epoxides, with

TABLE XIV
MUTAGENICITY OF POLYCYCLIC HYDROCARBONS AND "K-REGION" EPOXIDES IN *Drosophila*[a]

Compound	Induced mutations/10^3		Selectivity index $\frac{bb}{bb + X} \times 100$
	RNA gene mutations, *bb*	Other X chromosome mutations	
Benz[*a*]anthracene	0.2 ± 0.1	1.8 ± 0.8	11.4
Benz[*a*]anthracene 5,6-oxide	0.1 ± 0.1	3.4 ± 1.4	3.0
7-Methylbenz[*a*]anthracene	0.9 ± 0.3	0.4 ± 0.3	69.6
7-Methylbenz[*a*]anthracene 5,6-oxide	0.3 + 0.1	6.3 ± 1.5	4-5
7,12-Dimethylbenz[*a*]anthracene	5.6 ± 0.4	1.1 ± 1.1	83.2
7,12-Dimethylbenz[*a*]anthracene 5,6-oxide	0.7 ± 0.2	1.5 ± 0.2	31.3
3-Methylcholanthrene	3.0 ± 0.4	0.7 ± 0.7	80.9
3-Methylcholanthrene 11,12-oxide	0.5 ± 0.2	6.4 ± 1.7	7.2
Benzo[*a*]pyrene	0.6 ± 0.3	11.5 ± 3.5	4.6
Benzo[*a*]pyrene 4,5-oxide	0.5 ± 0.1	0.6 ± 0.1	45.9

[a] Data supplied by Dr. O. Fahmy.

the exception of benzo[*a*]pyrene 4,5-oxide and the related hydrocarbon, consistently produce more of the other types of X chromosome mutations than do the parent hydrocarbons.

The Fahmy's interpret these data as meaning that the "K-region" epoxides, with the possible exception of benzo[*a*]pyrene 4,5-oxide, are not the significant metabolically activated forms of the hydrocarbons since their selectivity for inducing RNA gene mutations is not as high as that of the hydrocarbons. Whatever the fate of this hypothesis concerning mutagenic selectivity, it is clear that both hydrocarbons and epoxides are mutagenic in *Drosophila*. The metabolic activation of polycyclic hydrocarbons by *Drosophila* tissue preparations has not been studied.

IX. Properties of Epoxides Formed on Olefinic Double Bonds Conjugated with Aromatic Ring Systems

In the course of the preparation of non-"K-region" epoxides of polycyclic hydrocarbons, a number of dihydro derivatives and their related epoxides became available for study (Sims, 1971, 1972b; Waterfall and Sims, 1972). The dihydro compounds all contain olefinic double bonds in conjugation with the aromatic ring system, and in the metabolism

EPOXIDE HYDRASE H_2O

GSH GLUTATHIONE TRANSFERASE

FIG. 18. The metabolism of 10,11-dihydrobenz[*a*]anthracene 8,9-oxide.

of the compounds by rat liver preparations, dihydrodiols and glutathione conjugates are formed; these arise by reactions at the olefinic double bonds (see Fig. 18) in the same way that 1,2-dihydronaphthalene is metabolized to products of these types (Section IV,A,2). The formation of epoxide intermediates at the olefinic double bonds seems probable since the dihydro derivatives and related epoxides are both metabolized by rat liver preparations to the same products. These metabolic reactions are summarized in Table XV.

Of the two dihydro compounds that have been tested for carcinogenicity by subcutaneous injection into mice (P. Sims, unpublished observations), 7,8-dihydrobenzo[*a*]pyrene (XLI) proved to be a power-

(XLI) (XLII)

ful carginogen whereas the isomeric 9,10-dihydrobenzo[*a*]pyrene (XLII) was virtually inactive. Similarly, the 7,8-dihydro derivative was active and the 9,10-dihydro derivative relatively inactive in inducing malignant transformation in mouse prostate cells (H. Marquardt, personal com-

(XLIII) (XLIV)

munication). When the related epoxides were tested in these cells, the 7,8-epoxide (XLIII) (i.e., that derived from the 9,10-dihydro compound) showed little activity, whereas the 9,10-oxide (XLIV) proved to be

TABLE XV

CHEMICAL AND BIOCHEMICAL REACTIONS OF EPOXIDES OF DIHYDRO DERIVATIVES OF POLYCYCLIC AROMATIC HYDROCARBONS

Epoxide	Formula	Product of the reaction of the epoxide with		Reference
		Water	Glutathione	
10,11-Dihydrobenz[*a*]anthracene 8,9-oxide		*trans*-8,9,10,11-Tetrahydro-8,9-dihydroxybenz[*a*]anthracene	*S*-(8,9,10,11-Tetrahydro-9-hydroxybenz[*a*]anthracen-8-yl)glutathione	Sims, 1971
10,11-Dihydrodibenz[*a*,*c*]anthracene 12,13-oxide		*trans*-10,11,12,13-Tetrahydro-10,11-dihydroxydibenz[*a*,*c*]anthracene	*S*-(10,11,12,13-Tetrahydro-11-hydroxydibenz[*a*,*c*]anthracen-10-yl)glutathione	Sims, 1972b
7,8-Dihydrobenzo[*a*]pyrene 9,10-oxide		*cis*- and *trans*-7,8,9,10-Tetrahydro-9,10-dihydroxybenzo[*a*]pyrene	*S*-(7,8,9,10-Tetrahydro-9-hydroxybenzo[*a*]pyren-10-yl)glutathione	Waterfall and Sims, 1972
9,10-Dihydrobenzo[*a*]pyrene 7,8-oxide		*cis*- and *trans*-7,8,9,10-Tetrahydro-7,8-dihydroxybenzo[*a*]pyrene	*S*-(7,8,9,10-Tetrahydro-8-hydroxybenzo[*a*]pyren-7-yl)glutathione	Waterfall and Sims, 1972

highly cytotoxic. The 7,8-epoxide was highly mutagenic in strains of *S. typhimurium* whereas the 9,10-epoxide was toxic even at very low concentration (B. N. Ames, personal communication). The high toxicity associated with the 9,10-epoxide may be related to its alkylating ability since, when the abilities of the two epoxides to alkylate 4-(*p*-nitrobenzyl) pyridine were compared (Waterfall and Sims, 1972), the 9,10-oxide was at least as active as 7,12-dimethylbenz[*a*]anthracene 5,6-oxide, the most active of all the hydrocarbon epoxides so far tested (Section VI,B,3), whereas the activity of the 7,8-oxide was low. It is not clear from the available evidence whether the carcinogenic activity of 7,8-dihydrobenzo-[*a*]pyrene involves epoxidation of the 9,10-bond or some reaction elsewhere on the molecule.

A closely related compound with an olefinic bond in conjugation with the aromatic rings system is 1,2-dehydro-3-methylcholanthrene (3-methylcholanthrylene) (XLV). This hydrocarbon is metabolized at the 1,2-bond by rat liver preparations to yield *trans*-1,2-dihydroxy-3-methyl-

CH_3 (XLV) O CH_3 (XLVI)

cholanthrene and 3-methylcholanthren-2-one, reactions that suggest that the 1,2-oxide (XLVI) is formed as a metabolic intermediate (Sims, 1966). Attempts to prepare this epoxide by the oxidation of the hydrocarbon were unsuccessful: acenaphthalene oxide has likewise been difficult to prepare (Kinstle and Ihrig, 1970). 1,2-Dehydro-3-methylcholanthrene is a potent carcinogen when tested either by subcutaneous injection or by skin painting (Sims, 1967c; Neumann and Thomas, 1967).

Other dihydro derivatives of polycyclic hydrocarbons, such as 5,6-dihydrodibenz[*a,h*]anthracene (Lijinsky *et al.*, 1965), 5,6-dihydrodibenz-[*a,j*]anthracene, and 11,12-dihydro-3-methylcholanthrene (Lijinsky *et al.*, 1970) and 5,6-dihydro-7,12-dimethylbenz[*a*]anthracene (Lijinsky and Garcia, 1972), possess carcinogenic properties. These compounds differ from those described above, however, in that they do not possess olefinic double bonds conjugated with the aromatic ring systems. No investigations appear to have been made into their modes of action.

X. Discussion

The available evidence indicates that oxidative metabolism at the double bonds of aromatic substrates leads to the formation of epoxides.

From what is known of the properties of intermediates of this type, it is therefore tempting to speculate that epoxide metabolites are responsible for many of the biological effects that have previously been attributed to the parent hydrocarbons. This suggestion appears to have something to commend it, since it then permits the formulation of a working hypothesis that associates epoxides with, for example, both the leukemogenic effects of benzene in man (Browning, 1965; Askoy *et al.*, 1972) and the mutagenicity of 7,12-dimethylbenz[*a*]anthracene in *Drosophila* (Fahmy and Fahmy, 1973). Similarly, the hepatotoxic effects of bromobenzene (Reid *et al.*, 1971; Jollow *et al.*, 1972) and the carcinogenicity of dibenz[*a,h*]anthracene (Hartwell, 1951) can also both be logically linked to epoxide formation. The main attraction of this hypothesis is, therefore, that the toxic, mutagenic, and carcinogenic effects of a whole range of compounds can all be associated with one type of metabolic modification. At present the hypothesis remains unproved, and results have been obtained in some investigations that do not appear to support it; however, there is considerable circumstantial evidence in favor of this mechanism.

Other types of reactive intermediate have also been proposed in order to account for the observed effects produced by hydrocarbons. These have included free radicals (Nagata *et al.*, 1967, 1968; Morreal *et al.*, 1968; Hoffmann *et al.*, 1970), radical cations (Fried and Schumm, 1967; Wilk and Girke, 1972), and carbonium ions (Dipple *et al.*, 1968; Cavalieri and Calvin, 1971); in each case, some evidence for their existence, and a discussion of the role that might be played by such intermediates in polycyclic hydrocarbon carcinogenesis, have been presented. These alternative mechanisms of activation remain unsubstantiated and often appear to be applicable to a much less extensive series of compounds than does the epoxide hypothesis.

Investigations of the properties of epoxides derived from polycyclic hydrocarbons have concentrated mainly on "K-region" derivatives. The reasons for this are, first, that "K-region" epoxides can be synthesized by much simpler routes than those that exist for the preparation of non-"K-region" epoxides; this also means that they can be more easily obtained labeled with tritium. Second, the fact that the "K-region" derivatives are more stable than their non-"K-region" counterparts has facilitated their isolation and purification for further study. Last, the epoxides that have been detected as microsomal metabolites have all been found, so far, to be "K-region" derivatives; the failure to detect non-"K-region" isomers is, however, probably due, at least in part, to the experimental methods that have been used.

Although "K-region" epoxides have been studied more extensively, the

principal hydroxylated products formed from polycyclic hydrocarbons in biological systems are frequently non-"K-region" derivatives, presumably also arising, if current thinking is correct, from epoxide precursors. Consequently, it seems quite likely that some of the biological effects of hydrocarbons are mediated through non-"K-region" epoxides; this is obviously an area that merits further investigation.

The hypothesis that the biological effects attributed to hydrocarbons are actually mediated through epoxide metabolites will be difficult either to prove or to disprove conclusively. In a given biological system that possesses a polycyclic hydrocarbon metabolizing capability, the factors governing the effects that are mediated through epoxide metabolites are likely to be complex. In any such situation, the amount of an individual epoxide liberated from the endoplasmic reticulum presumably depends on the difference between (a) the rate at which it is formed, by the action of the microsomal oxygenase, and (b) the rate at which that particular epoxide is converted to the corresponding dihydrodiol by the microsomal epoxide hydrase. If the rate of formation exceeds that of hydration, then free epoxide may leave the endoplasmic reticulum. In the cytoplasm, epoxides can be converted enzymatically into glutathione conjugates, or may isomerize into the corresponding phenols; these reactions presumably compete with those that lead to the covalent reactions of epoxides with cytoplasmic and with nuclear constituents. In addition, the chemical properties of epoxides formed on different double bonds of a hydrocarbon may vary considerably, as will the properties of comparable epoxide derivatives related to different hydrocarbons. If these factors are added to those associated with the specificity of hydrocarbons and of epoxides as substrates for the enzymes involved in their metabolism, a complex situation can be envisaged with regard to the rates at which covalent reactions of epoxides with cellular constituents may occur. These considerations are not made any easier by the present shortage of knowledge concerning (a) the biological effectiveness of the reactions of individual epoxides with various cellular constituents and (b) the critical cellular targets that are involved in hydrocarbon toxicity and carcinogenesis.

Similarly, with regard to the mutagenic effects of epoxides, it is not known at this stage whether phenanthrene 9,10-epoxide, for example, is inactive in reverting frameshift mutations in S. *typhimurium* because it does not react appreciably with the bacterial genome or because the reactions that do occur are not effective in reverting the mutation.

Another possible complicating factor concerns DNA repair. The available evidence suggests that hydrocarbon epoxide-alkylated DNA will be subject to DNA repair in test systems that possess such mechanisms

(Stich and San, 1973). However, the possibility that cellular DNA repair mechanisms are able to deal more efficiently with the damage caused by some hydrocarbons than with that due to others cannot be ruled out at present. If DNA repair mechanisms can effectively deal with DNA alterations caused by reactive hydrocarbon derivatives, and if this DNA damage is perpetuated in a nonrepairable form following the insertion or deletion of a normal base opposite to a hydrocarbon moiety during replication, then in theory, the stage in the cell cycle at which the DNA is damaged should be critical. Reactions with DNA just prior to or during the S phase should be more effective than those occurring at other stages of the cycle. This proposition is strongly supported by data from experiments carried out with polycyclic hydrocarbon epoxides in an *in vitro* transformation system (H. Marquardt, personal communication). The experiments showed that synchronized C3H mouse prostate cells could readily be transformed if treated during the S phase, but were not affected in this way if treated at some other stages of the cell cycle. If the initiation of malignancy is cell-cycle dependent, then it follows that the mitotic activity of a tissue should also affect the susceptibility of that tissue to carcinogenic stimuli. Results that can be interpreted as supporting this corollary have been obtained. Regenerating liver, for example, is known to be more susceptible to the carcinogenic effects of polycyclic hydrocarbons, aromatic amines and urethan (Marquardt *et al.*, 1971/1972; Warwick, 1967; Chernozemski and Warwick, 1970), and confluent cultures of rodent cells are resistant to polycyclic hydrocarbons (Chen and Heidelberger, 1969b) and to X-rays (Borek and Sachs, 1966) that can transform exponentially growing cultures of the same cells. Phorbol esters, which are the active principles present in croton oil (Hecker, 1968; Van Duuren and Sivak, 1968), are potent cocarcinogens (Berenblum, 1941; Berenblum and Shubik, 1947) that are also thought, by some workers to act by stimulating cell division (Ryser, 1971).

Very few of the factors discussed that can influence the formation, further metabolism and biological effectiveness of polycyclic hydrocarbon epoxides have been thoroughly investigated so far. Consequently it is difficult at this time, to make accurate assessments of the relative importance of all the data obtained in the various systems that have been used to test hydrocarbons and their epoxides and other derivatives for biological activity. Variations in these factors may account for the susceptibility of certain tissues in certain species to the biological effects of the hydrocarbons and the apparent resistance of others. For this reason detailed comparative studies of the metabolic activation and inactivation of polycyclic hydrocarbons in "target" and "nontarget" tissues would seem to be worthwhile.

Epoxides may prove to be the biologically important derivatives that are formed from polycyclic hydrocarbons by microsomal metabolism; alternatively, they may not. In either case, the exact molecular mechanism that leads to the initiation of malignancy by hydrocarbons remains obscure, although strong suspicions persist that somatic mutations induced by metabolites are involved. This suspicion has been strengthened as a result of work by Ames *et al.* (1973b). These authors showed that both rat and human liver microsomal preparations were capable of metabolizing several polycyclic hydrocarbons into metabolites that were frameshift mutagens in *S. typhimurium*. More importantly perhaps, Ames *et al.* (1973b) also showed that a variety of other types of chemical carcinogens including aromatic amines, stilbenes, azo dyes and aflatoxins could be converted by metabolism to frameshift mutagens. The feature that is common to all these types of carcinogens is a planar aromatic ring system, which is thought, from work on the acridines (Streisinger *et al.*, 1966) that also possess this type of structure, to be required for frameshift mutagenesis. The acridines form two types of complex with DNA, an internal, intercalated type with the acridine molecules inserted between the base pairs and an external type, where the acridine is stacked on the outside of the helix (reviewed by Gale *et al.*, 1973). The acridine mustards, which carry an alkylating side chain, are more potent mutagens than the acridines themselves (Ames and Whitfield, 1966), and they are thought to act by an initial intercalation into DNA, which is followed by the covalent reaction of the chloroethyl mustard groups on the side chain with an adjacent base (Creech *et al.*, 1972). This sequence of events, in which intercalation precedes covalent reaction, was first suggested by Lerman (1965) and may well apply to polycyclic hydrocarbon epoxides, to aromatic nitroso compounds, and to the microsomal metabolites of the aromatic carcinogens reported to be frameshift mutagens (Ames *et al.*, 1972a,b, 1973b).

The types of mutation induced in mammalian cells by hydrocarbon epoxides (Huberman *et al.*, 1971a) and aromatic amine derivatives (Huberman *et al.*, 1972a) have not been characterized. In *Drosophila*, the bobbed and minute mutations induced by hydrocarbons (Fahmy and Fahmy, 1970) and by aromatic amines (Fahmy and Fahmy, 1972a,b) result from large deletions in the genes for ribosomal and transfer RNA which could arise from frameshift mutations. The idea that frameshift mutagenesis is involved in the initiation of malignancy in some cases is an attractive one since, by the frameshift mechanism, a single molecule of the mutagen may inactivate a whole gene or series of linked genes subsequent to the point of reaction. Although this could mean that, in theory, other alkylating frameshift mutagens are likely to be effective

carcinogens, few data on this are available. While it is true, however, that some compounds, like aflatoxin and benzo[*a*]pyrene, whose metabolites have been found to be frameshift mutagens (Ames *et al.*, 1973b), are potent carcinogens, there are others, like the aliphatic nitrosamines and nitrosamides, which are also potent, but which are very unlikely to act by this mutational mechanism.

An alternative possibility that is gaining support is that, in general, reactions of carcinogens with DNA lead to single- or to double-strand breaks that are inaccurately repaired (Sugimura *et al.*, 1968; Sarma *et al.*, 1973; Cox *et al.*, 1973; Stewart *et al.*, 1973). In cell cultures growing exponentially, reactions with DNA that lead to strand breaks at the start of the DNA-synthetic stage of the cycle may leave insufficient time for repair to be completed before replication begins. Although other types of chemical carcinogens are known to cause breaks in DNA, the polycyclic hydrocarbons and their reactive derivatives have not been examined so far.

It has been reasoned, in reviews of heredity and cancer in man (Knudson, 1973; Knudson *et al.*, 1973), that cancer induction is at least a two-step process. Since there is evidence for hereditary forms of many types of human cancer, which can also arise in a nonhereditary manner, the first step is most probably mutational, using that term in its widest sense. The second step may also result from mutations presumably arising, in both hereditary and nonhereditary cases, either spontaneously or from environmental carcinogenic stimuli.

With regard to human respiratory cancer, Tokuhata (1964) has reported that a familial factor can markedly increase the risk of lung cancer in smokers. Indirect evidence strongly supports the proposal that this familial factor is associated with the inducibility of pulmonary oxygenases.

Nebert and his colleagues have shown in animals that the inducibility of the murine microsomal oxygenase that metabolizes polycyclic hydrocarbons into epoxides is genetically determined (Nebert and Gielen, 1972; Nebert *et al.*, 1972c; Thomas *et al.*, 1972). Using benzo[*a*]pyrene as substrate, these authors have shown that the inducibility of the enzyme is inherited as a simple autosomal dominant trait, and strains of mice in which this enzyme can or cannot be induced by xenobiotics have been described (Nebert *et al.*, 1972b).

In man, a pattern of enzyme induction in cultured lymphocytes that is consistent with Nebert's work on the inheritance of the inducibility of the enzyme in mice has been reported (Busbee *et al.*, 1972; Kellermann *et al.*, 1973c,d). The population can apparently be divided into those with either a low, an intermediate or a high lymphocytic enzyme induci-

bility (Kellermann *et al.*, 1973d). When enzyme inducibility was studied in lymphocytes obtained from subjects with and without pulmonary cancer, many more of the lung cancer patients (30%) were found to fall into the highly inducible group compared with the control subjects (9%). All the lung cancer patients in this study were reported to be heavy smokers. While nothing is known of the inducibility of pulmonary microsomal oxygenases in man, the hereditary pattern may follow that described in lymphocytes. The formation of polycyclic hydrocarbon epoxides by rat lung preparations can be enhanced by suitable pretreatment of the animals with an enzyme inducer (Grover, 1974). The metabolic formation and further metabolism of the "K-region" epoxide related to benz[*a*]anthracene by human lung preparations has also been described (Grover *et al.*, 1973) and benz[*a*]anthracene is a known constituent of tobacco smoke (Kennaway and Lindsey, 1958). Further support for the concept that hereditary variations in the inducibility of microsomal enzymes are important will no doubt be sought; if this is obtained it may strengthen the hypothesis that epoxides are the biologically active intermediates derived from hydrocarbons since epoxides appear to be the primary products of hydrocarbon metabolism.

With regard to carcinogenesis, the polycyclic hydrocarbons all appear to be metabolized by similar pathways to similar types of products, yet some polycyclic hydrocarbons are potent carcinogens whereas others are not. They therefore seem to be an ideal series of compounds in which to investigate structure–activity relationships. Recently, much more information has been obtained concerning their metabolic activation and the properties of the active metabolites that are so formed. This progress, which we have attempted to review in the preceding pages, may conceivably lead, in the near future, to an increased understanding of the molecular mechanisms involved in carcinogenesis.

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VIRION AND TUMOR CELL ANTIGENS OF C-TYPE RNA TUMOR VIRUSES

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I. Introduction

The etiological role of certain RNA-containing viruses in the development of tumors in animals has been proved in many laboratories. In particular, naturally occurring tumors from chicken, mouse, and cat contain virus particles which after cell-free experimental transmission are able to induce tumors in healthy animals. The pathology of such tumors ranges from leukemia through sarcoma and carcinoma. Interestingly enough, there is a close similarity between the various RNA tumor viruses with regard to structure, genome size, and biological behavior, independent of the animal species from which they were isolated. On the basis of minor differences in shape, morphology, and maturation, Bernhard (1960) distinguished between A-, B-, and C-type particles, and this terminology has been widely used since then. Most of the virus strains observed are of the C type. Morphologically different from these are the B-type particles, which so far have been found to have a very narrow range of oncogenicity, as they provoke only mammary carci-

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nomas. A third morphological type, the A-type particles, appear intracellularly and probably primarily represent immature forms of the B-type particles although they have also been observed in C-type particle-producing cells (Anonymous, 1966).

It must be mentioned that a closer analysis of the ultrastructure of RNA tumor viruses (RTV)[2] now allows a further subdivision of these viruses solely on a morphological basis (Dalton, 1972). Thus, for example, C-type viruses of chicken, murine, and feline origin can be distinguished from each other because of ultrastructural differences. Viruses found in the same species, on the other hand, also may not have quite the same morphology, as has become obvious from the study of the reticuloendotheliosis virus (REV). This agent must be classified in a separate virus group from that of the avian sarcoma and leukosis viruses because of its morphological, biochemical, and serological properties (Purchase *et al.*, 1973; Halpern *et al.*, 1973; Mölling *et al.*, unpublished). Furthermore, it is clear that some RNA tumor viruses cannot be classified as either B or C type, for example the Mason-Pfizer monkey virus (Chopra and Mason, 1970; Nowinski *et al.*, 1971) and several viruses detected in human cells (Hooks *et al.*, 1972; Bykovsky *et al.*, 1973; Gelderblom *et al.*, 1974).

Research on oncogenic RNA viruses has made much progress in recent years, and much more is now known about their structure, replication, and genetics, as well as the virus-induced molecular processes linked to the induction of cell malignancy. In addition to biological, biochemical, and genetic approaches, the seroimmunological assay for virus-directed macromolecules (antigens) has been of great importance throughout these studies for several reasons: The serological relationship of individual antigens among different viruses could be tested, and in this way a host specificity could be deduced. Virion antigens served as markers not only for studying productive infection, but also for the detection of inapparent virus-carrier systems.

Many questions about virus antigens remain to be answered. Although

[2] Abbreviations used in this chapter: RTV: RNA tumor viruses of the C type; LV: leukosis/leukemia viruses; SV: sarcoma viruses; prefixed with A (for avian), Mu (for murine) and Fe (for feline) the virus group is specified, for example, MuLV for murine leukemia virus; RSV: Rous sarcoma virus; BH-RSV: Bryan high titer of RSV; SR-RSV: Schmitt-Ruppin-RSV; Pr-RSV: Prague-RSV; G, Gi, F, M, and R, respectively: Gross, Graffi, Friend, Moloney and Rauscher, strain of MuLV; the letters A, B, C, D, and E, respectively in connection with ATV strains will denote the serological subgroup, for example LV-A for leukosis virus of subgroup A. REV: reticuloendotheliosis virus; TSTA: tumor-specific transplantation antigens; TSSA: tumor-specific cell surface antigens; EA, embryonic antigen; CEC: chick embryo cells.

the major protein components of the virion are known, the precise number of proteins that are essential for the building up of the virus structure is uncertain. Likewise, the virus specificity of the virion antigens, i.e., whether they are coded for by the virus or the cell genome, is unknown for most of the antigens. The most interesting question, which proteins are specified by the transforming viral genes, is unanswered. Thus, it is not known which antigens in virus-infected cells have functions other than those related to the virus replication, for example, a regulatory role controlling the expression of viral or cellular genes involved in the transformation process.

It is the purpose of this article to review the current knowledge about structure, function, and immunological properties of C-type virus-directed macromolecules that are immunologically active and therefore approachable by immunological techniques. Throughout this chapter the term antigen will often be used to designate such molecules, essentially because the chemical constitution of many of them is not yet known. The mechanism of virus assembly by a budding process suggests that the virus may contain many host cell constituents that are not essential to the virus structure, but rather represent virus-associated "contaminant." Further, it seems feasible that as a result of virus-induced changes in cell physiology new antigenic determinants appear either by exposure of hidden antigenic macromolecules or through synthesis of new antigens as a result of derepression of cellular genes that are inactive in a normal cell. Therefore, special attention will be given to the question of which of the virion constituents and which of the newly appearing antigens in the cell can be assumed, or have been proved, to be tumor cell coded, and which of them are probably coded for by the virus genome.

It became evident that C-type viruses of different species are closely related with respect to fundamental properties. They mature by budding from the cell membrane, are similar in morphology, size, and constitution of nucleic acid, proteins, lipids, and enzymes, and they require similar cell growth conditions for infection. Therefore, it will be the goal of this article not to review the total relevant literature of C-type particles, but rather to give a general outline of the present knowledge on the subject by reviewing essentially the most thoroughly investigated systems, namely avian (ATV), murine (MuTV) and feline (FeTV) tumor viruses.

All C-type viruses naturally occurring in a given species are commonly called a virus group because they share many properties in common. This definition may not remain entirely valid in the future, because several exceptions have now been found. For purposes of this discussion, the term group will be further used here to designate related C-type viruses of the same natural host species. The term strain or type will define

individual virus populations with either peculiar biological or immunological (virus envelope) properties.

II. Morphogenesis and Ulrastructure

Intact C-type viruses 1200–1400 Å in diameter can only very infrequently be found in the interior of the cell, and then only in certain cell types. In general, the virus is fully assembled during a budding process from the cell surface or into cytoplasmic vacuoles, as was first shown by Haguenau and Beard (1962) and Heine *et al.* (1962). This process may be summarized as follows, taking the avian tumor viruses as a model.

Thin sections of budding particles reveal the following morphology (Fig. 1a): A "unit"-membrane that is continuous with the cell plasma membrane, and is covered with surface projections, evaginates at the point where a crescent-shaped intermediate membrane structure contacts the cell membrane. This intermediate membrane structure is composed of two differentially stained layers. According to its staining behavior, the inner dense layer is associated with the nucleic acid and collapses later during maturation to the electron dense nucleoid in the case of ATV (Fig. 1b) (Feller *et al.*, 1971; Gelderblom *et al.*, 1972a). In mature MuTV, in contrast, this layer is not distinguishable from the core shell (Bader *et al.*, 1970). The outer layer represents the intermediate layer observed in thin sections of mature virus particles. Very rarely, preformed cores, i.e., A-type particles, are observed in the cytoplasm of ATV producing transformed chicken cells (Anonymous, 1966). Whether they are

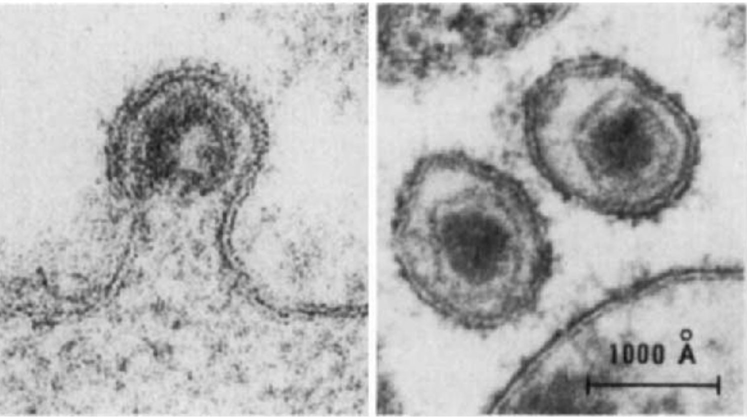

FIG. 1. Ultrathin sections of a budding (left) and mature (right) avian tumor virus particle.

due to an excessive production of virus structural components or to a misled assembly of virus constituents is unknown.

From Fig. 1a it appears that the virus envelope is almost entirely composed of host-derived membrane material while the core may trap cellular constituents. This feature probably explains why the virus contains such large amounts of cellular material.

More refined techniques have given further insight into the structure of the virion. By negative staining it was shown that the surface of the avian myeloblastosis virus (AMV) is covered by projections (Bonar *et al.*, 1963; Eckert *et al.*, 1963) 60 Å in length with a knoblike end 50 Å in diameter (Gelderblom *et al.*, 1972a). This can be assumed to be characteristic for all avian C-type particles (with the possible exception of REV-like particles) because the same has been reported for other ATV strains. This assumption is also based on the recent findings that the proteins carrying the virus type-specific antigens are constituents of virus surface projections. By treatment of purified virus with nonionic detergents, the projections could be released and isolated in a pure form as aggregates that possessed type-specific antigenicity and reacted with neutralizing antibody (Bolognesi *et al.*, 1972a). Likewise, the projections could be tagged by ferritin-labeled neutralizing antibody (Gelderblom *et al.*, 1972b; see also Section IV,A,1).

Murine and feline C-type particles reveal similar projections although these are less distinct (Nermut *et al.*, 1972) and possibly more fragile, characteristics that might explain the failure of some authors to have detected them with certainty (de Harven, 1968; Nowinski *et al.*, 1970). The length of the projections is a morphological parameter that allows the differentiation between different groups of these viruses. It has been proved that, like the avian C-type particles, the surface projections of C-type particles of mammalian species also carry the type-specific antigens (Witter *et al.*, 1973a; Moennig *et al.*, 1973).

Negative staining and freeze-etching shed further light on the structure of the virus core. Virus particles from which the outer membrane seems to have been removed are very infrequently observed. These reveal capsomere-like subunits in a hexagonal arrangement (Dourmashkin and Simons, 1961). On the basis of similar pictures, Padgett and Levine (1966) suggested a cubic symmetry of the virus capsids. Their suggestions have been confirmed recently by two other groups using different methods. In the case of murine viruses, the shadowing of freeze-etched particles reveals a hexagonal arrangement of capsomere-like structures (Nermut *et al.*, 1972). The capsomeres surround the core in a cubic symmetry. This is supported by the cubical appearance of some of the cores after isolation from AMV with nonionic detergent and ether;

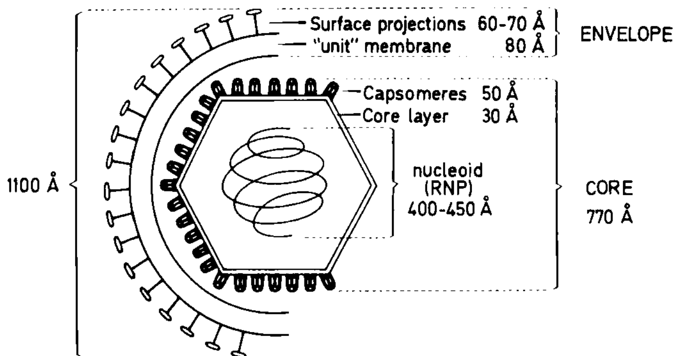

FIG. 2. Schematic representation of the structure of avian tumor virus.

these data also led to the assumption of a capsomere shell in the core (Bolognesi *et al.*, 1972b; Gelderblom *et al.*, 1972a).

A filamentous material, which has been assumed to be a ribonucleoprotein (RNP) complex similar to that of myxoviruses (Eckert *et al.*, 1963), can be eventually observed in the core of ATV (Bauer, 1970). Several groups have recently isolated and purified a RNP complex consisting of the high molecular weight viral RNA and a viral protein of 12,000 d molecular weight (Coffin and Temin, 1971; Quigley *et al.*, 1972a; Davis and Rueckert, 1972; Bolognesi *et al.*, 1973a). However, it is still uncertain whether either the morphologically or the biochemically defined complex is really helical in structure as in the case of myxoviruses. Helical structures have also been observed in murine viruses (Nermut *et al.*, 1972), but neither have as yet been analyzed biochemically.

The diameter of the electron dense nucleoid is 750 Å for murine and only 350–450 Å for avian viruses (Gelderblom *et al.*, 1970; Feller *et al.*, 1971). This, in addition to the morphology of surface projections and the distance between the two core layers of the developing bud (Dalton, 1972), allows further a differentiation between mammalian and avian C-type particles.

A diagrammatic scheme of the complete structure of avian C-type particles is shown in Fig. 2. It is similar to the one proposed for murine C-type viruses by Padgett and Levine (1966) and Nermut *et al.* (1972; see also Bolognesi, 1973).

III. Physical Properties and Chemical Composition

The value of 1.15 to 1.17 gm/cm^3 for the buoyant density in sucrose is characteristic for RTV. This density may vary within a virus strain, depending on the host cell in which the virus is grown. For example, the

buoyant density of AMV in CsCl is 1.20 gm/cm^3 when grown in myeloblasts and 1.22 gm/cm^3 when grown in fibroblasts (Bauer and Schäfer, 1967). This difference might be due a higher lipid content of the AMV-grown from myeloblasts.

As a first estimation of the overall chemical composition of the virion Bonar and Beard (1959) calculated 60% protein, 35% lipids, 2.2% nucleic acid and 0.5% carbohydrate. Besides ribonucleic acid (RNA) the virus also contains small amounts (0.04%) of deoxyribonucleic acid (DNA) which seem to be cellular in origin (Riman and Beaudreau, 1970; Levinson *et al.*, 1972).

The pattern of the lipids was investigated by several authors with divergent results. While Rao *et al.* (1966) found it similar to that of the cell nuclear membrane, others found it to be related to that of the outer cell membrane (Quigley *et al.*, 1972b,c).

The nucleic acid consists of single-stranded ribonucleic acid molecules of five different size classes. After phenol or SDS extraction, the bulk of the RNA sediments in a sucrose gradient with 60 to 70 S, depending on the virus strain studied (Bolognesi and Graf, 1971; Scheele and Hanafusa, 1971). From these studies, the variation in size did not seem to be correlated with biological virus properties. Duesberg (1968) has suggested that the high molecular weight (HMW) RNA consists essentially of subunits that seem to be linked by hydrogen bonds. The subunit size of the RNA after heating or dimethyl sulfoxide treatment is 30–40 S or about one-quarter of the 60–70 S RNA length. By gel electrophoresis, two size classes, "a" and "b," could be distinguished (Duesberg and Vogt, 1970), which seem to be charactesistic for sarcoma ("a") and leukosis ("b") viruses, respectively (Martin and Duesberg, 1972; Duesberg and Vogt, 1973a).

It has to be mentioned at this point that, for purpose of discussion, virus strains that transform chicken fibroblasts *in vitro* are called sarcoma viruses (SV), and viruses that replicate in chicken fibroblasts without morphological cell transformation are called leukosis viruses (LV). Mutant derivatives of SV, which in that respect behave similarly to LV (Toyoshima *et al.*, 1970; Goldé, 1970; Graf *et al.*, 1971), will also be called LV for this discussion, and indeed some of them have been tested *in vivo* and have in fact been found to induce nonsarcomagenic tumors (Biggs *et al.*, 1973).

Class "a" RNA of a given SV strain probably contains all information of class "b" RNA of the LV derived by mutation from that SV (Lai *et al.*, 1973). Class "a" RNA contains about 350,000 d nucleic acid, which in addition is presumed to contain the genetic information responsible for the transformation of fibroblasts by SV (Duesberg and Vogt, 1973b).

It cannot yet be decided whether the three to four RNA subunits of either class are unique or whether they consist of identical RNA sequences, i.e., whether the viral genome is haploid or polyploid.

The residual RNA species in the virion, which represent 30% of the total RNA sediment with molecular weights of 28 S, 18 S, 7–9 S, and 4–5 S. These RNA fractions are probably of cellular origin, as was stated for ATV (Bauer, 1966; Bonar *et al.*, 1967) and MuTV (Wollmann and Kirsten, 1968). Others did not detect this, probably because of short pulse labeling (Bader and Steck, 1969). The fractions cannot be removed from the virus by additional purification procedures and represent ribosomal (18 S, 28 S) (Bonar *et al.*, 1967; Obara *et al.*, 1971) and transfer (4–5 S) RNA (Travnicek, 1969; Carnegie *et al.*, 1969; Erikson and Erikson, 1970). The function of the 7–9 S RNA has not been determined (Bishop *et al.*, 1970).

Probably as a result of the maturation process, C-type viruses contain several cellular enzymes, some of which presumably have a function in the infectious cycle of the virus. After the reverse transcriptase had been detected in RNA tumor viruses (Temin and Mizutami, 1970; Baltimore, 1970), other enzyme activities, mostly involved in nucleic acid replication were detected (Mizutami and Temin, 1971; for review, see Temin and Baltimore, 1972; Sarin and Gallo, 1973). However, more studies are necessary to decide whether any of the enzymes other than reverse transcriptase are virus specific in the sense that it is virus coded or virus induced.

From a technical point of view, the detection of adenosine triphosphatase (ATPase) in AMV was of some importance (Mommaerts *et al.*, 1954) because a quantitative assay of that virus was possible by measuring the amount of enzyme in association with virus from leukemic chicken plasma. When the virus is grown in fibroblasts instead of myeloblasts, it is almost free from ATPase probably because the surface of fibroblasts is poor in that enzyme (de Thé, 1964; Bauer *et al.*, 1965). Similar results have been obtained with an RNA-digesting enzyme (Bauer, 1966).

From the problems described above in defining viral proteins and the physical nature of the viral genome, one can understand the special difficulties which arise in studying the structure and virus-specific constituents of these viruses and also why many problems relevant to that question are still unsolved. It is noteworthy that there is no general technique that could be applied to different viruses in order to isolate the viral subunits. For example, even within one and the same virus group great differences were found in the resulting virus substructures after detergent or ether treatment of leukosis and sarcoma viruses (Bolognesi *et al.*, 1972a).

IV. Virus-Specific Structural Proteins

The investigation of virus proteins by immunological and biochemical means requires large quantities of material. Most of the early work was performed in the avian system, since the recovery of up to 10^{12} AMV particles per milliliter in the plasma of AMV-leukemia diseased chickens is possible (Mommaerts *et al.*, 1954). It was from such virus that the solubilization and purification of a virus antigen was first achieved (Eckert *et al.*, 1964a,b). The antigen in question was protein in nature and group specific (gs) for the ATV (Bauer and Schäfer, 1965) deriving from the interior of the virion (Bauer and Schäfer, 1966). It was identical with a virus-induced antigen found in nonvirus-producing hamster tumors (Huebner *et al.*, 1964; Payne *et al.*, 1966; Kelloff and Vogt, 1966; Bauer and Janda, 1967).

The other serologically reactive component was the type-specific virus envelope (Ve) antigen, which was postulated on the basis of biological experiments to be serologically different in individual virus strains. It was mainly the work of Vogt and colleagues (Vogt, 1965; Vogt and Ishizaki, 1965, 1966; Vogt *et al.*, 1967), Hanafusa (1965), and Payne and Biggs (1966) which led to the subdivision of the avian RNA tumor viruses into several subgroups (now subgroups A through E) on the basis of related envelope antigens, a systematics which has greatly facilitated the study of these viruses. Viruses within one subgroup express cross-neutralizing antigens and have the same host range. This is determined by the presence of "receptors" on the host which are genetically defined (for review, see Crittenden, 1968; Payne *et al.*, 1971; Payne, 1972) and necessary for virus penetration (Steck and Rubin, 1966).

The situation was similar for the other RNA tumor virus systems. There was a group-specific antigen of murine viruses defined by immunoprecipitation (Geering *et al.*, 1966) and complement fixation (Huebner *et al.*, 1966), and there were strain-specific antigens which allowed a distinction between the naturally occurring AKR mouse strain or Gross(G)-virus group and the Friend–Moloney–Rauscher (FMR) virus group (Old *et al.*, 1965).

The development of high-titer virus strains, of virus-producing permanent cell lines in the mammalian system, and of new biochemical techniques stimulated the study of virus proteins, and during the last few years has resulted in the accumulation of a large amount of information, which today allows a fair understanding of the structure and the serological characteristics of these viruses.

For purposes of a coherent survey, a nomenclature will be used for this discussion which indicates some biological and biophysical proper-

ties of the individual antigens reported, and which was adapted at a colloquium held at the Sloan-Kettering Institute for Cancer Research, June 4–5, 1973. The antigens of protein nature will be termed p, with an index number for the approximate value of the molecular weight × 10^{-3} as estimated on a guanidine hydrochloride (GuHCl) column; and the glycoproteins will be designated gp, with an index number that indicates the MW range as estimated by SDS–polyacrylamide gel electrophoresis (PAGE). This is made possible by comparison of the serological and other properties of the protein isolates as reported by various laboratories.

A. Avian Viruses

1. *Envelope Antigens*

The isolation of an antigen with all the properties of type-specific Ve antigen was reported only recently (Bauer *et al.*, 1970; Tozawa *et al.*, 1970). By treatment of AMV with Triton X, an antigen was released that did not react with gs antibody but was precipitated in a double immunodiffusion test by neutralizing sera. This antigen induced neutralizing antibody and interfered with the infection of cells with virus of the same subgroup by blocking the respective cell receptors. Its subgroup- and type-specific reaction was further demonstrated by showing that Ve antigens isolated from viruses of subgroups A and B were not identical in the immunodiffusion test.

The biochemical analysis of the Ve antigen from AMV and other ATV strains including Rous sarcoma virus (RSV) revealed their glycoprotein nature (Duesberg *et al.*, 1970; Bolognesi and Bauer, 1970; Bauer and Bolognesi, 1970; Fleissner, 1971). The same studies showed that ATV contain at least two glycoproteins with molecular weights (MW) of 37,000 (gp 37) and 85,000–115,000 (gp 85) daltons (d), respectively, as estimated by SDS-PAGE in comparison with standard proteins.

Second (Hung *et al.*, 1971) and third (Scheele and Hanafusa, 1971) minor glycoprotein fractions in the high MW (HMW) region of PAGE were described for the Bryan high titer (BH) strain of RSV, which is defective for the synthesis of an envelope antigen conferring infectivity on the virus (Hanafusa *et al.*, 1963, 1964), and they seemed to represent the defective glycoprotein of that virus strain. The major glycoprotein (gp 85) of viruses from different subgroups differs in electrophoretic mobility when tested in the SDS-PAGE (Robinson *et al.*, 1970). A distinct difference has also been observed in the size of glycopeptides from sarcoma and leukosis viruses of the same subgroup (Lai and Duesberg, 1972). Glycopeptides of viruses released from SV-transformed cells are

larger than the respective peptides of viruses released from LV-infected cells. Interestingly, the respective glycopeptides of LV when synthesized in transformed cells are also of the large type. It could not yet be decided whether viral or cellular genes are responsible for that phenomenon. These data suggest that the carbohydrate part of the polypeptide does not significantly influence the antigenic properties of the Ve antigen, since no differences have been found in neutralization tests between SV-derived nontransforming viruses and the parental SV strains (Toyoshima *et al.*, 1970; Graf *et al.*, 1971). Although differing in the carbohydrate portion of the oligopeptides, such viruses exhibit identical Ve antigen. Immunological investigations with isolated glycoproteins of ATV (see below) are in agreement with the apparent independence of immunological specificity of Ve antigen from the carbohydrate structure.

The glycoproteins have been shown by several means to be the constituents of the virus envelope projections. By bromelain treatment of SV, the surface projections are removed with a concurrent loss of infectivity (Rifkin and Compans, 1971). After treatment of AMV with nonionic detergent rosettelike aggregates of the envelope projections from two LV and one SV strain were obtained in a morphologically homogeneous form (Bolognesi *et al.*, 1972a). They consisted only of the two glycoproteins gp 37 and gp 85 and contained, like the material isolated by Tozawa *et al.* (1970), all the properties of Ve antigen. In addition, the rosettes reveal further antigenic determinants that are not identical with the subgroup-specific Ve antigen. The specificity of these antigens does not follow the subgroup specificity of the virus, but rather the type of host cell. This second kind of antigen was identical for SV of subgroup A (SV-A) and LV of subgroup B (LV-B) strains, both grown in fibroblasts, and it was different for two LV-B strains, one of which was grown in fibroblasts and the other in myeloblasts (Bauer *et al.*, 1973). Furthermore, this second antigenic determinant of the glycoproteins was different in the gp 37 and the gp 85 of a given virus. It was assumed that this antigen is defined by the carbohydrate part of the virus projections, which might vary among different host cells. A similar conclusion was drawn from the biochemical analysis of host antigens in influenza virus (Laver and Webster, 1966).

Whether the two strain-specific antigens of a given virus as contained in gp 37 and gp 85 are identical or different—for example, one being type specific and the other subgroup specific—is not known. Two such envelope antigenicities were suggested on the basis of partial cross neutralization effects between different virus strains (Bauer and Graf, 1969). The antigenic properties of only the protein part of gp 37 and gp 85 have not been tested because it is difficult to remove quantitatively

the carbohydrates from the glycoproteins. Since some changes of the carbohydrate portion of the glycoproteins are not correlated with a change of the Ve antigenicity, one might suspect that the protein part of the glycoproteins carries the type-specific neutralizing determinant, and that this is virus coded. There is also some preliminary evidence derived from *in vitro* protein synthesis experiments that the viral HMW RNA serves as messenger for proteins of that size (Siegert *et al.*, 1972).

A virus membrane fraction was isolated by enzyme and detergent treatment of AMV that was free of type specific and internal gs-antigens, respectively. By analysis in SDS-PAGE it contained two components migrating faster than the 10,000 d protein, one of which was a polypeptide and the second a glycolipid, as was shown with specific staining (Ishizaki *et al.*, 1973). The serological analysis of this virus membrane (Vm) material indicated that it is host cell derived and not identical in viruses originating from cells of different host species.

Recent studies indicated that the smallest of the major polypeptide constituents of the virion (MW 10,000) is weakly glycosylated and may be situated in the virus envelope (Bolognesi *et al.*, 1973). It has not yet been precisely localized and attributed to a morphological substructure.

2. *Internal Virion Antigens*

The existence of more than one internal antigen was suggested (Berman and Sarma, 1965; Bauer and Janda, 1967), but convincing evidence was not presented until Duesberg *et al.* (1968) reported the separation of two major proteins and one minor one by SDS-PAGE after phenol extraction of SV proteins; the two major components fixed complement in the presence of gs antiserum. Up to 5 immunoprecipitation lines were described with viral or virus-induced cellular antigens by using gs antigen-reactive hamster and chicken sera (Roth and Dougherty, 1969; Armstrong, 1969).

Further analysis with respect to the number of antigens, their isolation and characterization by biophysical, biochemical, and serological methods followed soon afterward. After PAGE of phenol-SDS extracted proteins of several ATV strains, four major and several minor protein fractions were regularly observed (Bolognesi and Bauer, 1970). The major components, designated CF 1 through CF 4 with decreasing electrophoretic mobility had molecular weights between 13,000 d and 28,000 d (see Table I). They were isolated by preparative SDS-PAGE, and shown to be antigenically different from each other and from Ve antigen by gel-immunoprecipitation tests. The respective group-specific antisera were either prepared in rabbits against SDS-dissociated AMV or taken from Rous tumor-bearing hamsters (Bauer and Bolognesi, 1970).

TABLE I

NOMENCLATURE AND PROPERTIES OF STRUCTURAL PROTEINS OF AVIAN TUMOR VIRUS

Nomenclature used in this article	Allen (1968, 1969), Allen *et al.* (1970)	Bolognesi and Bauer (1970), Bolognesi *et al.* (1972a)	Duesberg *et al.* (1968, 1970)	Fleissner (1970)	Hung *et al.* (1971)	MW[c] (d)	Chemical nature	Immunological specificity
p 10		CF 1[a]	RSV 1[a]	p 5	P 8	10,000	(Glyco) protein	?
p 12		CF 2	RSV 2	gs 4	P 7	12,000	Protein	gs
p 15	gs b	CF 1[a]	RSV 1[a]	gs 3	P 6	15,000	Protein	gs
p 19		CF 3		gs 2	P 5	19,000	Protein	gs + type-specific
p 27	gs a	CF 4	RSV 3	gs 1	P 4	27,000	Protein	gs
gp 37		G I	I	m 1	P 3	37,000	Glycoprotein	Type + cell specific
gp 85		G II	II	m 2	P 2	85,000	Glycoprotein	Type + cell specific

[a] p 15 and gp 10 appear as one band in polyacrylamide gel electrophoresis (PAGE).

[b] Different nomenclatures were used by these authors for different isolation procedures; the one listed here was taken from Table II (BioGel chromatography).

[c] MW of p 10, p 12, p 15, p 19, and p 27 is indicated as determined by BioGel chromatography because of the better resolution obtained in comparison to SDS-PAGE; MW of gp 37 and 85 is indicated as determined by SDS-PAGE because after GuHCl extraction gp 37 appears to remain associated with the viral lipid envelope (K. Mölling and H. Bauer, unpublished).

The pattern of the major internal proteins is similar for all virus strains investigated (Bolognesi and Bauer, 1970; Robinson *et al.*, 1970; Scheele and Hanafusa, 1971; Fleissner, 1971; Nowinski *et al.*, 1972a) (Table I) and suggests an identical or closely similar structure for all viruses. In addition, the group specificity for some of these antigens has recently been clearly shown by comparison of the individual proteins from a subgroup B and a subgroup C virus, respectively, with monospecific antisera (Bolognesi, personal communication).

The proteins can be isolated with the same resolution as with PAGE by isoelectric focusing (Robinson *et al.*, 1970). Superior to both methods, however, is the separation by GuHCl-BioGel column chromatography as introduced by Fleissner (1971) in that field of research. The antigenicity of the proteins is better preserved and the resolution in the low MW range is more accurate. The fastest migrating band in PAGE appears to consist of two polypeptides with 10,000 d (p 10) and 15,000 d (p 15) MW, respectively, as estimated by molecular sieve chromatography. P 10 has not been obtained antigenically active thus far and seems to be glycosylated and a component of the virus envelope (Bolognesi *et al.*, 1973).

Until recently, little was known about the amino acid composition of the virus proteins. The terminal amino acids, the amino acid composition, and a sequence of about 20 amino acid residues of the polypeptides gs a and gs b have been determined showing that these two polypeptides are distinct by these criteria (Allen, 1968, 1969; Allen *et al.*, 1970). Gs a and gs b are probably identical with p 27 and p 15, respectively.

An extensive biochemical study of the four polypeptides p 12 through p 27 has been performed most recently by Bolognesi and co-workers (personal communication). The peptide pattern, the amino acid composition, and the terminal amino acids have been determined for the polypeptides isolated from AMV-B and Prague (Pr)-RSV-C. The various polypeptides of a given virus were distinct from each other, but the corresponding polypeptides of the two viruses were identical with the single exception of p 19, which contains one peptide that is unique for each virus strain. These results are in good agreement with immunological data; both immunological and biochemical analysis fail to give any support to the proposition that the smaller polypeptides are fragments of the larger.

The initial suggestion that the gs antigens are internal components of the virion was based on the observation that they do not react with neutralizing antibody and are not accessible to antibody in the intact virion (Payne *et al.*, 1966; Kelloff and Vogt, 1966; Bauer and Schäfer,

1966). The exact localization of gs antigens in the virion remained unknown until virion substructures were isolated and investigated for their constituents. By isopycnic centrifugation of RSV treated with various detergents, material was obtained that appeared in the electron microscope as core structures and contained HMW RNA and some gs antigen (Bader *et al.*, 1970). By combined treatment with nonionic detergent and ether, a homogeneous material was isolated from AMV at a density of 1.25 gm/cm^3 in sucrose gradient (Bolognesi *et al.*, 1972b; Gelderblom *et al.*, 1972b). After negative staining the isolated structures were of a similar size and morphology to the virus core. The material contained HMW RNA, most of the reverse transcriptase activity, and the polypeptide p 27. Interestingly, these cores were infectious for chick embryo cells and induced the synthesis of infectious progeny which had all the properties of intact virus (Bolognesi *et al.*, 1972b).

A more generally applicable method that allowed the quantitative isolation of morphologically homogeneous cores was described by Stromberg (1972; Stromberg *et al.*, 1973), who disrupted AMV with the surfactant Sterox-SL. Cores obtained with this treatment appear more electron dense within the core shell and contain more polypeptides (Bolognesi *et al.*, 1973) than cores isolated with the aid of ether, but have about the same buoyant density in sucrose (ρ = 1.25 gm/cm^3). Besides HMW RNA and reverse transcriptase, the major constituents of these cores are p 27 and an enriched fraction of p 35, which is also observed after ether detergent treatment (Bolognesi *et al.*, 1972b) and detectable only as a minor fraction on the total virus proteins. In addition, these cores contain p 15 and p 12, both of which are probably almost quantitatively released from the core by ether treatment. Further treatment of that material with Sterox SL yields a component with a higher density, a ribonucleoprotein complex consisting essentially of the HMW RNA, the reverse transcriptase complex, and p 12, the most basic polypeptide (Bolognesi *et al.*, 1973). It resembles the nucleoid and has also been isolated from Triton X (Coffin and Temin, 1971; Davis and Rueckert, 1972) or NP-40 treated RSV (Quigley *et al.*, 1972a; Fleissner and Tress, 1973b).

The significance of the several minor polypeptide components with MW above 35,000 d which have been found in the core or nucleoid preparations (Davis and Rueckert, 1972; Quigley *et al.*, 1972a; Bolognesi *et al.*, 1972b, 1973) has not been elucidated except for the two constituents of the reverse transcriptase (Kacian *et al.*, 1971; Watson *et al.*, 1973) (see next Section).

It has tentatively been suggested, that p 27 represents the major constituent of the core shell, and that p 15 with the highest percentage of

hydrophobic amino acid residues represents the capsomeres of the core (Bolognesi, 1973; Bolognesi *et al.*, 1973). The p 19 protein, which does not appear to be a constituent of the core at all and has not been detected at the virus surface, might be located beween core and virus membrane, connecting these two structural elements.

3. *Enzymes*

It was noted above that often the virus contains cellular enzymes such as RNase or ATPase. There is convincing evidence now that the virion bears also virus-specific enzymes in the sense that they are virus induced or virus coded. Since the discovery of the reverse transcriptase (Temin and Mizutani, 1970; Baltimore, 1970), the work of many authors has revealed that all infectious RNA tumor viruses contain such an enzyme, which is capable of transcribing RNA into DNA and also of synthesizing DNA with DNA as template. There is overwhelming evidence that this enzyme function represents a necessary step in the replication cycle of RNA tumor viruses, which allows the genome to become integrated as a DNA provirus into the cellular DNA (for review, see Temin and Baltimore, 1972). The significance of the polymerase for virus infectivity has been best demonstrated with a mutant of the BH-RSV strain [RSVα(O)], which seems to be defective for polymerase synthesis (Hanafusa and Hanafusa, 1971; Hanafusa *et al.*, 1973), and by two ts mutants of RSV, which are noninfectious at high temperature because their DNA-polymerase is temperature sensitive (Verma *et al.*, 1974).

The enzyme can be released after disruption of the virus (Temin and Mizutani, 1970; Baltimore, 1970) because of its location in the viral core (Bolognesi *et al.*, 1972b). More precisely, it is associated with the HMW RNA containing nucleoid (Coffin and Temin, 1971; Davis and Rueckert, 1972; Stromberg, 1972; Bolognesi *et al.*, 1973). Enzyme preparations which have been highly purified by various means contain only two polypeptides of 70,000 d and 110,000 d MW (Kacian *et al.*, 1971; Watson *et al.*, 1973; Grandgenett *et al.*, 1973). Immunological studies have shown the nonidentity of the enzyme with the 7 major structural polypeptides of AMV (Watson *et al.*, 1972; Nowinski *et al.*, 1972b). The polymerases are antigenically related among the individual ATV strains (Parks *et al.*, 1972), but distinct from the enzymes of mammalian RNA tumor viruses (Scolnick *et al.*, 1972b,c).

A ribonuclease (RNase H) that specifically degrades the RNA moiety in an RNA–DNA hybrid, but not single- or double-stranded RNA or DNA, has been detected in partially purified polymerase preparations from AMV (Mölling *et al.*, 1971). Its existence has been confirmed for avian viruses (Keller and Crouch, 1972; Baltimore and Smoler, 1972;

Leis *et al.*, 1973) and extended to RNA tumor viruses of other species (Grandgenett *et al.*, 1972). The RNase H could not be separated from the polymerase of AMV and is inhibited by antiserum prepared against highly purified enzyme (Keller and Crouch, 1972; Baltimore and Smoler, 1972; Leis *et al.*, 1973; Watson *et al.*, 1973).

Whether the two polypeptide constituents of the enzyme are both required for all the enzyme activities of that complex, or whether the subunits have individual functions is not quite clear as yet. A recent publication by Grandgenett *et al.* (1973) suggests that the smaller polypeptide alone is sufficient for all enzyme functions: besides a major fraction containing the $\alpha\beta$ complex of both polypeptides, the α-polypeptide of 70,000 d was isolated as a minor fraction by phosphocellulose chromatography and demonstrated to have the properties of both DNA polymerase and RNase H. Such a small enzyme fraction has also been found by others (Faras *et al.*, 1972; Hurwitz and Leis, 1972), but was not further investigated. These data need confirmation and extension. Likewise, a protein that stimulates the reaction rate and yield of DNA synthesized by the reverse transcriptase has been isolated from AMV by one group (Leis and Hurwitz, 1972), and its existence awaits confirmation.

The properties of the enzyme complex have been extensively studied by many laboratories (for review, see Temin and Baltimore, 1972), and especially the question of virus specificity has been the focus of much attention. It now appears to be clear that the polymerase of ATV as well as of other C-type viruses is different from similar enzymes found in uninfected cells (Weissbach *et al.*, 1972; Smith and Gallo, 1972; Robert *et al.*, 1972; Mizutani and Temin, 1973). Likewise the RNase H which has exonucleolytic activity has been found to be different from cellular or bacterial RNase H which have endonuclease activities (Keller and Crouch, 1972; Leis *et al.*, 1973; K. Mölling, unpublished).

B. Mammalian Viruses

When it was detected that mammalian C-type viruses share an internal antigen (Geering *et al.*, 1968), the hope arose that hypothetical human RNA tumor viruses might be related to mammalian viruses by sharing that same antigen and therefore be detectable with antisera prepared against the model animal viruses. This hope has provoked a great deal of effort in many laboratories with the goal of isolating and characterizing the antigens of mammalian C-type viruses. Since the murine system is the best developed of all mammalian systems, most of the energy was spent with murine C-type viruses. However, enough information has accumulated recently also with viruses of other mammalian

species, to indicate a close similarity of these agents with respect to number and biophysical and biochemical properties of the protein constituents for all known mammalian C-type viruses.

The progress made in the last few years is remarkable. However, comparison of the results reported by the various laboratory groups leads to a little confusion owing to the variety of isolation and characterization procedures, and also to the lack of a common nomenclature.

1. *Envelope Antigens*

C-type viruses of mammalian origin are far less definitively classified according to their envelope antigens as are the avian viruses. Only for feline viruses have three subgroups—A, B, and C—been distinguished by interference and cross neutralization tests (Sarma and Log, 1973). There is also supporting evidence for the proposition that feline RNA tumor viruses may enter the cell via receptor sites specific for individual viral envelope antigens (Jarret *et al.*, 1972).

Like ATV and FeTV in their respective host systems, the MuTV exhibit differing efficiencies of infectivity among the various mouse strains. However, these differences cannot serve as a useful classification, since host-range specificities are not Ve antigen dependent and are neither absolute nor stable (Hartley *et al.*, 1970; Huang *et al.*, 1973; Rowe *et al.*, 1973; Lilly and Steeves, 1973; Lilly and Pincus, 1973).

The G (for Gross virus) and the FMR (Friend, Moloney, Rauscher) virus serogroups have been distinguished (Boyse *et al.*, 1964; Old *et al.*, 1965) according to the type of virus-induced cell membrane antigens observed. These characteristic cell membrane antigens have been thought for years to be identical with the Ve antigen. Two recent systematic studies comparing a large number of virus strains in cross neutralization tests employing mainly murine antisera have confirmed the earlier neutralization experiments (Steeves and Axelrad, 1967) and thereby the de facto subdivision of murine C-type viruses into the G- and the FMR groups (Eckner and Steeves, 1972; Gomard *et al.*, 1973). Partial cross reactions and different patterns in cross neutralization tests within the FMR group as well as with other virus strains (Eckner and Steeves, 1972) led Gomard *et al.* (1973) to postulate two additional serogroups. This is probably not justified under the criteria used for the avian system (see Section IV,A), but rather reflect strain-specific virus envelope antigens. Other classifications can probably be obtained by the use of heterologous immune sera that might detect group-specific and interspecies-specific determinants at the virus surface (see below; McCoy *et al.*, 1968).

Glycoproteins of the murine viruses, the probable carrier molecules

of the type-specific Ve antigen, have been described by several groups, and the molecular weights estimated were from 42,000 d to 60,000 d for the smaller glycoprotein (gp 45) and from 70,000 to 93,000 d for the larger (gp 70) (Duesberg *et al.*, 1970; Oroszlan *et al.*, 1971a; Moroni, 1972; Schäfer *et al.*, 1972b; Nowinski *et al.*, 1972a; Witter *et al.*, 1973b; Shanmugan *et al.*, 1972; Bolognesi, 1973). Similar findings were reported for hamster and cat C-type viruses (Nowinski *et al.*, 1972a; Bolognesi, 1973). Only a few workers have focused on the isolation and further characterization of Ve antigen of mammalian, and specifically, murine C-type viruses. Both glycoproteins of R-MuLV have been identified as constituents of the viral membrane by immunoelectron microscopy (Nowinski *et al.*, 1972a). By the same bromelain method as that used by Rifkin and Compans (1971) for RSV, the glycoproteins of F-MuLV were identified precisely as the virus surface projections (Witter *et al.*, 1973a), which have escaped the notice of many investigators but were clearly demonstrated by Nermut *et al.* (1972). They probably contain three antigenic determinants, one that is type specific (II v in Table II), one that is group specific (II gs), and one that is demonstrable with nonneutralizing FeLV antiserum that is shared by viruses of different groups, i.e., among viruses isolated from different animal species (Schäfer *et al.*, 1972b; Witter *et al.*, 1973a,b; Moennig *et al.*, 1973). This latter antigen is therefore interspecies group specific, and hence has been also called interspec. An interspec determinant present in two R-MuLV proteins of HMW (69,000 d and 71,000 d, respectively) has been described by Strand and August (1973). It is likely that one or the other of these polypeptides could be identical with gp 70.

Two Ve antigen specificities, one strain specific and the other group specific for the G and the FMR viruses, are also suggested by cross neutralization and immunoferritin labeling tests (Gross, 1965; Geering *et al.*, 1966; Igel *et al.*, 1967; Fefer *et al.*, 1967b; Levy *et al.*, 1969; Aoki and Takahashi, 1972; Ferrer, 1973).

The isolation of the viral surface projections from mouse C-type viruses by Tween-80 ether treatment has resulted in material of low density that is probably contaminated with lipids (Schäfer *et al.*, 1972b; Witter *et al.*, 1973a). The tendency of the surface projections to be released spontaneously from the virus surface, which is probably the reason for the difficulty in detection, was used to recover this material in a fairly pure form which exhibited properties similar to the Ve antigen of ATV (Moennig *et al.*, 1973). The material consisted of two glycoproteins; it absorbed neutralizing antibody and interfered with cell adsorption of virus with related Ve antigen. When made multivalent by treatment with nonneutralizing antibody directed against the gs component (II gs) of

TABLE II
NOMENCLATURE AND PROPERTIES OF STRUCTURAL PROTEINS OF MuTV

Nomenclature used in this article	Bolognesi (1973); Green *et al.* (1973)	Duesberg *et al.* (1970)	Moroni (1972)	Nowinski *et al.* (1972a)	Schäfer *et al.* (1972a,b, 1974)	Shanmugam *et al.* (1972)	MW (d)	Chemical nature	Immunological specificity
p 10	P 1		I	p 4	I	I	10,000	Protein	gs
p 12	P 2			p 3	III	III	12,000	(Glyco) protein	Type?
p 15	P 3		II	p 2	III?	II	15,000	Protein	gs
p 31	P 4		III	p 1	IV, V	IV	31,000	Protein	gs; interspec a,b
gp 45		I	IV	m 1			45,000	Glycoprotein	Type specific
									gs
gp 70	G 2	II	V	m 2	II v, II gs		70,000	Glycoprotein	Interspec

[a] MW of p 10, p 12, p 15, p 31 is indicated as determined by BioGel-chromatography; MW of gp 45 and gp 70 is an average of the determinations performed by the various groups on SDS-PAGE.

the glycoproteins (Witter *et al.*, 1973b), the material was able to agglutinate erythrocytes. Hence the hemagglutinin was a virus type-specific envelope component of MuLV (Schäfer and Szántó, 1969). Its serospecificity follows the G and FMR subgroups, and it is associated with the (glycoprotein) virus envelope projections (Witter *et al.*, 1973a,b). Such a hemagglutinating viral glycoprotein has not been detected in the ATV or FeTV.

Like the ATV, a major low MW polypeptide of MuLV, p 12, is weakly glycosylated and seems to represent a further virus envelope glycoprotein, with type- and group-specific antigenicity (Green *et al.*, 1973).

In view of the experimental findings mentioned above, and in analogy to ATV, it is reasonable to assume that all mammalian C-type viruses contain two glycoproteins on their surface which morphologically appear as spikes or knoblike projections and which are virus type specific, i.e., induce neutralizing antibody.

2. *Internal Antigens*

Various antigens of MuLV were first postulated by Fink and Cowles (1965). The comprehensive work of Geering *et al.* (1966), later confirmed by Huebner *et al.* (1966), indicated clearly the serological group specificity (gs) of a major internal antigen of MuLV. Much attention was directed to the isolation and purification of that major gs antigen. Although the results were not uniform, it became obvious that it was represented by a 30,000–35,000 d protein (Schäfer *et al.*, 1969; Gregoriades and Old, 1969; Oroszlan *et al.*, 1970) henceforth to be referred to as p 31.

The more recent analysis of the total polypeptides of murine and other mammalian C-type viruses employing SDS-PAGE has revealed two further major polypeptides: one in the range of 14,000–15,000 d and the other in the range of 17,000–19,000 d (Franke and Gruca, 1969; Oroszlan *et al.*, 1971a,b, 1972a,b; Schäfer *et al.*, 1971a, 1972b; Moroni, 1972; Shanmugam *et al.*, 1972; Nichols *et al.*, 1973; Hoekstra and Deinhardt, 1974). Treatment of the virus with acetone prior to electrophoresis allows the resolution of one more polypeptide that had been occasionally observed by others, but which usually co-migrates with the second band (Bolognesi *et al.*, 1973). The pattern obtained by gel filtration in Gu-HCl (Nowinski *et al.*, 1972a; Green *et al.*, 1973) also has revealed 4 major polypeptides. As in the case of ATV, the MWs estimated by that method differ from those obtained by gel electrophoresis, but they will be used here to designate the individual polypeptides. Since there seem to be only minor differences in the MWs of

corresponding polypeptides of viruses from different groups, the MW's of 10,000, 12,000, 15,000, and 31,000 d will be attributed to the 4 polypeptides of a prototype mammalian C-type virus.

Of these four polypeptides, p 31 is best analyzed serologically. Two antigenic determinants were originally described by Geering and coworkers (1968, 1970): one (gs 1) that is group specific for RTV of a given species and which has been called gs spec antigen (Schäfer *et al.*, 1970), and one (gs 3) that cross reacts among RTV isolated from different mammalian species, called gs interspec antigen (Schäfer *et al.*, 1970). Both antigenic determinants coincide with the polypeptide p 31 of various mammalian C-type viruses, as was demonstrated with isolated and purified p 31 preparations from mouse (Gilden *et al.*, 1971; Gilden and Oroszlan, 1972), rat (Oroszlan *et al.*, 1972b), hamster (Gilden *et al.*, 1971; Oroszlan *et al.*, 1971c), pig (Moennig *et al.*, 1974), and monkey (Parks and Scolnick, 1972; Kawakami *et al.*, 1972; Schäfer *et al.*, 1973a,b) C-type viruses.

This suggests that all mammalian C-type viruses share gs interspec antigen. Although Ferrer (1972) was unable to detect interspec antigen in bovine C-type viruses, Schäfer *et al.* (1971b) detected interspec antigen in material banding at a buoyant density of 1.16 gm/cm^3 in sucrose that was harvested from bovine leukemic cell cultures producing C-type particles. In contrast to Gilden's group (1971), Schäfer and co-workers described a polypeptide from Gross-MuLV and FeLV that contained only gs interspec but no gs spec antigen (Schäfer *et al.*, 1971b, 1972b). Nowinski *et al.* (1972a), on the other hand, did not find any interspec activity in either of the polypeptides as isolated from the GuHCl column. It is uncertain at present, whether these results indicate two different polypeptides as substrate for the two antigenic specificities, whether the antigenic activities can be somehow inactivated to a different extent, or whether the explanation lies in the following observation of Oroszlan *et al.* (1972c). In their hands, the interspec antigen was detectable only in the presence of both IgG and IgM. Neither of the Ig fractions alone precipitated the antigen, and β-mercaptoethanol eliminated the precipitating antibody. It was therefore suggested that the antigen has to react first with IgM before this complex is precipitated by IgG.

A recent discovery of great interest indicates that small rodent and feline C-type viruses share two interspec determinants a and b which are apparently both contained in p 31, of which only one is found in C-type viruses of pig, monkey, and RD 114 (Schäfer *et al.*, 1973a,b). The latter virus had been thought to be of human origin (McAllister *et al.*, 1972; Oroszlan *et al.*, 1972a), but now seems to be an endogenous feline C-type virus (Long *et al.*, 1973; McAllister *et al.*, 1973; Gillespie *et al.*, 1973; Rupprecht *et al.*, 1973; Todaro *et al.*, 1973).

Interestingly, the RD 114 antigen which corresponds to the spec determinant of p 31 in the murine system has no antigenic correlate in other FeLV strains (Oroszlan *et al.*, 1972a).

The partial and weak serological cross reaction between C-type viruses from rodents and monkeys was also observed by Parks, Scolnick, and colleagues (Parks and Scolnick, 1972; Parks *et al.*, 1973a) and adds weight to the thus far unconfirmed detection of weak interspec antigen in 1.16 gm/cm^3 density gradient material from two human tumor cell lines (Schäfer *et al.*, 1970, 1971). The existence of human-C-type-particles has been suggested by electron microscopy (Dmochowski, 1965, 1970), by molecular hybridization studies (Baxt and Spiegelman, 1972; Kufe *et al.*, 1973) and by demonstration of reverse transcriptase with enzyme properties unrelated to cellular enzymes but similar to RTV reverse transcriptase (Baxt *et al.*, 1972; Sarngadharan *et al.*, 1972; Bhattacharyya *et al.*, 1973). The serological assay of such human viruses via interspec antigens might require antisera that detect the b interspec antigen if not other as yet unknown antigens of the interspec type.

A second group-specific determinant also associated with p 31 has been described by only one group, and its existence needs confirmation (Schäfer *et al.*, 1973b).

Little was known until recently about the serological properties of the remaining three p 10, p 12, and p 15. A murine antigen "gs 2" that was not interspecies specific (Geering *et al.*, 1970) has not been identified with any polypeptide and might be identical with p 10 because of its strong antigenicity. Likewise, two species-specific gs antigens of MuTV termed I and III were not identified with certainty but suggested to correspond to p 10 and p 12 or p 15 (Schäfer *et al.*, 1972a,b, 1974). For p 12 a group-specific determinant was demonstrated by Tronick *et al.* (1973).

Most recently, a comparative study dealing with the serological properties of highly purified proteins from both MuTV and FeTV has been reported (Green *et al.*, 1973). The proteins were isolated by gel filtration in GuHCl. P 10 contained a group-specific reactivity different from that present in p 31. P 12, which was associated with carbohydrate, revealed type or subgroup specificity. P 15 was not investigated in much detail.

Gs antigen of undefined size has been found associated with isolated cores of MuLV (Fink *et al.*, 1969). A more precise localization and of better characterized polypeptides has been performed very recently by Lange *et al.* (1973), who found p 10 and p 31 as constituents of morphologically pure core preparations from F-MuLV. Bolognesi *et al.* (1973), who performed a similar study, reported in addition the presence of p 15. After further degradation, p 10 is enriched and p 15 disappears from the nucleoid complex, but, in contrast to the cores from ATV, some

p 31 is still adherent to that material. This was explained by a closer association of the major polypeptide (p 31) with the ribonucleoprotein complex in the case of murine viruses (Bolognesi *et al.*, 1973). As with ATV cores, reverse transcriptase and HMW RNA are likewise constituents of the core (Lange *et al.*, 1973; Bolognesi *et al.*, 1973).

3. *Enzymes*

The general remarks made in Section IV,A,3 concerning avian virus enzymes hold true also for mammalian viruses. As first reported by Baltimore (1970) and confirmed by others (Green *et al.*, 1970; Hatanaka *et al.*, 1970; Spiegelman *et al.*, 1970), all C-type viruses of mammalian origin so far investigated contain DNA polymerase with enzyme properties similar to those found for avian viruses (for review, see Temin and Baltimore, 1972). Ribonuclease H activity (Mölling *et al.*, 1971) has also been detected in mouse and feline C-type viruses (Grandgenett *et al.*, 1972; Mölling, 1974).

The size and polypeptide composition of mammalian C-type reverse transcriptase is strikingly different from the avian system. The MW of the polymerase is between 70,000 d and 90,000 d, and the enzyme reveals only one polypeptide band of that size in the SDS-PAGE (Ross *et al.*, 1971; Tronick *et al.*, 1972; Hurwitz and Leis, 1972; Abrell and Gallo, 1973; Mölling, 1974), in contrast to the two components observed with ATV.

At present the question is under discussion whether that polypeptide contains both polymerase and RNase H, as has been suggested by the findings with the avian system (Grandgenett *et al.*, 1972). Whereas Wang and Duesberg (1973) could not detect RNase H activity in the purified polymerase of Kirsten (Ki) MuLV, in our laboratory both enzymes of F-MuLV copurified in the procedure described by Kacian *et al.* (1971) resulting in a single polypeptide of 70,000 d MW, as resolved by protein staining after SDS-PAGE (Mölling, 1974). Whether this discrepancy is due to the use of different virus strains must be further investigated.

Enzyme-inhibiting antisera have been prepared, and the antigenic property of polymerase from different viral species have been compared. Interestingly, the interspecies serological relationship of the polymerase follows the same pattern as the p 31 interspec antigen. A strong cross inhibition has been found within polymerases from rodent mammalian viruses on the one hand and within monkey viruses on the other, while the cross-reaction between these two groups is weak (Aaronson *et al.*, 1971a; Scolnick *et al.*, 1972a,b; Abrell and Gallo, 1973). No cross-reaction of antiserum to reverse transcriptase of mammalian viruses has been found with reverse transcriptase of avian C-type viruses (Scolnick

et al., 1972b; Parks *et al.*, 1972), the B-type and the viper C-type viruses (Oroszlan *et al.*, 1971b), and the Mason-Pfizer monkey virus (Abrell and Gallo, 1973), which is morphologically and serologically different from C-type viruses (Nowinski *et al.*, 1971), and from human leukemia cells (Todaro and Gallo, 1973). Thus, the serological properties of reverse transcriptase can be used in addition to the two other interspec antigens mentioned before in order to classify unknown C-type viruslike particles.

V. Virus-Directed Intracellular Antigens

A. Expression of Endogenous Viral Functions

Cells of the natural host are usually permissive for the replication of C-type virus progeny after experimental infection. Therefore, the appearance of new antigens in such cells may not necessarily be linked to the molecular events of transformation, but be only a result of the synthesis of virion constituents or enzymes for the replication of new viral progeny.

In nature, the presence of a virus genome is not always accompanied by virus synthesis and the serological assay of viral antigens has been used to demonstrate latent RNA tumor viruses. However, a correlation between the detection of viral gene products and the presence of a latent RTV infection is no longer meaningful since the fundamental discovery that probably each individual, as has been shown for avian and murine species, contains endogenous viruses (see below).

The widespread occurrence of MuLV among inbred mouse strains was recognized very early, but the incidence of infection varies with strain and age. Likewise, the expression of the gs antigen which was taken as a parameter for the presence of the MuLV genome was not always observed (Huebner *et al.*, 1970a,b). Direct evidence has been presented for host genetic control over the expression of individual endogenous virus genes in the murine and avian systems (Payne and Chubb, 1968; Meier and Huebner, 1971; Hanafusa *et al.*, 1972; Hilgers *et al.*, 1972). This host genetic control was shown to be inherited according to Mendelian laws. In the case of the murine system, a total "switch on" or "partial switch off" of the virus genome has been postulated (see also Huebner *et al.*, 1970b; Huebner and Todaro, 1969).

Partial expression of the endogenous virus genome has also been observed in the avian system. Hence, the expression of gs antigens need not correlate with the synthesis of virus particles (Dougherty and Di Stefano, 1966; Payne and Chubb, 1968; Allen and Sarma, 1972), but is generally in good correlation with the expression of a chicken helper factor (chf) which is responsible for the rescue of the BH strain of RSV

through complementation of the defective envelope proteins (Weiss, 1969a; H. Hanafusa *et al.*, 1970; Vogt and Friis, 1971; Weiss and Payne, 1971). The endogenous virus is present in the state of an integrated DNA provirus, as postulated earlier by Temin (1964a,b), with properties similar to the lysogenic state characteristic of temperate bacteriophages. The expression of provirus genetic information can be induced by superinfection of the cell with other RTV or by biophysical or biochemical methods with the consequent synthesis of virus (T. Hanafusa *et al.*, 1970; Weiss *et al.*, 1971; Lowy *et al.*, 1971; Aaronson *et al.*, 1971b; Klement *et al.*, 1971; Teich *et al.*, 1973). The viruses observed in chicken cells after induction all belong to the new subgroup E, as defined by their Ve antigen.

The chf seems to represent a partial expression of an endogenous virus genome rather than the production of whole virus particles. The detection of the chf activity in cells closely correlates with the detection in cells of gs antigen. Implicit in the ability of chf-positive cells to rescue the defective BH-RSV is the likelihood that Ve antigen can be expressed. This conclusion is considerably supported by the interference-like phenomenon observed in studies of host cell susceptibility to subgroup E viruses. The expression of the host gene e^s, which defines susceptibility to subgroup E viruses, is under the control of a second unlinked epistatic gene I^e (Payne *et al.*, 1971). Further work by Weiss and colleagues (Weiss, 1974; R. A. Weiss, R. R. Friis, and P. K. Vogt, personal communication) suggests that the effect of I^e on the e^s allele probably results from a blocking of the cell receptors with Ve antigen produced in cells expressing the chf, thereby producing an interference effect similar to that described originally by Rubin (1961). The existence of Ve antigen of the subgroup E type on the surface of I^e positive cells has been demonstrated by absorption of subgroup E neutralizing antibody (Hanafusa *et al.*, 1973; Weiss, 1973). The close correlation between the expression of chf and the Ve antigen suggests that these are two markers of an identical phenomenon.

The molecular background of these biological findings has been clearly established by molecular hybridization experiments. Those studies have confirmed the existence of DNA provirus in each individual chick embryo. The provirus may exist in a nonfunctional state, i.e., without synthesis of viral RNA, alternatively viral RNA, gs antigen, chf, and in rare cases even whole infectious virus particles may be produced (Bishop *et al.*, 1973; Hayward and Hanafusa, 1973; Varmus *et al.*, 1972, 1973).

B. Experimentally Infected Cells

For studies on virus-directed antigens that might be significant specifically for the transformation of the host cell, experiments with

nonpermissive (non-virus-producing) systems have been useful because virus structural antigens are not necessarily synthesized, and the tumor specificity of a new antigen may therefore be easier to prove. Unfortunately, this approach was not successful in the elucidation of intracellular tumor specific antigens, but gave valuable insight into virus replication.

Nonpermissive or nonproductive infection is the usual result when tumors are induced in a heterologous host, or when cells from a heterologous host are transformed *in vitro* by RTV. For example, mouse cells transformed by ASV and hamster cells transformed by MuSV usually yield no progeny virus. In these instances tumors not only fail to produce infectious virus but also physical noninfectious particles (Gelderblom *et al.*, 1970). Usually the virus genome can, however, be rescued by cocultivation with cells from the natural host (for review, see Svoboda and Hlozanek, 1970; Simkovic, 1972).

It was Huebner and co-workers who first reported that some non-virus-producing (NP) tumors, namely RSV-induced hamster fibrosarcomas, contain a new soluble intracellular antigen, because of the specificity of the antibodies developed in the host, they concluded that this antigen is group specific (gs) for ATV (Huebner *et al.*, 1964). This antigen is also present in productively infected cells (Huebner *et al.*, 1964) and has been shown to be an internal group-specific antigen of the virion (Bauer and Schäfer, 1966; Payne *et al.*, 1966; Kelloff and Vogt, 1966; Bauer and Janda, 1967; Bauer and Bolognesi, 1970; Fleissner, 1970). Similarly, MuLV-infected mouse cells have been shown to contain virion gs antigen (Hartley *et al.*, 1965). Intracellular Ve antigen has not been demonstrated in NP cells.

The RNA tumor virus gs antigens detectable in NP tumor cells are not analogous to the T-antigen invariably found in papovavirus-transformed cells (for review, see Huebner, 1967) for the following reasons: they are virion constituents as mentioned before whereas T-antigens are not found in the virion; they are not localized in the nucleus as the T-antigen, but instead in the cytoplasm of the cell (Fleissner, 1970; Hampar *et al.*, 1971; Dougherty *et al.*, 1972); they are not always found in NP tumors (Bubenik and Bauer, 1967; Thurzo *et al.*, 1969; Huebner *et al.*, 1970b) as T antigens usually are, and sometimes even disappear after a prolonged passage of tumor cells (Kurth and Bauer, 1972b).

Thus, neither the production of whole virus nor the expression of gs virion antigen seems to be essential for the persistence of the malignant behavior of a cell, although it is possible that in these studies "gs positive" and "gs negative" indicate a quantitative rather than a qualitative difference. The radioimmune assay (RIA), which has added new sensitivity to the quantitative assay of antigens and compares favorably with

previous standard techniques like the complement fixation (CF) test or immunofluorescence (Weber and Yohn, 1972; Stephenson *et al.*, 1973; Parks *et al.*, 1973b), might help to resolve this problem.

The immunoprecipitation of radioactively labeled intracellular antigens allows the component analysis of the proteins by column chromatography or PAGE. This method may be even more sensitive than the RIA and has the advantage of qualitative results even when complex sera, i.e., sera reactive to more than one antigen, are used. Application of this method has revealed two interesting phenomena. The bulk of the intracellular gs antigens appear to have molecular weights of 40,000 to 90,000 d and are therefore much larger than the antigenically analogous proteins that can be isolated from the virion. This was explained as a large precursor protein that must be cleaved later, prior to virus assembly (Vogt and Eisenmann, 1973), or by the aggregation of structural protein monomers with other proteins, probably of cellular origin, to which the monomers have high affinity (Fleissner and Tress, 1973). Interestingly, not all the monomeric proteins found in the virion have been demonstrated with this method (Shanmugam *et al.*, 1972; Fleissner and Tress, 1973; Vogt and Eisenmann, 1973), and it is reasonable to assume that part of the intracellular gs proteins may be in an antigenic form that is unrecognized by antisera prepared against proteins isolated from the virus. Thus, a "gs negative" result may be misleading.

C. Concluding Remarks

Several conclusions can be drawn from the findings reviewed in this section. (1) The failure to detect virion antigens in the cell is no proof of the absence of an RNA tumor virus genome. (2) Virion antigens can be synthesized without concomitant virus particle synthesis. (3) Synthesis of virus structural antigens is no prerequisite for the persistence of the malignant state of RTV-transformed cells. (4) No intracellular RTV antigen has been detected to date that is specific for a malignant cell, i.e., requisite for any RTV-transformed tumor cell but absent from productively infected, untransformed cells. As will be shown in the next section, however, virus-directed tumor-specific antigens can be found on the cell surface.

VI. Virus-Induced Cell Surface Antigens

Tumor virus-directed changes in the antigenic makeup of the surface of transformed cells have been demonstrated with certainty in practically all RNA tumor virus systems. The most recent articles which review comprehensively the immunological aspect are those by Pasternak

(1969), Habel (1969), Koldovsky (1969), Hellström and Hellström, (1969), and Haughton and Nash (1969). Earlier reviews on that subject were published by Klein (1966; 1969), Sjögren (1965), and Old and Boyse (1965).

Tumor cell surface antigens were first assayed by *in vivo* transplantation immunity experiments, and therefore these antigens have usually been termed tumor-specific transplantation antigens (TSTA). When tumor cell surface antigens have been assayed by *in vitro* techniques, such as cytotoxic tests, the term TSSA (tumor-specific cell surface antigens) has been widely used. For purposes of this discussion, tumor cell-specific cell surface antigens will be called TSTA when they have been assayed by *in vivo* transplantation experiments and TSSA when they have been assayed by any *in vitro* technique or when a general term is needed. Although there is evidence that in many cases TSTA and TSSA refer to the same antigens, one should be aware that the various *in vitro* techniques might detect antigens that are distinct from those involved in transplantation immunity reaction.

There is one common feature of tumor virus-directed cell surface antigens: they are virus specific in the sense that TSSA of all tumors induced by a given virus, even across host species boundaries, cross-react in immunological tests. This is in contrast to observations with chemically or physically induced tumors (see the reviews above), each of which expresses an individual TSSA pattern, showing only occasionally a weak cross-antigenicity (Southam, 1967; Basombrio, 1970). This observation has been taken as an argument for the proposition that virus-directed TSSA are indeed coded for by the respective viral genome.

The first virus-specific TSTA were described by Habel (1961) and Sjögren *et al.* (1961) in polyoma-virus tumors. Intensive studies of this and the SV40 system led to the conclusion that the respective TSTA were not identical with any structural proteins of the virion. The failure of the tumor cells to produce virus and the absence of neutralizing antibodies in the tumor-bearing animals were taken as evidence for this, upon the assumption that if any virion antigen acted as TSTA, it would probably be a virus coat protein (Habel, 1962; Sjögren, 1964).

TSTA induced by RNA tumor viruses were first described by Klein *et al.* (1962), Sachs (1962), and Pasternak *et al.* (1962) in the case of MuLV, and by Sjögren and Jonsson (1963) in the case of RSV. Since RNA virus-induced tumors produce virus particles in the natural, and in some rare cases even in the heterologous, host (see Svoboda and Hlozanek, 1970), it was discussed whether the Ve antigen acts as the only transplantation antigen in these systems or whether there were other such antigens. For example, it was strongly suggested that in RSV

chicken tumors the Ve antigen is the only TSTA (Rubin, 1962; Shimizu and Rubin, 1964). This conclusion was drawn from the nonimmunogenicity of certain chicken tumors, which had been induced by the defective (Hanafusa *et al.*, 1964) BH-RSV strain and were at that time believed, incorrectly as we know now, not to produce virus particles. As will be discussed below, more elaborately designed experiments and the use of more sensitive *in vitro* assays for cell surface antigens have shed some light on these problems.

The purpose of this section is to discuss the biology of RNA tumor virus-directed cell surface antigens rather than their immunological aspect, which has been extensively described in previous reviews. The newer experimental immunological information, such as the blocking serum factors and enhancement effects, are beyond the scope of this article. The interest here will focus on the following questions: (1) How many different types of TSSA coexist on a given cell? (2) Are TSSA virion constituents? (3) Are they virus strain specific? (4) Is their expression linked with the transformed state of a cell? (5) What is known about the biochemical nature of TSSA? (6) What is the origin of TSSA?

A. Avian Virus Group

1. *In Vivo Demonstration of TSTA*

In the very first reports on TSTA in RSV-induced mouse tumors (Sjögren and Jonsson, 1963; Koldovsky and Bubenik, 1964, 1965), the virus specificity of RSV-directed TSTA was demonstrated. After mice had been homografted or pretreated with cells of syngeneic RSV tumors under conditions that did not allow the outgrowth of a tumor, the animals proved to be resistent to challenge with syngeneic tumor cells that had been induced by infection with the same virus strain. These mice were not protected, however, when challenged with polyoma virus-induced or chemically induced tumor cells. These results were interpreted to indicate that all mouse tumors of the same virus etiology contain common TSTA. The RSV-induced TSTA were not virus strain specific, but rather group specific, since mice immunized with cells from either a Schmidt-Ruppin (SR)-RSV or Pr-RSV induced tumor became resistant to tumors induced by the SR-RSV, Pr-RSV, or BH-RSV as well, while they remained susceptible to methylcholanthrene-induced tumors (Bubenik *et al.*, 1967; Bubenik and Bauer, 1967). Likewise, Pr-RSV and SR-RSV-induced rat tumors cross immunized in transplantation experiments (Svoboda, 1967). In all these studies, however, contamination of the RSV strains with a common virus was not excluded.

Evidence was obtained through further *in vivo* experiments that the collection of viruses inducing a common TSTA included also avian leukosis viruses. Although no tumors were observed after injection of avian myeloblastosis virus into mice, a single injection of a large dose of AMV into newborn mice induced resistance against syngeneic RSV tumor cells injected 4–8 weeks later (Bauer *et al.*, 1969). Since the AMV was no longer immunogenic after treatment with the mutagen hydroxylamine, it was suggested that AMV had infected and transformed some mouse cells with the consequent production of group specific TSTA in sufficient amounts to immunize the animals.

None of these studies suggested that TSTA were virion constituents. No infectious virus could be recovered from these RSV mammalian tumors (see Svoboda, 1966, 1967), and no physical virus particles were detected by electron microscopy in thin sections of tumor tissue (Gelderblom *et al.*, 1970). Furthermore, no virus-neutralizing antibody was demonstrated in the serum of immunized or tumor-bearing animals (Jonsson and Sjögren, 1965, 1966; Svoboda, 1967; Koldowsky *et al.*, 1966; Bauer *et al.*, 1969). Thus, it seemed unlikely after all that virus envelope antigen played any role as transplantation antigen in non-virus-producing RSV-transformed mammalian cells.

The absence of gs antigen in some of these tumors, as determined by CF test, was taken as evidence that the virus internal gs antigen did not act as a transplantation antigen (Bubenik and Bauer, 1967). It must be kept in mind, however, that at that time only one gs antigen was recognized to be a virus constituent, and those experiments did not rule out the possibility that another virion gs antigen, unknown at that time, acts as a TSTA.

The question was asked whether RSV tumors in different animal species share antigenically related TSTA. This was first suggested by Svoboda (1967), who reported the protection against syngeneic Rous sarcoma challenge of rats that had been preimmunized with RSV tumor cells from the mouse. In order to avoid unspecific immunization effects by heterologous cells, Jonsson (1966), in similar experiments, X-irradiated the recipient mice shortly before challenge with syngeneic tumor cells. It was assumed that this procedure prevented any primary immune response of the time of challenge and that immune effects under those conditions would be due to a secondary immune response against TSTA. The results showed that mice were protected against syngeneic RSV tumor challenge after pretreatment with RSV rat tumor cells, but not after pretreatment with hamster or rabbit RSV tumor cells. In earlier experiments a protective effect had also not been observed after immunization of adult mice with chicken RSV tumor cells (Jonsson and

Sjögren, 1965). It is difficult to understand why of the four animal species tested, only tumors from rats should cross-react with mouse tumors, and it has been suggested that this technique might not be appropriate to detect all types of heterologous TSTA cross-reaction. On the other hand, it was not proved with certainty that the hamster, rabbit, and chicken RSV tumors contained TSTA at all. Svoboda (1967) reported a protective effect of chicken RSV sarcoma cells in mice; however, it was not clear from this study whether the effect was due to true cellular TSTA or to secondary infection of mouse cells with virus produced by the chicken cell inoculum.

For many years only indirect evidence was available for the existence of TSTA in the natural host of the ATV, the chicken. This evidence derived essentially from the observation that certain BH-RSV tumors regress. The time of regression was found to be reciprocal to the virus dose applied, and immunity in chickens was not achieved after injection of the so-called BH-RSV NP cells. Injection of infectious virus, however, had an immunizing effect (Rubin, 1962; Shimizu and Rubin, 1964). The conclusion was that Ve antigen was the only antigen acting as transplantation antigen in the chicken system. The basis of this interpretation was the belief at that time that BH-RSV-NP cells did not produce virus; this, however, has been subsequently demonstrated (Dougherty and Di Stefano, 1965; Robinson, 1967; Vogt, 1967; Weiss, 1967, 1969a,b, 1972; Hanafusa and Hanafusa, 1968; H. Hanafusa *et al.*, 1970).

Several subsequent reports were in contrast to those by Rubin. A cellular antigen in lymphoid tumors of chickens was demonstrated by immunofluorescence with antibody from chickens hyperimmunized with a transplantable lymphoid tumor (Tekeli and Olson, 1965; Kakud and Olson, 1967). The antigen did not react with virus neutralizing antibody but, on the other hand, was also not found on tumor cells transformed by other ATV strains.

Meyers *et al.* (1972) presented indirect evidence for the existence of TSTA that were distinct from the Ve antigen in ATV-chicken tumors. Infection of nonsarcomatogenic LV into chickens protected animals that were subsequently challenged with RSV of a subgroup different from that of the immunizing LV. For example, chickens immunized with LV of subgroup A do not develop a sarcoma when challenged with BH-RSV (RAV-2) which is of subgroup B. As an explanation for this effect, it was suggested that in the animal appropriate target cells were transformed by the LV. These LV-transformed cells produced an antigen also contained in SV-transformed cells, which is group specific for all subgroups of ATV. Hence, the injected LV was protective by provoking an immune response to the group-specific TSTA. These results and their

interpretation are in full agreement with recent *in vitro* experiments that will be described in the following section.

The *in vivo* experiments described above have demonstrated the existence of new cell surface antigens that are specific for transformed cells, are common to host cells of a variety of species transformed by biologically different LV or SV, and are not identical with the Ve antigen.

2. *In Vitro Studies of TSSA*

Many questions concerning TSTA remained unsolved and unapproachable by *in vivo* transplantation methods. Especially the biophysical and biochemical characterization of TSTA may be expected to require *in vitro* methods that allow the antigenic activity to be followed during the isolation procedure. Such methods would also permit the immunological comparison of antigens induced by a given virus or group of viruses in different species without interference by xenogeneic immune reactions that are not directed against the tumor-specific antigen. On the other hand, it is reasonable to suppose that TSSA detected by some *in vitro* methods, such as immunofluorescence- or immunoferritin staining, need not necessarily be identical to TSTA as detected by transplantation experiments. Antigens demonstrated by cytotoxicity assays, however, are likely to be identical to TSTA.

a. *Demonstration of TSSA in Chicken Cells.* In contrast to the other tumor virus systems, only a few reports have appeared dealing with the subject of *in vitro* studies of AMV-induced TSSA. The initial demonstration of TSSA with appropriate controls was by Sjögren and Jonsson in 1970. This study posed with special emphasis the question of whether primary tumor-bearing chickens could develop cellular immunity to the tumor cell. It was established with the colony inhibition test (Hellström, 1967) that thymus cells of SR-RSV tumor-bearing chickens could reduce the efficiency of colony formation by cells from a SR-RSV induced sarcoma. Because the target cells used in these experiments were of mouse origin, it was further possible to postulate an immunological cross reaction between SR-RSV sarcomata of chicken and mouse origin. However, since chicken target cells were not used and the reciprocal experiment (mouse lymphocytes versus chicken cells) was not performed the extent of that cross-reaction was not defined. The RSV specificity of that reaction was shown, however, because colony formation by cells from several other tumors of differing etiology was not inhibited.

A more extensive *in vitro* study on the nature and tumor specificity of ATV-induced TSSA was performed recently in our laboratory. By comparing chick embryo cells (CEC) infected by LV or SV of different

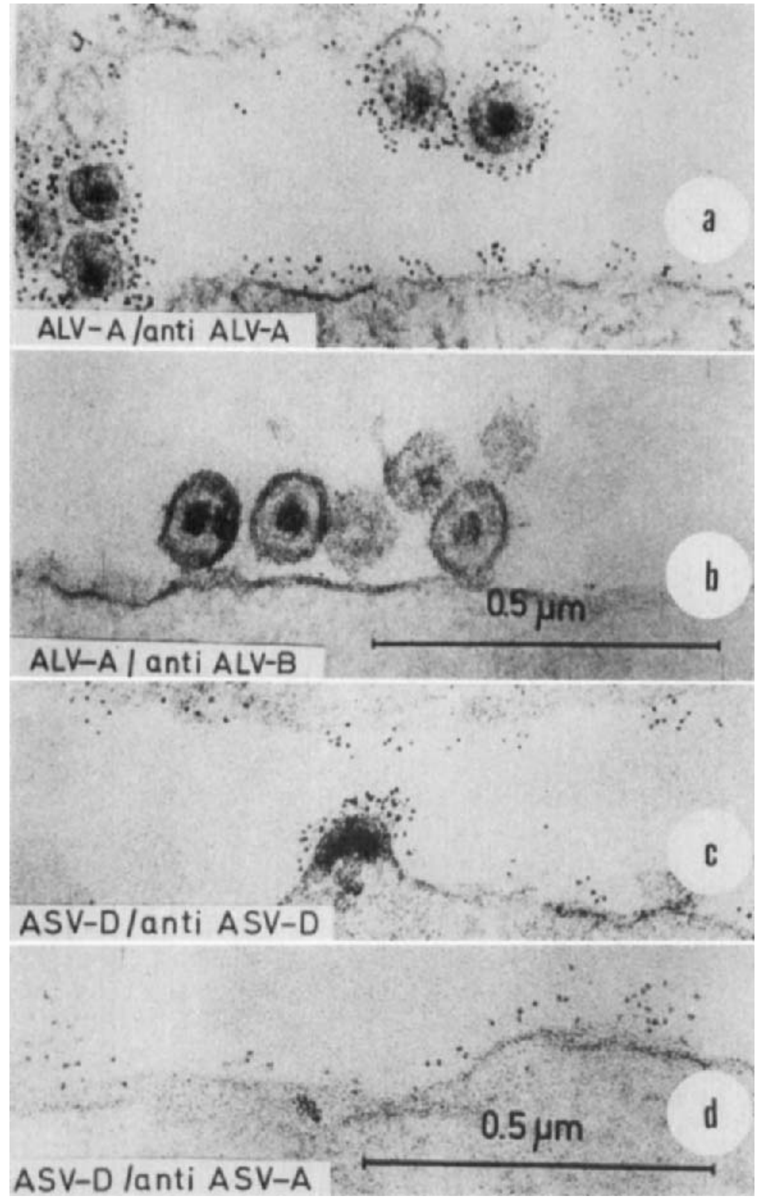

FIG. 3. Thin sections of chick embryo cells infected or transformed by avian leukosis virus (ALV) and avian sarcoma virus (ASV), respectively, after staining with ferritin-labeled hybrid antibody. (a) Subgroup A leukosis virus infected cells stained with homologous antiserum; (b) the same cells as in (a) stained with antiserum against LV of a heterologous subgroup (anti-ALV-B); (c) subgroup D sarcoma virus transformed cells stained with homologous antiserum; (d) the same cells as in (c) stained with antiserum against SV of a heterologous subgroup (anti-ASV-A).

subgroups, it is possible to study the role of Ve antigen in the tumor immunity of the natural host and to evaluate the contribution of any other new cell surface antigen. This system takes advantage of the fact that LV in CEC establish a productive infection without host cell transformation whereas the SV infection results in transformation as well as progeny virus production.

In an analytical approach, chicken immune sera were reacted in the hybrid antibody immunoferritin technique (Hämmerling *et al.*, 1968) with CEC infected with LV or SV (Gelderblom *et al.*, 1972b). Some of the results are shown in Fig. 3. Antisera against LV or LV-like mutant derivatives from SV strains (Graf *et al.*, 1971) were shown to label only the surface of cells infected by viruses of the homologous subgroup. In addition they tagged the envelope of the respective viruses either during the budding process or after release from the cell.

In contrast, chicken antisera against SV strains of different subgroups not only exhibited a homologous reaction, but also stained cells transformed by SV of a heterologous subgroup. Since these SV antisera did not react with cells productively infected by LV of a nonhomologous subgroup, the antigen in question was considered to be tumor cell specific (TSSA) (see Table III).

A parallel investigation concerned itself with the biological question of which of the ATV-induced cell surface antigens react with specific immune reagents to produce a cytotoxic effect (Kurth and Bauer, 1972a). Spleen lymphocytes from chickens that had been immunized with virus several times were tested for their cytotoxic potential. Chicken target cells were infected with various strains of LV and SV. The results were similar to those obtained with the immunoferritin technique (Table IV).

TABLE III
CELL SURFACE STAINING OF LEUKOSIS VIRUS (LV)-INFECTED OR SARCOMA VIRUS (SV)-TRANSFORMED CHICKEN EMBRYO FIBROBLASTS BY THE IMMUNOFERRITIN TECHNIQUE

Chicken antibody prepared against	Infecting viruses			
	LV-A	SV-A	LV-D	SV-D
LV-A	+[a]	+	0	0
SV-A	+	++[b]	0	+
LV-D	0	0	+	+
SV-D	0	+	+	++

[a] The antigens reacting at the LV-infected cells are Ve antigen.
[b] The surface of SV-transformed cells reveals in addition to Ve antigen tumor-specific cell surface antigen, which is detectable only by SV antisera.

TABLE IV
CYTOTOXIC EFFECT OF CHICKEN IMMUNE LYMPHOCYTES AGAINST LEUKOSIS VIRUS (LV)-INFECTED OR SARCOMA VIRUS (SV)-TRANSFORMED CELLS

Immune lymphocytes prepared against	Infecting viruses			
	LV-A	SV-A	LV-D	SV-D
LV-A	+	++[a]	0	+
SV-A	+	++	0	+
LV-D	0	+	+	++[a]
SV-D	0	+	+	++

[a] In contrast to the LV antisera (see Table III), immune lymphocytes obtained after several LV injections also detect tumor-specific cell surface antigen.

Lymphocytes from chickens immunized with a sarcoma virus were cytotoxic not only for CEC transformed by this virus, but also for CEC transformed by sarcoma viruses from other subgroups, hence confirming the existence of a TSSA that is group specific and not identical with Ve antigen. Likewise such lymphocytes reacted with CEC infected by LV viruses of the homologous subgroup, but not with CEC infected by LV of heterologous subgroups. This subgroup-specific reaction indicates strongly the capability of the host to exert a cell-killing effect via the Ve antigen present at the cell surface. From the cytotoxic experiments, it became obvious, however, that the destructive effect on the cell resulting from the reaction against TSSA was stronger than that against the Ve antigen. Whether the TSSA is indeed a stronger antigen than the Ve antigen or whether it is simply present in greater amounts needs further studies.

Lymphocytes from chickens with regressed myeloblastic leukemia were reported to exert a cytotoxic effect against leukemia cells (Silva and Moscovici, 1973), but the target antigen in those studies was not known.

b. *Common TSSA in Leukemia and Sarcoma Cells.* The *in vivo* experiments described in Section VI,A,1 suggested that common TSSA appeared in cells transformed either by LV or SV. This conclusion was further supported by experiments described immediately above. There was a single discrepancy between the results obtained with chicken immune lymphocytes (Kurth and Bauer, 1972b) and those obtained with the immunoferritin technique (Gelderblom *et al.*, 1972b). Immune lymphocytes prepared against LV reacted not only with CEC infected by viruses of the same subgroup, but were also capable of killing CEC transformed by SV of a different subgroup. This reactivity was explained

by the fact that immune lymphocytes—in contrast to immune chicken sera—were obtained after repeated booster injections of virus. The prolonged immunization procedure apparently allowed the leukosis viruses to transform the appropriate target cells of the host with concomitant induction of TSSA, thereby provoking a cellular immunity. A long latency period for the induction of tumors by LV is well known (see Biggs *et al.*, 1973). This LV-induced cellular immunity against transformed cells further suggests the existence of common TSSA on leukemia and sarcoma cells of the chicken.

A more direct proof for this was most recently obtained by antibody absorption experiments. Radiolabeled TSSA-specific antibody obtained from SV-infected chickens was shown to be specifically absorbed by chicken myeloblastosis cells which had been transformed by avian myeloblastosis virus (Kurth *et al.*, 1974).

c. *Common TSSA on Cells of Different Species.* By combining the immunoferritin and the cellular cytotoxicity techniques with those of immunofluorescent staining and humoral cytotoxicity (Kurth and Bauer, 1972b), the relationship of the TSSA induced by ATV in different host species was also investigated (Kurth and Bauer, 1972b; Gelderblom and Bauer, 1973). Chicken immune sera and lymphocytes were reacted with RSV-transformed mouse and hamster cells, and immune lymphocytes and sera from mice were tested against transformed chicken and hamster cells. In all cases a clear-cut immunological cross-reaction between tumors of the three species were observed; however, the cytotoxicity tests revealed only a partial cross-reaction which will be discussed below.

Mouse immune serum prepared against RSV-induced TSSA (Kurth and Bauer, 1973b) were also cytotoxic against chicken myeloblasts, thus confirming not only the existence of a common antigen in chicken and mouse cells transformed by ATV, but also underlining the induction of a common TSSA by LV and SV (Kurth *et al.*, 1974).

Since TSSA-specific mouse immune sera or lymphocytes did not kill CEF infected with a leukosis virus of the homologous subgroup (Kurth and Bauer, 1972b), these results incidentally confirmed the previous suggestion that Ve antigen is not involved as TSSA in mammalian Rous sarcoma cells.

d. *On the Origin of the ATV-Directed TSSA.* The origin of tumor cell surface antigens is the subject of intense discussion. In the case of virus-induced tumors it was reasonable to assume that TSSA might be coded for by the virus genome (Klein, 1966). In favor of this proposition, as stated previously, are findings in several different virus systems that, in contrast to chemically or physically induced tumors, tumors of the same viral etiology and in various animal species share common TSTA or

TSSA. These tumor antigens, in turn, differ in tumors of different viral etiology.

The findings in the ATV system as discussed in the foregoing sections meet the concept that tumor viruses code for TSSA, which are therefore common among tumors induced by a given virus in different animal species. There might be differences in the TSSA as directed by a given virus in different host species (Kurth and Bauer, 1972b). For example, the dilution end point for 50% cytotoxicity with a standard mouse anti-TSSA serum was at 1:512 against mouse tumor cells, but at 1:64 against chicken cells. Furthermore, chicken immune sera stained RSV-transformed mouse cells more heavily than transformed chicken cells, by the immunoferritin and immunofluorescence techniques. Such findings gave some hint of the existence of antigens characteristic of particular cell species transformed by a given virus and of other antigens held in common among the transformed cells of different species. Species specific antigens might be induced by virus infection through the derepression of cellular genes, and one possible hypothesis is that they are embryonic antigens (EA), i.e., antigens that are expressed in a particular embryonic stage of life and repressed in the differentiated cells of adults. Since EA need not be expressed during the entire embryonic period, it might be difficult to verify their presence in the embryonic cells grown *in vitro*, which are most often used as controls in TSSA experiments.

Svoboda (1966) reported a state of tolerance with respect to Rous sarcoma cells which he had induced in chickens by injection of uninfected chicken material. He concluded that ATV-directed TSTA in the chicken is similar to a normal chicken antigen, and it is plausible that embryonic antigens were involved in this observation, though a contamination of apparently normal chicken antigenic material with an RNA tumor virus was not excluded.

A closer analysis of the expression of embryonic antigens was performed recently in our laboratory. Experiments were designed to identify possible tumor cell surface antigens of embryonic origin and to learn more about the correlation between expression of TSSA and EA (Kurth and Bauer, 1973c). Inbred mice, as well as randomly inbred chickens, were immunized with the respective embryonic cells from these animal flocks, and the immune lymphocytes and sera were tested in cytotoxicity and radioimmune assays. A cytotoxic effect with mouse sera and specific antibody binding with chicken sera was observed not only against normal embryonic cells cultured *in vitro* but also, and to a greater extent, against RSV tumor cells of mouse and chicken. By means of extensive cross absorption experiments, the existence of at least three antigens of embryonic

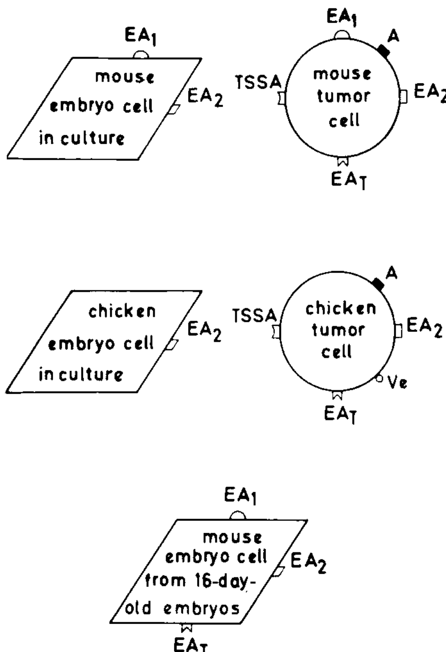

FIG. 4. Schematic diagram of avian tumor virus (ATV)-induced cell surface changes. A, agglutinability by lectins; EA, embryonic antigen (EA_1, mouse specific; EA_2, interspecies specific; EA_T, detectable only on RSV-transformed cells); TSSA, tumor-specific cell surface antigen. Taken from Kurth and Bauer (1973c).

origin was established (Fig. 4). Of these, one was mouse specific (EA_1), one interspecies-specific, hence demonstrable on both normal embryonic mouse and embryonic chicken cells (EA_2), and a third was likewise interspecies specific, but was detectable only on RSV-transformed cells (EA_T). None of these antigens was identical with the previously described TSSA, according to these absorption experiments.

On the basis of the experiments cited above, TSSA do not seem to be derepressed EA though the possibility must remain open that TSSA are identical to EA that can be detected only at a particular time of gestation. It remains to be considered whether TSSA could be identical to some virus structural constituents because TSSA are group specific for ATV, and this virus group has several gs structural antigens. The failure to detect gs antigen in some TSSA-positive RSV mammalian tumors (see Section V) is in conflict with this assumption, though it must be admitted that the assay of gs antigen by complement fixation test as it was performed in those studies might not have been sensitive enough to exclude the pres-

ence of small amounts of gs virion antigen. Preliminary experiments performed in our laboratory with the aim to absorb TSSA-specific antibody with virus extracts yielded equivocal results. Likewise, a very weak cytotoxic effect against sarcoma cells by rabbit antisera prepared against the highly purified four major gs components of ATV (p 12 through p 27) has been observed, although uninfected cells were also killed to some degree.

In summary, the experiments with EA antisera have revealed a variety of tumor cell surface antigens capable of stimulating an immune reaction by the host, and quantitative differences in their expression can well be the reason for conflicting results obtained by different authors, using different techniques and different systems.

B. Mammalian C-Type Viruses

Of the mammalian C-type virus systems, only the murine has been investigated in great detail with respect to tumor cell surface antigens, and it will therefore be the main subject of this section. A few reports which have appeared on the feline system will also be discussed.

The existence, in MuTV infected, untransformed, or transformed cells, of new cell surface antigens capable of provoking a specific immune reaction in the host was well documented by several methods some years ago. Resistance against an otherwise lethal dose of syngeneic Gross virus (G-MuLV)-induced lymphoma cells (Gross, 1951) was obtained by allogeneic preimmunization with tumor cells. The immunity was specific, since the animals were not resistant against challenge with polyoma cells (Klein *et al.*, 1962). Likewise, cytotoxic antibodies in these animals were found to be specific for Gross lymphoma cells (Slettenmark and Klein, 1962). In separate experiments transplantation immunity against viable Moloney virus (M-MuLV)-induced lymphoma cells (Moloney, 1962) in mice was achieved by preinjection of M-MuLV (Sachs, 1962). New cellular antigens in Graffi virus (Gi-MuLV)-induced myeloid leukemia cells (Graffi *et al.*, 1954) were demonstrated by *in vivo* transplantation (Pasternak *et al.*, 1962) and *in vitro* cytotoxicity tests (Pasternak and Hölzer, 1965). These earlier reports were substantiated and have been extended to other MuTV strains (E. Klein and Klein, 1964; Glynn *et al.*, 1964; Klein *et al.*, 1966; Rich *et al.*, 1965; Bianco *et al.*, 1966; Kobayashi and Takeda, 1967; Fefer *et al.*, 1967a).

The existence of new cell surface antigens in FeSV or FeLV transformed or infected cells has also been established recently by immunofluorescence, immunoferritin, cytotoxic, and radioimmune techniques (Oshiro *et al.*, 1971; Essex *et al.*, 1971a,b, 1972; Jarrett *et al.*, 1973, 1974; Essex, 1974).

1. *Are the Virus-Induced Cell Surface Antigens Identical with the Ve Antigens?*

A series of papers has been devoted to the question of whether MuTV directed cell surface antigens are virus type specific, and therefore probably determined by the viral envelope, or rather group specific, i.e., common to several or all MuTV strains. The evaluation of the often contradictory results, of earlier years especially, is a difficult task for several reasons. The sensitivity of the applied methods was rarely comparable. The different techniques used in the individual studies, such as immunofluorescence, immunoferritin labeling, humoral and cellular cytotoxicity, and *in vivo* transplantation experiments, might well have revealed individual antigens that would not have been detectable by all these methods. The choice of the animal in which immune sera were prepared was also very critical since mice seem to exhibit tolerance or immune paralysis toward certain virus-directed antigens. Furthermore, it can be argued that the virus preparations used in these studies, though derived originally from individual virus stocks, do not represent clone-derived virus preparations. The virus strains were in fact often passaged and grown in mice that are known to carry a latent infection of the "wild type" Gross virus, for example, which appears to be widely distributed in most strains of inbred mice (Gross, 1966).

Evidence was early presented for at least two antigenic specificities, the G and the FMR types, detectable by mouse immune sera. By using cytotoxicity techniques an antigenic cross reaction was found between Friend (1957), Moloney, and Rauscher (Rauscher, 1962) leukemia cells (Old *et al.*, 1963a, 1964; Boyse *et al.*, 1964; Glynn *et al.*, 1964), which led to the assumption that a common FMR antigen existed. This was found to be distinct from the analogous G antigen of Gross leukemia cells which could be detected by immunofluorescence, cytotoxicity, and transplantation tests (Wahren, 1963; E. Klein and Klein, 1964; Old *et al.*, 1963b, 1965; Glynn *et al.*, 1968). The cell surface antigens of the Graffi (Gi) leukemia cells do not include the G antigen and Graffi virus has been shown to belong to the FMR system (Pasternak and Hölzer, 1965; Levy *et al.*, 1968, 1969).

In contrast to some of the above results, Rich *et al.* (1965) reported the lack of cross-protection between Rauscher and Moloney, or Rich and Moloney leukemia cells using *in vivo* transplantation techniques. On the other hand, Rich leukemia immune sera exerted an *in vitro* cytotoxic effect not only against FMR, but also against Gross leukemia cells. A weak immunological cross-reaction between some FMR and G type leukemia cells and lymphoma cells, respectively, has also been observed

(E. Klein and Klein, 1964; Old *et al.*, 1965; Levy *et al.*, 1968). The significance of these cross-reactions between FMR and G tumor cells will be discussed in the next section.

A soluble antigen has been described in the plasma of either FMR (Stück *et al.*, 1964) or G (Aoki *et al.*, 1968) type virus-infected mice which is capable of absorbing to certain normal cells, thereby rendering them sensitive to leukemia cell-specific cytotoxic antibody. The antigenic specificity of this soluble antigen is of the same type as the infecting virus, i.e., it is either FMR or G type, and is therefore thought to be antigenically identical with the FMR- or G-specific antigen which has been detected at the surface of malignant cells (Old *et al.*, 1964; Aoki *et al.*, 1966).

FMR strains of MuLV cross-react with each other to a substantial extent in virus neutralization experiments, but not with G-MuLV, provided mouse antisera are used (Levy *et al.*, 1968; Eckner and Steeves, 1972; Gomard *et al.*, 1973). The question has therefore been asked by many workers in the field, whether the FMR- and G-specific reactions of leukemia cells are due to the virus envelope antigen being exposed at the cell membrane. The additional finding that the Ve antigen of MuTV has a rather labile association with the virion suggests also that part of the soluble FMR or G antigen is identical with Ve antigen. Furthermore, this would be consistent with the ability of formalin-inactivated virus to immunize against leukemias (Pasternak, 1965; Rich *et al.*, 1965; Cohen and Fink, 1969). Arguing against the existence of new cell surface antigens other than of the Ve antigen type was the invariable finding that when virus neutralizing antibody was absent, then antibody against tumor cells was also absent (Klein and Klein, 1966). This was taken as suggestive, but not conclusive, evidence for the presence of only one new antigen, i.e., the Ve antigen at the tumor cell surface. This conclusion seemed to be further supported by the reports on the correlation between virus release and immunogenicity (Fenyö *et al.*, 1968, 1969; Ferrer and Gibbs, 1969).

In spite of the foregoing conclusion, additional studies have given the following opposing results (G. Klein and Klein, 1964; Klein *et al.*, 1966; Fenyö *et al.*, 1971; Fenyö, 1974). It was found that the degree of virus release is parallelled by immunogenicity but not by sensitivity of the tumor cell to cytotoxic effects, immunofluorescent staining, or established isograft resistance. Based on these results, a virus-induced cell surface antigen distinct from Ve antigen was postulated (G. Klein and Klein, 1964; Klein *et al.*, 1966).

The isolation of antisera with only one antibody specificity has provided clarification. Mouse G typing sera had been reported which had

little or no virus neutralizing activity (Geering *et al.*, 1966); suggesting that not all virus-directed cell surface antigens were constituents of the viral envelope. The fact that, in most instances, both virus-neutralizing and anticellular immunity coexisted in the FMR-type mouse sera, was very unfortunate for the clarification of this question in the FMR system. The removal of one or the other antibody specificity was therefore necessary. This was made possible by absorption of serum with cells productively infected, but obviously not transformed, by Gi-MuLV (Pasternak, 1967; Pasternak and Pasternak, 1968b) or with virus concentrates of F-MuLV (Steeves, 1968). Both procedures removed all detectable virus-neutralizing antibody but left tumor cell surface reactivity. By comparing the antibody absorption capacity of M-MuLV leukemia cells and L-cell virus-infected untransformed cells, Fenyö *et al.* (1974) was also able to distinguish between Ve antigen and a further antigen detectable only in transformed cells.

It is significant to note that both the Ve antigens and the nonvirion tumor cell surface antigens conform to the seggregation of FMR and G-type viruses into separate types.

A most convincing and very elegant proof that a cell surface antigen absent from the virus envelope was present in Gross leukemia cells was obtained by employing the hybrid-antibody immunoferritin method (Hämmerling *et al.*, 1968) for localizing cell surface antigens in the electron microscope. High-titer cytotoxic G-MuLV leukemia specific antisera obtained from C57Bl/6 mice which had little or no virus-neutralizing activity (Old *et al.*, 1968) stained certain distinct areas of the surface of Gross leukemia cells, but not the virion itself (Aoki *et al.*, 1970). This antigen has been referred to as G(a) or GSCA(a) in the literature and will be further discussed below. The converse result can be obtained with NZB mouse antibodies, which bind to the virion surface but not at all to the tumor cell surface, hence proving the specificity of the reaction.

In the feline system a differentiation between Ve antigen and other new cell surface antigens has not been achieved with certainty (Oshiro *et al.*, 1971; Essex *et al.*, 1971b, 1972). Only seroepidemiological studies have suggested the existence of cell surface antigens other than Ve antigen (Jarrett *et al.*, 1974).

The results reviewed in this section clearly indicate the existence of at least two kinds of MuTV-induced cell surface antigens, one of which is Ve antigen.

2. *Can Ve Antigen Act as a Transplantation Type Antigen?*

The question can be asked whether Ve antigen can indeed act as a transplantation antigen at all. The observation cited above that NZB

mouse antisera used in the immunoferritin technique stain budding virus, but not other areas of the cell surface (Aoki *et al.*, 1970), suggests the exposure of Ve antigen in that system mainly at the virus budding sites, but not otherwise on the cell surface. It has not been excluded, however, that in certain mammalian tumors Ve antigen is expressed at nonbudding sites in a manner similar to that observed in the avian system (Gelderblom *et al.*, 1972b). In a report that argues for this hypothesis, mice have been described that were immune against Gi-MuLV leukemia and likewise resistant to the transplantation of Gi-MuLV-producing Landschütz-sarcoma cells (Pasternak and Pasternak, 1968a); these cells, however, were unable to absorb antibody against FMRGi-type cell surface antigen (Pasternak, 1967). Thus, antiviral antibody or lymphocytes may combine with the budding virus or with Ve antigen molecules at nonbudding cell surface areas with the consequence of cell lysis.

3. *Cell Surface Antigens Common within a Virus Group*

Like ATV, the C-type virus groups of other species share many antigens in common, such as several antigens of the virus core and the virus reverse transcriptase. Therefore, and in analogy to the findings with ATV, the existence of nonvirion cell surface antigens that are group specific for all C-type viruses isolated in a given species might be suspected.

The observation of immunological cross reactions between virus-producing murine sarcoma and leukemia cells induced by the FMR group viruses (Fefer *et al.*, 1967a) may have been entirely trivial, because the common antigenicity could have been the Ve antigen of the helper leukosis virus as contained in excess in the MuSV stock used (Huebner *et al.*, 1966). Cross absorption experiments with MuSV and MuLV antisera indicated the immunological identity of all new cell surface antigens detectable on MuSV- and MuLV-transformed cells (Chuat *et al.*, 1969). It was not established, however, whether or not the sera used could have detected antigens other than of the Ve and the FMR type, all of which could have been provided by MuLV contained in the MuSV stock. A cross reaction, which was observed between cells infected by viruses of the G and the FMR type (E. Klein and Klein, 1964; Rich *et al.*, 1965; Levy *et al.*, 1968), seems to provide better evidence for the presence of common cell surface antigens other than of the virus envelope, because these viruses are distinct with respect to Ve antigen when tested in the mouse.

The use of rat immune sera has brought light in this darkness of ambiguous results. When rat and mouse immune sera were compared by different immunological techniques, the rat serum was found to detect additional tumor cell surface antigens that were not recognized by the

mouse serum, and which are therefore distinct (Geering *et al.*, 1966; Micheel *et al.*, 1972; Levy *et al.*, 1969; Aoki *et al.*, 1970; Herbermann, 1972; Aoki *et al.*, 1973; Kuzumaki *et al.*, 1973). The antigens detected by sera of G-MuLV infected rats were defined as follows: GCSAa, a Gross leukemia cell-specific antigen, and GCSAb, a group-specific antigen common to both G and FMR type leukemias. GCSAa can also be detected by mouse sera and corresponds to the G-type antigen described above; the recognition of the GCSAb is only possible with rat antiserum.

The analysis of the G-type soluble antigen (GSA) in mice (Aoki *et al.*, 1968) was undertaken by comparing mouse and rat G type antisera by antibody blocking techniques in immunofluorescence tests and immunoferritin electron microscopy, and has revealed the same pattern of antigens (GSAa and GSAb) on the plasma and ascitic fluid of mice. In addition, a third antigen GCSAc, which is specific for G type leukemias, was detected only by mouse antisera (Aoki *et al.*, 1972).

An antigen, which was localized not only at the cell surface but also on the virus envelope, has been demonstrated by immunoferritin staining to be common to G-MuLV and Gi-MuLV leukemias (Micheel *et al.*, 1972). Since the group- and the type-specific antigens were localized on the cell as well as on the virus surface, these authors have assumed that both antigens reside on the same molecule. The sera used neutralized Gi-MuLV as well as G-MuLV; therefore the common antigen is probably identical with the group-specific Ve antigen described by others (Aoki and Takahashi, 1972) and possibly causes the cross-neutralization results mentioned in Section IV,B,1.

Common cell surface antigen has also been reported with several FeTV strains; however, it was not determined whether this was a trivial result due to the presence of common Ve antigen (Essex *et al.*, 1973).

4. *Common Antigens in Cells of Different Species*

In analogy with the studies of other systems, there is benefit in considering common antigens induced by a given mammalian C-type virus in different cell species. Such studies have mainly been performed with MuTV-infected rat and mouse cells. One must be aware, however, that in comparing MuTV-induced tumors from these two host species, a cross reaction could be due to the Ve antigen, since for example, MuTV replicate in both mouse and rat cells.

Cross-reaction in cytotoxicity tests between rat and mouse virus-induced lymphomas has been reported with the radiation-induced leukemia virus (RadLV) that is probably identical with the wild-type Gross virus (Ferrer and Kaplan, 1968). This result as well as a cross reaction observed by Doell and Mathieson (1971) could well be caused by the Ve

antigen produced by tumors of both species. Chuat *et al.* (1969) reported a cross reaction between MuSV-transformed rat and mouse cells, but detected little if any common antigens on mouse and hamster MuSV cells with the exception of one experiment, in which hamster MuSV-cells induced a weak transplantation immunity in mice against MuSV-transformed mouse cells. In the cytotoxicity tests of these studies, mainly mouse immune sera were used. These, however, are limited in their ability to recognize MuTV-induced antigens other than Ve antigen or cell-surface antigens associated with virus synthesis and release, and therefore might have been unable to detect certain new cellular antigens on the nonproducing hamster cells.

Graffi leukemia antisera from rats which contained antibody against cell surface antigen other than Ve antigen (Micheel and Pasternak, 1972) detected also a new cell surface antigen on Gross leukemia cells of mouse. That this serum did not neutralize Gross-MuLV suggests a MuLV-specific cell surface antigen different from Ve antigen and common to mouse and rat Graffi leukemias (Micheel *et al.*, 1972).

5. *On the Tumor Specificity of Cell Surface Antigens*

In the previous sections, ample evidence has been reviewed for the existence of new cellular antigens at the surface of mammalian RTV-induced leukemia and sarcoma cells (Table V), which are not identical with Ve antigen. It remains to be considered which of these are inti-

TABLE V
MuTV-INDUCED CELL SURFACE ANTIGENS

Specification of the antigen	Immunological properties	Tumor cell specificity according to both definitions discussed in Section VI,C,3
$GCSA_a$(=GSA_a)	Gross leukemia specific	∅
FMR	FMR leukemia specific (analog to $GCSA_a$)	∅
$GCSA_b$(=GCS_b)	Group specific for G and FMR type leukemias and sarcomas	+
$GCSA_c$(=GSA_c)	Gross leukemia specific	?
Ve antigen	With either G or FMR type specificity as well as group specificity	∅
Embryonic antigens (EA)	Whether different virus strains induce the same or different EA, is unknown	?
GIX, TL, GT, G_L	Specificity unknown	∅

mately associated with the malignant character of a cell, and which are ony concomitant to virus production.

Although further investigation is necessary to determine the degree to which the Ve antigen is exposed at nonbudding cell surface areas, it is clear that Ve antigen is expressed in all cells releasing infectious virus.

It is somewhat surprising that the G type-specific GCSAa is not restricted to leukemia cells, but is also found in normal lymphoid tissue (Old *et al.*, 1965; Aoki *et al.*, 1966). Thus, this antigen can hardly be considered as tumor specific. It is likely, but has not been proved, that this also holds true for the corresponding FMR type antigen. The obvious correlation between the expression of these antigens and the synthesis of virus particles indicates perhaps that these are virion constituents, though not located on the virus envelope. That a virus constituent other than Ve antigen can indeed appear at the cell surface is suggested by the finding of viral antigen of the FMR type which is incapable of absorbing neutralizing antibody, as has been found in purified F-MuLV preparations (F. Lilly, personal communication).

The specificity of the GCSAc (Aoki *et al.*, 1972), is not known. It is unclear whether it is expressed in rat cells and recognized by the rat as a foreign antigen.

The GCSAb that is common for G- and FMR-type viruses and similar in that respect to the avian TSSA has not been detected in normal nonmalignant cells so far. Therefore, this antigen can be considered at present as the best candidate for tumor-specific cell surface antigen (TSSA). If this is indeed true, one would predict that this antigen should also be expressed on virus-transformed, but nonproducer, cells. The close similarity of avian and mammalian C-type viruses and the existence of nonvirion TSSA in ATV-transformed NP cells also makes it likely that a similar phenomenon exists in mammalian systems. Several groups have developed data bearing on this hypothesis.

As mentioned above, Chuat *et al.* (1969) could not substantiate TSSA in MuSV-induced hamster tumors. Likewise, two other groups failed to detect any new cell surface antigen in non-virus-producing MuSV mouse tumor cell lines. Stephenson and Aaronson (1972) investigated a Kirsten MuSV-transformed BALB/3T3 NP cell line with transplantation experiments and by immunofluorescence and cytotoxic tests for the presence of TSSA. The results were interpreted to show that MuSV NP cells lack detectable virus-specific surface antigens and that the immunogenicity of virus-producing MuSV-transformed cells is due to the presence of virion antigens at the cell surface. Strouk *et al.* (1972) studied M-MuSV-transformed NIH/Swiss mice 3T3 cells which, although they release a small amount of noninfections C-type particles, failed to produce infectious

virus. These cells did not react with MuLV or MuSV mouse immune sera in a mixed hemadorption test. Furthermore, a cell line mixture of these cells plus normal cells (D56) was not sensitive to the cytotoxic action of lymphocytes from MuSV-infected mice or rats. These results were taken as evidence against the proposition that MuSV-specific cell surface antigens other than Ve antigen served as TSSA in that system. The authors went even farther in the interpretation of their data to suggest that the appearance of TSSA is not intrinsic to the malignant behavior of a cell.

The inaccuracy of these conclusions and of certain methods used for the detection of TSSA was demonstrated recently by Aoki *et al.* (1973). By using the immunoferritin electron microscopic technique, these authors could demonstrate MuSV-associated cell surface antigen on NP cells, which had been found to be negative when tested by other techniques (Stephenson and Aaronson, 1972). This new antigen was distinct from the Ve antigen. TSSA in NP-MuSV-mouse cells has also been demonstrated by transplantation experiments (Law and Ting, 1970).

The expression of several alloantigens in leukemia cells has been described: the TL antigen (Boyse *et al.*, 1967), the G_{IX} antigen (Stockert *et al.*, 1971), and most recently the G_L and G_T antigens (Nowinski and Peters, 1973). None of these, however, can be considered as tumor specific because they are also expressed in normal cells. Some of them have been shown to be regulated by genes closely linked to loci controling synthesis of virus-specific antigens (Ikeda *et al.*, 1973; Nowinski and Peters, 1973), and this may be the reason for their characteristic expression in leukemia cells.

6. *On the Origin of the Virus-Directed Cell Surface Antigens*

As pointed out in the preceding section, the best candidate for a murine TSSA is the group-specific GCSAb. Two recent reports suggested the expression of group-specific virus structural antigens of MuTV and FeTV at the tumor cell surface. Ferrer (1973) found that a guinea pig antiserum prepared against purified p 31 virion antigen was cytotoxic, and that this activity could be completely absorbed by tumor cells, and Yoshiki *et al.* (1973) demonstrated that the cytotoxic effect of a rabbit serum prepared against ether-extracted FeLV could be completely absorbed with purified p 31. Furthermore, the cytotoxic effect of spleen cells from mice with regressing M-MuSV-tumors could also be blocked by treatment with p 31 (K. Knight, personal communication). Nowinski *et al.* (1972a) observed a weak cytotoxic effect exerted by p 31 and p 15 antisera against leukemia cells. Thus, the evidence for internal virion antigens being expressed at the cell surface seems rather strong. It will be interesting to see whether this expression is restricted to trans-

formed cells or is also demonstrable on productively infected, but untransformed, cells. The possibility that p 31 is really identical with GCSAb needs further analysis.

As with the other tumor systems, embryonic antigens have also been detected on the surface of MuTV transformed cells (Hanna *et al.*, 1971; Ishimoto and Ito, 1972; Ting *et al.*, 1972; Fenyö *et al.*, 1974), but none of these EA could be identified with tumor-specific cell surface antigens. However, the same argument applies here as with the avian system, namely, that the embryonic cells used for comparison might not have been from the stage of gestation in which the putative TSSA is normally expressed as an embryonic antigen.

An answer to the question of which of the virus-directed cell surface antigens are virus coded can probably be provided with certainty only by *in vitro* protein synthesis experiments. It is promising that the virus coding of some of the major virion proteins has also been demonstrated recently in the murine system using the viral RNA as messenger (Gielkens *et al.*, 1972; Twardzik *et al.*, 1973). Although it will be difficult to identify small amounts of *in vitro* products, this method when improved might be useful for that purpose.

C. General Discussion of TSSA

1. *Biochemical Constitution of TSSA*

TSSA are immunologically rather weak antigens, scarcely detectable in comparison to normal histocompatibility antigens. Convenient methods for a sensitive *in vitro* assay of TSSA, as will be necessary to follow the antigen through isolation procedures, have been described only recently. This is probably the reason why there are almost no reports on the isolation and biochemical characterization of RNA tumor virus-specific TSSA. A glycoprotein with a MW of 70,000 d that has been isolated from murine leukemia cells was apparently identical with all or part of the Ve antigen (Kennel *et al.*, 1973). A transplantation type antigen isolated from R-MuLV cells was assumed not to be a virion constituent, although this was not proved with certainty (Law and Apella, 1973). Most recent experiments in our laboratory have led to the isolation of ATV-specific TSSA from transformed mouse (Pauli *et al.*, in preparation) and chicken cells (Rohrschneider *et al.*, in preparation). The complete analysis has not yet been performed, but there is evidence that the TSSA isolated from chicken cells is slightly larger than the gp 85 Ve antigen and glycoprotein in nature. The latter might be expected in analogy to histocompatibility (H-2) antigen (Reisfeld and Kahan, 1970; Muramatsu and Nathenson, 1970; Davies *et al.*, 1969) and to the TSTA of a chemically induced tumor

(Harris *et al.*, 1973), both of which have been highly purified and shown to be glycoprotein.

A fucose-containing glycopeptide is present in relatively higher concentrations on the surface of RTV-transformed cells as compared to normal cells (Buck *et al.*, 1970, 1971a,b). It is not known whether this observation has anything to do with TSSA. Remarkably, this glycopeptide is found only in cells infected at permissive temperature by a temperature-sensitive mutant of RSV (Warren *et al.*, 1972a). At nonpermissive temperature (40°C), this virus still replicates in CEC but cannot maintain the state of transformation (Martin, 1970). Absorption experiments with TSSA specific antibody can now be performed to determine whether this glycopeptide has tumor-specific antigenic properties.

The characteristic agglutinability of malignant cells by low doses of various plant lectins has been considered a property of tumor cell (for review, see Tooze, 1973), and it seems to be associated with enhanced cell growth, since the growth pattern of transformed cells could be restored to that of normal cells by covering lectin receptors with protease-treated lectin molecules (Burger and Noonan, 1970). The chemical nature of the lectin receptors is not precisely known but involves carbohydrate residues (Burger, 1969; Inbar and Sachs, 1969a), which are also present in normal cells but apparently in a differently arranged conformation (Inbar and Sachs, 1969b; see also Tooze, 1973). Agglutinability of RTV-transformed cells has likewise been observed (Bicquard and Vigier, 1972; Burger and Martin, 1972; Kapeller and Doljanski, 1972; Lehman and Sheppard, 1972; Salzberg and Green, 1972; Kurth and Bauer, 1973a), and the question can therefore be asked as to whether the agglutinin receptor molecules have any relationship to TSSA. This, however, seems to be unlikely because the expression of TSSA and agglutinability of tumor cells did not seem to be correlated (Kurth and Bauer, 1973a,d). When the intracellular concentration of cyclic adenosine 3′:5′ monophosphate (cAMP) is increased by addition of dibutyryl cAMP and theophylline, tumor cells take on a normal morphology and growth pattern (for references, see Tooze, 1973) and the agglutinability of the cells by plant lectins is distinctly reduced (Sheppard, 1971; Hsie *et al.*, 1971; Kurth and Bauer, 1973a). The expression of TSSA is, however, not reduced (Gazdar *et al.*, 1972) and has been found even to increase (Kurth and Bauer, 1973a,d). These findings and the characteristic association of certain TSSA with tumors of specific viral etiology do not support the assumption that TSSA and lectin receptors are related.

2. *The Pattern of TSSA at the Cell Surface*

Several studies have shown that TSSA appears in discrete areas and is not randomly distributed on the cell surface. Immunoelectron mi-

croscopy techniques revealed that TSSA and histocompatibility antigens were exposed at different cell surface areas (Aoki *et al.*, 1970; Micheel *et al.*, 1971). The expression of TSSA is also not constant over the whole cell cycle and is maximal in the G_1 period (Cikes and Friberg, 1971; Cikes and Klein, 1972). It is possible that TSSA replaces normal cell surface constituents because the amount of some other cell surface molecules decreases in transformed cells. It has been recently reported that certain proteins of normal cells which have been characterized by electrophoresis cannot be detected in transformed cells (Bussell and Robinson, 1973; Wickus and Robbins, 1973). Likewise, glycolipids which are increased at high cell density in normal cell populations are sharply decreased after viral transformation of cells (Hakomori *et al.*, 1971).

3. *Are TSSA Functionally Linked to the Oncogenic Properties of the Transformed Cell?*

It seems as though in the discussions about the significance of tumor antigens, either of two definitions have been used in order to define tumor specificity. One definition says that an antigen is tumor specific if it is present only on tumor cells, but not on untransformed cells. According to the other definition, an antigen is considered to be tumor specific if it is expressed in all tumors of the same virus etiology.

With respect to the second definition, a note of caution must be sounded. As has been pointed out, various of the antigens as expressed on the tumor cell surface are also detectable in normal cells; for example, the GCSAa and the TL antigen in the mouse system. In productively infected but untransformed cells, the virus envelope antigen itself would fulfill this criterion for a tumor-specific antigen. These examples almost certainly deal with antigens that have no significance for the malignant state of a cell, since they are only concomitant to virus replication. In fact, both Ve antigen, and GCSAa of the mouse system have so far not been detected in MuSV NP cells. It has not been excluded, on the other hand, that antigens like GCSAa have some function in the malignant process which is only necessary and functional in cells of the natural host.

Antigens satisfying the criterion of the first definition seem more appropriate for consideration as candidates for an essential functional component of the tumor cell, because cell transformation more closely parallels their expression. It appears that such antigens have been unambiguously demonstrated in both ATV (TSSA) and MuTV (GCSAb) systems. The proof was more difficult in the MuTV system because of the absence of antibody in the natural host, the mouse, and due to the lack of a convenient assay for the appearance of transformation in cells infected *in vitro* with LV. The latter problem, in particular, made it impossible to compare transformed and productively infected, untrans-

formed cells, as had been done in the avian system. It is not known why mice appear to be tolerant to GCSAb. In that connection it is interesting to note, that mice are also apparently tolerant to the p 31 virion antigen that has been proposed as a TSSA of leukemia cells (Ferrer, 1973; Yoshiki *et al.*, 1973).

The TSSA of the ATV and GCSAb of the MuTV are tumor specific according to both the first and the second definition, i.e., they are present uniquely in transformed cells and are expressed in all cells transformed by viruses of a given group. Various exceptional reports on the failure to detect TSSA in such virus transformed cell systems might have been a result of technical considerations. Thus, for example, Pasternak (1967) has demonstrated by *in vitro* methods TSSA in leukemia cells which could not be demonstrated as a TSTA with *in vivo* experiments.

4. *Control of TSSA Expression*

Evidence for the regulation of TSSA expression by the host cell, irrespective of the question of whether these antigens are virus or cell coded, comes from the experimental finding, that LV can replicate in fibroblasts in culture without producing TSSA, although TSSA is invariably expressed, after *in vivo* LV infection of the appropriate target cell gives rise to transformed leukosis cells. Because expression of TSSA appears as a prerequisite for and is thus closely linked to transformation, its expression may be the most approachable parameter to use in beginning the study of the transformation process.

In the case that TSSA are coded for by the virus genome, one must postulate a regulation mechanism functioning in fibroblasts to repress the TSSA-gene of LV, but not of SV. On the other hand, if TSSA are cell coded, LV may simply fail to derepress the appropriate cellular gene in fibroblasts. A third intermediate model may also be considered, that the expression of TSSA and cell transformation could depend on the integration site of the viral DNA, which in turn might be influenced by the structure of the virus genome. This integration site could well be linked with cell genes controlling the expression of cellular antigens like EA, the function of which could also be essential to malignant cell growth behavior. It is interesting in this connection that hydroxylamine-induced deletion mutants of SV (Graf *et al.*, 1971), which behave biologically like LV in that they produce leukemia *in vivo* (Biggs *et al.*, 1973), also fail either to transform or to induce TSSA in CEC *in vitro*.

The mouse is far better genetically defined than the chicken and is probably the best system for the study of these questions. In fact, cell genes have already been defined in that system which influence the leukemogenic activity of different virus strains by mechanisms that do not

simply involve the presence of cell surface receptor sites allowing virus penetration (Huang *et al.*, 1973; Lilly and Pincus, 1973; Lilly and Steeves, 1973; Rowe *et al.*, 1973).

5. *Function of TSSA*

It is generally assumed that changes in the tumor cell surface allow the cell to escape the effects of growth control mechanisms exerted by adjacent cells. It is reasonable therefore to attribute such alterations to the presence of new antigenic molecules that are found consistently on the surface of tumor cells. Although an increasing body of data on TSSA is accumulating, it appears still rather premature to speculate too far on the possible biological function of these antigens; however, a few statements seem to be justified.

Because of the close correlation between the appearance of the transformed state and the expression of TSSA, it seems likely that TSSA is somehow essential to the maintenance of the malignancy (see Table VI). However, there is enough information available to exclude the possibility that the expression of TSSA alone is sufficient to induce the transformed state of a cell (see Table VI). Once TSSA have been produced, they may or may not remain present after tumor cells have reverted to normal. TSSA does not disappear, after phenotypic reversion to normal of transformed cells is induced by cAMP (Kurth and Bauer, 1973a,d). Furthermore, different RSV temperature-sensitive (ts) mutants exist (Wyke and Linial, 1973), some of which exhibit a correlation between the absence of TSSA in cells reverted to the normal state at the nonpermissive tem-

TABLE VI
CORRELATION BETWEEN THE EXPRESSION OF TSSA AND CELL TRANSFORMATION IN THE AVIAN SYSTEM

Virus host system	Cell transformation	Appearance of TSSA
SV_{wt}[a] 37°C in CEC	+	+
SV_{ts23}[b] 35°C in CEC	+	+
SV_{ts23} 41°C in CEC	∅	+
SV_{ts29} 35°C in CEC	+	+
SV_{ts29} 41°C in CEC	∅	∅
SV Mouse cells	+	+
SV Mouse cells at high cAMP conc.	∅	+
LV CEC	∅	∅
LV Hematopoietic cells	+	+

[a] wt, Wild type.
[b] ts, Temperature sensitive.

perature, and others of which appear to be constitutive for the production of TSSA even when at the nonpermissive temperature the cells lack any other functions of the transformed state (Kurth *et al.*, 1974b). Therefore, a second, or even several other molecular events, must be postulated as prerequisites for malignancy. Studies with ts mutants of RSV have indicated the participation of at least four cistrons present in the viral genome for the expression of the transformed state (Wyke, 1973). Whether these possible virus cistrons may specify information for the expression of TSSA, the increased sugar transport across the host cell membrane (for review, see Hatanaka, 1974), the enhancement of cell protease (Schnebli and Burger, 1972) and fibrinolysin (Unkeless *et al.*, 1973), or the derepression of EA, is a matter of pure speculation. Much effort will have to be expended in order to make the difficult proofs of identity between particular genetic loci and the expression of tumor cell-specific functions.

VI. Concluding Remarks

The answers to all the questions raised in Section I are not yet known, in spite of the vast literature that has accumulated on the subject. Nevertheless, our knowledge about RTV directed antigens has been extended, and some intriguing clues have been obtained. Thus, it appears that the biochemical investigation of the major constituents of purified virus, at least for the avian and the murine systems, has almost reached a final and successful stage. On the other hand, several minor protein components of the virion have not yet been studied because they have not been accumulated in sufficient amounts. These certainly deserve attention because they may represent macromolecules of high biological significance. Furthermore, an understanding of the pathway of intracellular synthesis of even the major constituents remain to be achieved.

A direct approach to the question of which of the virus-directed proteins are coded for by the virus genome seems now to be possible, since it has been demonstrated that the viral RNA itself acts as a messenger for the synthesis of viral structural proteins (Hlozanek *et al.*, 1967; Riman *et al.*, 1967; Siegert *et al.*, 1972; Gielkens *et al.*, 1972; Twardzik *et al.*, 1973). *In vitro* protein synthesis with such viral RNA as messenger has already revealed that at least some of the major gs antigens of ATV and MuTV are coded for by the virus.

There is no doubt that a more thorough biochemical investigation of the virus-directed antigens in the cell, especially at the cell surface, is now urgently needed. The immunological approach to the study of virus host cell interactions is certainly insufficient for the understanding of the molecular mechanisms of malignant transformation. However, immunological methods have served and will certainly continue to serve as

the basis for further biochemical investigations because they allow the identification, and aid in the purification, of tumor-specific molecules.

A biochemical study of tumor cell surface antigens promises to be helpful for the rational development of immunoprophylactic techniques against cancer. The immunological "blocking" or "enhancement" effects which are known to occur in tumor-bearing hosts, and which seem to interfere to a large extent with the capability of the host to reject tumor cells, can probably be studied more effectively if the antigenic molecules involved in those reactions could be prepared in a biochemically pure form. Furthermore, the development of immune reagents, for example immune lymphocytes, that could be used for cancer therapy or other immunological techniques, which at our present state of knowledge cannot even be visualized, can probably best be produced with biochemically pure tumor cell antigens. Preparations of tumor cell surface antigens can even be considered as vaccines, now that it has been clearly demonstrated at least in one system (feline) that cancer can be horizontally transmitted, probably by virus (Jarrett *et al.*, 1973; Hardy *et al.*, 1973), and that there is an inverse relationship between tumor development and the presence of tumor-specific antibodies (Essex *et al.*, 1973). Because of the existence of TSSA that are common to all viruses of a given group, attention should be focused on the group-specific TSSA, which may allow a simultaneous immunological attack against a variety of potential tumors occurring within a given species. An initial step for this program in human research must be the isolation and development of a biological system for the growth of transforming human RNA tumor viruses. It is the hope that such a situation may also exist in man and that the animal systems will be proved to be valid models for human cancer research.

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ADDENDUM TO "MOLECULAR REPAIR, WOUND HEALING, AND CARCINOGENESIS: TUMOR PRODUCTION A POSSIBLE OVERHEALING?"[1]

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Nicolai, you must remember, what is broken here on earth, will be made whole in heaven.

"The Birds Fall Down," Rebecca West

Cimented with a fast balme.

"The Extasie," John Donne

I. Introduction

Current interest in the physiopathology of repair, at all levels from the molecular to the cellular, is so great that the writer has found it advisable to add a brief note to the article entitled "Molecular Repair, Wound Healing, and Carcinogenesis: Tumor Production a Possible Overhealing?" which appeared in Volume 16. While the treatment is by no means complete, he has been impressed, as before, by the relevance of much of the older literature as well as the new, and especially by the comparison between malignancy and wound repair, and the possibility that the former may be construed as a species of overhealing. I am specially indebted to my friend Professor Hermann Druckrey of Freiburg for many references to the older contributions of German pathology

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and particularly to those of Virchow.[3] Thus, overhealing seems to correspond in some way to the views of Virchow on disturbances of chronic regeneration in carcinogenesis (see Virchow translation, 1971). Furthermore it appears that the process would necessarily involve heritable changes, directly or secondarily. Virchow's notion was originally restricted to chronic irritation and exhaustion of regeneration in carcinogenesis. Later this was interpreted as overhealing by the pathologist Fischer-Wasels (1928, 1932), who, early in the century, carried out some of the first, if unsuccessful, experiments in chemical carcinogenesis, using the dyestuff Biebrich Scharlach R, a derivative of *o*-aminoazotoluene, which, in its acetylated form (Pellidol), has been used for stimulating wound healing. Fischer-Wasels was tempted to regard the lesions he observed (in the rabbit) as tumors, but this view was soon refuted by others. However, there seems to be little doubt that it was the phenomenon of wound healing which led Fischer-Wasels to these attempts. Other relevant papers are Cleland (1868), Arnold (1869), Wadsworth and Eberth (1870), Hoffmann (1870), Klebs (1874), and Cohnheim (1874).

It is now evident that repair in the widest sense can be effected through a variety of mechanisms. Thus, Crew (1928, p. 402) wrote in his Milroy Lecture: "Certain defects and derangements can even now be repaired, the cretin can be made normal, hereditary disharmonies in many instances can be made good by modern therapeutic interference. . . . surely insulin and thyroxin are but forerunners of a great list of synthetic chemicals which can be used to cancel and correct the action of undesirable genes." According to Lorrain Smith (1932): "The procession starts from the impregnated ovum. Just because this cell shares the lives of the parent cells, but has cast loose from their realised organic relationships with surrounding cells, it proceeds to reproduce these relationships step by step; and the normal growth-procession expresses this reproduction, which we can thus compare with the process of healing." A special case of repair is perhaps to be found in the reversibility of tumors, and particularly the reversibility frequently observed in skin carcinogenesis (Roe *et al.*, 1972). Again, Smith (1972) wrote: "Without entering more deeply into speculation as to whether or not carcinogenesis is accompanied by a reversal of some wound healing processes, it is not unreasonable to suppose that, at some stages at least, the pseudopodia observed in carcinogen-treated tissue are part of a wound healing response." And in the same volume, Tarin (1972) expressed the view: "It has been known for some time that following simple skin incision there

[3] A genius in pathology, Virchow nevertheless seems to have become somewhat autocratic in old age. He was opposed not merely to Bismarck, but also to Pasteur, Robert Koch, and Emil von Behring.

is considerable local tissue disorganisation and the epidermis invades the underlying connective tissue (Ordman and Gillman, 1966). However, in this situation the epithelial invasion is directional, in that the ingrowths from each side of the wound grow towards each other, and it is also limited for it ceases after three to four days, a short time after the two ingrowths unite. Comparison of the ultrastructural features of epithelial invasion in wound healing and in carcinomas was therefore considered likely to be instructive." In a paper at the First International Congress of Biorheology, Weissenberg (see Seaman and Chien, 1973) gave it as his interesting opinion that prior to the occurrence of irreversible changes, such as the fracture of a bone, or the rupture of a blood vessel or blockage of a blood vessel, there is a transition period during which remedial action may be effective. On the more chemical level, in a symposium (Anonymous, 1973a) on free radicals in pathology, further consideration was given to the role of scavenging agents in repair. The fact that the sequel to trauma is occasionally neoplasia, not repair (Anonymous, 1973b; see also Behan, 1939), is suggestive of an analogy between the two. Finally, Hanawalt (1972) has provided a review of the repair of genetic material in living cells.

II. Repair Replication

Increasing interest in the general functions of repair, and particularly in its biochemical mechanisms, has been reflected in an increasing flood of publications, of which the following are only a few. A characteristic of the lethal effect of ionizing radiation on Hcr^- bacterial strains was described by Alper (1967). Eight strains, selected by various workers for ultraviolet sensitivity (six of them being Hcr^-), were exposed to ionizing radiation in the presence and in the absence of oxygen. In all cases the sensitivity in anoxic conditions of the UV-sensitive strains was greater than that of their parents, and the oxygen enhancement ratio was considerably less. These data support the concept that the mode of X-ray damage which is bypassed or repaired in the resistant parents is of a different type from that which is mainly responsible for the oxygen effect. Bleichrodt *et al.* (1972) examined the involvement of the excision repair system in recovery of bacteriophage. Bacteriophages T1 and λvir irradiated with gamma rays under anoxic conditions show a higher survival when assayed on an *Escherichia coli* Hcr^+ strain than when assayed on a Hcr^- derivative (uvr A, B, or C). Hardly any difference in sensitivity is seen for phage irradiated in the presence of oxygen. Assayed on a strain deficient in DNA polymerase I, the survival of irradiated T1 is lower than on the wild-type parent, for irradiation under both oxic and anoxic conditions. It was concluded that the damage sustained under oxic conditions, which is repaired by the excision repair system, does

not need the products coded by the uvr genes, whereas part of the damage inflicted under nitrogen does. This conclusion is supported by the observation that the sensitivities of bacteriophages T4D and T4Dv_1 for gamma rays differ under nitrogen, but are identical under oxygen.

In a study of the effect of hypoxia on the repair of sublethal radiation damage in cultured mammalian cells, Hall (1972) used Chinese hamster cells synchronized with hydroxyurea and then stored at room temperature. These G_1/S cells were uniform in sensitivity, did not progress through the cell cycle at room temperature, and their response to a single acute exposure of X-rays remained constant for at least 24 hours of room temperature storage. These cells were used to investigate the repair of sublethal damage under aerated and hypoxic conditions. Under aerated conditions, a recovery factor of 3 was observed; under conditions of extreme hypoxia, defined by the presence of less than 16 parts per million of oxygen in the gas phase, repair of sublethal damage appeared to be completely inhibited.

UV inactivation of transforming *Bacillus subtilis* markers was studied by Bron and Venema (1972) with the aid of an 8-fold auxotrophic recipient and its excision-repair-deficient derivative. The results allow the following conclusions: Wild-type *B. subtilis* cells are able to repair approximately 80% of the UV-induced lesions causing inactivation of transforming activity in UV-sensitive recipients; saturating amounts of donor DNA increase the apparent marker sensitivities, this phenomenon being most pronounced in transformation of UV-sensitive recipients; various markers are inactivated to different degrees, both when assayed on the wild-type as well as on the UV-sensitive strain; various markers are repaired to different degrees in the wild-type recipient. The results favor the hypothesis that in this system differential UV inactivation of transforming markers is mainly due to differences in the frequency or distribution of potential photochemical lesions in the transforming segments of DNA.

The effect of temperature on the repair mechanism of radiation-induced damage in *E. coli* was reported on by Iyer (1972). Some strains of *E. coli* B have enhanced survival if initially incubated at 18°C after irradiation with X-rays or gamma rays. This process is referred to as low temperature reactivation. B/r and B_{s-1} cells were studied for the manifestation of low temperature reactivation under a variety of pre- and postirradiation incubation conditions. The radiation-induced DNA degradation at 18° and 37°C for cells grown in the presence and in the absence of glucose was also examined. On the basis of the results, Iyer then discussed the possible repair mechanism in *E. coli* B/r.

According to Resnick and Setlow (1972a) in their study of repair of pyrimidine dimer damage induced in yeast by UV light, crude extracts

from UV-irradiated yeast cells compete with UV-irradiated transforming DNA for photoreactivating enzyme. The amount of competition is taken as a measure of the level of cyclobutyl pyrimidine dimers in the yeast DNA. A calibration of the competition using UV-irradiated calf thymus DNA indicates that an incident UV dose (1500 ergs/mm^2) yielding 1% survivors of wild-type cells produces between 2.5×10^4 and 5×10^4 dimers per cell. Wild-type cells irradiated in the exponential phase of growth remove or alter more than 90% of the dimers within 220 minutes after irradiation. Pyrimidine dimers induced in stationary-phase wild-type cells appear to remain in the DNA; however, with incubation, they become less photoreactivable *in vivo,* although remaining photoreactivable *in vitro.* In contrast, exponentially growing or stationary-phase UV-sensitive cells (rad 2–17) show almost no detectable alteration of dimers. It was concluded that the UV-sensitive cells lack an early step in the repair of UV-induced pyrimidine dimers. In a second paper Resnick and Setlow (1972b) found that the amount of photoreactivating enzyme in tetraploid cells of *Saccharomyces cerevisiae* and the ability of the cells to be photoreactivated after UV irradiation are directly proportional to the number of genes per cell involved in the synthesis of photoreactivating enzyme. Also in the field of photoreactivation, Fortuin (1971) has studied photoreactivation of UV damage in uvs D and uvs E mutants. Photoinhibition of respiration in yeast and bacteria and its recovery in yeast were investigated by Ninnemann (1971), who concluded that addition of substrate to irradiated yeast allows resynthesis of proteins which were destroyed by blue light. Paterson and Roozen (1972) examined the photoreactivation, excision, and strand-rejoining repair in R-factor-containing minicells of *E. coli* K12.

Ley and Setlow (1972) reviewed repair replication in *E. coli* as measured by the photolysis of bromodeoxyuridine. The ability selectively to photolyse bromouracil-containing repaired regions in cellular DNA allowed these authors to estimate the average size of repaired regions in UV light-irradiated *E. coli.* Cells were labeled with thymidine-^{3}H, irradiated at 254 nm, and incubated in nonradioactive bromodeoxyuridine. After incubation the cells were exposed to 10^6 ergs·mm^{-2} at 313 nm, lysed, and sedimented in alkaline sucrose gradients so as to measure the average molecular weight of single DNA strands. In strains that had excised ~45 cyclobutane pyrimidine dimers/10^8 daltons, the 313 nm treatment resulted in ~6 single-strand breaks/10^8 daltons. In an excisionless strain, the same treatment resulted in only 1.5 breaks/10^8 daltons. From the determination of the sensitivities of fully substituted DNAs to 313 nm light, it was concluded that the repaired regions in excising strains of *E. coli* contain an average of 4–6 bromouracil residues. Photoreactivation experiments indicate that the excision of pyrimidine

dimers in the presence of bromodeoxyuridine is the primary source of repaired regions selectively photolysed by 313 nm radiation.

The effect of mitomycin C or nitrogen mustard in *E. coli* K12 strains T71 arg^- and AB2463 (rec^-) was studied by Schjelderup *et al.* (1972) under conditions where the cellular DNA remains constant. After exposure of T71 arg^- and AB2463 to mitomycin C (10 μg/ml) or nitrogen mustard (300 μg/ml), an incorporation of labeled thymidine into DNA (no increase in cellular DNA) corresponding to about 20% of that in control culture was found in T71 arg^-. No incorporation took place in the case of AB2463. There was no incorporation of thymidine-methyl-^{3}H into the cellular DNA of the T71 arg^- when arginine was absent or chloramphenicol was added during exposure to mitomycin C or nitrogen mustard. The results could be interpreted to mean that repair replication after treatment with alkylating agents is dependent on protein synthesis. When T71 arg^- and AB2463 were prelabeled with thymidine-methyl-^{3}H, a decrease with time in the total radioactivity of cellular DNA was found after exposure to mitomycin C. The degradation of DNA was not dependent on protein synthesis.

The formation and repair of thymine base damage was investigated, by Hariharan and Cerutti (1972), in *Micrococcus radiodurans* after exposure to gamma rays. The radiolysis products of thymine were determined in the cytoplasm and the culture medium after their release from the DNA during postirradiation incubation. It is estimated that the thymine product is formed in the DNA of *M. radiodurans* with an efficiency of about 1.2×10^{-6}/rad, 10^6 daltons for irradiation in air and 10^{-6}/rad, 10^6 daltons for irradiation in nitrogen. Damaged residues are released from the DNA of irradiated *M. radiodurans* into cytoplasm and medium during postirradiation incubation in a biphasic, selective process. On the average, about 300 undamaged thymine residues are removed from the DNA per damaged residue during 240 minutes postirradiation repair after irradiation in air. The first phase of removal (0 to 30 minutes at 37°C) and postirradiation degradation may be part of the same process. Selectivity of product removal may be due to product clustering. Degradation is assumed to start at nearby radiation-induced breaks. After a temporary halt, a second phase of product removal is observed (60–200 minutes). Hoffman (1972) studied the characteristics of repair mechanisms operating subsequent to genetic damage induced by ethyl methanesulfonate and gamma irradiation in *Bracon hebetor*.

The thermal deactivation of DNA was described by Woodcock and Grigg (1972). Their results show that a large number of breaks is produced in 15 minutes at 52°C, most scoring as double-stranded breaks.

They could, however, find conditions in which the breaks were fully repaired, and this must include most of the double-strand breaks. The process might be analogous to the conversion of linear lambda DNA to its circular form: complementary overlapping regions allow a ligase covalently to join the adjacent ends of the phage chromosome. Presumably DNA polymerase I would also be associated with repair since some exonuclease degradation accompanies thermal induction of DNA strand breakage. While it is widely held that double-strand breaks cannot be repaired, Kitayama and Matsuyama (1968) and Dean *et al.* (1966) have suggested that such breaks are repaired in *M. radiodurans,* which is resistant to UV light, ionizing radiation, and thermal inactivation (Bridges *et al.,* 1969). The discovery of an effective process for repairing thermally induced breaks in DNA suggests that similar breaks may occur at lower, physiological temperatures, but that they are repaired with high efficiency. Their presence would become apparent only if the repair system were inhibited or saturated (Gross *et al.,* 1971). Of the mechanisms of DNA dark repair, excision-insertion may be blocked at the excision stage (uvr mutants of *E. coli*) or at a later stage (pol I mutants). Recombination repair, less well understood, is deficient in the mutants rec A, rec B, rec C, and exr A. Pyronin Y (0.16 m*M*), coumarin (4 m*M*), 6,9-dimethyl-2-methylthiopurine (2 m*M*) completely inhibit excision repair with little effect on recombination repair (Grigg, 1972). The repair of thermally induced DNA breaks was completely blocked by pyronin Y, whereas coumarin and methylthiopurine had no effect whatever. Thus this repair system inhibited by pyronin Y appears to be different from either of the two repair systems previously described. That the rec A but not rec B or C function may also be involved in the repair of thermally induced lesions is suggested by the strong synergism of pyronin Y and temperature in a rec A-13 strain only.

Bridges (1972) presented evidence that a third DNA dark repair process for UV damage, reinitiation recovery, exists in addition to excision repair and postreplication (recombination) repair; that it involves the repair and removal of a "stalled" replication point and the initiation of a new one at the chromosomal origin; that it is inhibited by chloramphenicol and acriflavine; that it is dependent upon both rec^+ and uvr^+ functions; and that it is responsible for the shoulders on some UV survival curves.

In a study of excision repair properties of isogenic rec mutants of *E. coli* K12, Shlaes *et al.* (1972) found that a rec B^- rec C^- strain excises dimers at a rate nearly that of the rec^+ parent, reaching the same extent of excision after a 1-hour postirradiation incubation. Rec A^- and rec A^- rec B^- strains excise 75–80% of the dimers excised by their rec^+ parent,

whereas a uvr B^- strain excises no dimers during a 1-hour incubation. The doses of UV light (254 nm) required to reduce survival to 37% of the original population are 8 ergs/mm^2 for rec A or rec A rec B mutants, 5 ergs/mm^2 for uvr B^- strain, 30 ergs/mm^2 for the rec B rec C mutant, and 230 ergs/mm^2 for the wild-type parent. From these data one cannot account for the UV light sensitivity of rec^- strains on the basis of their excision repair properties. It was concluded that rec gene products play no significant role in the early steps of excision repair. To show the properties and validity of their method, the authors present results of experiments with thymine dimers formed *in vitro* and *in vivo* in *E. coli* K12 and claim the method to be reproducible and sensitive to 0.005% of the total radioactive thymine present in thymine-containing dimers.

In an electron microscope study of substrate specificity of T4 excision repair endonuclease, Friedberg and Clayton (1972) noted that, following infection of a suitable host by bacteriophage T4, a phage-specific endonuclease that makes single-strand breaks in UV-irradiated DNA is produced (see Yasuda and Sekiguchi, 1970), and presumed that this enzyme is involved in pyrimidine dimer excision, since a mutant phage $T4v_1$ (which does not produce detectable enzyme) is abnormally sensitive to UV light and fails to excise dimers from its DNA. Previous *in vitro* studies utilizing sedimentation velocity of DNA in sucrose density gradients showed UV dose-dependent reduction in sedimentation velocity of UV-irradiated T4 and T7 DNA following incubation with the purified enzyme. The average number of enzymatically induced nicks per molecule had been found to correlate well with the calculated number of dimers per molecule (Friedberg and King, 1971), and these studies suggested that the only substrate sites in UV-irradiated DNA were pyrimidine dimers. Unirradiated DNA was unaffected by the enzyme, and it was concluded that such DNA either does not contain areas of distortion in the secondary structure such as might be produced by pyrimidine dimers, or if such distortions are present they are not recognized as substrate sites by the endonuclease. Friedberg and Clayton go on to describe the use of a technique which provides extraordinary sensitivity for the detection of phosphodiester bond scissions in DNA and also allows the determination of the pyrimidine dimer content of UV-irradiated DNA by a totally independent method which does not require radioactive labeling. The procedure involves the differentiation of supercoiled circular SV40 DNA from relaxed circular DNA, a task that is readily accomplished by electron microscopic examination. As the change from the supercoiled to the relaxed state is dependent on a single phosphodiester bond break, this technique provides the required sensitivity for examination of the substrate specificity of the T4 excision repair

endonuclease. SV40 DNA UV-irradiated at 300 ergs/mm^2 without enzyme addition showed no evidence of radiation-induced phosphodiester bond breakage. Incubation of unirradiated SV40 DNA with endonuclease did not show any reduction in the fraction of form I molecules. This experiment was performed with and without added $MgCl_2$ with the same result. Purified T4 excision repair endonuclease clearly does not nick unirradiated DNA. On the other hand, UV-irradiated DNA is attacked by the endonuclease, resulting in the conversion of form I to form II molecules. No linear DNA molecules were ever observed, confirming the observation that the endonuclease makes only single-strand breaks. After complete reaction, the fraction of form I molecules follows a Poisson distribution as a function of the UV dose. According to this distribution, 37% of the molecules are in the form I state when there has been an average of one endonucleolytic incision per molecule. If every endonucleolytic incision occurred at a pyrimidine dimer, the UV dose at which 37% of the molecules are in the form I state should have produced an average of one dimer per molecule. Assuming a molecular weight of 2.8×10^9 for *E. coli* DNA, 1 erg/mm^2 would produce an average of $[(2.8 \times 10^9)/(3.2 \times 10^6)] \times 1/172.8$ or 5.06 dimers per *E. coli* genome. The dimer content per erg of the *E. coli* genome has been previously quoted in the literature as 5.0, 5.2, and 6.0 (respectively: Boyle and Setlow, 1970; Setlow *et al.*, 1963; Howard-Flanders and Boyce, 1966) and for the T4 genome (molecular weight of 130×10^6) as 0.23 (Friedberg and King, 1971). As the T4 genome is 21.53 times as small as that of *E. coli* the value of 0.23 extrapolates to 5.0 for a molecular weight of 2.8×10^9. The correlation between the expected and observed number of dimers per SV40 DNA molecule suggests very strongly that every endonucleolytic incision occurred at a pyrimidine dimer site. At the very low doses of UV irradiation used, the probability of photoproducts other than dimers producing the same statistical coincidence is extremely unlikely.

Studying the role of bacteriophage T4 genes in radiation repair, Maynard-Smith and Symonds (1973) suggested that the polymerase activity associated with this repair synthesis is provided by the bacterial Kornberg polymerase pol I.

The DNA in the cells of *E. coli* B/r Hcr^+ thy^- trp^-, prestarved for amino acid and irradiated by UV light, was investigated by Sedliakova and Bugan (1973) by ultracentrifugation in alkaline sucrose gradients. Owing to irradiation, molecular weight of the parental DNA was lowered. However, the values were not different in both the prestarved and the exponentially growing cells during 120 minutes of postincubation. After 3 hours of postincubation, the former displayed values like those of the

unirradiated cells; the values of the latter remained negligibly lower. The molecular weight values for DNA synthesized after irradiation became similar to those of unirradiated cells after 2 hours of postincubation in both types of cells. Thus the return of the parental DNA of prestarved cells to the normal molecular weight values observed after 3 hours of postincubation seems to be rather a consequence than a cause of the high resistance to UV light. This is also in agreement with a more rapid growth and higher thymine incorporation, observed in the prestarved cells during the second hour of postincubation. The data indicate that the enhancement of resistance to UV light, observed after amino acid prestarvation, cannot be ascribed to a more efficient excision repair of the dimers.

The isolation of a UV-specific endonuclease was described by Caputo and Zupi (1973), especially from the cells of radiosensitive hamster's plasmocytoma. Their system represents a simple and valuable method for the quick detection of damage specifically induced by irradiation of DNA.

The excision of pyrimidine dimers from the DNA of *Neurospora crassa* was examined by Worthy and Epler (1973). Postirradiation incubation in the presence of several chemicals known to inhibit various repair systems indicated that caffeine reduced the rate of excision by half, but did not inhibit excision completely as did proflavine and quinacrine. Examination of the time course of excision showed that repair occurs at a relatively rapid rate: approximately 60 dimers excised per minute after 500 ergs/mm^2. Further evidence for rapid excision was obtained by sedimentation analysis of DNA; the maximum number of breaks introduced during repair was three, suggesting that breaks are repaired almost as fast as they are made and that only a few dimers are repaired at a time. Repair synthesis was measured by prelabeling the DNA with ^{15}N and D_2O, and then subjecting the DNA to equilibrium density gradient centrifugation after postirradiation incubation with ^{32}P. Accumulation of single-strand breaks with increasing dose of UV irradiation suggests that the limiting step was subsequent to the incision and excision steps of repair. Equilibrium CsCl centrifugation demonstrated that the limiting step in excision was repair synthesis.

B. W. Fox and Fox (1973a) have compared the numbers of single-strand breaks and their rates of rejoining in two Yoshida cell lines showing similar sensitivity to X-rays (D_0 85 rads, $n = 2.5$) and two L5178Y cell lines showing differential sensitivity, the sensitive cell line having a D_0 value of 55 rads, $n = 2.5$, and the resistant line a D_0 value of 160 rads and $n = 3.0$. After doses of 5 and 10 krad, no differences in the numbers of single-strand breaks or in their rates of rejoining were

observed when measured by the alkaline sucrose gradient technique. The findings confirm that single-strand breaks in themselves are not lethal events, since similar numbers are produced and virtually all are rejoined in the cell lines studied. However, a deficiency in some of the processes involved in repair replication could be the cause of their differential sensitivity by allowing damage to be fixed. B. W. Fox and Fox (1973b) have also studied the effect of UV irradiation on Yoshida sarcoma cells, sensitive and resistant to UV light, by examining changes in the sedimentation rate of the DNA on an alkaline sucrose gradient. The whole cells were lysed on the gradient at different periods of time after UV irradiation and at different doses. After an immediate initial decrease, the sedimentation rate increased to a maximum at about 3–4 hours after treatment. The UV-irradiated DNA sedimented faster than the untreated control after an equivalent amount of lysis time. The component which sedimented more quickly, then decreased in amount between 4 and 6 hours after irradiation and appeared as a component that sedimented very slowly near the surface of the gradient. The authors suspected the formation of a DNA-containing complex, which is more pronounced in the sensitive cell line than in the resistant line. The sedimentation patterns occurring on more prolonged incubation of the cells were considered to be due to changes in DNA before the death of the cells. It was suggested that this faster-sedimenting complex might be associated with the excision system and that the efficient dissociation of this complex might be an intrinsic part of the repair process.

Paton (1973) has considered the role of metals in carcinogenesis and mutagenesis, with special reference to the possibility that certain metal ions may damage repair enzymes or induce lesions which cannot otherwise be repaired.

An excellent and comprehensive review has been provided by Howard-Flanders (1973), considering repair mechanisms involving a single DNA strand only; photoreactivation; repair mechanisms dependent upon the complementary strand; repair processes (including every aspect of twin breakages) and an account of those hereditary changes involving DNA repair in man.

III. Wound Healing

In his biographical memoir on the late Emmanuel Fauré-Fremiet, it is interesting to note that Willmer (1973) describes him as regarding the process of wound healing as a modification of the normal processes of growth and thus likely to be governed by the same or similar laws, basing this conclusion mainly on the study of the cicatrization of wounds (Fauré-Fremiet, 1920, 1930; Fauré-Fremiet and Vles, 1919a,b). The lag

period in healing wounds was the subject of a paper by Whipple (1940). The histogenesis and repair of liver cirrhosis in rats produced on low protein diets and preventable with choline was described by Lillie *et al.* (1942). The healing and repair of tegumentary wounds was examined by Leslie-Roberts (1941; see also Holmes, 1942; Rhoads *et al.*, 1942; Bowers, 1943; Localio, 1943; Localio *et al.*, 1943; Mann, 1943; Apperly and Cary, 1944; Bruger, 1944; Laugier and Dugal, 1943; Ross, 1969), while the special action of thiourea in wound healing was described by Fearon (1942). The role of cell movements in the healing of micro wounds *in vitro* was described by Wilbur and Chambers (1942), and Cook and Fardon (1942) posit the concept of a wound hormone in wound healing.

Arey and Covode (1943) gave further study to mitosis in corneal wound healing (see also Buschke *et al.*, 1943; Friedenwald and Buschke, 1944). The influence of hypophysectomy on the epithelization of wounds was the subject of a paper by Mueller and Graham (1942). The rate and nature of epithelization in wounds with loss of substance was discussed by Howes (1943). Besser and Ehrenhaft (1943) described the relation of acute anemia to wound healing. The general role of nutrition in wound healing was examined by Lund and Crandon (1943; see also Anonymous, 1944).

The following references, not hitherto noted, may be added to the pioneering contributions of Paul Weiss and his school on wound repair, nerve healing, and the morphogenetic capacity of tissues: Weiss (1944, 1966), Weiss and Taylor (1943), Overton (1948, 1950a,b), Chiakulas (1952), and Lash (1955, 1956). Young (1944–1945) also reported on the structure, degeneration, and repair of nerve fibers.

Nickel pectinate has been used in wound repair by Hamilton (1944), who regarded it as an excellent agent because of its capacity to inhibit proud flesh, which leads to an acceleration of epithelization. According to Flynn *et al.* (1973), bilateral adrenalectomy and long-term corticosteroid therapy produced a sustained decrease in serum zinc. In ten patients adrenalectomy resulted in serum-zinc deficiency within 1 week, and this decrease was maintained for at least 6 months. Six patients on long-term corticosteroids presented with delayed wound healing; these were given oral zinc supplements (660 mg zinc sulfate per day), and their wounds healed completely. These results are important clinically because corticosteroids are widely used drugs and because the maintenance of adequate zinc levels is essential for proper wound healing.

According to Tarin (1972), epithelial pseudopodia and associated vesicles are present at later stages in wound healing and are remarkably similar to those observed at the dermoepidermal junction in human

tumors (Sugár and Faragó, 1966; Frithiof, 1969) and experimental tumors (Frei, 1962; Tarin, 1967; Woods and Smith, 1970).

A perennial, if unsolved, problem concerns the relationship of ascorbic acid to wound healing generally, and references not hitherto noted are Lund and Crandon (1941), Bartlett *et al.* (1942), Harris (1943), and Kodicek and Murray (1943).

The original review to which this is an Addendum drew attention to the physiology of regeneration and hypertrophy in its relationship to wound healing, and the phenomena of mitosis and regenerative growth in Amphibia have been dealt with by Litwiller (1939, 1940), Poole (1966), and Kingman (1972).

The important bearing of scar formation in wound healing and carcinogenesis is reported by Halford and Gotshalk (1941) from the aspect of epitheliomatous degeneration (see also Anonymous, 1942). A description of cicatricial carcinoma arising 65 years after burn scars is given by Reichman (1971), who raised the question of regarding it as an avoidable sequel. Scar cancers of the lung were considered by Kennaway (1957) in their possible relation to cholesterol. The cancers of the lung associated with scars and with deposits of cholesterol described by the German pathologists Rössle (1943) and Lüders and Themel (1954) have been investigated also by Raeburn (1955), who stated: "Further cases of lung cancer associated with scarring have been studied in detail. The scars are of various aetiology—collapse pneumonia, tuberculosis, and even foreign body. The common factor is proliferation of bronchial epithelium within the fibrous framework of the scarred area. . . . These scars are rich in cholesterol." The juxtaposition of cholesterol and a carcinoma was, of course, acknowledged by Kennaway as being far from constituting proof that one is the cause of the other.

The concept of the wound as a self-healing tumor is further supported in the contributions of Dameshek (1965). Having classified the immunoproliferative disorders into lymphoproliferative, plasmoproliferative, and reticuloproliferative, he divides the lymphoproliferative into acute and chronic. Of the acute self-limited he writes: "Of the many conditions in which a well-defined acute disturbance characterised by lymphoid proliferation and abnormal proteins or antibodies is found, infectious mononucleosis is most striking." The self-healing *Molluscum contagiosum* represents another case (see Haddow, 1973). The influence of the hair cycle in the rabbit was investigated by Whiteley (1957), who thought that the self-healing tumor developed from the germinal bud which forms the new hair and that the squamous papilloma developed from the surface epithelium. This experimental self-healing tumor of rabbit skin also has its human counterpart in *Molluscum sebaceum,* a

keratoacanthoma, or histologically invasive tumor, that undergoes spontaneous regression and only occurs on hair-bearing skin.

IV. Xeroderma Pigmentosum

It is evident that continued experience of the nature of xeroderma pigmentosum has involved some complication, from the recognition of variants of the disease with differing biochemistry. While the most common form of xeroderma pigmentosum manifests reduced capacity for the excision of damaged DNA, two minor variants have been described by Cleaver (1973a). In one, excision of damaged DNA is present with evidence of neurological involvement. In the other, there is apparently normal excision repair and normal UV sensitivity *in vitro*. In J. E. Cleaver's opinion (personal communication, 1972) these variants probably represent cases in which the clinical picture blends so closely with a true xeroderma pigmentosum that a clinician could not discriminate between them. Cleaver (1972) had reported on three patients with distinct symptoms of xeroderma pigmentosum in which the cultivated fibroblasts were different from those usually found in this disease. Ordinarily, xeroderma pigmentosum fibroblasts are extremely sensitive to UV light and perform reduced amounts of repair replication during the repair of damage to DNA. Cells from these three variants of xeroderma pigmentosum were indistinguishable from normal cells: their sensitivity to UV light was normal and they performed normal amounts of repair replication. Because of this normal sensitivity, it was unlikely that a defect in any DNA repair mechanism was present in these cases; in microorganisms, defects in repair are invariably associated with increased sensitivity. These results implied that a minority of those cases which are clinically diagnosed as xeroderma pigmentosum constitute a biochemically distinct condition, and Cleaver emphasized that possible relationships previously inferred between DNA repair and carcinogenesis should be cautiously evaluated. Cells from parents (heterozygotes) of a xeroderma pigmentosum patient with reduced DNA repair also showed reduced DNA repair when subjected to high doses of UV light, which presumably exceeded the repair capacity of the partial (heterozygous) repair defect (see also Cleaver and Carter, 1973). The recognition of these variant clinical forms obviously entails modification of earlier views concerning excision. If the situation is more complex than was at first appreciated, it is very much to be hoped that this will not prove a bar to study of the extent to which repair processes may be damaged in carcinogenesis. In Cleaver's (1973a) view, carcinogenesis in xeroderma pigmentosum may develop through the unrepaired damage in DNA which leads to genetic changes or viral transformation.

Regan (1969; see also Regan *et al.*, 1970) had already indicated that individuals with xeroderma pigmentosum are the human analog of the excisionless, UV-sensitive bacterial mutants so well known in microbial photobiology. Cleaver (1973b) has also described some of the damage and repair processes following carcinogen treatment and makes some preliminary classification of carcinogenic agents on the basis of the repair which they induce (see also Setlow and Regan, 1972; Stich *et al.*, 1972). Cleaver *et al.* (1972) described epithelial cells and fibroblasts from Hereford and black Aberdeen Angus cattle, the former of which is subject to cancer of the eyelid; there was no indication of enzymatic deficiency or lack of repair in Hereford cows, such as is found in xeroderma, and no difference was established between the two breeds. It is of interest that a reduced repair synthesis of DNA has been observed by Stich and San (1971) in xeroderma cells exposed to the oncogenic 4-nitroquinoline 1-oxide and 4-hydroxyaminoquinoline 1-oxide. The role of DNA repair in aging cells from xeroderma and controls was discussed by Goldstein (1971). Robbins and Kraemer (1972a,b) reported abnormal rate and duration of UV-induced thymidine incorporation into lymphocytes from patients with xeroderma pigmentosum and associated neurological complications and described its relation to hydroxyurea. The relationship of DNA repair to carcinogenesis was also dealt with by Robbins and Burk (1973), and the differences in repair replication in various rodent cell lines are described by M. Fox and Fox (1973).

V. The Implication of DNA, and the Bacterial Analogy

At the molecular level, DNA is clearly involved in repair processes following damage with X irradiation, UV irradiation, and with carcinogenic and other chemical reagents. Also, much of the evidence is dependent on the similar behavior observed in the bacteria and other related lower forms. Thus Burrell *et al.* (1971) have described the membrane-associated DNA in relation to double-strand breaks in *M. radiodurans*. Fox and Ayad (1971) studied the characteristics of repair synthesis in P388 cells treated with methyl methanesulfonate. Plant and Roberts (1971a) described a novel mechanism for the inhibition of the DNA synthesis following methylation and the effect of *N*-methyl-*N*-nitrosourea in HeLa cells. The same authors (Plant and Roberts, 1971b) also examined the extension of the pre-DNA synthetic phase of the cell cycle as a consequence of DNA alkylation in Chinese hamster cells, a possible mechanism of DNA repair. Roberts *et al.* (1971a) produced evidence for the inactivation and repair of the mammalian DNA template after alkylation by mustard gas and half mustard gas.

VI. Repair and Carcinogenesis

In spite of the complication of our knowledge of xeroderma pigmentosum, and especially of the recognition of subtypes which lack the repair defect present in the main group, it is evident that the relationship of repair to carcinogenesis still remains of central importance. The relationship, if any, has been dealt with by Cleaver (1973c), and the connection between UV light, DNA, and carcinogenesis in man has been investigated by Epstein *et al.* (1971). The need for caution is shown in the description by Robbins and Burk (1973) of a case of xeroderma pigmentosum in whom radioautograms of intact UV-irradiated epidermis showed no detectable abnormality in UV-induced thymidine-^{3}H incorporation. This result is consistent with findings in this patient's UV-irradiated skin fibroblasts and lymphocytes and contrasts with findings in cells from typical xeroderma pigmentosum patients, all of which exhibit an impaired ability to repair UV-damaged DNA. The development of numerous tumors in the presence of apparently normal DNA repair suggests that some mechanism other than enhancement of UV carcinogenesis by defective DNA repair was responsible for skin tumor formation in this patient and perhaps in all patients with xeroderma pigmentosum.

The action of a variety of cocarcinogens in inhibiting DNA repair was studied by Gaudin *et al.* (1971). Hydroxyurea was used to reduce background incorporation as a result of ordinary DNA synthesis, and the cocarcinogens employed included croton oil, Tween 80, Arlacel A, diethylstilbestrol, estradiol, testosterone, 7-hydroxyacetylaminofluorene, and azobenzene, 7-hydroxyacetylaminofluorene being the most effective. It was concluded that inhibition of DNA repair may be an important mechanism in the action of cocarcinogens (see also Gaudin *et al.*, 1972). Stone and Anthony (1970) reported that the application of 5% crude coal tar produced a 45.9% delay in wound healing in experimental animals, possibly due to enhancement of bacterial infection. The high sensitivity of xeroderma pigmentosum cells to 4-nitroquinoline 1-oxide was described by Takebe *et al.* (1972), and the action of the same substance on human lymphocytes was described by Jacobs *et al.* (1972).

Lieberman *et al.* (1971b) studied deoxyribonucleoside incorporation and the role of hydroxyurea in a model lymphocyte system in its relation to DNA repair in carcinogenesis, while Lieberman and Dipple (1972) described the removal of bound carcinogen during DNA repair in nondividing human lymphocytes. DNA repair in normal and malignant cells was investigated by Lieberman and Forbes (1973) after treatment with proximate chemical carcinogens and UV irradiation. Non S-phase label-

ing or unscheduled DNA synthesis had been shown to correspond to repair synthesis in a variety of cultured mammalian cells, in tissues, and in bacteria (Pettijohn and Hanawalt, 1964; Evans and Norman, 1968; Cleaver, 1971; Epstein *et al.*, 1971; Lieberman *et al.*, 1971a,c; Roberts *et al.*, 1971b; Roberts, 1972; Lieberman and Dipple, 1972). To the extent that this is so, it seems that a variety of cells from normal tissues can repair damage to DNA by both chemical carcinogens and UV irradiation, and this suggests that whatever role repair plays in modifying the carcinogenic process, it is probably complex and not an all-or-none phenomenon. A paper by Norman *et al.* (1972) is regarded by Lieberman and Forbes (1973) as supporting evidence of their finding that repair is retained during neoplastic transformation. Roberts (1973) described the relationship between alkylation-induced cell death, mutations, chromosome aberrations, and effects on DNA synthesis in Chinese hamster cells. Since the three former effects are enhanced by an agent like caffeine, which it has been shown inhibits the repair of alkylated DNA, then it follows, not only that all three effects are likely to arise as a consequence of the direct reaction of agents with DNA, but also that there are common step(s) in the pathway by which cells attempt to repair the DNA damage responsible for these effects. Moreover, since caffeine interferes with the repair of gaps in newly replicated DNA, it is also postulated that it is the failure to repair gaps or the faulty repair of such gaps which is responsible for these separate phenomena. Roberts envisaged therefore that the ends of DNA strands on either side of one of the postulated postreplication gaps in DNA can join with similar ends of other contiguous DNA molecules to produce the various chromosomal aberrations which may be induced by these agents. A comparable mechanism to this would also account for the gene loss required to produce deletion mutations in mammalian cells consequent upon alkylation which could result in cell transformation. The carcinogen-induced aberrations are therefore also likely to arise by the above mechanisms, since, when it has been investigated, carcinogens not only react with DNA but also affect DNA replication. Whether or not such effects on DNA replication and the appearance of chromosome aberrations are linked causally to the malignant transformation of cells is therefore a question of current vital importance (see also Anonymous, 1972). Venitt and Tarmy (1972) have examined the selective excision of arylalkylated products from the DNA of *E. coli* treated with the carinogen 7-bromomethylbenz[*a*]anthracene. Bosch *et al.* (1972) discussed the possible role of the repair process in central nervous system carcinogenesis.

The writer (Haddox, 1938) had previously suggested that the malig-

nant race embodies a kind of drug resistance to the carcinogen, and related carcinogens, employed in tumor induction; and a similar concept is evident in papers by MacNider (1941, 1942) on morphological changes accompanying repair and the chemoresistance to mercuric bichloride which may be developed in the kidney.

VII. Therapeutic Possibilities

The previous review (Haddow, 1972) entertained various possibilities which, in spite of their speculative nature, seemed deserving of further experiment. These arose mainly from consideration of the nature of the cell surface (Ambrose, 1956, 1962, 1967; Abercrombie and Ambrose, 1958; Ambrose and Ellison, 1968; Ambrose *et al.*, 1956, 1958; see also Robertson, 1973), from the lessons learned from plant and animal wounds, and from the striking discovery made by the late Dr. E. M. F. Roe (1959) of the extraordinary inhibitory action of tragacanth gum on the growth of separated cells on various ascites tumors in mice. Many observations now attest the significance of the cell surface for cancer research. Thus, Puck (1973) has described the importance of genetic biochemistry and its relevance to the cancer cell. Mason and Lee (1973) described the process of resealing in relation to natural biological membranes. Increasing recognition of cell contacts and cell inhibition was the subject of a Gordon Conference in 1973. It is of some interest in relation to cell adhesion and wound healing that the action of pyroxylin has been recognized for some 100 years and is the effective basis of "Newskin," dissolved in ethyl acetate, ethyl alcohol, butyl alcohol, and amyl acetate, to give a solution which rapidly evaporates, when applied to the skin, to yield a film which is plasticized by castor oil and camphor.

If cell adhesion is important, much interest would attach to the nature of the chemical agents involved. However, the role of one agent which had been considered a candidate was studied by Steinberg and Gepner (1973). They found that combination of concanavalin A with receptor sites does not in fact play any part in the process of cell adhesion. This demonstration that the adhesion-dependent processes of cell aggregation, cell sorting, and tissue spreading are little affected by the masking of concanavalin A receptor sites rules out the possibility that the particular sugar groupings play a major role, in the cells studied, as sites of intercellular adhesion. At the same time, these experiments illustrated an approach that should in principle be able to identify such sites if the "right" lectins are found. It is interesting to compare these results with those of Burger and Noonan (1970), who found that exposure of polyoma-transformed 3T3 (mouse) cells to univalent concanavalin A returned to these cells their competence to display post-

confluence inhibition of cell division (Martz and Steinberg, 1972), as shown by normal 3T3 cells. Since the specific binding of univalent concanavalin A to chick embryonic cell surfaces have been found to have no appreciable effect on reaggregation, cell sorting, or tissue spreading, the theory that loss of growth control is a consequence of decreased intercellular adhesion (Coman, 1944; Baker and Humphreys, 1971) is open to doubt. Rowlatt *et al.* (1973) have also considered the influence of concanavalin A receptors on hamster embryo and adenovirus tumor cell cultures.

The earlier review (Haddow, 1972) had also suggested the possible role of glycoprotein for cell-to-cell adhesion, and this aspect has been studied by Shier (1973) in a paper on the modification of tumor growth with a defined glycoprotein antigen. [See also Rosenberg (1973) on cartilage proteoglycans.] Polymethacrylic acid had also been considered, and this has now been studied by Franchi *et al.* (1973) in relation to tumor metastasis.

It is of much relevant interest that the ova of hamsters can be rendered infertile through the action of wheat germ agglutinin by the induction of changes in the zona pellucida. It is a property of the zona pellucida that it is impenetrable to foreign sperm and that penetration by homologous sperm is rendered impossible by fertilization. Oikawa *et al.* (1972) described a structural change associated with nonpenetration which can be detected by light-scattering changes in the surface. Wheat germ agglutinin is of interest as distinguishing between cancer cells and normal cells, the former being agglutinated while there is no effect on the latter. It also agglutinates embryonic cells—a factor of importance in its support of the embryonic analogy, suggesting that the cancer cell constitutes an atavistic or regressive variation from the parent cell type, and that the capacity of malignant cells to grow and divide is reflected in some manner in the physical properties of their surfaces.

VIII. Conclusion

The concept of a wound as a self-healing tumor, and the interpretation of neoplasia as a species of overhealing, still seem to merit investigation, and it has been judged useful to review these additional papers with a bearing on these problems. It seems more than possible that further insight may be gained from the study of the healing process in plant and animal tissues. The phenomena of contact inhibition and their apparent absence in cancer cells appear to point to the cell surface as very likely to be of special importance. In addition, it seems desirable that the biological action of natural gums should be further explored in an endeavor to induce cellular agglutination and to reestablish that

cellular inhibition which is lost. This process would involve the natural axiom which has long been known—namely, the need to understand not merely the source of the malignant cell's loss of growth regulation, but why the growth of normal cells is so precisely regulated and controlled.

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