

ADVANCES IN
Immunology

EDITED BY

F. J. DIXON, JR.

*Division of Experimental Pathology
Scripps Clinic and Research Foundation
La Jolla, California*

J. H. HUMPHREY

*Division of Immunology
National Institute for Medical Research
Mill Hill
London, England*

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LIST OF CONTRIBUTORS

Numbers in parentheses indicate pages on which the authors' contributions begin.

- K. FRANK AUSTEN, *Department of Medicine, Harvard Medical School, and Massachusetts General Hospital, Boston, Massachusetts* (1)
- DAN H. CAMPBELL, *Division of Chemistry and Chemical Engineering, The California Institute of Technology, Pasadena, California* (261)
- JUSTINE S. GARVEY, *Division of Chemistry and Chemical Engineering, The California Institute of Technology, Pasadena, California* (261)
- JOHN H. HUMPHREY, *National Institute for Medical Research, Mill Hill, London, England* (1)
- M. R. IRWIN, *Division of Genetics, University of Wisconsin, Madison, Wisconsin* (315)
- C. R. JENKIN, *Department of Microbiology, University of Adelaide, Adelaide, South Australia* (351)
- D. S. NELSON, *The Department of Experimental Pathology, John Curtin School of Medical Research, Australian National University, Canberra, Australia* (131)
- D. R. STANWORTH, *Department of Experimental Pathology, The Medical School, University of Birmingham, Birmingham, England* (181)
- CHANDLER A. STETSON, *Department of Pathology, New York University School of Medicine, New York, New York* (97)
- W. H. STONE, *Division of Genetics, University of Wisconsin, Madison, Wisconsin* (315)

PREFACE

The third volume of *Advances in Immunology* appears under the editorship of Frank J. Dixon and J. H. Humphrey, the senior editor, William F. Taliaferro, having retired last year. On behalf of ourselves and of the many readers who may have found the first two volumes useful and stimulating we wish to record our gratitude to Dr. Taliaferro for the wisdom, care, and energy that he devoted to initiating the series and to producing the first volumes. Immunology continues to develop rapidly and to have an increasing impact on biology and medicine. Whatever doubts may have been expressed three years ago about the need for *Advances in Immunology* must have been dispelled by the reception it has received and by the readiness with which authors have been willing to take the time and trouble required to survey their special fields. With the continued cooperation of our scientific colleagues and the expert assistance of Academic Press, we foresee that the serial publication will remain of a high standard for many years to come.

The present volume contains four chapters concerned with biological effects of antigen-antibody interactions. The first, by Frank J. Austen and J. H. Humphrey, reviews *in vitro* studies of anaphylaxis in the several model systems which have been investigated during recent years, and attempts to draw these together so as to make clear the extent to which the underlying mechanisms follow common or divergent patterns. The fourth chapter, in which D. R. Stanworth summarizes current knowledge of reaginic antibodies in Man and puts forward his ideas about how their peculiar properties might be explained, is to a considerable extent complementary to the first chapter. In the second chapter Chandler A. Stetson takes up the continuing controversy about whether or not homograft rejection is mediated by humoral antibodies, and marshals a powerful set of arguments and of experimental evidence in favor of the thesis that antibodies play an essential part. David S. Nelson, in the third chapter, reviews the history and current concepts of immune adherence, a phenomenon that not only provides an exceedingly sensitive method for detecting antibodies but that may have a hitherto largely unrecognized importance for the destruction of sensitized cells.

The remaining three chapters concern three different aspects of immunology. Dan H. Campbell and Justine S. Garvey from the wealth of

their own experience discuss the very topical problem of the fate and intracellular persistence of antigen in relation to its capacity to provide a continuing antigenic stimulus. Irving S. Stone and Malcolm R. Irwin review the knowledge of blood groups in species other than Man, and some of its genetic implications. Readers who are unfamiliar with this field may be surprised to learn how much is known, for example, about the blood groups of cattle. Finally, C. S. Jenkin outlines a new approach to parasitism based on the idea that when a parasite and its host share important antigenic determinants, the capacity of the host to give an immunological response to the parasite may be seriously impaired.

In conclusion we wish once again to thank the authors for their cooperation and Academic Press for smoothing the path of the editors and for arriving at the technically excellent production for which they are reputed.

September, 1963

F. J. DIXON, JR.
J. H. HUMPHREY

***In Vitro* Studies of the Mechanism of Anaphylaxis**

K. FRANK AUSTEN¹ AND JOHN H. HUMPHREY

*Department of Medicine, Harvard Medical School and Massachusetts General Hospital,
Boston, Massachusetts, and the National Institute for Medical Research,
Mill Hill, London, England*

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The symptom complex exhibited during systemic anaphylaxis varies from species to species. During systemic or local anaphylaxis direct damage to a variety of cells in the body may occur, but an increasing volume of evidence suggests that the main part of the symptom complex is caused by the release of pharmacologically active agents or their activators. These materials are released from a rather small number of cell types, and when other cells or tissues are involved they become so, secondarily. Some of the factors contributing to species variation include: the *relative proportion* of the four, presently recognized, pharmacologically active products of the anaphylactic reaction, namely, histamine, slow reacting substance, plasma kinins, and serotonin; the particular host organ subjected to the highest concentration of these materials, the so-called "*shock*" organ; the susceptibility or *reactivity* of host smooth muscle and vascular tissue to each of these substances; the rate at which each species *degrades* them; and the extent of direct participation by antigen-antibody *complexes*. Despite the variation in the anaphylactic symptom complex, it is a tenable thesis that the mechanism whereby the chemical mediators are released is very similar even in different species.

Quantitative experiments of the type needed to study the possible steps in the anaphylactic release of the chemical mediators of anaphylaxis *cannot* be performed in the whole animal. However, their release *in vitro* from replicate samples of sensitized tissue can be quantitated and has been used by numerous workers to investigate the mechanism of anaphylaxis. *In vitro* experiments in which the tissue is thoroughly washed permit a study of the reaction between antigen and antibody fixed to tissue without the complicating presence of free serum factors. It is even more convenient to investigate *in vitro* anaphylaxis in a homogeneous suspension of sensitized cells capable of giving detectable evidence of their response, since this eliminates non-participating cell types. On the other hand, with each refinement of the *in vitro* test system one must exert greater caution in applying the findings to systemic anaphylaxis. The mere observation that cytotoxic antibody and serum or that preformed antigen-antibody complexes can damage or release some pharmacological material from cells *in vitro* is not evidence that a similar mechanism is important for anaphylaxis *in vivo*. Furthermore, as systemic anaphylaxis in a given species may involve several chemical mediators arising from different cell types, an *in vitro* system may eliminate one or more important materials. With these reservations, *in vitro* anaphylaxis or, more correctly, the antigen-induced release of chemical mediators from tissue or cells, may be a useful means of

investigating the mechanism whereby antigen-antibody interaction produces systemic anaphylaxis.

The *in vitro* tissue or cell systems to be considered in detail include the chopped guinea pig lung, the rat or guinea pig mesentery, the rat peritoneal mast cell, the rabbit platelet, and the human white blood cell. Data obtained with similar systems in other species will also be included when available. Studies with other *in vitro* systems, such as guinea pig ileum, rat or guinea pig uterus, dog or rabbit liver, guinea pig, dog, or rabbit skin, and guinea pig heart, will be considered only to the extent that the available data contribute to our understanding of the mechanism of anaphylaxis. Cytotoxic systems—immune hemolysis or the lysis of ascites tumor cells by antibody against a cell surface constituent and complement—will be considered only for purposes of comparison, since the details of the complement system have recently been reviewed by M. M. Mayer (1956, 1961a) and by Osler (1961). There is no convincing evidence one way or the other that cytotoxic reactions are really suitable models for anaphylaxis. Complement will again be considered in the section on serum factors in anaphylaxis; this section will also include anaphylotoxin and antigen-antibody activated serum enzyme activities. Immunological reactions in tissue culture will not be reviewed. Studies with chemical histamine or serotonin releasers will not be included since there are data with the guinea pig lung (Mongar and Schild, 1957a; Yamasaki *et al.*, 1960) and rabbit platelet (Humphrey and Jaques, 1955) that point out important differences between histamine release by the *p*-methoxyphenyl-ethylmethylamine and formaldehyde condensation product (48/80) and by antigen. In systems involving the rat mast cell the similarity between histamine release by 48/80 and by antigen is considerable (Hogberg and Uvnas, 1960). The limits set for this review result from our wish to evaluate data strictly pertinent to the mechanism of anaphylaxis *in vitro*. Accordingly, studies on the variety of para-anaphylactic (anaphylactoid) phenomena will be largely neglected.

Before proceeding to a detailed consideration of anaphylaxis *in vitro*, it will be necessary to review briefly the characteristics of the pharmacological materials used as indicators of this reaction. We shall also consider the *in vivo* evidence for the release of these materials during anaphylaxis.

I. Pharmacology

A. HISTAMINE

Histamine has been of great interest to workers in the field of anaphylaxis since Dale and Laidlaw (1910) and Dale (1929) pointed

out the many resemblances, and some differences, between its effects and those of anaphylaxis in the guinea pig. In 1932, direct evidence was presented that it could be released from tissue *in vitro* or *in vivo* by an anaphylactic reaction (Bartosch *et al.*, 1932; Dragstedt and Gebauer-Fuelnegg, 1932). Histamine is widely distributed in mammalian tissue (Feldberg, 1956) but the concentration in a given organ shows great species variation. Much of the tissue histamine is contained in mast cells (Riley and West, 1953, 1955). The histamine content per mast cell shows surprising constancy in normal tissue as illustrated in Table

TABLE I
THE HISTAMINE CONTENT PER MAST CELL IN NORMAL TISSUE
FROM DIFFERENT SPECIES

Species	Histamine content per mast cell ($\mu\text{g.}$)
Dog tissue	7 - 16
Beef liver capsule	32
Guinea pig tissue	21 - 34
Rat (isolated cells)	10 - 40

I which summarizes the data from several groups of investigators. Graham and her associates (1955), based on mast cells counts and total tissue histamine analyses, concluded that the histamine content of the dog mast cell ranged from 7 to 16 $\mu\text{g.}$, whereas that of the beef liver capsule mast cell was 32 $\mu\text{g.}$ Boreus and Chakravarty (1960a), using a similar method, reported that the histamine content of guinea pig mast cells ranged from 21 to 34 $\mu\text{g.}$ The histamine content of isolated, rat, peritoneal mast cells ranged from 10 to 20 $\mu\text{g.}$ in the experiments of Humphrey *et al.* (1963), whereas Archer (1960) reported a value of 40 $\mu\text{g.}$

The mast cells, which are located mainly in connective tissue in relation to blood vessels (Riley, 1953), are *not* the only cells containing histamine. Histamine has been found in platelets (Humphrey and Jaques, 1955) and in basophilic leucocytes (VanArsdel *et al.*, 1958); histamine is present in high concentration in fetal liver (Kahlson, 1960) and in the parietal cell region of the stomach (Feldberg, 1956) even though mast cells are virtually absent from these latter sites. It is of interest that in the rat, at least, the histamine that resides in mast cells is released by 48/80, whereas that in non-mast cell areas such as the fundus and duodenum is not (Mota *et al.*, 1956). Regardless of whether or not tissue histamine is contained in mast cells, it is formed from L-histidine (Schayer, 1959). Schayer (1960) has recently presented evidence that histidine decarboxylase in tissues not necessarily

rich in mast cells increases in activity in response to a variety of stimuli. Schayer refers to the product of this "adaptive form" of enzyme activity as induced histamine to distinguish it from the stored histamine of the mast cells and suggests that this induced histamine is formed by vascular endothelium and may be an important intrinsic regulator of the microcirculatory system.

Some of the established pharmacological effects of histamine include capillary vasodilatation with increased permeability, bronchiolar and other smooth muscle constriction, and stimulation of the glands of exocrine secretion. Despite this diverse activity and the widespread distribution of histamine, the physiological significance of this material is still obscure. The evidence that parietal cell stimulation is a physiological function of histamine has been summarized by Code (1956); Whelan (1956) has concluded that it is doubtful whether histamine plays a role in the regulation of blood flow during reactive or post-exercise hyperemia; and Miles (1959) is not convinced that histamine is primarily responsible for the increased capillary permeability of inflammation. Recently, Kahlson (1960) has suggested that histamine is connected with anabolic events such as growth, development, and repair.

B. SLOW REACTING SUBSTANCE

The term "slow reacting substance" (SRS) refers to material or materials that contract smooth muscle, usually guinea pig ileum, more slowly than histamine or acetylcholine. Feldberg and Kellaway (1938) introduced this term to describe a substance obtained from lung during perfusion with cobra venom, and 2 years later, in 1940, Kellaway and Trethewie (1940) reported the presence of a similar material in the effluent collected during anaphylactic shock of the perfused guinea pig lung. The evidence for the presence of a slow reacting material was indirect and based on the observation that the effluent caused a more prolonged contraction of the guinea pig ileum than did histamine alone. In 1953, Brocklehurst (1953, 1960), using antihistamines to abolish the response of the ileum to histamine, presented direct and conclusive evidence for the anaphylactic release of slow reacting material from guinea pig lung. This material was termed SRS-A to indicate that it was obtained as a consequence of antigen-antibody interaction.

The chemical properties of the slow reacting material produced in guinea pig lung by the lecithinase A in cobra venom are consistent with those of an unsaturated fatty acid (Vogt, 1956). The material released from guinea pig lung by anaphylaxis has not been characterized chemically,

but the properties of the partially purified preparation suggest that it is acidic and lipid. It is always obtained rather firmly associated with some protein (Brocklehurst, 1962).

Slow reacting substance contracts only a limited number of isolated smooth muscle preparations. These include the guinea pig ileum, the rabbit jejunum, the fowl rectal cecum, and the human bronchiole; it does not contract tissues such as rat colon, rat uterus, or the bronchiole of the guinea pig, dog, rabbit, or cat (Brocklehurst, 1962). On intravenous injection it did not depress the blood pressure of the cat or rabbit. It is easily differentiated from bradykinin or serotonin, as shown in Table II. Bradykinin contracts the rat uterus, is inactive on the human

TABLE II
THE DIFFERENTIAL BIOLOGICAL ACTIVITY OF SRS-A, BRADYKININ, AND SEROTONIN

Parameter	SRS-A	Bradykinin	Serotonin
Contraction of guinea pig ileum	+	+	+
Contraction of rat uterus	—	+	+
Contraction of human bronchiole	+	—	—
Reduction of arterial pressure (cat)	—	+	+
Tachyphylaxis	—	—	+
Destruction by chymotrypsin	—	+	—

bronchiole, depresses arterial pressure, and being a polypeptide is destroyed by chymotrypsin. Serotonin contracts the rat uterus, has no effect on the isolated human bronchiole except in high doses which cause relaxation rather than constriction, depresses arterial pressure, and produces tachyphylaxis of the guinea pig ileum.

Whereas the antigen-antibody reaction releases *preformed* tissue histamine, SRS is both *formed* and *released* as a result of antigen-antibody interaction. This was demonstrated by Brocklehurst (1960) who found appreciable amounts of SRS-A in shocked tissue and the effluent therefrom, but only trace amounts in unshocked tissue and, of course, none in the effluent.

The predominant source of SRS as well as histamine in the guinea pig is lung tissue. As will be discussed shortly, SRS has also been obtained *in vitro* from rabbit, monkey, and human lung but not from horse or goat lung (Brocklehurst, 1960). The *in vivo* release of SRS has recently been demonstrated in the rat (Rapp, 1961). Although Uvnas and Thon (1959) and Boreus and Chakravarty (1960b) have presented evidence that SRS originates in the mast cell, there are data to the contrary which will be considered later.

C. PHYSIOLOGICALLY ACTIVE POLYPEPTIDES

In the early 1930's, Frey and his associates (1930, 1933) demonstrated that the intravenous injection of pancreatic juice into dogs produced hypotension; the active principle in the pancreatic extract was termed "kallikrein." *In vitro* experiments several years later (Werle *et al.*, 1937) revealed that kallikrein reacted with blood to form a smooth muscle stimulating principle, but the realization that kallikrein was an enzyme which acted on a protein substrate in blood to produce the muscle stimulating principle was delayed for more than 10 years. In 1949, Rocha e Silva *et al.*, showed that a muscle stimulating and vasodilator polypeptide, which they termed bradykinin, was produced by the enzymatic action of trypsin or snake venom on the plasma proteins.

Apart from trypsin, there are now several enzymes of possible physiological significance capable of elaborating a plasma kinin in varying degrees of proficiency. Two of these, plasmin and salivary gland kallikrein, are known to arise from an inactive precursor state, termed, respectively, plasminogen and kallikreinogen (Hilton and Lewis, 1957). Although both these enzymes act on a substrate in the plasma proteins, split the synthetic ester *p*-toluenesulfonyl-L-arginine methyl ester (TAME), and are inhibited by diisopropylfluorophosphate (DFP) (Webster and Pierce, 1961), some important differences in their action have been demonstrated, and these must be kept in mind because of their pertinence to the subsequent discussion of antigen-antibody activated serum enzymes. Salivary gland kallikrein, in contrast to plasmin, forms a kinin from the plasma proteins with great rapidity, is resistant to inhibition by the soybean trypsin inhibitor, and does not split casein (Lewis, 1959, 1960).

A kinin-forming enzyme which arises from a precursor in plasma has now been delineated (Elliott, 1963). It is differentiated from plasmin by virtue of its marked kinin-forming and poor fibrinolytic capacity (Schachter, 1963). These two plasma enzymes may also be distinguished by differential activation (Margolis, 1963). Indeed, the studies of Margolis (1958, 1963) indicate that the initial step in the elaboration of plasma kallikrein involves activation of the Hageman factor, and it has even been suggested that the permeability factor of Miles is synonymous with plasma kallikrein.

Purification of bradykinin obtained from the action of crystalline trypsin on pseudoglobulin from ox blood was achieved by Elliott *et al.* (1960a, 1960b) who found that it was a nonapeptide. Synthesis was accomplished by Boissonnas and associates (1960, 1963). Recent studies

indicate that human plasma kallikrein, like trypsin, produces a nonapeptide (kallidin I), whereas salivary kallikrein and an analogous enzyme in urine produces a decapeptide (kallidin II) which differs from the nonapeptide only in the addition of an *N*-terminal lysine (Webster and Pierce, 1963).

Using the pure nonapeptide (bradykinin or kallidin I), Elliott *et al.* (1960c) demonstrated five pharmacological activities: smooth muscle stimulation, vasodilatation, increase in capillary permeability, migration of leucocytes, and stimulation of pain fibers. On a molar basis (Fox *et al.*, 1961) bradykinin is an extremely active vasodilator, and this could prove to be a significant consequence of its activation during anaphylaxis. The decapeptide (Webster and Pierce, 1963) has similar pharmacological activity which differs in degree depending on the assay. There is some evidence that these kinins produce bronchoconstriction when given intravenously or intraperitoneally to the guinea pig, but this action is absent on inhalation (Collier, 1963).

The possibility that a plasma kinin might play a role in systemic anaphylaxis was first suggested by Beraldo's finding of bradykinin-like activity in dog blood during anaphylactic shock (Beraldo, 1950). Recently Brocklehurst and Lahiri (1962) have shown that significant amounts of plasma kinin appear in the blood of sensitized guinea pigs, rats, and rabbits within 2 to 5 minutes of injecting antigen intravenously. These workers also found that the effluent from perfused, shocked, guinea pig lung contained no detectable bradykinin, but would produce bradykinin when incubated with plasma pseudoglobulin which had been heated to destroy kinin-inactivating enzymes. Since the effluent contained the kinin-forming capacity but not kinin, it is reasonable to assume that the kinin-forming enzyme or some precursor thereof was derived from the lung tissue. Diniz and Carvalho (1963) have reported that the plasma substrate for bradykinin formation is depleted during systemic anaphylaxis in the rabbit.

D. SEROTONIN

In 1955 Humphrey and Jaques showed that serotonin was released from rabbit platelets *in vitro* by antigen-antibody interaction, and subsequently Waalkes and his associates (1957) demonstrated serotonin release *in vivo* during rabbit anaphylaxis. These observations naturally stimulated further investigation into the possible role of serotonin in the anaphylactic reaction.

In mammalian tissue, serotonin is localized primarily in the mucosal layer of the gastrointestinal tract, and to a lesser extent in brain tissue

(Erspamer, 1954). Species variation in the serotonin content of the remaining organs is marked. Rat, mouse, and rabbit lung contain significant amounts of serotonin, whereas it is virtually absent from dog, cat, guinea pig, or human lung (Parratt and West, 1957; Udenfriend and Waalkes, 1959). Only the rat and mouse have a significant skin concentration. In the rat and mouse, the mast cells contain serotonin as well as histamine (Benditt *et al.*, 1955; Bhattacharya and Lewis, 1956), but serotonin is apparently not contained in the mast cells of most other mammals, such as man, dog, cat, and cow (Sjoerdsma *et al.*, 1957; West, 1959b). On the other hand, serotonin has been identified in the platelets of a number of species, including horse, ox, goat, dog, guinea pig, and rabbit. It is noteworthy that the nature of the cells which carry the bulk of the tissue serotonin, those in the gastrointestinal tract and brain, is not clearly established.

Serotonin is derived from the amino acid, tryptophan, by the introduction of a hydroxyl group in the 5 position and decarboxylation (Clark *et al.*, 1955). Detoxification by deamination to 5-hydroxyindole acetic acid is accomplished by amine oxidase, an enzyme found in many tissues and plasma (Udenfriend *et al.*, 1956).

Although serotonin is considered to be a constrictor of smooth muscle, the species variation is striking and has been useful in distinguishing it from other smooth muscle constrictors. Smooth muscle in the rat is very sensitive to serotonin and relatively insensitive to histamine; for this reason, the isolated rat colon (Dalglish *et al.*, 1953) or the estrus rat uterus (Amin *et al.*, 1954) have been widely used as indicators of serotonin activity. Pharmacological identification of serotonin is also facilitated by the demonstration of inhibition with minute amounts of lysergic acid (Gaddum and Hameed, 1954). The circulatory response to serotonin in the intact animal is usually depressor followed by pressor (Page and McCubbin, 1953; Schneider and Yonkman, 1954). Table III, taken from the published work of Parratt and West (1957), Udenfriend and Waalkes (1959), and Brocklehurst (1958a), compares the lung concentration of histamine and serotonin in various species with the reactivity of the bronchiolar tissue in these same species to these amines. The species variation in bronchiolar reactivity to serotonin is almost the converse of that observed with histamine. The cat and rat are very sensitive to serotonin but rather resistant to histamine, whereas man and rabbit are very sensitive to histamine but resistant to serotonin. The guinea pig and dog are reasonably sensitive to both. Only in the case of the rat is an appreciable lung serotonin concentration correlated with a significant bronchiolar sensitivity.

TABLE III
SPECIES VARIATION IN SEROTONIN AND HISTAMINE CONTENT AND REACTIVITY

Species	Lung content ($\mu\text{g./gm.}$)		Bronchiolar sensitivity (minimal effective dose in $\mu\text{g.}$)	
	Serotonin	Histamine	Serotonin	Histamine
Cat	<0.2	34	0.01	2
Rat	2.3	5	0.01	>5
Dog	<0.1	25	0.05	0.3
Guinea pig	<0.2	5-25	0.4	0.4
Rabbit	2.1	4	>8	0.5
Man	<0.3	2-20	>20	0.2

The physiological role of serotonin is not yet clearly established. There is no convincing evidence that it plays an important role in hemostasis (Zucker, 1959), and its possible role in central nervous system function (Whitelock, 1957) and gastrointestinal physiology (West, 1959b) is still being elucidated.

Before concluding the discussion of these four pharmacological materials, it is of interest to point out that the evidence that they were released during anaphylaxis *in vivo* or *in vitro* came at 5 to 10 year intervals—histamine in 1932, SRS in 1940, plasma kinin in 1950, and serotonin in 1955. Thus, it seems not unlikely that still other substances will be involved. The suggestion that acetylcholine contributes to the anaphylactic reaction in the guinea pig was based on the apparently beneficial effect of atropine. However, this could have been nonspecific, and Brocklehurst (1958b) was unable to detect acetylcholine in the effluent from perfused, shocked guinea pig lung using the leech muscle assay, even when the acetylcholine was protected from choline esterase by DFP. The incoagulability of dog blood during anaphylactic shock has been recognized since 1909 (Arthus, 1909; Biedl and Kraus, 1910), and the responsible substance, heparin, was isolated from dog blood in crystalline form in 1941 (Jaques and Waters, 1941). Nonetheless, there is no evidence that this material contributes in an important way to the symptom complex of anaphylaxis.

An important point to keep in mind from the summary in Table IV is that two of these materials, histamine and serotonin, exist in tissue in their *final form*, whereas the other two, SRS-A and plasma kinin, must be *produced as well as released* by the anaphylactic reaction. It is also necessary to remember that there are very active mechanisms in tissue and blood that inactivate each of these materials when they are set free as a result of antigen-antibody interaction.

TABLE IV
SUMMARY OF PHARMACOLOGICAL MATERIALS

Material	Cell source	Form	Chemistry	Identification
Histamine 1932	Mast cell platelet unknown	Final	Amine	Inhibition by antihistamine
SRS-A 1940	Unknown	Precursor	Acidic & lipid	Constriction of human bronchiole Resistant to antihistamine Resistant to chymotrypsin Inactive on rat uterus
Kinin 1950	Unknown	Precursor	Nonapeptide	Contraction of rat uterus Destruction by chymo- trypsin
Serotonin 1955	Platelet mast cell unknown	Final	Amine	Contraction of rat uterus Inhibition by lysergic acid

II. Role of Pharmacological Materials in *in Vivo* Anaphylaxis

This section emphasizes the marked clinical and pathological variation in the systemic anaphylactic reaction and illustrates that the principal mediator for a given species can vary from histamine, to some non-histamine pharmacological mediator (possibly SRS-A, serotonin, or plasma kinin), to an antigen-antibody complex *per se*. This species variation must be considered in evaluating studies with *in vitro* systems since the tissue or mediator involved need not necessarily be pertinent to the systemic reaction of that species.

A. GUINEA PIG

Anaphylactic shock in the guinea pig can exhibit different courses depending on the route of the challenging parenteral antigen. If the antigen is administered intravenously, dyspnea due to acute bronchiolar constriction is the predominant sign; at post-mortem examination the lungs are markedly distended and bloodless due to the severe acute emphysema (Auer and Lewis, 1910). That the lung is the "shock organ" and the circulatory collapse secondary is shown by the fact that bronchiolar constriction and acute emphysema can be produced *in vitro* in the perfused guinea pig lung (Dale, 1912). If a large amount of the same antigen is administered subcutaneously or intraperitoneally the guinea pig does not experience respiratory distress; instead, there results a protracted shock which leads to death over several hours (Rocha e Silva, 1955; Stone, 1958). At post-mortem examination the lung shows *no*

emphysema, but the abdominal viscera reveal a marked hyperemia and hemorrhage.

In 1932, Bartosch *et al.* demonstrated the antigen-induced release of histamine from sensitized, perfused guinea pig lung, and in 1939, Code detected the release of histamine *in vivo* during anaphylactic shock in the guinea pig. The initial conviction of many workers that histamine accounted for virtually all the manifestations of the anaphylactic reaction in the guinea pig has been supported by the finding that the antihistaminics give significant protection (Staub and Bovet, 1937; Reuse, 1949, 1956) and by the demonstration of *in vivo* mast cell degranulation during this reaction (Boreus, 1960a). Boreus (1960a,b) performed quantitative mast cell counts in the guinea pig nasal mucosa, and observed a gross correlation between the "disappearance" (loss of diagnostic metachromasia) of mast cells and the intensity of the anaphylactic shock. Desensitization with sublethal doses of antigen and the subsequent restoration of sensitization also correlated with the loss and reappearance of mast cells in the nasal mucosa. Certainly the data shown earlier (Table III) on the histamine content of guinea pig lung and the reactivity of the guinea pig bronchiole are consistent with the belief that in the guinea pig the lung is the "shock" organ and histamine is the principal mediator.

Several discordant observations (Schild, 1936a,b) were explained by Dale (1948) on the assumption that histamine might be present in both an extrinsic and intrinsic form. The first clue that substances other than histamine might be involved came from the observation of Kellaway and Trethewie (1940) that the effluent from perfused, shocked lung contained a slow reacting substance. As already mentioned, Brocklehurst (1953, 1960) subsequently demonstrated that this material was resistant to inhibition by antihistamines. The importance of SRS-A in guinea pig anaphylaxis is difficult to estimate. The isolated guinea pig bronchiole is rather resistant to its action (Brocklehurst, 1962), but intravenous infusion of a crude preparation causes increased resistance of the lungs to inflation (Berry *et al.*, 1963).

At the same time that the anaphylactic release of SRS was first reported (Kellaway and Trethewie, 1940), Campbell and Nicoll (1940) reported that anaphylaxis in nonperfused, guinea pig lung produced a substance which, unlike histamine or SRS-A, contracted the rat uterus. Recently, Brocklehurst and Lahiri (1962) have shown that bradykinin is produced *in vivo* during anaphylactic shock especially in the protracted form, and that a kinin-producing enzyme can be released *in vitro* by antigen from perfused shocked guinea pig lung. They also found that

in protracted shock, the plasma substrate from which bradykinin is formed was depleted (Brocklehurst and Lahiri, 1963). It seems possible that the rat uterus contracting material noted by Campbell and Nicoll (1940) was actually plasma kinin.

Although Humphrey and Jaques (1955) have demonstrated the *in vitro* release of serotonin from guinea pig platelets by antigen-antibody interaction, they found no evidence that the absence of platelets altered the picture of acute systemic anaphylaxis in this species. The guinea pig bronchiole is moderately sensitive to this amine (Brocklehurst, 1958a), but the concentration of serotonin in guinea pig lung is negligible (Udenfriend and Waalkes, 1959), and serotonin has not been detected in the effluent from perfused shocked guinea pig lung (Brocklehurst, 1962).

In summary, present evidence suggests that the acute anaphylactic reaction in the guinea pig which follows intravenous antigen is mediated by the action of histamine and possibly SRS-A on the bronchiole. The mediators of the protracted shock syndrome which follows intraperitoneal antigen have not been firmly established but bradykinin may be implicated.

B. RAT

Circulatory collapse and increased peristaltic activity are prominent during anaphylactic shock in the rat, and West (1959a) has suggested that the gut is the "shock" organ. Although hyperemia of the gut is often a prominent sign at post-mortem examination, in some rats hemorrhage into the pulmonary tissue is equally impressive.

Mota (1957) has marshalled considerable evidence to implicate histamine in active anaphylaxis of the rat sensitized with horse serum. This evidence includes: a rise in plasma histamine concentration; *in vivo* mast cell degranulation, especially in the skin; and partial protection against anaphylaxis by antihistamine or by prior mast cell degranulation with 48/80. Systemic anaphylaxis of rats sensitized with *Bacillus pertussis* vaccine plus horse serum resulted in much more extensive mast cell disruption than anaphylaxis in rats sensitized with horse serum alone (Mota, 1958). However, in passively sensitized rats, exhibiting a similar but perhaps less severe clinical picture of prostration and respiratory distress, Mota (1962) observed no rise in plasma histamine and no mast cell degranulation. This difference was confirmed by *in vitro* experimentation. When mesentery from actively sensitized rats (*B. pertussis* and horse serum) was exposed to antigen *in vitro* there was mast cell degranulation and histamine release (Mota and Ishii, 1960), but when mesentery

from passively sensitized rats was exposed to antigen there was no effect (Mota, 1962).

In an attempt to use a more clearly defined antigen than horse serum and to avoid the use of *B. pertussis* vaccine, Austen and Humphrey (1961, 1962) immunized rats to bovine serum albumin, human γ -globulin, or hemocyanin in Freund's adjuvant and followed with intravenous booster doses of the same antigen absorbed to alum. After the first booster dose these rats consistently developed a transient mild-to-severe prostration to each subsequent injection of antigen. The circulating antibody titer ranged from 0.9 to 4.2 mg./ml. Despite this clinical and laboratory evidence of sensitization, the addition of antigen to isolated peritoneal mast cells or to preparations of mesentery failed to release histamine or to produce mast cell degranulation. Thus, in the rat the mast cell does not seem to participate in passive systemic anaphylaxis, or usually in active anaphylaxis when *B. pertussis* has not been used. By the same token one can argue that histamine is probably not important in these latter reactions.

The recent finding that SRS can be released by antigen in the absence of mast cell damage is particularly pertinent to this discussion. Rapp (1961) found that the intraperitoneal injection of antigen into rats, previously passively sensitized by the intraperitoneal injection of rabbit antibody, consistently released large quantities of slow reacting material that was pharmacologically indistinguishable from guinea pig SRS-A. Neither mast cell damage nor histamine release accompanied the appearance of this slow reacting material in these passively sensitized rats, and it is not known from what cells or even from what tissue it arose. Clinically the animals showed only mild discomfort and macroscopically the only change was hyperemia of the omentum, mesorchium, and peripancreatic tissue. It is important to realize that to date there is no evidence that slow reacting material causes vascular collapse and that rat smooth muscle (bronchiolar, colonic, and uterine) is rather resistant to contraction by guinea pig SRS-A (Brocklehurst, 1962).

Serotonin has received some consideration in relation to anaphylaxis in the rat because of its relatively high concentration in certain rat tissues, such as lung and skin (Parratt and West, 1957; Udenfriend and Waalkes, 1959), and because rat smooth muscle such as uterus (Amin *et al.*, 1954), colon (Dalglish *et al.*, 1953), and bronchiole (Brocklehurst, 1958a) is very responsive to it. Although there is some disagreement as to whether or not serotonin inhibitors prevent the anaphylactic contraction of the rat uterus (Brocklehurst, 1958a; West, 1959a), present evidence shows that they are of no *in vivo* benefit (West, 1959a).

Because of the high concentration of both histamine and serotonin in rat skin and the finding that these materials can be released by 48/80 (Bhattacharya and Lewis, 1956) or dextran (Rowley and Benditt, 1956), the possible participation of these amines in cutaneous anaphylaxis in the rat has been investigated in some detail. Here again there is controversy, but the bulk of the evidence favors the view that these two amines are not critical to the production of passive cutaneous anaphylaxis (Brocklehurst *et al.*, 1955, 1960).

Recently, Brocklehurst and Lahiri (1962) have demonstrated the appearance of a plasma kinin within 2 to 5 minutes after challenging a sensitized rat with intravenous antigen.

In summary, histamine does not seem to be significant except in active anaphylaxis of animals sensitized with both foreign protein and *B. pertussis* vaccine. Serotonin is present in high concentration in many rat tissues, and rat smooth muscle is very responsive to it, but there is no evidence that it participates in the anaphylactic reaction in this species. Slow reacting substance and plasma kinin are newcomers to the rat scene and both are candidates for the role of mediator in this species.

C. MOUSE

Anaphylaxis in the mouse is characterized by respiratory distress (Waalkes and Coburn, 1960a) and prostration (Gershon and Ross, 1962) and is associated with mast cell degranulation (Carter *et al.*, 1957). The mast cells of the mouse contain both histamine and serotonin. Although Waalkes and Coburn (1960a) have observed an increase in blood and lung histamine during systemic anaphylaxis in the mouse, there are several points which argue against the significance of histamine in mouse anaphylaxis. These include the inability of anti-histamines to protect against systemic anaphylaxis (Malkiel and Hargis, 1952), the relative insensitivity of normal mouse tissue to histamine (R. L. Mayer and Brousseau, 1946, Pittman, 1951), and the failure of antigen to release histamine *in vitro* from mouse lung (Waalkes and Coburn, 1960a).

Fink (1956) has implicated serotonin in the anaphylactic contraction of the mouse uterus by obtaining inhibition with lysergic acid, but these data have not yet been confirmed, and others (Udenfriend and Waalkes, 1959) have been unable to demonstrate the presence of serotonin in normal or sensitized mouse uterus. Serotonin has also been implicated by *in vivo* studies (Fox *et al.*, 1958; Tokuda and Weiser, 1961), and those of Gershon and Ross (1962) are particularly pertinent

since they demonstrate that serotonin depletion by two different types of reagents, reserpine and 3,4-dihydroxyphenyl-1- α -methylalanine (methyl DOPA), suppresses the anaphylactic reaction. Reserpine depletes by releasing serotonin and the catecholamines (Brodie, 1958), whereas methyl DOPA prevents serotonin synthesis because of inhibition of 5-hydroxytryptophan decarboxylase (Smith, 1960). B-TM₁₀ (McLean *et al.*, 1960), a compound which depletes the tissues of catecholamine but not serotonin, offered no protection. Administration of a monamine oxidase inhibitor, TB 516 (Horita, 1958), changed the behavioral pattern of systemic anaphylaxis from hypoactive to hyperactive. This was attributed to an augmentation of the central nervous system effects of serotonin. The reserpine protection was associated with a depletion of chromaffin material in the enterochromaffin cells of the bowel, and a similar depletion was associated with systemic anaphylaxis, suggesting that material from this site mediated the reaction. Waalkes and Coburn (1960a) reported that serotonin was released by the addition of antigen to intestine *in vitro*, but they observed no reduction in intestinal serotonin content after systemic anaphylaxis.

Waalkes and Coburn (1960a) detected no increase in blood or lung serotonin during systemic anaphylaxis. Malkiel and Hargis (1960) found that mice with a mastocytoma excreted large quantities of the serotonin degradation product, 5-hydroxy indoleacetic acid, but were neither more nor less sensitive to systemic anaphylaxis than non-tumor-bearing mice. This is noteworthy since Donaldson *et al.* (1960) observed that the normal tissue of the tumor-bearing mice had a marked increase in serotonin content, and that, in contrast to the tumor, anaphylaxis greatly diminished the serotonin concentration of these normal tissues. If serotonin were an important mediator one might well expect these animals to be more susceptible to anaphylaxis. The studies of Malkiel and associates (Malkiel and Hargis, 1960; Donaldson *et al.*, 1960) and the analytical data of Waalkes and Coburn (1960a) seem inconsistent with the depletion data of Gershon and Ross (1962). Since the latter is circumstantial, the role of serotonin in mouse anaphylaxis remains to be rigorously proven.

Zweifach and associates (1961) have recently reported that ϵ -aminocaproic acid (EACA) suppresses the anaphylactic reaction produced in the mouse by soluble complexes. Since this inhibitor might well be effective against the type of trypsin-like enzyme implicated in plasma kinin formation, the latter may be important in mouse anaphylaxis. In contrast, failure of the same inhibitor to suppress the anaphylactic

reaction in the guinea pig (Zweifach *et al.*, 1961) fits with the observation that it is a poor inhibitor of antigen-induced histamine release *in vitro* in guinea pig lung (Austen and Brocklehurst, 1961b).

In summary, histamine does not seem to be an important mediator of systemic anaphylaxis in the mouse. Circumstantial evidence seems to implicate serotonin, but rigorous proof is still lacking. The possible participation of plasma kinin requires further investigation.

D. RABBIT

As early as 1911 (Auer, 1911; Scott, 1911) post-mortem data suggested that the anaphylactic circulatory collapse of the rabbit (Arthus, 1909) was related to acute dilatation of the right heart. Initially, the right heart dilatation was attributed to an increase in pulmonary artery pressure (Airila, 1914; Drinker and Bronfenbrenner, 1924) secondary to pulmonary artery constriction (Coca, 1919), but later, based on several pieces of evidence (Abell and Schenck, 1938; Dragstedt *et al.*, 1940; Rose, 1941a; Kopeloff and Kopeloff, 1941), it was realized that mechanical plugging of the pulmonary capillaries due to microthrombi might be even more important. When Katz (1940) demonstrated that the addition of antigen to sensitized rabbit blood caused a shift in histamine from cells to plasma, the obvious conclusion was that pulmonary leucocyte-platelet thrombi released histamine and that the summation of mechanical and pharmacological events produced the right heart dilatation (Rocha e Silva, 1955).

This formulation has received impressive support in subsequent years. Waalkes and his associates (1957) observed that anaphylactic shock in the rabbit was associated with an increase in plasma histamine, a reduction in total blood histamine because of a diminution in circulating platelets and leucocytes, and an increase in lung histamine in association with platelet-leucocyte clumps (Udenfriend and Waalkes, 1959; Waalkes and Coburn, 1959, 1960b). The only real additions to the earlier formulation have been a better understanding of the etiology of the microthrombi and the recognition of serotonin release by the same mechanism.

The observation (Humphrey and Jaques, 1955) that antigen-antibody interaction *in vitro* can release serotonin as well as histamine from rabbit platelets prompted Waalkes and his associates (1957) to study this amine during rabbit anaphylaxis *in vivo*. Not only is there a significant pulmonary serotonin concentration in normal rabbit lung (Udenfriend and Waalkes, 1959), but also it increases during anaphylaxis (Waalkes *et al.*, 1957). This increase is associated with a

rise in plasma serotonin and a fall in total blood serotonin due to the reduction in platelet count (Waalkes *et al.*, 1957; Waalkes and Coburn, 1959). However, since neither antihistaminics (Reuse, 1951, 1956) nor serotonin depletion by reserpine administration (Fisher and Lecomte, 1956) ameliorate anaphylaxis in the rabbit, it may be that the microthrombi are more important than the release of these two amines.

Brocklehurst (1960) has observed the release of SRS along with histamine from perfused rabbit lung, and more recently Brocklehurst and Lahiri (1962) have reported the appearance of plasma kinin in sensitized rabbits within 2 to 5 minutes after the intravenous administration of antigen. Others have observed that the plasma protein substrate for bradykinin formation is depleted during systemic anaphylaxis in the rabbit (Diniz and Carvalho, 1963).

It has been recognized for some time that anaphylactic shock in the rabbit requires a high titer of circulating antibody (Jackson, 1935) and is associated with eosinophilic masses in the pulmonary capillaries (Gregory and Rich, 1946). However, the full significance of these masses was not recognized until Dixon (1953) demonstrated that they contain radioactivity if the antigen is labeled with I^{131} ; this led to the suggestion that obstruction by antigen-antibody precipitates might play an important role in rabbit anaphylaxis. This view has been confirmed by studies with fluorescein-labeled antigen (McKinnon *et al.*, 1957; McKinnon, 1959) and by the finding that similar clinical and pathological events can be produced in normal rabbits by the intravenous administration of preformed immune precipitates. On the other hand, typical anaphylactic reactions have been produced in rabbits with soluble antigen-antibody complexes in the absence of obvious pulmonary embolization (Weigle *et al.*, 1960).

In summary, although all four pharmacological materials could be implicated in rabbit anaphylaxis and although histamine and serotonin are probably released directly into the pulmonary circulation, it is possible that pulmonary emboli composed of immune precipitates may be more important than the appearance of these chemical mediators. The effects of emboli are likely to be exaggerated by action of such mediators on the lung capillary bed.

E. Dog

Anaphylaxis was first described by Portier and Richet (1902) who observed a lethal reaction in the dog to a second dose of an extract of sea anemones. Circulatory collapse is the predominant clinical manifestation of anaphylaxis in the dog (Dale, 1912), and a grossly congested

liver without stasis in the kidney or spleen is the most striking post-mortem finding (Pearce and Eisenbrey, 1910). That the hepatic congestion is the primary event in the initiation of the circulatory collapse was suggested by Manwaring (1910) and confirmed by Voegtlin and Bernheim (1911) both of whom observed that exclusion of the liver from the general circulation aborted the anaphylactic reaction.

In 1925, Manwaring *et al.* in an ingenious set of cross-circulation experiments demonstrated the antigen-induced release of a vaso-depressor, smooth muscle stimulating principle from dog liver. Dragstedt and his associates (1932, 1936) subsequently identified histamine in the thoracic duct lymph and peripheral blood, and extraction of the histamine from the peripheral blood of dogs in anaphylactic shock was accomplished by Code (1939). Support for the contention that the liver is the "shock" organ and histamine the mediator comes from several further observations: the finding that the dog liver contains a relatively large amount of histamine (8-110 $\mu\text{g./gm.}$) in comparison to that found in most other species (Feldberg, 1956); the demonstration that this histamine can be released from liver *in vitro* by the appropriate antigen (Ojers *et al.*, 1941; Scroggie and Jaques, 1949); and the clinical observation that pretreatment of sensitized dogs with a concentration of 48/80 sufficient to disrupt liver mast cells and reduce liver histamine by more than 40% abolishes the usual anaphylactic reaction to an antigen challenge for at least 17 days (Akcasu and West, 1960).

A discordant note to the conclusion that histamine is the sole mediator of anaphylaxis in the dog comes from the observation that the antihistamines are unable to prevent the antigen-induced increase in portal pressure or the clinical picture of anaphylaxis (Reuse, 1951, 1956). The old finding of Code (1939) that some dogs died with a delayed shock despite the return of the blood histamine to normal also suggests that some other mediator might be involved. Plasma kinin activity was detected in dog blood during anaphylaxis by Beraldo (1950) but the magnitude of kinin activity did not correlate well with the severity of the shock. The fibrinolysin which appears during anaphylaxis in the dog (Rocha e Silva *et al.*, 1946) could act on plasma proteins to elaborate a kinin, but it seems more likely that any kinin activity that appears during anaphylaxis is produced by a kallikrein, plasma or glandular.

A slow reacting substance has apparently been produced in the dog with 48/80 (Paton, 1951, 1957) but release of SRS from dog tissue by antigen has not yet been reported.

Although dog liver contains an appreciable amount of serotonin,

especially in comparison to the negligible concentration in dog lung or skin, systemic anaphylaxis does not diminish the liver serotonin content (Akcasu and West, 1960). This holds true even though the same reaction causes a considerable reduction in liver histamine. Sensitized dog liver also fails to release serotonin *in vitro* when exposed to antigen (Akcasu and West, 1960). Evidently, an abundance of a pharmacological substance in a so-called "shock" organ does not necessarily imply its release during an anaphylactic reaction. By the same token, the release of heparin from the "shock" organ does not mean that it contributes to the symptom complex. The finding that serotonin can be released from platelets *in vitro* (Humphrey and Jaques, 1955) by the antigen-antibody interaction and the observation that a leucothrombocytopenia can appear during anaphylaxis in the dog (Rocha e Silva, 1955) might mean that serotonin is actually released in this reaction, but there is no evidence as yet that this occurs.

In summary, the evidence to date certainly points to the liver as the "shock" organ and to histamine as an important mediator. However, for the reasons already stated, it is unlikely that histamine is the sole mediator, and a plasma kinin may well participate. There is no data at present to implicate either serotonin or SRS in the dog.

F. MAN

There is only limited clinical and meager post-mortem data on acute anaphylactic shock in man. Rocha e Silva (1955) reviewed the literature in his book, and concluded that the post-mortem findings of anaphylactic shock in man were not uniform and at times resembled those of the guinea pig, the dog, or even the rabbit. More recently, James and Austen (1963) reviewed the clinical and post-mortem findings in six cases of fatal systemic anaphylaxis in whom data on the antigen, the interval between injection and the onset of symptoms leading to death, and complete autopsy findings were available. In five instances death could be attributed to laryngeal edema and/or acute pulmonary emphysema. The extent of the laryngeal edema was more significant than anticipated and this may represent an important species characteristic. In the sixth case neither laryngeal edema nor pulmonary emphysema was a prominent finding and the anatomical cause of death was not evident.

Rose (1941b) studied patients with various forms of allergy including asthma and observed that the blood histamine level was unstable relative to normals but did not increase during the induction of an asthmatic attack when measured in blood samples taken from the femoral artery or right heart (Rose *et al.*, 1950). The values reported

make no distinction between histamine in the plasma and that still bound in the cells. Although data on plasma histamine during anaphylactic shock in man are not available to our knowledge, some insight into the possible role of histamine in acute allergic reactions in man comes from the studies of Lecomte (1956) on endogenous histamine liberation in man with chemical histamine releasers. The reaction is characterized by pruritus, skin erythema, a reduction in blood pressure, supra- and infraorbital edema, and finally, full-blown generalized angioneurotic edema with or without nausea, vomiting, or colic. Circulatory collapse was not observed, but even more striking was the complete absence of bronchospasm. The reaction was associated with a threefold rise in plasma histamine and could be aborted by pretreatment with antihistamine. Thus, at least, the pruritus, urticaria, and some portion of the hypotension seen during anaphylaxis in man can probably be attributed to histamine. *In vitro* experiments have demonstrated that antigen capable of eliciting immediate skin reactions can release histamine from the whole blood (Katz and Cohen, 1941) or the lung tissue (Schild *et al.*, 1951) of allergic patients.

In view of the resistance of antigen-induced human bronchoconstriction to antihistamines and the failure of chemical histamine releasers to evoke a bronchoconstrictor response in normal man, it is reasonable to speculate that SRS may play an important part in the bronchoconstrictor reaction. Brocklehurst (1956, 1960) has demonstrated the release of SRS from perfused segments of lung of two pollen-sensitive individuals. In addition, using a biologically similar material obtained from sensitized guinea pig lung, he has shown that the most sensitive bronchiolar tissue known is human; in fact, its responsiveness is almost as exquisite as that of the guinea pig ileum (1962).

There is at present no information as to whether or not serotonin release occurs during anaphylaxis in man (Udenfriend and Waalkes, 1959). Human lung, like guinea pig lung, contains virtually no serotonin (Table III), and the mast cells of man, like those of most species, are devoid of this amine (Sjoerdsma *et al.*, 1957). The disappearance of human platelets during antigen-antibody reactions has been demonstrated, but there is no evidence that this is associated with the release of pharmacologically active amines (Storck *et al.*, 1955).

As in the case of the dog, fibrinolytic activity has been demonstrated in man following intravenous administration of antigen (Lowell *et al.*, 1956). Again, the significance of this proteolytic activity is not established, but it could lead to plasma kinin formation. Landerman and his associates (1962) have presented evidence that a patient with hereditary

angioneurotic edema lacked a serum inhibitor of kallikrein, and these workers feel that an excessive production of plasma kinin underlies the syndrome. Donaldson and Evans (1963) have noted that patients with hereditary angioneurotic edema lack an inhibitor of the esterase activity of the first component of complement (C'1 esterase), and Kagen and Becker (1963) have found that the C'1 esterase inhibitor is also effective against kallikrein.

In summary, it seems evident that histamine accounts for only a limited number of the manifestations of anaphylaxis in man—pruritus, urticaria, angioneurotic edema, and some hypotension. Slow reacting substance may well play a role in the appearance of bronchospasm, whereas plasma kinin could conceivably mediate the profound vascular collapse that characterizes some anaphylactic reactions. At present there are no data to implicate serotonin.

III. The Site and Nature of Antigen–Antibody Interactions Involved in Anaphylaxis

A. ADSORPTION OF ANTIBODY AND ITS RELATION TO SENSITIZATION

The arguments between the proponents of the cellular and humoral theories of anaphylaxis have largely died away. There is more interest now in discovering why certain kinds of antibody can apparently become attached to tissues in such a way that interaction with antigen is highly effective in eliciting anaphylaxis, whereas other kinds of antibody, even against the same antigen, are much less effective, or even inactive in this respect. The question has been considered for many years in terms of the “fixation” of antibody onto tissues, and the “acceptability” of a particular kind of antibody to the tissues of the animal in question. These terms, though meaningful in their context, are essentially vague. The study of “fixation” of antibody comprises two operationally separate steps. The first involves measuring attachment of antibody to tissue, and the second detecting whether the antibody is attached in such a manner that the tissue is *sensitized*, i.e., that subsequent contact with antigen elicits a detectable anaphylactic response, such as histamine release or smooth muscle contraction. Evidence will be outlined later that antibodies probably become attached to tissue by *adsorption*, but that this, although necessary, is not a sufficient condition for sensitization. The finding that only certain antibodies appear able to be adsorbed in the right way or at the right sites so as to sensitize tissue has begun to acquire a more precise significance in the light of experiments based on recently acquired knowledge of the structure and heterogeneity of γ -

globulin. More of the experimental work involved has been conducted in whole animals than *in vitro*, and in the section which follows, both types of work will be discussed where necessary for the argument.

The type of evidence which indicates that antibody needs to become attached to tissues is well illustrated by the quantitative studies of Benacerraf and Kabat (1949) on passive anaphylaxis in the guinea pig. These authors ascertained the minimal amounts of rabbit antiovalbumin that must be injected intravenously in order to produce lethal anaphylaxis when a fixed quantity of 1 mg. ovalbumin was administered by the same route after varying time intervals. When the antigen was injected immediately, about 2 mg. antibody N was required; when the intervals were 1, 5, and 48 hours, the amounts of antibody were 0.24, 0.06, and 0.03 mg. N, respectively. It is evident that interaction of antigen and antibody in the bloodstream was much less effective than their interaction after at least part of the antibody had had time to become attached to the tissues in some appropriate manner. Similarly, if mesentery, removed from guinea pigs at varying times after they have received a constant dose of rabbit antibody intravenously, is washed to remove readily diffusible antibody, and then incubated with antigen under standard conditions *in vitro*, the extent of mast cell degranulation increases markedly with time (Humphrey and Mota, 1959b). When contact with antibody has been allowed to proceed *in vivo* for 48 hours, the amount of passively transferred antibody required to sensitize tissues so as to give an *in vitro* anaphylactic response to antigen is found to be very small. Using I¹³¹-labeled rabbit antibody, the concentrations of antibody on the tissue reported to be sufficient for anaphylaxis to occur are as follows: per gram wet weight of ileum, < 0.6 μ g. (Ishizaka *et al.*, 1957) and 0.02 μ g. (Brocklehurst *et al.*, 1961); and per gram wet weight of mesentery, < 0.2 μ g. (Humphrey and Mota, 1959a).

For quantitative study of the uptake of antibody, experiments *in vivo* are complicated both by the need for antibody to become distributed between blood and tissue fluid and by the presence of the normal plasma proteins. Since tissues can be equally well and demonstrably sensitized by contact with antibody after removal from the body, more informative data may be obtained from experiments *in vitro*. Dale (1912) showed that the uterus of a normal guinea pig could be sensitized by perfusion for 3 to 5 hours with serum containing antibody, and P. Hartley (1939) later found that perfusion was unnecessary, in that prolonged soaking of uterine tissue in antibody would produce sensitization, even at 4°C. Using the chopped lung preparation of

Mongar and Schild (1953) but after pre-perfusion to remove blood, Brocklehurst *et al.* (1961) studied the uptake of a radiolabeled partly purified rabbit antibody concentrate and related this to the degree of sensitization. Sensitization was measured by antigen-induced histamine release after excess antibody had been washed off in a standard manner. It was found that antibody became adsorbed to the tissue quite rapidly (approximately exponentially with time), the rate at 37°C. being about twice that at 0°C. At high external antibody concentrations (400 µg./ml.) some degree of sensitization occurred within 20 seconds, and a degree of sensitization almost equal to the maximum attained under the given experimental conditions was reached within about 5 minutes, even at 0°C. At lower antibody concentrations (0.4 µg./ml.) maximal sensitization was not reached until after 40 minutes at 0°C. At both concentrations, sensitization was maximal when the lung fragments had taken up less than 1 µg. antibody/gm. wet weight, which represented only a small fraction of the total antibody finally taken up by the tissue. The uptake of antibody appeared to be a process of adsorption and to obey a Langmuir isotherm. The globulin concentrates used in these experiments contained 50% or more of specific antibody—a fact of some significance inasmuch as unlabeled γ -globulin was shown to displace labeled antibody. Attempts were made to discover whether different chromatographic fractions of γ -globulin (containing about the same proportions of antibody) would differ in their capacity to sensitize lung tissue, and it was concluded that no significant differences were demonstrable. The levels of antibody used (20–40 µg./ml.) were, however, too high to exclude the presence of some contaminating specifically sensitizing antibody, although there was no evidence for this.

The sensitization *in vitro* of guinea pig ileum by rabbit antibodies has been studied in detail by Halpern *et al.* (1958, 1959b), by Binaghi *et al.* (1959, 1961), and by Nielsen *et al.* (1959). The degree of sensitization was measured by relating the height of contraction elicited by excess antigen to standard histamine contractions. Nielsen *et al.* (1959) used radiolabeled antibody and also found that uptake obeyed a Langmuir isotherm, and that after contact with antibody at 27°C. for 60 minutes the smallest amount that must be taken up to give a definite response to antigen was about 0.8 µg./gm. of tissue. Contact for 1 hour at 37°C. with relatively low concentrations of antibody and subsequent challenge with antigen could be repeated five times without abolishing the capacity to respond, although the response diminished by about 25% on each occasion. Halpern *et al.* (1959b) treated the ileum with undiluted whole rabbit serum and found that over a temperature range

10°–38°C. there existed a constant relationship between the concentration of antibody Ab and the time t to reach a given degree of sensitization, namely, $(Ab) \cdot t^2 = k$.

By making use of this relationship they were able to investigate factors affecting uptake of rabbit antibody and sensitization. The rate of sensitization was little affected by pH over the range 5.8–8.2, except where CO₂ was used in the buffer (in which case sensitization was markedly diminished). The Q₁₀ over the range 30°–38°C. was 2.5, and over the range 10°–30°C. was 1.6. Sensitization was inhibited by the addition of nonspecific, rabbit γ -globulin, but not of α - or β -globulins or of albumin, and washing with γ -globulin would reverse a pre-existing sensitization (Halpern *et al.*, 1959b). The rate of sensitization was much increased, or the concentration of antibody required to sensitize in a given time greatly decreased, by lowering the ionic strength of the medium (Binaghi *et al.*, 1961) or by addition of 0.8 M urea (Binaghi *et al.*, 1959). It was tentatively suggested that the effect of urea was produced by the release of antibody from hydrogen-bonded water.

It seems clear that sensitization of guinea pig tissues by rabbit antibody involves the absorption of γ -globulin onto the tissues rather than active uptake into the cells and that antibody and nonspecific γ -globulin are not distinguished. However, adsorption of antibody per se is not a sufficient condition for sensitization of tissue. As already mentioned (Brocklehurst *et al.*, 1961), experiments on the *in vitro* uptake of labeled rabbit antibody by chopped guinea pig lung showed that sensitization was maximal by the time only a small fraction of the antibody ultimately adsorbed had become attached to the tissue. Furthermore, the maximum degree of histamine release by antigen at 37°C. was much less when antibody had been taken up in the cold rather than at 37°C., despite the fact that the total amount of antibody adsorbed in the cold was not less than that associated with a higher degree of sensitization when adsorbed at 37°C. Feigen *et al.* (1962) have recently compared the uptake of labeled rabbit antiovalbumin by strips of guinea pig ileum at temperatures ranging from 20° to 30°C. with the degree of sensitization achieved, as measured by release of histamine on subsequent contact with antigen at 37°C. Although in this system there was no essential difference in the rates of adsorption of the labeled γ -globulin at difference temperatures, the quantity of histamine released by antigen for a given amount of physically adsorbed antibody was greatly increased as the temperature of the adsorption process was raised. A temperature effect on the adsorption step, as previously reported in the lung system (Brocklehurst *et al.*, 1961), was

only apparent in the ileal system when the antibody in the bulk phase was low, and was obscured when the concentration was sufficient to ensure maximum sensitization over the whole temperature range. It is clear from these results that sensitization, as distinct from adsorption, depends upon the uptake of antibody in a manner which requires considerable activation energy. Feigen *et al.* (1962) calculate this energy to be 18 kcal. Whether, as these authors suggest, a reaction is required involving a change in covalent bonding or whether, as suggested by Mongar and Schild (1962), antibody must achieve a multipoint attachment to the relevant binding site, remains to be determined.

No direct studies have been reported on the effective sites of attachment of the antibodies to cells. Since desensitization by antigen *in vitro* can occur in circumstances where active uptake can be precluded (e.g., in the cold or in the presence of metabolic inhibitors) and in which large molecules do not diffuse into living cells, the antibody must presumably be on or near the cell surface. Humphrey and Mota (1959b) emphasized the regular correlation between anaphylaxis and mast cell damage, and pointed out that the interaction of Forssman antibody with vascular endothelium, although damaging the endothelial cells, does not affect mast cells even though these may be situated only a few microns away. Since antibody against guinea pig γ -globulin damages mast cells very effectively in reversed anaphylaxis, they argued that the relevant γ -globulin (or antibody) for mast cell damage to occur by antigen-antibody interaction must be adsorbed on or very near to the mast cell surface. This does not preclude the possibility that combination of antigen with antibody adsorbed elsewhere may effect other cells, or that larger amounts of antibody reacting with antigen in free solution may activate, less effectively, the same or some other mechanisms which result in cell damage. For example, in Rapp's (1961) study of the release of SRS by antigen in the peritoneal cavity of rats passively sensitized with rabbit or rat antibody, the extent of SRS release was closely correlated with the extent of uptake of labeled antibody onto the tissues of the peritoneal cavity, but there was no accompanying mast cell damage.

Two other facts may be relevant to the question of where and how antibody is "fixed." One is the finding of White *et al.* (1963), by means of the indirect fluorescent antibody technique, that 7S γ_1 - but not γ_2 -globulin from guinea pig serum sticks rather firmly to mast cells in frozen sections of mouse tongue. The γ_1 fraction corresponded to that containing passive cutaneous anaphylaxis (PCA)-active antibodies for the guinea pig, whereas the γ_2 fraction contained blocking rather

than sensitizing antibody. The second is the observation mentioned by Humphrey *et al.* (1963) that after treatment of sensitized, guinea pig lung with trypsin or ethylenediamine tetraacetate (EDTA), individual cell suspensions are obtained which still contain histamine but which no longer release histamine on contact with antigen. Such cells are also no longer able to be sensitized passively *in vitro*. Although these results could mean that something essential for the anaphylactic reaction had been lost, it is also possible that trypsin or EDTA treatment has altered the receptor sites for antibody.

B. REVERSED ANAPHYLAXIS

If antigen-antibody interaction at or near a cell surface were sufficient to elicit anaphylaxis it might be expected that it would be immaterial whether antigen or antibody were adsorbed in the first place. Van den Ende's (1940) studies of reversed anaphylaxis using impure antigens suggested that reversed anaphylaxis occurred only when the antigen contained γ -globulin and the antiserum subsequently injected contained antibody against the γ -globulin. Humphrey and Mota (1959a), using various highly purified antigens confirmed this suggestion and found that, to be effective, the γ -globulin must be derived from a species whose antibodies were themselves able passively to sensitize guinea pigs (see following). Ovary (1960) obtained similar results in studies of passive cutaneous anaphylaxis. Thus, reversed anaphylaxis falls into line and is shown to depend upon the special property of certain γ -globulins to become "fixed" in the right way onto tissues. It has already been mentioned that antibody against guinea pig γ -globulin will damage guinea pig mast cells. The use by Austen and Humphrey (1961, 1962) and Keller (1962) of antibody against rat γ -globulin to damage washed, rat peritoneal mast cells constitutes clear evidence that γ -globulin is adsorbed on these cells in such a manner that antigen-antibody interaction is biologically effective.

C. SPECIES SPECIFICITY OF SENSITIZING ANTIBODIES

A further distinction between adsorption of antibody onto tissues and sensitization is apparent from the differences in sensitizing capacity between antibodies from different species. It has been known for some time that antisera from certain species are generally unable to effect passive sensitization of guinea pigs for systemic anaphylaxis or the PCA reaction, even though the amounts of antibody and the nature of antigens used would have been fully adequate had the antisera been derived from rabbits or guinea pigs (Kabat and Mayer, 1961, Table 7,

p. 250). Whereas all ordinary rabbit antibodies appear to be effective, as are most antibodies from guinea pig, and 7 S antibodies from man, monkey, or dog, the antibodies from horse, cattle, goat, rat, or fowl are wholly or almost inactive in this respect. It has been suggested (Humphrey and Mota, 1959a) that those antigen-antibody systems that do not passively sensitize guinea pigs are those that are inefficient at fixing guinea pig complement; although there is a general correlation between the two properties, there are too many exceptions for such a generalization to be tenable. It appears, rather, to be a question of whether or not the globulin from these species can become attached in the right manner at the right sites on the guinea pig tissue.

Few investigations have been reported on the detailed mechanisms underlying species variations in antibody fixation. Humphrey and Mota (1959a), working with guinea pig mesentery, found no gross differences between the degree of uptake or ease of washing off of I^{131} -labeled rat or goat antibody, which do not sensitize, and rabbit antibody, which does. It is probable that any significant differences were obscured by nonspecific adsorption onto irrelevant tissues, and more detailed cytological studies are needed.

Passive anaphylaxis in other species has received relatively little attention. Rabbit antibodies fail to sensitize rats (but so do most rat antibodies; see Section III, D, 2) insofar as mast cell damage and histamine release on contact with antigen are concerned, although SRS may nevertheless be released (Rapp, 1961). Keller and Schwarz-Speck (1961a) have shown, however, that rat peritoneal mast cells pretreated with human γ -globulin (HGG) *in vitro* are damaged by rabbit anti-HGG, and therefore, HGG can presumably be effectively attached to these cells. Mice pretreated by intraperitoneal injection of *Hemophilus pertussis* can be passively sensitized by rabbit or mouse antibody so as to show signs of generalized anaphylaxis or PCA reactions, but since the severity of the reactions is maximal when antigen is given within a short time after antibody, fixation of antibody on the tissues probably does not play an important role. Species specificity is evident, however, insofar as mouse antibody was found to be 25-50 times as effective as rabbit antibody to the same antigen (Munoz *et al.*, 1958; Munoz and Anacker, 1959). In man, there are several reports that besides sera from humans, antisera from laboratory animals such as the rabbit (Winkenwender *et al.*, 1939; Vaughan and Kabat, 1953; Farah *et al.*, 1960) and guinea pig (Sherman *et al.*, 1939; Sherman and Coulson, 1951) can passively sensitize human skin to give weal and flare reactions. Most workers observed no correlation between precipitating

antibody content and skin-sensitizing activity, but Farah *et al.* (1960) found that if rabbit antibody to a 2,4-dinitrophenyl conjugated antigen was specifically separated from inert γ -globulins it could be used to elicit a weal and flare reaction, and that this property was correlated with the precipitin content. After giving 130 μ g. of pure antibody, skin reactivity to antigen persisted for > 3 and < 5 days, the half-life of labeled antibody in the skin being about 12 hours. This indicates a relatively poor persistence compared with that of human reaginic antibodies.

D. VARIATION IN SENSITIZING ANTIBODY WITHIN A SPECIES

1. Man

It is well known that most human antibodies do not sensitize human skin, and that a tendency readily to form skin-sensitizing antibodies appears to be genetically determined. However, with prolonged exposure to suitable antigens (e.g., pollens, animal dander) the proportion of persons who eventually become sensitized is quite high, e.g., from 30 to 40% of bakers give skin reactions to grain extracts and over 20% of cavalrymen (when cavalry used horses) reacted to horse dander, even though few had clinical symptoms (Hill, 1940). It is not the place of this article to review the findings of workers who have attempted to separate human skin-sensitizing antibodies, and it is sufficient to state that such antibodies are not associated with the γ_2 -globulin fraction, in which "blocking" antibody activity is found. There is a general correlation between the physicochemical properties of sensitizing antibodies and those of β_2 A-globulins. Heremans and Vaermon (1962) were able to prepare a fraction from human serum which contained predominantly β_2 A-globulin, with little γ -globulin or γ_1 -macroglobulin, and which retained most of the skin-sensitizing activity of the original serum. A summary of the distinguishing features of human skin-sensitizing antibody has been given by Kabat and Mayer (1961, Table 12, p. 297), and the subject is reviewed by D. H. Stanworth in this volume.

Human antibodies also differ in their capacity to sensitize guinea pig tissues. Thus, although there are many reports in the literature that protective or blocking antibodies will give rise to passive anaphylaxis in the guinea pig, sera containing human skin-sensitizing antibodies only have failed to do so. Inasmuch as there are no quantitative methods for measuring skin-sensitizing activity, it could be argued that the amount of antibody present was insufficient, although this seems unlikely in view of the sensitivity of the test. Ovary *et al.* (1960), however, have recently shown clearly that human γ_1 -macroglobulin antibodies are

unable to sensitize guinea pigs in reversed or direct PCA reactions, although their 7 S counterparts would do so. It must be concluded that human γ_1 -macroglobulin does not "fix" on guinea pig tissues. Specific tests with human β_2 A-globulin along the same lines have not yet been reported.

2. Rat

As mentioned in an earlier section describing systemic anaphylaxis in the rat, this reaction is regularly accompanied by mast cell damage and histamine release when sensitization is produced by the intraperitoneal injection of various antigens, usually horse serum, together with 10^9 killed *Bacillus pertussis* in the smooth phase (Mota, 1958). On the other hand, passively sensitized rats pretreated with *B. pertussis* undergo passive systemic anaphylaxis without showing mast cell damage, even when the antiserum is obtained from rats actively immunized with antigen plus *B. pertussis* (Mota, 1962). Mesentery from rats actively sensitized with antigen plus *B. pertussis* showed mast cell damage when exposed to antigen *in vitro*, whereas mesentery from passively sensitized rats did not (Mota and Ishii, 1960). This prompted Mota to speculate that *B. pertussis* directed the synthesis of an antibody which was not present in appreciable amounts in the circulation but had a particular affinity for the mast cell (1962). This view was supported by the finding of Austen and Humphrey (1962) that mesentery or peritoneal mast cells from rats, sensitized without *B. pertussis* but with antigen emulsified in Freund's adjuvant, failed to show mast cell damage and histamine release when exposed to antigen *in vitro*, even though the donor rats had good titers of precipitating antibody and regularly experienced collapse with each intravenous booster dose of alum-precipitated antigen. The finding that mast cells from these actively sensitized rats showed damage and histamine release when reacted with rabbit antirat γ -globulin (Humphrey *et al.*, 1963) is not inconsistent with the studies of Mota in that such antibody would react with the whole spectrum of rat γ -globulin. Either these cells do not carry γ -globulin identical with that circulating as precipitating antibody or such antibody is inert in terms of initiating histamine release after interaction with antigen. Further evidence that the administration of *B. pertussis* with specific antigen results in the elaboration of at least two types of antibody comes from a recent finding of Mota (1963). The capacity of antiserum to sensitize rat mast cells to subsequent antigen-induced histamine release appears early and transiently during the immunization process and is destroyed by heating at 56°C., whereas the hemagglutinating titer of the same antiserum to the same antigen is heat stable and does not correlate with histamine release.

3. Guinea Pig

It has recently been reported that guinea pig 7 S antibodies may belong to two populations of molecules with quite distinct electrophoretic mobilities (Yagi and Maier, 1962; Ovary and Benacerraf, 1962; White *et al.*, 1963). These can be separated by chromatography or electrophoresis, one population behaving as slow and the other as fast γ -globulin. Ovary and Benacerraf (1962), Ovary *et al.* (1963), and Bloch *et al.* (1963) found that although both kinds of antibody precipitated with antigen, only the slower migrating fraction fixed guinea pig complement and only the faster fraction was capable of producing the PCA reaction. The slower fraction, which appeared earlier in immunization with antigen in Freund's adjuvant, was not only ineffective in eliciting the PCA reaction but, in addition, blocked the action of the faster fraction. The finding of White *et al.* (1963) that γ_1 but not γ_2 guinea pig serum γ -globulin sticks to mast cells was mentioned earlier in this section.

E. COMPETITION OF γ -GLOBULINS

It is evident from the material already reviewed that the uptake of specific antibody by tissue under any given circumstance takes place in competition with host γ -globulin already present and other nonspecific γ -globulins in the antibody preparation used for sensitization. If all the globulins present compete on equal terms for the relevant sites, then the amount of specific antibody adsorbed would be directly related to the proportion of total γ -globulin which was specific antibody. In practice, such conditions would be unusual. More likely the competition will be between specific antibody and other globulins present in the antiserum and in the tissues with varying affinities for the sites.

Biozzi *et al.* (1959) measured the threshold amounts of rabbit antibody required to elicit a positive PCA reaction in guinea pigs when the antibody was diluted before injection in physiological saline or in saline containing various concentrations of other proteins. They found that the presence of albumin had no effect, but that the sensitizing capacity of a solution containing 0.2 $\mu\text{g./ml.}$ of rabbit antiovalbumin was abolished by the simultaneous presence of nonspecific rabbit γ -globulin (100 $\mu\text{g./ml.}$), guinea pig γ -globulin (20 $\mu\text{g./ml.}$), or human γ -globulin (1000 $\mu\text{g./ml.}$). Horse γ -globulin had no effect at concentrations up to 10,000 $\mu\text{g./ml.}$ Mongar and Schild (1960) studied *in vitro* sensitization of chopped guinea pig lung by unfractionated rabbit or guinea pig antisera at various temperatures. They found that sensitization was slowed by the simultaneous addition of normal serum or γ -globulin from rabbit, man, or guinea pig but not by human serum albumin or bovine γ -globulin. They also showed that sensitization which

had occurred *in vitro* or *in vivo* could be reversed *in vitro* at 38°C. in the presence of sufficient HCG or of undiluted guinea pig serum. Halpern *et al.* (1961) washed the guinea pig ileum for 24 hours at 5°C. in the presence of varying concentrations of normal guinea pig γ -globulin and found that the rate of subsequent sensitization by rabbit antibody was inversely proportional to the concentration of γ -globulin in the washing fluid. These studies were extended by Binaghi *et al.* (1962) to show that sensitization by rabbit antibody was inhibited by γ -globulins from various species in the following order of effectiveness: rabbit > man > dog > guinea pig > rat > horse > cattle > pig > chicken > goat. This order resembles the order of effectiveness of antibodies from the same species for passive sensitization of guinea pig tissues, but even globulins that themselves consistently fail to sensitize produced some degree of inhibition.

A direct consequence of the competition of globulins for receptor sites on the tissues is that the effectiveness of antibody for passive sensitization depends upon the ratio of antibody to nonspecific globulins in the system. At a constant total γ -globulin concentration, the relationship between the antibody concentration and the time t necessary to obtain a given level of sensitization of guinea pig ileum is expressed by $(Ab) t = k'$, instead of $(Ab) t^2 = k$, which holds when the concentration of antibody and total γ -globulin vary simultaneously (Binaghi *et al.*, 1962). This probably explains why Brocklehurst *et al.* (1961), using a globulin concentrate containing more than 50% antibody and chopped tissue from pre-perfused guinea pig lung, found that sensitization was complete in a much shorter time than did others who used crude antiserum and unwashed lung tissue. It also implies that the sensitivity of the PCA reaction for demonstrating antibody will be less when the proportion of antibody to nonspecific globulin in the antiserum is low than when it is high.

There are numerous observations in the early literature (Weil, 1913; Kellaway and Cowell, 1922) indicating that administration of large amounts of homologous or heterologous serum to guinea pigs rendered them incapable of becoming passively sensitized, or desensitized animals already sensitized, provided that the amount of antibody used for sensitization was not too great. These findings are explicable in terms of competition of globulins for receptor sites. Halpern (1961) and Halpern and Frick (1962) have shown that passive sensitization of guinea pigs or mice by rabbit antibody can be prevented by administration of 100 times as much normal rabbit or human γ -globulin but not of horse or bovine γ -globulin. The normal globulin was effective

when given between 18 hours before and 1 hour after the antibody but not when given 15 hours after (9 hours before antigenic challenge).

F. PHYSIOCHEMICAL FACTORS INVOLVED IN BIOLOGICAL ACTIVITY OF γ -GLOBULINS

1. *Passive Sensitization by Antibody*

a. *The Nature of the Antibody.* Progress in the understanding of antibody adsorption and tissue sensitization comes not only from the studies of species and intraspecies variation but also from the new knowledge concerning the structure of γ -globulin.

It is now well established that γ -globulin from several species can be split by limited digestion with various proteolytic enzymes and disulfide bond reduction into three subunits. These have been named Fragments I, II, and III when derived from rabbit γ -globulin and slow (S and S') and fast (F) fragments when derived from human or mouse γ -globulins. Slow (I and II) fragments each contain one antibody-combining group and are immunologically very similar or identical. Fast (III) fragments are immunologically distinct and are not associated with antibody activity. By limited digestion without disulfide bond reduction, γ -globulin molecules can be split into two unequal fragments, one of which contains both the antibody-combining groups and consists of the S-S' (I-II) portions still linked together. Although the S (I or II) fragments of antibodies are univalent and nonprecipitating, the S-S' (I-II) portions are bivalent and retain their precipitating capacity unimpaired. A full discussion of the chemical and immunological properties of such fragments has been given by Fahey (1962).

Although the enzymatically produced subunits are in a sense artifacts and do not exactly correspond to the actual polypeptide chains from which γ -globulins are composed, their use has contributed to the understanding of the requirements for fixation of antibody onto tissues in such a way as to cause sensitization. Hartley reported in 1951 that "refined," i.e., pepsin-digested, guinea pig antibodies would neither cross the guinea pig uterus nor sensitize guinea pig tissues. Such antibodies still precipitated with antigen and presumably corresponded to rabbit Fragments I-II. The fragments produced by papain-cysteine treatment of rabbit antibody are also unable to sensitize guinea pig tissues (Humphrey and Mota, 1959a). By means of the reversed PCA reaction, elicited with antibody against whole rabbit γ -globulin, Ovary and Karush (1961) showed that Fragment III was capable of sensitizing the skin but that Fragment I was not. The specific antibody used to elicit the reactions was capable of reacting with either fragment.

In the direct PCA reaction, Fragment I again failed to sensitize, but it could be shown to combine with antigen by its ability to inhibit the reaction due to the intact parent antibody if the two were mixed before injection. Ovary and Karush (1961) concluded that Fragment III was necessary for γ -globulin to fix to guinea pig tissues. Taranta *et al.* (1962) have also reported that, although pepsin-digested rabbit antibody against HGG does not sensitize the skin for PCA, it will elicit a reversed PCA reaction when HGG is already fixed on the skin.

It has already been mentioned that human 7 S γ -globulin will "fix" on guinea pig tissues but that γ_1 -macroglobulin will not. It is probably significant in this connection that only the former contains the F groupings which are immunologically equivalent to Fragment III (Fahey, 1962).

Ishizaka *et al.* (1961b) have treated human and rabbit γ -globulins with mercaptoethanol followed by iodoacetate so as to reduce and alkylate some of the disulfide bonds. Such treatment only slightly diminished their immunological activities as antigen or antibody, respectively, but abolished their capacity to sensitize guinea pig skin for reversed or direct PCA reactions. The molecular changes that accompany such treatment are not yet clear.

b. The Nature of the Antigen. Leskowitz and Ovary (1962) studied the relation between the molecular weight of various antigens and their ability to elicit PCA reactions in guinea pigs sensitized with equal amounts of rabbit antibody and found over a wide range of molecular weights that very approximately equal numbers of antigen molecules were required to elicit minimal reactions. In the case of antihapten antibodies, Ovary and Karush (1960) concluded that the interaction of fixed antibody and hapten did not give rise to any biologically detectable effect. When a hapten molecule containing two haptenic groups, namely, α , ϵ -dinitrophenyl lysine, was used in conjunction with rabbit or guinea pig antibodies to dinitrophenyl protein, both Ovary (1961) and Cohen (1962) report that good PCA reactions were obtained, provided that the antibody was of rabbit origin.

2. *The Use of Antigen-Antibody Complexes*

The biological activities of antigen-antibody complexes have been discussed in recent reviews by Weigle (1961), Dixon (1962), and Campbell (1962). Only those aspects will be briefly mentioned that have a bearing on the nature of antibody fixation and the mechanism of anaphylaxis. Certain antigen-antibody complexes, in moderate antigen excess, will mimic anaphylactic reactions, in the sense that they

cause contraction of isolated guinea pig smooth muscle or increased capillary permeability in the skin. They do so in quite small amounts, although these are substantially greater (of the order of 100-fold) than the smallest quantities of antibody required to produce the same effects in anaphylactic reactions in which the tissue is passively sensitized. With few exceptions, rabbit antibody-antigen complexes were found by Ishizaka *et al.* (1959) to possess skin irritating activity, but complexes formed with horse or chicken antibodies did not. Skin-reactive complexes were inhibited when mixed with normal rabbit or human γ -globulin prior to intracutaneous injection, but were unaffected by the presence of horse or chicken γ -globulins. Injection of reactive complexes into the skin prevented subsequent sensitization of the same skin sites by an unrelated rabbit antibody when the latter was tested by the PCA reaction. Most inactive complexes had no such blocking effect, and Ishizaka and Campbell (1959) therefore suggested that combination of the soluble antigen-antibody complexes with some tissue constituents was necessary for their reactivity and that this combination occurred in competition with other γ -globulins.

The skin-irritating activity of the soluble complexes required the presence of Fragment III in the antibody molecule, since Ishizaka *et al.* (1962) found that complexes formed with Fragments I or II or I-II were inactive. Aggregation of Fragment III by cross linking via bisdiazotized benzidine imparted both skin-reactive and complement-fixing activity, whereas treatment of Fragment I or II in the same fashion did not.

IV. *In Vitro* Studies of the Mechanism of Anaphylaxis in Tissue or Cells

A. GUINEA PIG LUNG

The *in vitro* anaphylactic release of histamine (Bartosch *et al.*, 1932), SRS (Kellaway and Trethewie, 1940), and kinin-forming enzyme (Brocklehurst and Lahiri, 1962) was first appreciated in experiments with perfused, shocked, whole guinea pig lung. However, neither the perfused whole tissue nor the isolated whole tissue technique (Ungar and Parrott, 1936; Schild, 1937) is as suitable for studying anaphylaxis *in vitro* as the chopped lung method developed by Mongar and Schild (1953). Austen and Brocklehurst (1960a) modified the latter in two respects: the lung tissue was perfused free from blood prior to chopping so as to minimize any nonspecific contribution from serum factors not firmly adsorbed onto the tissue, and antigen was added 10 seconds after the inhibitor rather than 15 minutes later, so as to diminish any non-

specific effects as a result of prolonged contact between the tissue and the inhibitor prior to the addition of antigen.

1. *Time Course of the Anaphylactic Release of Histamine and Slow Reacting Substance*

The time course of the release of histamine and SRS after the addition of antigen to sensitized, perfused, chopped guinea pig lung is brisk (Austen and Brocklehurst, 1961a; Austen and Humphrey, 1962). Histamine began to appear within 10 to 15 seconds after adding antigen, and two-thirds of the total amount to be released was in the supernatant by the end of the first minute. Slow reacting substance did not appear in the supernatant until 30 to 60 seconds after the addition of antigen and did not attain a maximal release rate until the end of the first minute. That the time course of SRS release is slower than that of histamine has also been observed in nonperfused lung slices (Chakravarty, 1960a) and in perfused whole lung (Brocklehurst, 1960).

2. *Effect of Anoxia, Carbon Monoxide, and Cyanide*

The view that anoxia due to a nitrogen atmosphere prevents the anaphylactic release of histamine from guinea pig lung has been accepted for some time (Parrot, 1942; Mongar and Schild, 1957a) and has recently been confirmed again (Yamasaki *et al.*, 1960; Chakravarty, 1960b; Moussatche and Provoust-Danon, 1960; Edman and Mongar, 1961), but not by all workers (Austen and Brocklehurst, 1960b). The possibility that inadvertent changes in ionic strength or pH might account for these and other discrepancies in regard to nitrogen inhibition has been discussed (Austen and Brocklehurst, 1961c; Diamant, 1962a). The findings of Provoust-Danon and Moussatche (1961), Diamant (1962a,b), and Chakravarty (1962) that the anaphylactic release of histamine is only depressed by anoxia in the absence of glucose or when the glycolytic cycle is blocked provide an additional explanation for such contradictory results and support the view that aerobic metabolism *per se* is not a prerequisite for antigen-induced histamine release. The latter conclusion had been drawn from earlier studies in which carbon monoxide, a highly specific inhibitor of cytochrome oxidase, and 2-heptyl-4-hydroxyquinoline-*N*-oxide, which inhibits between cytochrome b and c, failed to inhibit anaphylactic histamine release in guinea pig lung in the usual glucose-containing Tyrode's buffer (Austen and Brocklehurst, 1960b, 1961c). Nonetheless, the ability of glucose to reverse a nitrogen inhibition is consistent with a requirement for energy which can be supplied by the glycolytic cycle when the cytochrome system is unavailable.

It has also been reported that cyanide inhibits antigen-induced histamine release in guinea pig lung. However, the concentration used, from 1 to 10 mM (Mongar and Schild, 1957a; Yamasaki *et al.*, 1960) was sufficient to inhibit many enzymes other than cytochrome oxidase (Dixon and Webb, 1958). Austen and Brocklehurst (1961c) found that 1 mM KCN inhibited the anaphylactic release of histamine most effectively if the duration of contact between the tissue and the cyanide prior to antigen addition was minimal; the inhibition after 10 seconds preincubation was 53% whereas that after 15 minutes preincubation was only 16%. This reversal of inhibition with preincubation of tissue and inhibitor could not be explained by loss of HCN from the tightly stoppered breakers because the cyanide concentration in the supernatant at the end of the experiment was 80% of that added whether or not the preincubation period was 10 seconds or 15 minutes. It is possible that during preincubation the tissue was becoming adapted to anaerobic glycolysis. However, cyanide inhibition could also be caused by inactivation of some metal-requiring enzyme other than cytochrome oxidase or could be produced by the reduction of some essential disulfide bond.

3. *Inhibition by Thiol Alkylating or Oxidizing Agents*

Mongar and Schild (1957a) first demonstrated that the sulfhydryl inhibitor, iodoacetate, could prevent the antigen-induced release of histamine from chopped guinea pig lung. Prolonging the contact between the tissue and the inhibitor prior to adding antigen increased the inhibition. The effectiveness of iodoacetate as an inhibitor of histamine release has been confirmed by other workers (Chakravarty, 1960b; Austen and Brocklehurst, 1961a) and its ability to prevent SRS release has also been reported (Chakravarty and Uvnas, 1959). Other sulfhydryl inhibitors such as chloromercuribenzoate (Mongar and Schild, 1957a), *N*-Ethylmaleimide or oxidized glutathione (γ -glutamylcystinylglycine) (Austen and Brocklehurst, 1961a) are also active. In contrast, the thiol compounds, cysteine, thioglycollic acid, and reduced glutathione (γ -glutamylcysteinylglycine) have no effect (Austen and Brocklehurst, 1961a).

Despite the abundant evidence that sulfhydryl inhibitors prevent the anaphylactic release of histamine from guinea pig lung, there is *no evidence* that any of these compounds are acting on an antigen-antibody activated step. When chopped lung is incubated with 1 mM of *N*-ethylmaleimide for 15 minutes and then washed four times to remove "unreacted" inhibitor before adding antigen, there is still maximal inhibition (Austen and Brocklehurst, 1961a). Either the thiol-inhibiting agent is

acting in some nonspecific way or some state of the cell essential to the anaphylactic reaction is susceptible to alteration by *N*-ethylmaleimide irrespective of the occurrence of antigen-antibody interaction. This may involve the integrity of the glycolytic cycle and could be related to the evidence that sensitization of tissue involves activation energy (Feigen *et al.*, 1962).

4. Effect of Temperature

The observation that the anaphylactic release of histamine from guinea pig tissue (aorta) is influenced by temperature was first made by Schild in 1939. A detailed study with lung tissue was carried out some years later by Mongar and Schild (1957b). Preheating lung tissue at temperatures greater than 42°C. for 25 minutes prior to reacting the tissue with antigen at 37°C. greatly reduced the histamine release. The rate of heat inactivation increased as the preheating temperature was elevated to 45°C. Inactivation was permanent at 45°C., whereas preheating at 43°C. was followed by some recovery on prolonged standing at 37°C. The inactivation was due to an effect on the tissue and not on the antibody. Antibody heated at 45°C. was still capable of passively sensitizing normal lung tissue, whereas normal or actively sensitized lung tissue heated at 45°C. could not be passively sensitized by unheated antibody.

When antigen was added to sensitized tissue at 17°C. there was no histamine release, but on warming the reaction mixture to 37°C. the histamine release varied depending on the duration of contact between tissue and antigen at 17°C. A contact period of 15 minutes at 17°C. did not diminish histamine release on warming, whereas a contact period of 1 hour reduced the release by more than 80%.

These experiments on the effect of temperature demonstrate that (1) the anaphylactic release of histamine from guinea pig lung requires activation of a factor that is heat labile in the precursor state, and (2) contact of sensitized tissue and antigen at 17°C. activates a labile, temperature-dependent factor which, though unable to act at 17°C., is gradually dissipated.

5. Effect of Ions

That the anaphylactic release of histamine from guinea pig lung requires calcium was demonstrated by Mongar and Schild (1958). Omission of calcium chloride from the medium in which the sensitized tissue was suspended greatly reduced histamine release, irrespective of whether the medium was calcium-free Tyrode's, 0.16 *M* sodium chloride

or potassium chloride in isotonic sucrose. Addition of 0.5 mM of EDTA to the reaction mixture virtually abolished antigen-induced histamine release. Chakravarty (1960b) using a veronal buffer containing EDTA found that the addition of excess calcium within a reasonable time interval to antigen-treated tissue gave histamine release without further addition of antigen.

The omission of magnesium or potassium from the Tyrode's solution did not diminish histamine release, nor did the addition of these ions to normal saline restore the capacity for histamine release (Mongar and Schild, 1958). The failure to demonstrate a magnesium effect does not exclude the possibility that a magnesium requirement is fulfilled by tissue stores. Even when the tissue had remained in calcium-free Tyrode's solution for 30 minutes histamine release was still 30% of that achieved in normal Tyrode's solution (Austen and Brocklehurst, 1961b). In experiments in which anaphylaxis has been prevented by chelation of calcium and magnesium with EDTA (Mongar and Schild, 1958; Chakravarty, 1960b), the restoration of the anaphylactic reaction by the addition of calcium cannot be attributed solely to a calcium requirement because calcium displaces magnesium from the chelating agent.

A pH curve for the anaphylactic release of histamine from guinea pig lung has been obtained in phosphate, bicarbonate (Mongar and Schild, 1958), or veronal buffer (Chakravarty, 1960b), and the optimum is in the range of 7.5 to 7.9. The effects of calcium and pH are somewhat interdependent in that the depression of release at low pH can be significantly reversed by increasing the calcium concentration (Mongar and Schild, 1958). A possible interpretation is that the *in vitro* anaphylactic reaction requires bound, unionized calcium.

Austen and Brocklehurst (1961c) found that the anaphylactic release of histamine from perfused, chopped guinea pig lung is very sensitive to changes in the NaCl concentration of the containing medium. An increase in the molarity of Tyrode's solution from 0.161 to 0.178 by the addition of NaCl *reduces* the histamine release by 32%, whereas a reduction in molarity to 0.143 by NaCl depletion *increases* histamine release by 45%. These results were obtained when antigen was added to the sensitized tissue 10 seconds after the suspending medium had been changed from normal to NaCl-augmented or NaCl-depleted Tyrode's solution. In an attempt to determine whether the effect of NaCl variation was due to ionic strength or osmolarity, Austen and Brocklehurst (1961c) investigated the comparative effect on histamine release of adding sucrose, NaCl, or MgCl₂ to NaCl-depleted Tyrode's solution. When the molarity of NaCl-depleted Tyrode's solution was

increased by adding MgCl_2 much greater inhibition was achieved at each final molarity than when the same final molarity was produced by adding NaCl . Increasing the final molarity by adding sucrose had little effect. Since the number of solute molecules or ions contributed by equimolar amounts of sucrose, NaCl , and MgCl_2 are in the ratio of 1:2:3, it is necessary to consider the relative effectiveness of these compounds in terms of osmolarity. This reveals that ionic strength is more important than solute concentration in modifying antigen-induced histamine release and that the enhancement and inhibition are not merely the result of cell swelling and contraction, respectively. The antigen-antibody interaction itself is not significantly influenced by changes in ionic strength of this magnitude (Oncley *et al.*, 1952), and it seems likely that some other surface protein-protein interaction, such as attachment of antibody, is responsible for the sensitivity of antigen-induced histamine release to changes in ionic strength. Irrespective of the mechanism, these studies point out that in investigations with inhibitors care must be taken to avoid inadvertently increasing ionic strength and thereby misinterpreting the cause of the inhibition.

These studies with ions clearly indicate that the anaphylactic reaction in guinea pig lung requires calcium and is very sensitive to changes in ionic strength. Definitive studies regarding a magnesium requirement have not been feasible.

The sensitivity of the anaphylactic release of histamine to changes in the NaCl concentration of Tyrode's solution (Austen and Brocklehurst, 1961c; Austen and Humphrey, 1962) is remarkably similar to the effect of variation in NaCl concentration on immune hemolysis (M. M. Mayer *et al.*, 1946) or the lysis of the sensitized red cell—carrying the first, fourth, and second components of complement ($\text{EAC}'_{1,4,2}$)—by the third component of complement ($\text{C}'3$) (Becker and Wirtz, 1959). As the reaction between the $\text{EAC}'_{1,4,2}$ cell and $\text{C}'3$ is also prevented by salicylaldoxime (Mills and Levine, 1959) or phlorizin (Rodriguez and Osler, 1960), Austen and Brocklehurst (1961c) investigated the effect of these two compounds on the anaphylactic reaction in guinea pig lung. Salicylaldoxime was an effective inhibitor, producing 50% inhibition at a concentration of 0.5 mM, but phlorizin was only a weak inhibitor. The inhibition produced by salicylaldoxime was not as a result of irreversible alteration of the sensitized tissue, because tissue washed free of the inhibitor responded to antigen in the usual fashion. It seems probable that salicylaldoxime is acting on some antigen-antibody activated step, but the mechanism of its inhibition is not known.

6. Inhibition by Diisopropylfluorophosphate

Since metabolic inhibitors, such as iodoacetate, *N*-ethylmaleimide, or cyanide, influence a wide range of enzymes and alter a variety of tissue metabolic processes, the observation that such compounds inhibit antigen-induced histamine release offers only limited insight into the type of enzymatic sequence which might be involved in anaphylaxis. Austen and Brocklehurst attempted to use inhibitors which were more selective in action, and most of their studies were done with competitive rather than noncompetitive inhibitors.

The finding of Levine (1955) that immune hemolysis could be prevented by the esterase inhibitor, diisopropylfluorophosphate (DFP), prompted a study of the effect of DFP on *in vitro* anaphylaxis. DFP inhibited the anaphylactic release of histamine and SRS from perfused (Brocklehurst, 1959) or chopped guinea pig lung (Austen and Brocklehurst, 1960a, 1961a). A concentration of 2 mM consistently reduced the anaphylactic release of histamine and SRS in chopped lung by more than 50%. That DFP, which is capable of inhibiting purified chymotrypsin on a 1:1 molar basis, was not more effective may be attributed to the following: the binding of DFP at tissue sites not participating in the reaction; the very short time interval between addition of antigen and release of histamine, during which DFP must combine with the antigen-antibody activated enzyme; and the fact that it must do this in the presence of natural substrate. Additional support for the contention that an esterase is involved comes from the finding that diethyldi-*n*-butylpyrophosphate and *o,p*-nitrophenyl-*o*-ethyl-*n*-propylphosphonate are inhibitory—the latter being approximately ten times as active as DFP (Austen and Brocklehurst, 1962).

Since DFP inhibits esterases irreversibly, it was possible to determine whether the DFP-inhibited esterase essential to anaphylaxis in guinea pig lung was present before or only subsequent to addition of antigen (Austen and Brocklehurst, 1961a). Incubation of sensitized tissue with 10 mM of DFP reduced the antigen-induced histamine release by more than 90%. When samples of the same DFP-treated tissue were washed four times with Tyrode's solution to remove unreacted DFP prior to adding antigen, there was *no* inhibition. Since DFP inhibited the anaphylactic release of histamine only when present at the time of antigen addition, DFP must be acting exclusively on an antigen-antibody activated esterase which exists in lung tissue in a DFP-resistant precursor state until its activation by the antigen-antibody interaction. Such a situation would be consistent with the fact that the proenzymes, chymo-

trypsinogen and trypsinogen, are resistant to DFP whereas chymotrypsin and trypsin are susceptible (Jansen *et al.*, 1949).

7. Inhibition by Specific Peptidase Substrates

The conclusion that activation of a DFP-sensitive esterase is a necessary condition for the anaphylactic reaction in guinea pig lung, initiated a search for its substrate specificity. DFP inhibits a variety of esterases including true and pseudo cholinesterases, lipases, aliesterases, and peptidases such as trypsin, plasmin, and chymotrypsin (Becker, 1962). Austen and Brocklehurst (1960a, 1961a) elected to study the effect of synthetic peptidase substrates in the hope that they would compete with the natural substrate for the enzyme involved. The esters, amides, or hydrazides of arginine or lysine, which are typical trypsin substrates, were not inhibitory; the trypsin inhibitor from soybeans also was not inhibitory. Substrates of leucine aminopeptidase or carboxypeptidase were not inhibitory.

In contrast, substrates of chymotrypsin were consistently capable of inhibiting the *in vitro* anaphylactic release of histamine and SRS from guinea pig lung. The most active substrates, *N*-acetyl-L-phenylalanine ethyl ester and *N*-acetyl-L-tryptophan ethyl ester, regularly produced greater than 50% inhibition at a concentration of 1.0 and 0.5 mM, respectively. The levorotatory form of the aromatic amino acids had no effect at 20 mM, and the peptides, *N*-acetyl-DL-phenylalanine and *N*-acetyl-L-tryptophan, required 20 mM to produce 50% inhibition. The finding that the aromatic amino acid esters were better inhibitors than the amide, and the observation that in each instance the acetylated ester was a better inhibitor than the nonacetylated aromatic amino acid ester (Austen and Brocklehurst, 1961a) suggested that ability to inhibit anaphylaxis correlated with the affinity of the synthetic substrate for pancreatic chymotrypsin (Green and Neurath, 1954). However, the relative effectiveness of all the acetylated and nonacetylated aromatic amino acid esters against anaphylaxis was not exactly the same as their susceptibility to chymotrypsin.

Chymotrypsin substrates are termed trifunctional because they interact with chymotrypsin at three sites—the aromatic side chain, the secondary peptide bond or free amino group, and the susceptible ester bond. Whereas trifunctional compounds can be either substrates or competitive inhibitors, compounds presenting the enzyme with only two such sites, namely, an aromatic residue and a polar group separated from it, may bind even more firmly and are termed bifunctional competitive chymotrypsin inhibitors. Compounds containing only an aromatic

residue may be monofunctional inhibitors (Green and Neurath, 1954). Inhibition of *in vitro* anaphylaxis was also achieved with bifunctional and monofunctional chymotrypsin inhibitors (Austen and Brocklehurst, 1961a). The most active bifunctional inhibitors, β -phenylpropionic acid, β -phenylcinnamic acid (*trans*), β -indoleacetic acid, and β (β -indole) propionic acid, consistently produced 50% inhibition at a concentration of 2.5 mM. The results obtained with the four monofunctional inhibitors tested are noteworthy because they were more active than the bifunctional inhibitors and the relative order of their effectiveness (indole > skatole > phenol > nicotinamide) was the same as that observed by Huang and Niemann (1953) against pancreatic chymotrypsin. While studying the effect of a number of antipyretics and related compounds on the anaphylactic reaction in chopped guinea pig lung, Mongar and Schild (1957a) first noted that phenol was an effective inhibitor; phenol did not denature the antibody and did not prevent effective antigen-antibody interaction, and it was suggested that phenol inhibited some enzymatic process initiated by the antigen-antibody interaction.

Austen and Brocklehurst (1961a) also excluded the possibility that the various classes of chymotrypsin inhibitors used in their work destroyed or released antibody or otherwise impaired the ability of the sensitized tissue to respond to antigen. Sensitized tissue was incubated with several representative inhibitors for 15 minutes at 37°C. and then washed four times with Tyrode's solution to remove the inhibitor prior to the addition of antigen. On the addition of antigen, such tissue gave virtually the same histamine release as control tissue which had never been exposed to the inhibitor. In each of the experiments the concentration of inhibitor selected was sufficient to have given more than 80% inhibition had it been present at the time of antigen addition, and so these studies indicate that the inhibitors act on an antigen-antibody activated step and not in some nonspecific manner. Furthermore, when tissue in which the anaphylactic release of histamine had been prevented by the presence of inhibitor was washed free of inhibitor and treated a second time with antigen, there was no histamine release. This desensitization without histamine release shows that the inhibitor has not prevented effective antigen-antibody interaction, but rather has acted beyond this point. A similar desensitization without histamine release was observed by Mongar and Schild when sensitized tissue was exposed to antigen in the cold (1957b) or in the presence of EDTA (1958), but there is no evidence as yet that the same labile factor is lost under each circumstance.

It seems probable that the DFP-inhibited esterase involved in

anaphylaxis has a chymotrypsinlike substrate specificity (Austen and Brocklehurst, 1961a), but there is no rigorous direct evidence. Ungar and his associates (1961), using a manometric technique, have reported the appearance of esterase activity against *N*-acetyl-L-tyrosine ethyl ester on the addition of antigen to sensitized guinea pig lung slices. However, the significance of these data is not established: the antigen-induced increment in esterase activity was less than one-third the control activity; the lung was not perfused prior to slicing, and so both baseline and antigen-induced activity could have arisen, in part, from gross serum constituents; and the ethylene glycol used to dissolve the esterase substrate has been found by others to inhibit the anaphylactic release of histamine from guinea pig lung (Austen and Brocklehurst, 1960c).

8. Inhibition by Monobasic Fatty Acids

While evaluating ϵ -aminocaproic acid, an inhibitor of plasminogen activation (British Patent Specification, 1957; Alkjaersig *et al.*, 1959), for its effect on the anaphylactic reaction in chopped guinea pig lung, Austen and Brocklehurst (1961b) found that caproic acid (hexanoic) was a more potent inhibitor than ϵ -aminocaproic acid. It was further observed that the ability of the unsubstituted fatty acids to inhibit increased with increasing chain length from valeric to dodecanoic. For example, the concentration of caproic acid (hexanoic) required to produce a 50% reduction of the anaphylactic release of histamine and SRS was 4 mM, whereas the same inhibition was achieved with only 0.6 mM of capric acid (decanoic). The introduction of a polar group, amino or carboxyl, into the hydrocarbon residue of caproic acid diminished or abolished the inhibitory capacity, whereas unsaturation or terminal branching had no effect.

The inhibition produced by the fatty acids was not caused by binding of essential calcium, because calcium excess produced no detectable reversal. Nor was it produced by irreversible alteration of fixed antibody, because sensitized tissue washed free from the fatty acid responded to antigen in the usual fashion. Since fatty acids bind strongly to human albumin (Goodman, 1958), and by so doing can alter the physiochemical properties of albumin (Schmid, 1959), it was possible that the inhibition was as a result of modification of the antigen. However, increasing the concentration of ovalbumin ten or even fifty times did not yield detectable reversal of inhibition, and the fatty acids inhibited as strongly when the tissue was sensitized to human γ -globulin as when the tissue was sensitized to ovalbumin. The finding that tissue in which the anaphylactic release of histamine and SRS had been prevented by the

presence of a fatty acid was desensitized to a second dose of antigen indicates that the acid did not prevent effective antigen-antibody interaction (Austen and Brocklehurst, 1961b). It seems probable that the monobasic fatty acids inhibit a reaction set off by the union of antigen and antibody, in a common pathway leading to the release of both histamine and SRS.

9. *Enhancement of the in Vitro Anaphylactic Reaction by Certain Dibasic Acids*

In 1957, Moussatche and Provoust-Danon reported that the anaphylactic release of histamine from guinea pig lung slices could be enhanced by the addition of succinic acid to suspending buffer solution; they attributed this to be an effect on the tricarboxylic acid cycle. Diamant (1962c) confirmed the finding of Yamaski *et al.* (1960) that sodium succinate would not enhance anaphylactic release under a nitrogen atmosphere and also concluded that enhancement under aerobic conditions was due to stimulation of some energy-requiring step. While studying the relationship of structure to the inhibitory capacity of the monobasic fatty acids, Austen and Brocklehurst (1961b) confirmed the remarkable enhancing effect of succinic acid and extended the findings sufficiently to conclude that the enhancement was not produced by an effect on the Krebs tricarboxylic acid cycle.

In twenty consecutive experiments with different tissue samples of perfused, chopped guinea pig lung, the addition of 0.5 mM of succinate to the reaction mixture 10 seconds before the antigen almost doubled the anaphylactic release of histamine; the percentage of total tissue histamine released by antigen increased from an average of 19.5 to an average of 37 when succinate was added (Austen and Brocklehurst, 1961b). Enhancement of the anaphylactic release of both histamine and SRS was definite with as little as 0.05 mM of succinate and reached near maximum with 0.5 mM. Increasing the concentration to 5 or even 20 mM did not give significantly more enhancement than was achieved with 0.5 mM. Succinate alone did not release either histamine or SRS.

The presence of succinate in the reaction mixture did not alter the time course of histamine release (Austen and Brocklehurst, 1961b). The augmentation of histamine release was due to a quantitative increase in release during the first minute and not to a prolongation of the period of maximal release. A detectable increase in histamine formation was not the basis of the increased release because the sum of the histamine released and that left in the tissue after anaphylaxis was not greater than the histamine content of control tissue exposed to neither succinate nor

antigen. The time course of SRS release was also the same as that observed in normal Tyrode's solution. Since SRS cannot be detected in tissue until after the addition of antigen (Brocklehurst, 1960), these data suggest that succinic acid increases both the formation and release of SRS without changing the time course.

In an attempt to elucidate the mechanism of this enhancement, Austen and Brocklehurst (1961b) studied other dibasic acids and certain Krebs cycle intermediates. Malonic acid, whose carbon atom chain is one shorter than succinic, had no effect even at 20 mM. Glutaric acid, which is one carbon atom longer than succinic, required 5 mM to produce the same enhancement as 0.5 mM of succinic acid. α -Ketoglutaric acid had approximately the same activity as glutaric. Adipic acid, which is two carbon atoms longer than succinic, had no effect even at 20 mM. Fumaric acid, the *trans* isomer of butenedioic acid was inactive at 20 mM, but maleic acid, the *cis* isomer of butenedioic acid, was as active as succinic in enhancing the anaphylactic release of histamine and SRS. These studies were all carried out with tissue from guinea pigs actively sensitized to ovalbumin. Similar results were obtained with lung tissue from guinea pigs passively sensitized with rabbit antihuman γ -globulin.

Since fumaric acid and other Krebs cycle intermediates, such as pyruvic, malic, or citric, are without effect (Yamasaki *et al.*, 1960; Austen and Brocklehurst, 1961b), whereas maleic acid, which is not a Krebs cycle intermediate, is as active as succinic (Austen and Brocklehurst, 1961b), the enhancement cannot be attributed to a general effect on the tricarboxylic acid cycle. In view of the fact that neither succinic nor maleic acid release histamine or SRS by themselves, the enhancement is probably caused by potentiation of some step activated by the antigen-antibody interaction and common to the release of both histamine and SRS. The structural configuration required for a dibasic acid to enhance the *in vitro* anaphylactic reaction in guinea pig lung is rather specific; the carboxyl groups should be separated by a two carbon chain and must be free or fixed in the *cis* position. Both the enhancement and the structural specificity have been confirmed *in vivo* in guinea pigs by Brocklehurst (1961).

Since both the inhibiting monobasic fatty acids and the enhancing dibasic aliphatic acids seemed to be acting on an antigen-antibody activated step, it was possible that they were competing for the same site in the reaction sequence. Studies were carried out to determine whether the inhibition of the *in vitro* anaphylactic reaction by varying concentrations of caproic or decanoic acid could be reversed by a fixed

concentration of succinic acid. Within the limits of the experiment a dose-related reversal was achieved (Austen and Brocklehurst, 1961b). This does not necessarily imply competition for the same site, but is consistent with this possibility. The inhibition produced by the chymotrypsin substrate, L-tryptophan ethyl ester was more resistant to reversal by succinic acid than the inhibition due to the fatty acids. This suggests that the ester and the acids may inhibit at different steps in the reaction sequence, or at least in a different way.

10. A Tabulation

The known characteristics of the mechanism responsible for the *in vitro* anaphylactic release of histamine and SRS in chopped guinea pig lung may be summarized as follows: the reaction requires calcium, a heat labile factor, and free sulfhydryl groups; the reaction involves the activation of an esterase which can be inhibited by DFP—indirect evidence suggests that this esterase has a chymotrypsin-like substrate specificity; the reaction can be affected markedly by compounds normally present in mammalian tissue—certain dibasic aliphatic acids, such as succinic or maleic, produce enhancement whereas the monobasic fatty acids from valeric to dodecanoic are inhibitory; the reaction apparently needs the glycolytic cycle but is not dependent on cytochrome-mediated aerobic metabolism; and the reaction resembles the step in immune hemolysis at which the EAC_{1,4,2} cell reacts with C'3 insofar as it is very sensitive to small changes in the NaCl concentration of the containing medium and can be inhibited by salicylaldehyde.

B. LUNG OF OTHER SPECIES

The *in vitro* anaphylactic release of histamine and SRS has also been demonstrated with human (Schild *et al.*, 1951; Brocklehurst, 1956), monkey, and rabbit lung (Brocklehurst, 1960). Although Brocklehurst (1960) failed to obtain histamine or SRS release from the lung tissue of rats sufficiently sensitized with ovalbumin or human serum albumin to exhibit anaphylactic shock, Chakravarty (1959, 1960a,b) did observe both histamine and SRS release from the lung tissue of rats sensitized with horse serum and *Hemophilus pertussis* vaccine. The latter worker has investigated the effect of various inhibitors on the antigen-induced release of histamine from rat lung and has observed inhibition with EDTA, iodoacetate, or 2,4-dinitrofluorobenzene. Anoxia due to a nitrogen atmosphere failed to inhibit unless glucose was omitted from the buffer (Diamant, 1962a).

C. MAST CELLS—GUINEA PIG OR RAT

The effect of antigen-antibody reactions on mast cells has been studied in tissues of the guinea pig or rat by morphological and/or pharmacological techniques. Experiments on isolated peritoneal mast cells have been confined to the rat since it has not been possible to obtain isolated peritoneal mast cells from the guinea pig by a similar technique.

I. Guinea Pig Mast Cells (*Tissue*)

Mota and Vugman (1956) observed that the mast cells in guinea pig lung are located in the alveolar walls, in the pleura, and surrounding the bronchioles; fatal systemic anaphylaxis following intracardiac antigen (ovalbumin) produced a pronounced reduction in the stainable mast cell population of the pulmonary tissue. The remaining identifiable mast cells showed partial degranulation. Guinea pigs dying with a similar degree of emphysema from histamine administration did not show a significant decrease in pulmonary mast cell concentration, whereas the lung tissue of animals protected against fatal systemic anaphylaxis by antihistamine showed mast cell degranulation, without much emphysema when the animals were subsequently sacrificed. Boreus (1960a,b) performed quantitative mast cell counts on biopsy specimens of guinea pig nasal mucosa before and after systemic anaphylaxis and observed a gross correlation between the disappearance of mast cells and the intensity of the shock. The disappearance of mast cells was already maximal within 1 minute of intra-arterial antigen administration. The mast cell degranulation produced by antigen *in vivo* can also be produced *in vitro*. Incubation of lung or mesentery from sensitized guinea pigs with antigen *in vitro* significantly diminishes the number of detectable tissue mast cells—presumably caused by a disappearance of their metachromatic content (Mota, 1959a). The mast cell degranulation which followed the addition of antigen to guinea pig tissue sensitized passively (Mota, 1959a; Humphrey and Mota, 1959a) was indistinguishable from that observed in actively sensitized tissue. When mesenteries, obtained from animals sensitized passively against two distinct antigens, were treated *in vitro* successively with each antigen, the proportion of the mast cell population damaged increased with each treatment but did not exceed 80% (Humphrey and Mota, 1959b).

In vitro mast cell degranulation lends itself to inhibition studies. Such studies have shown that anaphylactic mast cell degranulation and histamine release in guinea pig mesentery can be prevented by EDTA, iodoacetate, phenol, or preheating the tissue at 45°C. (Mota, 1959a). Phenol did not irreversibly alter either the antibody fixed to the tissue

or the mast cells, because the removal of phenol from the mesentery by washing prior to antigen addition permitted the usual mast cell degranulation. Antigen failed to produce mast cell degranulation when added to mesentery at 15°C., but degranulation occurred when the tissue was warmed to 37°C. Mast cell degranulation was apparent within 15 seconds of adding antigen, and from 50 to 70% of the maximal effect was achieved by the end of the first minute. More recently, Mota and associates (1960) have observed that nicotinamide inhibits mast cell degranulation in guinea pig mesentery. These data on the effect of calcium lack, heat, iodoacetate, phenol, and nicotinamide on mast cell degranulation and the observed time course of mast cell degranulation are in good agreement with the observations on antigen-induced histamine release in guinea pig lung (Mongar and Schild, 1957a,b, 1958; Austen and Brocklehurst, 1961a).

2. Rat Mast Cells (Tissue)

The finding that tissues from rats sensitized with *Bacillus pertussis* and horse serum, or some other foreign protein, consistently show mast cell disruption and histamine release when challenged with antigen *in vitro* or *in vivo* has already been considered in Sections II and III. Studies on the inhibition of mast cell degranulation in the rat have mostly been carried out with skin or mesentery from rats sensitized with *B. pertussis* and horse serum (Mota and Ishii, 1960; Hogberg and Uvnas, 1960). Histamine release from sensitized rat skin or mast cell disruption in rat mesentery is prevented by iodoacetate, *N*-ethylmaleimide, cyanide, or phenol. EDTA or preheating mesentery at 45°C. for 5 minutes abolishes antigen-induced histamine release or mast cell disruption (Mota and Ishii, 1960). In parallel studies, Hogberg and Uvnas (1958, 1960) also observed that antigen-induced mast cell disruption was prevented by sulfhydryl inhibitors, cyanide, phenol, or calcium lack. In addition, these workers (Hogberg and Uvnas, 1960) followed the time course of mast cell disruption, determined the pH optimum, and studied the effect of temperature on the reaction; disruption was about two-thirds completed at the end of the second minute, the pH optimum was broad but centered around 7.5, and disruption was abolished below 10°C. or above 45°C. Inhibition was also noted with dinitrofluorobenzene (DNFB) but inhibition was still maximal when the tissue was washed free of "unreacted" DNFB prior to adding antigen. Therefore, the inhibitor may not be acting specifically on the anaphylactic reaction. A magnesium ion requirement was not demonstrable.

The results of the available inhibition studies with the rat mesentery

(Mota and Ishii, 1960; Hogberg and Uvnas, 1960) are virtually identical with the findings in guinea pig mesentery (Mota, 1959a) or lung (Mongar and Schild, 1957a,b, 1958; Austen and Brocklehurst, 1961a). It may be that mast cell disruption (extrusion of metachromatic material) in the rat and mast cell degranulation (disappearance of metachromasia) in the guinea pig are mediated by some similar steps. The reason for the morphological difference following exposure to antigen is unknown. It must, however, be recalled that the significance of mast cell disruption and histamine release in rat anaphylaxis is questionable and that serotonin release has not yet been directly demonstrated during anaphylaxis in this species.

3. Rat Mast Cells (*Peritoneal*)

Padawar and Gordon (1955) separated rat peritoneal mast cells from peritoneal washings by centrifugation into a high density sucrose solution. During this procedure the cells lose much of their histamine and so are not suitable for studies on the mechanism of histamine release. This difficulty was avoided by Mota and Dias Da Silva (1960) and by Uvnas and Thon (1959) who substituted albumin and Ficoll (a highly water-soluble colloid), respectively, for sucrose. Mota and Dias Da Silva (1960) observed morphologic changes by phase microscopy when mast cells from rats sensitized with *B. pertussis* and horse serum were exposed to horse serum *in vitro*. A few seconds after the addition of antigen there was a slight increase in cell size followed by a sudden appearance and disappearance of successive vacuoles so as to give the cell a "bubbling" appearance. On occasion, these authors observed extrusion of granules, but there was no apparent discontinuity of the cell wall. Uvnas and Thon (1959) observed that the addition of horse serum to a suspension of isolated mast cells from rats sensitized with *B. pertussis* and horse serum released 36% of the total histamine in the suspension. Thus, it seems likely that the washed mast cells used by both groups of workers retained reacting antibody.

Archer (1960) suspended normal rat peritoneal mast cells in rat antiserum and studied the effect of adding antigen. For the most part, the rat peritoneal cell suspensions were used without further concentration of the mast cell population; the results were apparently reproducible when the mast cells were concentrated. Mast cell disruption and histamine release were observed with the addition of antigen (human red cells) to antiserum from rats sensitized by two injections of washed human red blood cells. Disruption even occurred when the peritoneal cells were added from 3 to 10 minutes after the antigen-antibody inter-

action had already lysed the red cells. A longer time interval or treating the immune serum by heat or hydrazine prevented mast cell disruption. The reaction between antiserum to human γ -globulin or bovine serum albumin and the appropriate antigen also caused disruption of the peritoneal mast cells provided the latter were added within 10 minutes of the antigen-antibody interaction. Washed preformed immune precipitates were incapable of disrupting the mast cells.

As discussed in Sections II and III, mesentery or isolated peritoneal mast cells from rats sensitized solely with a protein antigen failed to release histamine when exposed to antigen *in vitro*, even though the donor animals had a significant titer of circulating antibody and exhibited collapse with each intravenous booster injection (Austen and Humphrey, 1961, 1962). This refractoriness to specific antigen was not overcome by adding fresh guinea pig serum or by transferring unwashed mesentery directly from the killed animal to a buffered preparation of the antigen (Humphrey *et al.*, 1963). The discrepancy between these results and those obtained with *B. pertussis*-horse serum sensitized rats (Uvnas and Thon, 1959; Mota and Dias Da Silva, 1960) supports the view of Mota (1962, 1963) that *B. pertussis* directs the synthesis of a special kind of "mast cell lytic" antibody. It is also possible that some essential rat serum factor, similar to that postulated by Archer (1960), was lacking in the *in vitro* experiments involving the protein antigens (human γ -globulin, bovine serum albumin, or hemocyanin). Inasmuch as mast cell disruption was not even produced *in vivo*, the latter explanation seems remote.

The effect of rabbit antibody against highly purified rat γ -globulin was investigated by Austen and Humphrey (1961, 1962) and Humphrey *et al.* (1963). Three different test preparations were used: rat mesentery; unfractionated suspensions of rat peritoneal mast cells (such preparations contained about 3% mast cells, the remaining cells being mainly macrophages, lymphocytes, and a variable proportion, around 8%, of eosinophiles); or concentrated mast cells obtained from the peritoneal cell suspension by centrifugation into high density albumin as described by Mota and Dias Da Silva (1960). The latter contain from 60 to 80% mast cells. Rabbit antirat γ -globulin (anti-RGG) consistently disrupted mast cells and released histamine and serotonin from each of these preparations, and was equally effective when used on the tissues of the sensitized rats even though the latter failed to respond to specific antigen *in vitro*. When the reaction was followed by phase microscope, the findings in reversed anaphylaxis resembled those described by Mota and Dias Da Silva (1960) in direct anaphylaxis. That the histamine

release in reversed anaphylaxis was due to the antibody in the antiserum was indicated by the inactivity of normal rabbit serum, and by the fact that removal of the antibody by absorption of the antiserum with rat γ -globulin in slight excess over the equivalence ratio reduced the histamine releasing activity to 1% of that in the original antiserum when tested on the mesentery. Fractionation of the anti-RGG on columns of diethylaminoethyl (DEAE) cellulose showed that the activity resided in the γ -globulin fraction of the antiserum.

For studies of inhibition it is necessary to have a quantitative *in vitro* system, and the unfractionated rat peritoneal cell suspension offers a readily available source of histamine (the average value for the cells harvested from a single 250–350 gm. hooded rat of the Mill Hill strain was 7.3 μ g. of histamine), and permits study of histamine release from replicate samples with a negligible sampling error (Humphrey *et al.*, 1963). Histamine release from washed peritoneal cells was definite at an antibody concentration of 0.21 μ g./ml. and increased gradually with an increasing concentration of antibody. With an antibody concentration of 4.5 μ g./ml., histamine was detected in the supernatant within 5 to 10 seconds of adding antigen, and all the histamine to be released was in the supernatant within 30 seconds. It was also possible to demonstrate histamine release using a concentrated mast cell preparation washed four times and treated with the γ -globulin fraction of anti-RGG. When fresh dialyzed rabbit serum was present, the effect of low concentrations of antibody was greatly potentiated. The critical factor in normal rabbit serum was inactivated with heating at 56°C. for 30 minutes.

In studying inhibition of reverse anaphylaxis, these authors used washed cells in the absence of fresh normal rabbit serum so as to limit the number of variables. This does not exclude the possibility that factors other than the reacting γ -globulin were still adsorbed to the cells.

DFP, indole, and salicylaloxime were found to be effective in preventing histamine release by anti-RGG. DFP and indole were only active when present at the time of anti-RGG addition and had no effect if the cell suspension was washed "free" from them prior to adding the antiserum. Salicylaloxime produced irreversible inhibition. Histamine release did not occur in the absence of calcium or in the presence of sufficient EDTA to bind the available calcium, but release was normal if the ionized calcium concentration was restored before adding the antiserum. It was not possible to demonstrate a magnesium ion requirement. The addition of antiserum to the peritoneal cell suspension in the cold did not produce histamine release and, if the contact period in the cold was sufficient, there was no histamine release on warming to 37°C.

Further addition of anti-RGG showed the cells to be desensitized. Desensitization without histamine release was also produced by adding the anti-RGG in the presence of EDTA at 37°C. In all these respects, reversed anaphylaxis of the rat peritoneal cell suspension (Humphrey *et al.*, 1963) resembles direct anaphylaxis in chopped guinea pig lung (Mongar and Schild, 1957a, 1958; Austen and Brocklehurst, 1961a). The chymotrypsin substrates, L-tryptophan ethyl ester or L-phenylalanine ethyl ester, could not be studied because they released histamine in the absence of anti-RGG, but the acetylated ester substrates of chymotrypsin have been shown to be inhibitory (Keller and Beeger, 1963).

The two systems differed with respect to the effect of ionic strength or the mono- and dibasic acids. It was not possible to demonstrate enhancement of histamine release from the rat cells by succinic acid or inhibition by caproic acid in a range of concentrations which were effective in the guinea pig (Austen and Brocklehurst, 1961b). The release of histamine from the rat peritoneal cell suspension was not significantly influenced by changes in the NaCl concentration of the containing medium which would have had a pronounced effect on the anaphylactic release of histamine from guinea pig lung (Austen and Brocklehurst, 1961c).

Keller and Schwarz-Speck (1961b) have reported that isolated rat peritoneal mast cells passively sensitized with rabbit and antihuman γ -globulin release histamine on contact with human γ -globulin. This reaction is inhibited by iodoacetate or by DFP. Since it is inhibited by DFP it is reasonable to speculate that the same esterase is required for histamine release from rat mast cells by reversed or passive anaphylaxis.

Although with a high concentration of whole anti-RGG it was possible to release up to 80% of the histamine present in the concentrated rat peritoneal mast cell suspension, no slow reacting material was detected. The gut contracting activity of the supernatant was abolished by 10^{-6} M mepyramine (Humphrey *et al.*, 1963). On the other hand, Rapp (1961) has demonstrated that a slow reacting material indistinguishable from that of the guinea pig can be produced *in vivo* in rats by an appropriate intraperitoneal antigen-antibody interaction; neither histamine release nor mast cell disruption accompanied the appearance of this slow reacting material. These findings do not support the view that SRS formation accompanies mast cell damage (Uvnas and Thon, 1959) and indicate that in the rat the pathways leading to the anaphylactic release of histamine and slow reacting material need not necessarily share a common step.

4. Mast Cell Enzymes

Since anaphylactic mast cell alteration and histamine release have been observed *in vivo* (Mota and Vugman, 1956; Mota, 1957; Boreus, 1960a,b) and *in vitro* (Mota, 1959a; Mota and Ishii, 1960) in rat or guinea pig tissue, the enzyme activities which have been identified in or attributed to mast cells are of considerable interest. Peptidase activities resembling chymotrypsin (Benditt and Arase, 1959), trypsin (Glenner and Cohen, 1960), and leucine aminopeptidase (Braun-Falco and Salfeld, 1959) have been recognized.

Gomori (1953) observed by histochemical methods that the mast cells of the rat, mouse, rabbit, dog, and man are capable of splitting 3-chloroacetoxy-2-naphthoic acid anilide, and Benditt and Arase (1959) recognized that this anilide was a chymotrypsin substrate. Using the method of Padawar and Gordon (1955) to obtain isolated rat peritoneal mast cells, Benditt and Arase (1959) compared the enzymatic activity of the mast cell with pancreatic chymotrypsin and observed the following: both preparations hydrolyzed 3-chloroacetyl-2-naphthoic acid anilide; the hydrolysis of this substrate by either the mast cells or chymotrypsin was prevented by DFP; the mast cells split the acetylated esters of tyrosine, tryptophan, or phenylalanine which are typical chymotrypsin substrates; and neither preparation split *p*-toluenesulfonyl-L-arginine methyl ester (TAME) which is a typical trypsin substrate. The DFP-inhibited enzyme implicated in the anaphylactic release of histamine from guinea pig lung (Austen and Brocklehurst, 1961a) or the rat mast cell (Austen and Humphrey, 1962) exists in sensitized tissue or cells in a DFP-resistant precursor state, whereas the chymotrypsinlike enzyme of the rat mast cell described by Benditt and Arase (1959) was inhibited by DFP in the absence of an antigen-antibody interaction; this discrepancy might be explained by activation during the isolation procedure. In the guinea pig there are data to implicate a chymotrypsinlike esterase in anaphylactic histamine release (Austen and Brocklehurst, 1961a) but because some chymotrypsin substrates themselves release histamine, similar data were obtained only in part in the isolated rat mast cell preparation (Humphrey *et al.*, 1963; Keller and Beeger, 1963). Indole and phenol, which are monofunctional competitive chymotrypsin inhibitors, do, however, prevent disruption of the rat mast cell.

A trypsinlike enzyme capable of hydrolyzing *N*-benzoyl-DL-arginine- β -naphthylamide has been demonstrated in the mast cells of the skin of man and dog by histochemical methods (Glenner and Cohen, 1960). The activity was inhibited irreversibly by DFP and competitively by *N*-benzoyl-L-arginine amide; it was not affected by iodoacetate, cyanide,

or the trypsin inhibitor from soybeans. The trypsinlike activity was not detected in mast cells from the rat, mouse, rabbit, or guinea pig but the enzyme could have been present in a precursor form. There are no convincing data at present to implicate this trypsinlike activity in histamine release, but an enzyme of this type might well be implicated in plasma kinin formation.

Braun-Falco and Salfeld (1959) noted that mechanical irritation of the skin of a patient with diffuse mastocytosis increased the serum leucine aminopeptidase activity. Leucine aminopeptidase activity was identified in the mast cells in the skin of the patient, and also in the mast cells of rat mesentery by histochemical techniques. Tissue enzymes of this type are resistant to DFP or iodoacetate but are inhibited by EDTA or cyanide—presumably because of a metal requirement (Burstone and Folk, 1956).

It has been known for some time that nicotinamide is capable of inhibiting diphosphopyridine nucleotidase (DPNase) (Zatman *et al.*, 1953), and Alivisatos (1958, 1959a,b) has recently reported that histamine can be exchanged for the nicotinamide moiety of diphosphopyridine nucleotide (DPN) in the presence of DPNase. This prompted Mota *et al.* (1960) to study the effect of nicotinamide on the anaphylactic release of histamine from guinea pig lung or rat skin and on the mast cell alterations in guinea pig or rat mesentery. These workers and Austen and Brocklehurst (1961a), who studied nicotinamide for a different reason, observed inhibition of the anaphylactic reaction. Middleton and Devi (1963) found that nicotinamide not only prevented antigen-induced histamine or SRS release from guinea pig lung, but also prevented depletion of an orcinol-reactive substance assumed to be DPN. These data in the guinea pig and rat could mean that DPNase is involved in some way in histamine release, but it is equally likely that the histamine-nicotinamide exchange limits the anaphylactic reaction by increasing the tissue concentration of nicotinamide, which in turn inhibits some other enzyme essential to the reaction, such as the chymotrypsinlike enzyme implicated in guinea pig lung (Austen and Brocklehurst, 1961a).

Hogberg and Uvnas (1957) tested the effect of approximately 30 hydrolytic enzymes on the rat mesentery and observed that only lecithinase A caused mast cell disruption; this enzyme was inhibited by some of the same compounds which prevented mast cell disruption by the condensation product of *p*-methoxyphenethyl-methylamine and formaldehyde (48/80). In studies with one of these lecithinase A inhibitors, 1,3-diphosphoimidazole (DPI), these workers used Pk 3010aa rather than 48/80 because the former is the corresponding tertiary amine.

This was necessary because DPI apparently phosphorylates amino groups (Rathlev and Rosenberg, 1956) and so would have interacted with 48/80. When mesentery was exposed simultaneously to Pk 3010aa and DPI there was no mast cell disruption, and when this tissue was washed and exposed to 48/80 there was still no histamine release. It was postulated that Pk 3010aa had removed an inhibitor permitting activation of a lytic cell enzyme which was immediately inactivated by DPI. Incubation of this tissue with phosphoamidase to dephosphorylate the hypothetical lytic cell enzyme initiated mast cell disruption. These ingenious experiments do suggest that Pk 3010aa acts via a lytic cell enzyme with an essential amino group, but the view that this lytic enzyme is a lecithinase A cannot be accepted without reservation; DPI may well act on other types of enzymes; the toxicity of lecithinase A is not limited to the mast cell; and the temperature of lecithinase A inactivation is significantly different from that which prevents the mast cell from responding to 48/80. Even if the interpretation of Hogberg and Uvnas (1957) is correct, these observations may be valid only for the rat, for Boreus (1960c) has observed that the guinea pig mast cell is virtually resistant to disruption by lecithinase A. Riley (1958) favors the view that a type C phospholipase is involved in mast cell disruption in the rat. This is based on the finding that rat mast cells contain phosphatidyl serine and phosphatidyl ethanolamine, and the observation that addition of ethanolamine to a freshly exteriorized portion of rat mesentery produces swelling, vacuolation, and disruption of the mast cells. Irrespective of whether or not the lecithinase theory is a valid explanation for one of the steps in histamine release from rat mast cells by compounds such as Pk 3010aa or 48/80, there is no experimental data to implicate such an enzyme in anaphylactic mast cell disruption.

D. RABBIT PLATELETS

The observation that histamine can be released from the formed elements of rabbit blood by an *in vitro* antigen-antibody reaction was reported by Katz in 1940. This was confirmed almost immediately (Dragstedt *et al.*, 1940; Rose and Browne, 1941), and the platelets were recognized to be the major source of the histamine in rabbit blood (Minard, 1941; Code, 1952). McIntire *et al.* (1950, 1952), who studied the antigen-induced release of histamine from sensitized rabbit blood in an effort to determine if a fibrinolytic enzyme was involved, failed to implicate such a protease. Release of histamine was not accompanied by the appearance of detectable fibrinolytic activity, and the trypsin inhibitor from soybeans did not prevent antigen-induced histamine

release even when present in a concentration sufficient to abolish release by an optimal trypsin concentration. Histamine was not detected until 1½ to 2½ minutes after adding antigen, egg white, to the sensitized rabbit blood (McIntire, 1956), and this lag phase is somewhat greater than that observed in *in vitro* studies of histamine release or mast cell degranulation in the guinea pig (Mota, 1959a; Austen and Brocklehurst, 1961a) or rat (Hogberg and Uvnas, 1960; Humphrey *et al.*, 1963). The lag phase was not shortened by reacting antigen and antiserum prior to adding the normal cells, and so cannot be attributed to time required for some entirely fluid phase reaction. McIntire (1957) obtained inhibition of histamine release with phenol and with a variety of cationic substances, several of which were quarternary ammonium compounds; however, it still remains to be shown that these inhibitors do not alter the reactivity of platelets washed "free" from them prior to adding antigen.

Whereas previous workers studied the antigen-induced release of histamine in blood taken from actively sensitized rabbits, Humphrey and Jaques (1955) investigated the effect of a heterologous antigen-antibody interaction on platelets from a normal donor. Histamine and serotonin were released in parallel by antigen (type III pneumococcal polysaccharide or recrystallized ovalbumin) when the reaction mixture included heparinized plasma and platelets (twice washed buffy coat) from a normal rabbit, the γ -globulin fraction of rabbit antisera, and a calcium-containing buffer. Histamine release did not occur in the absence of plasma and was reduced 75% when the plasma was heated to 56°C. for 30 minutes. Treatment of plasma with a cation exchange resin or potassium oxalate or EDTA also abolished histamine release but this effect was completely reversed by furnishing a calcium excess of approximately 5 mM. Supernatant plasma after removal of the antigen-antibody precipitate did not affect platelets in six of eight trials. Histamine and/or serotonin could be released from human, dog, or guinea pig platelets under similar experimental conditions (Humphrey and Jaques, 1955).

Clotting is known to release histamine and serotonin from rabbit platelets (McIntire *et al.*, 1949; Humphrey and Jaques, 1955), and addition of antigen can accelerate blood coagulation in the presence of heparin (Humphrey and Jaques, 1955; Shore and Tidball, 1962). Several workers have attempted to determine whether or not the clotting mechanism is involved in histamine release by antigen-antibody interaction. Plasma from Dicumarol-treated rabbits permitted the expected histamine release despite a marked prolongation of the clotting time, and heparin was not inhibitory unless the concentration greatly exceeded

that which prevents clotting (McIntire, 1957). Either serum, which excludes fibrinogen involvement, or heparinized plasma treated with barium sulfate to remove prothrombin can be substituted for fresh plasma without impairing histamine release (Humphrey and Jaques, 1955). These data argue that the clotting mechanism is not involved in a gross way, but they do not rule out a more subtle participation—perhaps by the direct action of a small amount of one of the intermediates.

Barbaro (1961a,b) studied the release of histamine from rabbit platelets (washed buffy coat) in the presence of plasma and calcium by washed preformed antigen-antibody aggregates of known weight and composition. When the ratio of antibody to antigen was varied while the total nitrogen content of the precipitate remained fixed, histamine release increased as the antibody to antigen ratio became greater. The extent of antibody excess was not the only determinant of histamine release. Increasing the size of the precipitate at any given ratio increased release, and the nature of both the antigen and the antibody were additional factors (Barbaro, 1961a). Whereas precipitates made with human serum albumin and horse and sheep antihuman serum albumin failed to release histamine, precipitates made with human serum albumin and rabbit antihuman serum albumin or ovalbumin and horse or sheep antiovalbumin did release histamine. With this information, Barbaro (1961a) examined the relationship of complement fixation to histamine release and found *no* correlation. Fixation of rabbit complement was maximal at equivalence or in slight antigen excess and was minimal in the antibody excess range which was optimal for histamine release. Furthermore, under conditions in which aggregates formed by human serum albumin and sheep antihuman serum fixed complement as effectively as aggregates formed by ovalbumin and sheep antiovalbumin, only the latter released histamine from rabbit platelets. Preheating plasma for 30 minutes at 56° or 64°C. destroyed all the whole complement activity, but 63 and 40%, respectively, of the histamine-releasing capacity remained (1961b). Removal of all detectable whole complement (C') or third component (C'3) activity by zymosan reduced the histamine-releasing potential of the plasma by only half. Reduction in whole complement or fourth-component (C'4) titer by hydrazine or ammonium hydroxide did produce a parallel change in histamine release by the preformed antigen-antibody aggregates, but as a matter of logic, it is difficult to implicate C'4 and not C'3. The inhibition produced by hydrazine or ammonium hydroxide (Barbaro, 1961b) may be related to that obtained by previous workers (McIntire *et al.*, 1957) with cationic substances. The apparent inability to destroy all the histamine-releasing capacity at 56°C. is consistent with

an earlier observation of Humphrey and Jaques (1955). Recently Barbaro (1962a) has obtained inhibition of histamine release with DFP and a number of other organophosphorus compounds known to inhibit the hemolytic activity of rabbit complement. Barbaro (1962b) has also shown that when plasma is treated with the preformed immune precipitate in the cold, neither the supernatant plasma nor the washed precipitate alone releases histamine from platelets, but that full activity is restored when the two are recombined. Nonetheless, to date the discrepancies between immune hemolysis and histamine release from platelets by preformed immune precipitates seem as prominent as the similarities.

Recently, Gocke and Osler (1961) have studied the reaction between well-washed rabbit platelets and antiplatelet serum prepared in the monkey or rat. Although some histamine is released in the absence of added serum, the addition of fresh guinea pig serum markedly increases the rate of histamine release as well as the total amount in a 1-hour time period. Preheating serum at 56°C. for 30 minutes or the presence of EDTA depresses histamine release. Definitive studies as to whether or not this is a complement-requiring reaction are not yet available; however, since, like immune hemolysis, it is a cytotoxic reaction, complement may well participate.

Histamine has been released from rabbit platelets by at least four different techniques: addition of antigen to sensitized rabbit blood (Katz, 1940; McIntire *et al.*, 1950, 1952); a heterologous antigen-antibody interaction in the presence of normal plasma and platelets (Humphrey and Jaques, 1955); exposure of normal platelets in the presence of plasma to a preformed immune precipitate (Barbaro, 1961a,b); and addition of antiplatelet serum to a suspension of normal platelets (Gocke and Osler, 1961). Certain mechanisms of release seem unlikely for any of these reactions. The participation of the last steps in the clotting mechanism is not a prerequisite for anaphylactic histamine release (Humphrey and Jaques, 1955; McIntire, 1957); entanglement of platelets in the immune precipitate is not an adequate explanation—neither a heterologous antigen-antibody interaction with precipitation (Humphrey and Jaques, 1955) nor a preformed immune precipitate (Barbaro, 1962b) will release histamine from platelets in the absence of plasma; and immunological activation of a serum protease capable of splitting fibrin (McIntire *et al.*, 1950, 1952) or casein (Humphrey and Jaques, 1955) has not been demonstrable.

It is noteworthy that histamine release by a heterologous antigen-antibody interaction (Humphrey and Jaques, 1955), a preformed immune

precipitate (Barbaro, 1961b), or a cytotoxic antiserum (Gocke and Osler, 1961) requires both a heat-labile factor and calcium. Unfortunately, most of the other information on the requirements of these *in vitro* systems is limited to a particular system. Phenol, an established inhibitor of the *in vitro* anaphylactic reaction in guinea pig tissue (Mongar and Schild, 1957a; Austen and Brocklehurst, 1961a) or rat tissue (Mota and Ishii, 1960), inhibits histamine release when antigen is added to sensitized rabbit blood (McIntire, 1957). DFP, which inhibits both anaphylactic histamine release (Austen and Brocklehurst, 1961a; Austen and Humphrey, 1962) and immune hemolysis (Levine, 1955) prevents histamine release by preformed immune precipitates (Barbaro, 1962a). Phlorizin, an inhibitor of the reaction between the EAC_{1,4,2} cell and C'3 (Rodriguez and Osler, 1960), prevents histamine release by cytotoxic antibody (Gocke, 1962). The fragmentary data available, especially that with DFP, are consistent with the suggestion that histamine release follows activation of some enzymatic sequence. As already mentioned, Humphrey and Jaques (1955) found that the supernatant plasma after treatment with an immune precipitate gave histamine release in two of eight instances. It is difficult to know whether such data argue for or against the participation of an antigen-antibody activated serum enzyme in histamine release. The bulk of the data suggest that the platelets, plasma, antigen, and antibody must be present simultaneously to elicit significant histamine release. Although this meets the requirements for platelet agglutination by the antigen-antibody-complement complex as set forth by Siqueira and Nelson (1961), the complement requirement is not completely consistent with the available data on histamine release. Furthermore, the antigen to antibody ratios for maximum immune adherence resemble those optimal for complement fixation.

E. HUMAN WHITE BLOOD CELLS

The addition of ragweed extract to whole blood of ragweed-sensitive humans produces a shift in histamine from cells to plasma. This finding was reported in 1941 by Katz and Cohen, and was confirmed in 1954 by Noah and Brand. These latter workers noted that the leucocytes contained about 65% of the histamine in human blood, and that histamine could be released from the cells of sensitive patients by a variety of inhalent and food allergens (Noah and Brand, 1955). VanArsdel and associates (1958) obtained dose-response data on antigen-induced histamine release; release was generally initiated by a crude antigen concentration of 1 μ g. of protein nitrogen per liter and reached a peak with an antigen concentration of 4 to 10 μ g. of protein nitrogen per liter. These

workers noted some inhibition with antigen excess, suggesting a possible relationship to the inhibition of the precipitin reaction which occurs in the zone of antigen excess. Using a highly purified ragweed antigen, Lichtenstein and Osler (1963) obtained release with an antigen concentration of 10^{-3} μ g. of protein per liter; maximal release required only about 1 μ g. of protein per liter. Recently Middleton (1960) has reported *in vitro* passive sensitization of non-allergic human leucocytes by plasma from ragweed-sensitive patients. In ten attempts with six sensitive plasma and seven normal bloods, three pairings were successful as measured by histamine release from the passively sensitized cells on exposure to appropriate antigen.

VanArsdel and associates (1958) determined the time course of histamine release by adding EDTA at various intervals after the antigen. The EDTA prevented further histamine release during the time the plasma was being separated from the white cells by centrifugation. The rate of release was apparently linear and did not approach maximum until about 30 minutes after adding antigen. The apparent absence of even a short initial lag period requires confirmation. Based on the available data the time course of histamine release from human white cells is unlike that observed with guinea pig lung (Austen and Brocklehurst, 1961a), rat mast cells (Hogberg and Uvnas, 1960; Humphrey *et al.*, 1963), or rabbit platelets (McIntire, 1956). In the latter three systems, there is a lag period after adding antigen, and histamine release reaches virtual completion within 5 minutes of adding antigen.

The available studies on the mechanism of histamine release from leucocytes (whole blood) of allergic patients indicate that gross plasma factors are not required (Middleton and Sherman, 1960; Mathews *et al.*, 1961). Cells washed three times and resuspended in buffer released from 56 to 84% as much histamine as aliquots of the same washed cells resuspended in their own plasma prior to adding antigen. Neither the treatment of plasma with heat, zymosan, or ammonia to destroy complement components, nor the presence of phlorizin, which interferes with C'3 activity, appreciably diminishes antigen-induced histamine release. In addition, histamine release was not accompanied by detectable complement consumption, and iodoacetate, which did not alter the complement titer, prevented antigen-induced histamine release. Although these studies (Middleton and Sherman, 1960) do argue against the participation of free plasma factors in histamine release, they do not exclude the possibility of plasma factors being firmly adsorbed to the well-washed leucocyte.

In studies of inhibition, Middleton and associates (1960), added

the antigen immediately after the inhibitor so as to diminish any non-specific effects resulting from prolonged contact between the inhibitor and the cells prior to adding antigen. Inhibition was achieved with EDTA and was reversed by restoring the calcium ion concentration before adding antigen. Iodoacetate prevented histamine release but another sulfhydryl inhibitor, *p*-chloromercuribenzoate, did not. Phenol was inhibitory but unfortunately no studies were carried out to show that cells washed "free" of phenol reacted to antigen in the usual fashion. Histamine release was virtually abolished when the reaction was carried out at 45°C.; cyanide, fluoride, and azide were not inhibitory at the concentrations tested. The finding that EDTA, iodoacetate, phenol, and heating to 45°C. inhibit antigen-induced histamine release from leucocytes of allergic humans is consistent with the effect of these materials on histamine release from sensitized guinea pig or rat tissues, as described earlier in this section.

Recently, additional similarities as well as some important differences between histamine release in the human leucocyte and other *in vitro* systems have been demonstrated. Mathews (1962b) has obtained inhibition of the human leucocyte system with salicylaldehyde, an inhibitor of histamine release in guinea pig lung (Austen and Brocklehurst, 1961c) or the rat mast cell (Austen and Humphrey, 1962). Noah (1963) has observed enhancement of antigen-induced histamine release from the leucocytes of allergic humans with succinic or maleic acid; the ability of these dibasic acids to enhance the anaphylactic release of histamine and SRS from guinea pig lung was discussed in detail earlier in this section. On the other hand, the release of histamine from human leucocytes is insensitive to changes in ionic strength (Mathews, 1962b) which would markedly alter histamine release from guinea pig lung (Austen and Brocklehurst, 1961c). The studies with DFP are contradictory. Mathews failed to find inhibition with 2.5 mM DFP or four other organophosphorus esterase inhibitors (1962a), whereas Lichtenstein and Osler did observe effective suppression (1962).

F. OTHER SYSTEMS

1. Guinea Pig Ileum

The *in vitro* anaphylactic contraction of the ileum of the sensitized guinea pig was reported in 1910 by Schultz. Dale (1912) used the ileum to confirm his findings regarding desensitization of guinea pig uterus and noted that a pronounced anaphylactic contraction of either tissue left the smooth muscle unresponsive to further additions of the same antigen. Some years later, Schild (1939) found that, in contrast

to most other guinea pig tissues, the sensitized ileum released a negligible to minimal amount of histamine on exposure to antigen. The histamine content of the ileum is appreciable, averaging 12 to 27 $\mu\text{g./gm.}$ of tissue, but the per cent released by antigen is 2 or less as compared to a 10–36% release from such tissues as lung, uterus, aorta, heart, or skin (Mongar and Schild, 1952; Chakravarty, 1959). In Chakravarty's studies (1959) the finding of poor histamine release was supported by the absence of mast cell degranulation. On the other hand, antigen-induced mast cell degranulation has been reported with ileum passively sensitized *in vitro* (Mota, 1959a). Hawkins and Rosa (1956) found that from 10^{-7} to 10^{-5} M mepyramine gave a somewhat dose-related suppression of the first portion of a maximal anaphylactic contraction but did not alter the slow second portion of the ileal contraction. The minimal histamine release and the resistance of the contraction to antihistamine could be explained by the exquisite sensitivity of the ileum to the intrinsic release of undetectable quantities of histamine, but it is also possible that some other mediator is involved. This latter view is supported by the recent finding that the time course of release of "active" materials from the shocked guinea pig ileum is biphasic when estimated by bioassay, and monophasic when assayed for histamine by chemical methods (Nielsen and Feigen, 1962). Neither Brocklehurst (1960) nor Chakravarty (1959) detected SRS in the diffusate from shocked chopped ileum, but Brocklehurst (1960) did obtain SRS from the effluent of perfused shocked ileum. The tissue content of SRS in shocked ileum has not been reported.

In 1942, Kulka demonstrated that antigen-antibody mixtures of unknown ratio contracted the guinea pig ileum, and more recently, Trapani *et al.* (1958) showed that there is a quantitative relationship between the antigen to antibody ratio of the complex and the magnitude of the ileal response. Principal activity occurred with complexes formed in slight antigen excess. The time lag between the addition of the complex and the ileal contraction was from 30 to 90 seconds, which is considerably longer than that observed when antigen is added to actively sensitized ileum.

Since the indicator of *in vitro* anaphylaxis in the guinea pig ileum is a contraction rather than histamine release, the technical considerations have apparently limited the number of studies with inhibitors. Moussatche and Provoust-Danon (1956) found that *p*-chloromercuribenzoate prevented the anaphylactic contraction of the ileum. Although a higher concentration of the inhibitor or a more prolonged exposure depressed the response of the ileum to a standard histamine dose,

the concentration required for inhibition of the anaphylactic contraction did not depress the histamine response. In the studies reported by Mongar and Schild (1957a), 10 mM of phenol abolished the anaphylactic contraction of the ileum and, when the ileum was washed "free" of phenol and antigen, it was desensitized to a subsequent antigenic challenge. Ileum washed "free" from phenol prior to adding antigen, responded in the usual way. By analogy with the studies of histamine release in guinea pig lung, one might assume that phenol inhibited some step initiated by antigen-antibody interaction. Unfortunately, 10 mM of phenol virtually abolished the response of the ileum to a standard histamine dose, and so the effect of phenol on the anaphylactic contraction was not necessarily specific.

Heating the ileum at 45°C. for 15 minutes or at 43°C. for 80 minutes abolished the response to antigen (1957b). Tissue heated at 43°C. recovered its responsiveness to antigen almost completely after 2½ hours at 37°C. but there was no recovery after 4 hours at 37°C. in the tissue heated at 45°C. The irreversible inhibition was not due to an impaired contractile mechanism, for the latter was rapidly restored when the tissue returned to 37°C. Normal unheated ileum was readily passively sensitized, but ileum which had been preheated at 45°C. would not undergo passive sensitization. As with guinea pig lung, these studies indicate that heating inactivates some tissue component critical to the anaphylactic reaction.

The ileum of guinea pigs sensitized to ovalbumin fails to respond to either histamine or antigen when the ileum is washed and suspended in calcium-free Tyrode's solution (Huidobro and Valette, 1960). Washing the ileum within 1 to 3 minutes after adding the antigen and resuspending in Tyrode's solution containing approximately 3.6 mM of ionized calcium elicits an anaphylactic contraction without further addition of antigen. This was true even when the washing period lasted 30 minutes. Replicate samples of ileum in the same calcium-free Tyrode's solution were not exposed to antigen and did not contract when the same concentration of ionized calcium was added. In both instances, the addition of calcium restored the responsiveness of the ileum to histamine. Since no new antigen was needed to evoke the anaphylactic contraction, these studies by Huidobro and Valette (1960) indicate that calcium lack did not prevent antigen-antibody interaction. It seems likely that some step in the anaphylactic reaction immediately subsequent to the antigen-antibody interaction cannot be initiated in the absence of calcium. Chakravarty obtained somewhat similar results for the anaphylactic release of histamine from guinea pig lung (1960b).

The finding that the anaphylactic contraction of the guinea pig ileum requires calcium, a heat-labile factor, and free sulfhydryl groups, is consistent with the data on anaphylactic histamine release from guinea pig lung.

2. Uterus

Dale (1912), using the perfused, isolated, uterus of the sensitized guinea pig, demonstrated that the contraction on exposure to the sensitizing protein was a specific response and could not be duplicated by higher concentrations of indifferent protein. A maximal anaphylactic contraction to a specific antigen abolished further responses to the same antigen but did not necessarily desensitize to an unrelated antigen. Schild (1939) found that the uterus of the sensitized guinea pig released significant quantities of histamine *in vitro*, and this was confirmed by others (Mongar and Schild, 1952; Chakravarty, 1959). Antigen-induced mast cell degranulation has been observed in actively (Boreus and Chakravarty, 1960b) or passively (*in vitro*) (Mota, 1959a) sensitized uterine tissue. Despite the observations on mast cell degranulation and histamine release, there are still some discrepancies in the argument that histamine is primarily responsible for the anaphylactic contraction of the guinea pig uterus. Guinea pig uterus, made refractory to histamine by immersion in a high concentration of histamine, responds maximally to antigenic challenge (Schild, 1939, 1956). Disagreement exists as to whether or not antihistamines suppress the anaphylactic contraction of the guinea pig uterus—Jadassohn (1950) has reported failure whereas Sanyal and West (1957) have found effective suppression. Both Brocklehurst (1960) and Chakravarty (1959) have detected SRS in the diffusate from shocked uterine tissue of the guinea pig. Again, the choice seems to lie between contraction by intrinsic histamine alone or by the additional participation of some other chemical mediator.

Kulka (1942) has demonstrated that antigen-antibody mixtures of unknown ratio will contract the guinea pig uterus *in vitro*. In certain instances, the contractile potential of the antigen-antibody mixture was not realized unless fresh serum was added to the isolated uterus. The interpretation of these data must await further investigation.

The insensitivity of the rat uterus to histamine (Kellaway, 1930) was recognized in 1930 and ever since there has been speculation regarding the participation of some other mediator in the anaphylactic contraction of this tissue. Rat uterine tissue is extremely sensitive to serotonin (Amin *et al.*, 1954), but there is disagreement regarding the ability of serotonin antagonists to prevent anaphylactic contraction.

Brocklehurst (1958a), using uteri from animals passively sensitized to egg albumin, failed to obtain inhibition with lysergic acid diethylamide (LSD), whereas Sanyal and West (1957) claimed inhibition with a smaller concentration of the bromo derivative of LSD. The latter workers employed uteri from rats actively sensitized to *Hemophilus pertussis* and horse serum or egg albumin. The uterus of the mouse is also extremely responsive to serotonin (Fink and Rothlauf, 1955) and relatively resistant to histamine. Fink (1956) has observed that LSD or reserpine abolish the anaphylactic contraction of the mouse uterus, but Udenfriend and Waalkes (1959) have been unable to demonstrate serotonin in uterine tissue from normal or sensitized mice.

Inhibition studies of the anaphylactic mechanism per se in uterine tissue are scarce and limited to the guinea pig. A 20-mM solution of phenol completely suppressed the anaphylactic contraction of the guinea pig uterus and prevented detectable histamine release (Mongar and Schild, 1957a). The tissue was desensitized to a second dose of antigen, and presumably the phenol blocked some antigen-antibody activated step in the sequence leading to contraction and histamine release. The authors do not comment on the effect of this high phenol concentration on the response of the uterus to histamine. Heating the uterus at 47°C. for 5 minutes prior to contact with antigen at 37°C. abolished the anaphylactic contraction and the associated histamine release (Mongar and Schild, 1957b). The histamine response was not impaired on cooling to 37°C. and, as in the lung, ileum (Mongar and Schild, 1957b), or mesentery (Mota, 1959a) of the guinea pig, a heat-labile tissue factor is critical.

3. Skin and Mucosa

Mast cell damage during systemic anaphylaxis has been observed in the nasal mucosa of the guinea pig (Boreus, 1960a,b) and in the skin or the mucosa of the snout, lips, and tongue of the rat (Mota, 1958). Antigen-induced histamine release has been demonstrated by a variety of different techniques in the skin of the dog (Feldberg and Schachter, 1952), rabbit (Schachter, 1953), rat (Mota and Ishii, 1960), or man (Katz, 1942), and recently Brocklehurst and Lahiri (1962) have reported the release of kinin-forming enzyme from shocked rabbit skin. Passive cutaneous anaphylaxis in the guinea pig (Ramsdell, 1928; Ovary and Bier, 1953) or rat (Ovary, 1952), being a local *in vivo* reaction, is not considered in detail in this review except as it contributes directly to our understanding of *in vitro* systems. Antihistamines were initially felt to suppress passive cutaneous anaphylaxis in the rat (Ovary, 1952),

but it was subsequently demonstrated that depletion of rat skin of histamine and serotonin by 48/80 and reserpine treatment plus the parenteral administration of antihistamine and antiserotonin compounds failed to prevent passive cutaneous anaphylaxis (Brocklehurst *et al.*, 1955, 1960). It was concluded by Brocklehurst *et al.* (1960) that at best histamine and serotonin played a secondary role in this reaction in the rat. Mota (1963) has recently demonstrated that the effectiveness of antihistamines in suppressing PCA in the rat depends on the nature of the rat antibody mediating the reaction. The PCA reaction produced with early antiserum from rats sensitized with *B. pertussis* and antigen can be greatly inhibited by antihistamines while that elicited with antiserum from rats sensitized with antigen in Freund's adjuvant is not. As discussed in Section III, D, 2, only the antiserum obtained early during sensitization with *B. pertussis* and antigen is capable of passively sensitizing rat mast cells *in vitro*. The studies of Halpern *et al.* (1959a) showing a linear relationship between the systemic mepyramine dose and the concentrations of antigen or antibody required, respectively, to elicit minimal active or passive anaphylactic reactions in the guinea pig skin, support the view that such skin reactions in the guinea pig are histamine mediated (Biozzi *et al.*, 1948). On the other hand, Alberty and Takunen (1957) have reported that mepyramine produced only partial suppression of more intense cutaneous anaphylactic reactions in the guinea pig.

The only data on inhibition of *in vitro* anaphylaxis in skin is that obtained by Mota and Ishii (1960) using abdominal skin of the actively sensitized rat. Antigen-induced histamine release was effectively prevented by 1 mM of the sulfhydryl inhibitors, iodoacetate, *p*-chloromercuribenzoate, or *N*-ethylmaleimide, and in this regard the skin resembles rat mesentery (Hogberg and Uvnas, 1960; Mota and Ishii, 1960) or lung (Chakravarty, 1960b) or a variety of guinea pig tissues (Moussatche and Provoust-Danon, 1956; Mongar and Schild, 1957a; Mota, 1959a). The inhibition achieved with 5 mM of sodium cyanide cannot be attributed solely to an effect on cytochrome oxidase because, as discussed earlier in this review, the concentration is excessive. Phenol in a concentration of 10 mM also prevented histamine release but the ability of skin washed free from phenol before antigen addition to respond to antigen in the usual fashion was not documented (Mota and Ishii, 1960).

4. Liver

Histamine has been released by antigen *in vitro* from isolated rabbit (Schachter, 1953) or dog liver (Scroggie and Jaques, 1949). Al-

though in early experiments (Rocha e Silva and Grana, 1946a,b) it was not possible to demonstrate *in vitro* that antigen released histamine from the liver of sensitized dogs, modifications introduced in subsequent experiments by Scroggie and Jaques (1949) did result in antigen-induced histamine release. These latter workers observed that perfusion of the isolated dog liver with siliconized blood and antigen resulted in appreciable histamine release whereas none occurred with antigen alone. In experiments with dogs sensitized with two injections of alum-precipitated horse serum instead of the usual single injection, antigen-induced histamine release did not require the presence of siliconized blood. The nature of the factor in whole blood which was required for the anaphylactic release of histamine and heparin from the livers of the less well sensitized dogs has not been established but improvement in the physiological state of the organ may well have been responsible.

5. Heart

Feigen *et al.* (1960) have recently reviewed the data showing that the anaphylactic reaction in heart tissue has a primary effect on cardiac function which is independent of anoxemia. Anaphylaxis of the isolated guinea pig or rabbit heart was convincingly demonstrated in 1911 by Cesaris-Demel. Feigen *et al.* (1960), using the isolated, perfused heart of the guinea pig sensitized to ovalbumin, noted that antigenic challenge was followed by an acceleration in heart rate, an increase in the amplitude of contraction, a decrease in coronary blood flow, and, in intense shock, an A-V conduction block. These data are in agreement with the earlier studies of guinea pig or rabbit heart (Wilcox and Andrus, 1938). Feigen *et al.* (1960) also examined the effect of *in vitro* anaphylaxis on isolated guinea pig atrial tissue. Although the atrial tissue was stimulated electrically at a rate exceeding the natural frequency of the pacemaker, antigen administration initiated a new spontaneous rhythm which at times progressed to fibrillation. Such an arrhythmia has also been noted with rabbit atria (Rijlant, 1924; Penna *et al.*, 1959).

The perfusate obtained during the height of the anaphylactic reaction in the isolated guinea pig heart was shown to contain histamine by bioassay and chromatographic study (Feigen *et al.*, 1960, 1961). That histamine could be released from guinea pig heart tissue by anaphylactic shock *in vitro* had been demonstrated previously by Schild (1939), whereas both Brocklehurst (1960) and Chakravarty (1960a) have observed SRS release. There is no evidence that 5-hydroxytryptamine or acetylcholine are released during *in vitro* anaphylaxis of the guinea

pig heart. Since the changes in rhythm, amplitude, or coronary flow characteristic of anaphylaxis in the perfused guinea pig heart or the isolated atrial tissue can be produced by histamine alone, it has been proposed that this material is the primary mediator (Wilcox and Andrus, 1938; Feigen *et al.*, 1960).

V. Serum Factors Implicated in Anaphylaxis

Although reversed anaphylaxis of rat peritoneal mast cells is potentiated by fresh normal serum, the rabbit platelet system is the only one of the currently popular *in vitro* systems for studying anaphylaxis which has an absolute serum requirement. However, even in the other systems the possibility that essential serum factors in addition to antibody are already adsorbed onto the cell or tissue or manufactured therein cannot be excluded. For example, the first and second components of complements have been identified in specimens of well-washed guinea pig ileum (Borsos, 1962), and in preliminary experiments the second component has been found in high titer in perfused, homogenized, guinea pig lung and liver (Rapp, 1960). It is also possible that serum factors, such as those which constitute the anaphylotoxin system, offer an alternative and independent pathway to anaphylactic histamine release. Finally, it must be noted that histamine, and to a lesser extent SRS, were used as indices of the *in vitro* anaphylactic reactions just discussed. Had more data been available on the anaphylactic production of plasma kinin, serum would have been implicated as a source of substrate. The ability of immune complexes to produce the symptom complex of anaphylaxis in the guinea pig (Weigle *et al.*, 1960) or rabbit (McKinnon *et al.*, 1957) is well established. This subject was reviewed in detail in Volume 1 (Weigle, 1961) and will be considered here only in regard to the capacity of immune aggregates to fix complement and activate certain serum enzyme activities. The complement system was also exhaustively reviewed in Volume 1 of this series (Osler, 1961), but certain points will be reiterated in detail because of their pertinence to the final synthesis of the presently available information on anaphylaxis *in vitro*.

A. COMPLEMENT

1. Mechanism of Complement Action in Cytotoxic Reactions

Recent studies of immune hemolysis have been mainly concerned with elucidating the successive steps in the cytotoxic hemolytic reaction (M. M. Mayer, 1956, 1961a), determining whether or not cell

lysis occurs from a single effective hit or requires cumulative damage by multiple hits (Borsos *et al.*, 1961a; Mayer, 1961b), and establishing the enzymatic nature of the first component (Becker, 1959b, 1962; Lepow, 1959) of complement.

The current status of the lytic sequence based on studies of guinea pig complement is shown in Fig. 1. The first step is the union of

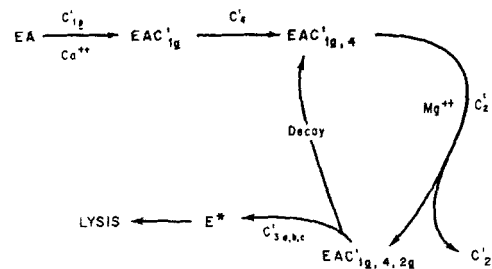


FIG. 1. Sequence of component action in immune hemolysis.

certain antigenic sites (S) on the red cell (E) with Forssman antibody (A). These sensitized cells (EA) then react with complement (C') in a definite sequence. The reaction with the first component (C'1) requires calcium (Levine *et al.*, 1953; Wirtz and Becker, 1961) and apparently converts the first component from an inactive (C'1p) to an active (C'1a) form (Becker, 1956a). The fourth component (C'4) acts only after the first (Becker, 1960). The addition of the second component (C'2) requires magnesium but other cations may be substituted at a lower efficiency (Levine *et al.*, 1953). The interaction of C'2 with the EAC'12,4 cell splits the former into an active fragment (C'2a) which is fixed to the cell (EAC'12,4,2a) and a hemolytically inactive fragment (C'2i) which can be identified in the supernatant fluid by immunological methods (Mayer *et al.*, 1962). At this stage, the first component can be removed from the reactive site without preventing the subsequent steps in the reaction sequence (Becker, 1960).

The fate of the cell in the EAC'12,4,2a state is determined by two competing reactions. Either the cell decays back to the EAC'12,4 state (Mayer *et al.*, 1954; Borsos *et al.*, 1961b) or it reacts with the C'3 complex, which represents at least three factors (Rapp, 1958, 1963), to produce an irreversibly damaged cell (E*) which will lyse spontaneously. In the event of decay, the EAC'12,4,2a state can be regenerated by adding more C'2 (Borsos *et al.*, 1961b). Since the over-all titer of hemolytic complement, with certain qualifications, depends on the supply of C'2 and the concentration of the C'3 complex, a fall in whole comple-

ment titer can be caused by either decay or effective utilization of the whole sequence. The evidence that the immune hemolytic reaction is produced by a single effective interaction between the sensitized cell and the complement sequence rather than by the damage resulting from multiple effective interactions has been presented by Mayer (1961b) and by Borsos, *et al.* (1961a). Accordingly, Fig. 1 could be rewritten so that a single antigenic site (S) is substituted for the cell (E). S* would then refer to a damaged site which is sufficient to give an irreversibly damaged cell (E*). The studies of Goldberg and Green (1959) and Green and his collaborators (1959) with Krebs ascites tumor cells or sheep erythrocytes suggest that the damaged cell (E*) has impaired osmotic regulation which results in an osmotic lysis.

Studies on the enzymatic nature of guinea pig complement were greatly facilitated when Levine (1955) reported that DFP inhibited the lysis of the sensitized sheep cell by guinea pig complement owing to inactivation of 1,4 sites. Becker (1956a) subsequently observed that DFP did not inactivate C' unless sensitized cells were also present and that it was the first component (C'1) which was inactivated. This suggested that C'1 existed in serum in a precursor form (C'1p) resistant to DFP until activated to an esterase (C'1a) by interaction with the sensitized cells. DFP is known to inhibit a number of esterases by phosphorylating the hydroxyl group of a critically located serine (B. S. Hartley, 1960), and it has been shown that nicotinohydroxamic acid is capable of regenerating the active enzyme site (Wilson and Ginsburg, 1953). Nicotinohydroxamic acid restored some of the hemolytic activity of DFP-inactivated C'1 (Becker, 1956a). Sensitized cells carrying activated C'1 were capable of splitting the synthetic amino acid ester substrates, *p*-toluenesulfonyl-L-arginine methyl ester (TAME) and *N*-acetyl-L-tyrosine ethyl ester (ATEE), and these same substrates protected C'1 from DFP inactivation as measured by the hemolytic reaction (Becker, 1956b). An eluate prepared by EDTA treatment (Laporte *et al.*, 1957) of cells carrying the activated first component had the same substrate specificity as the cells (Becker, 1959a). Not only was the eluate capable of transferring esterase activity back to sensitized cells (EA) but it also supplied hemolytic activity if the other components of complement (C'4, C'2, C'3a,b,c) were subsequently added.

Prior to the studies of Levine (1955) and Becker (1956a) with DFP and guinea pig complement, Pillemer *et al.* (1953) and Lepow *et al.* (1954) based on indirect evidence postulated that the cytotoxic reaction with human complement involved activation of C'1 to an enzyme. These

workers subsequently obtained more direct evidence by comparing partially purified, spontaneously activated C'1 with an eluate obtained from antigen-antibody aggregates treated with human serum (Lepow *et al.*, 1956). Both products destroyed C'4 and C'2 and exhibited esteratic activity against ATEE and TAME.

Recent support for the view that C'1a does, indeed, have enzymatic activity comes from the finding that the reaction of the EAC'_{1a,4} cell with C'2 splits the latter into two fragments, C'2a and C'2i (Mayer *et al.*, 1962). Stroud *et al.* (1963) have observed that cells inactivated with DFP fail to carry out this split and that TAME competes with C'2 for the active sites on the EAC'_{1a,4} cell. Thus, the available data does seem to suggest that C'1a can function as a TAME esterase and that C'2 is a natural substrate.

2. Complement and Anaphylactic Reactions

The observation that the serum complement titer is reduced during systemic anaphylaxis prompted Friedberger and Hartoch to suggest in 1909 that complement might be responsible for the cell damage. The association of a reduced serum complement level with systemic anaphylaxis has been confirmed by many workers including Ecker and co-workers (1939), Stavitsky and associates (1949, 1954), and Rice (1955), and has recently been reviewed by Osler (1961). Whether these systemic reactions are really mediated in part by complement or whether complement utilization is merely an incidental by-product of the antigen-antibody interaction is unknown. The experimental data on both sides are handicapped by the complex of variables present in almost any *in vivo* experiment and by the limited knowledge of complement existent at that time and to a lesser extent today.

On the affirmative side are the studies of Bier *et al.* (1955) and Osler *et al.* (1957) which implicate complement by showing that PCA in the rat can be correlated with the circulating C' level in the rat and with the capacity of the antigen-antibody system to fix C' *in vitro*. The reduction in C' titer subsequent to the parenteral administration of antibody followed by antigen was associated with an impaired PCA response to a second, unrelated antigen-antibody system. Responsiveness to the second system was restored when fresh serum was injected into the C'-depleted animals, and a similar injection into normal rats actually enhanced the PCA reaction. A critical constituent of the injected serum seemed to be C'3, and it is reasonable to assume that, if complement is involved, the whole sequence is needed. A homologous pneumococcal rabbit antipneumococcal system was superior to one in-

volving a heterologous antigen in terms of C' fixation and PCA production. Similarly, whereas a ribonuclease-rabbit antiribonuclease system fixed complement and effectively elicited the PCA reaction, acetylation of the enzyme greatly diminished both capacities (Osler *et al.*, 1957). In view of the correlation of the PCA response in the rat with the C'-fixing capacity of the eliciting antigen-antibody system and the *in vivo* C' titer, it is of interest that the PCA reaction can be suppressed by the parenteral administration of EDTA (Osler *et al.*, 1959a) or phlorizin (Osler, 1959). The former inhibits the metal requiring steps in immune hemolysis (Levine *et al.*, 1953), whereas the latter (Rodriguez and Osler, 1960), like salicylaldehyde (Mills and Levine, 1959), prevents the interaction of cells in the $EAC'_{1a,4,2a}$ state with the C'3 complex. Both salicylaldehyde and phlorizin suppress passive systemic anaphylaxis in the guinea pig (Mills *et al.*, 1959).

A number of studies have been interpreted as inconclusive or as evidence against the participation of C' in anaphylactic reactions. Weigle *et al.* (1960) observed that the severe anaphylactic reaction produced in guinea pigs by the intravenous injection of soluble complexes is indistinguishable clinically or pathologically from severe active or passive systemic anaphylaxis, and can be suppressed by antihistamine. The hemolytic C' titer fell 95% or greater in the animals receiving soluble complexes but only from 11 to 24% in those undergoing passive systemic anaphylaxis. The conclusion that complement fixation per se does not relate to the severity of the anaphylactic reaction is correct, but the change in C' titer does not necessarily reflect the extent of *effective* C' participation. The animals injected with soluble complexes received 1.65 mg. Ab N whereas those undergoing passive systemic anaphylaxis received only 0.1 mg. Ab N. In the former animals, the complex would rapidly achieve the $AgAbC'_{1a,4,2a}$ state, and the reaction of the complex in this state with C'3 must then compete with a decay reaction whereby C'2 is wasted. Since the over-all hemolytic titer, with certain qualifications, depends on the supply of C'2 and the concentration of the C'3 complex, the profound fall in hemolytic titer experienced by these animals could be the result either of decay or of effective C'3 utilization. In contrast, in the passively sensitized animals receiving only one-sixteenth as much Ab N, there would be much less wasting of C'2 and the fall in titer might well reflect effective C'3 utilization at some tissue site. Thus, these interesting experiments with soluble complexes and passive systemic anaphylaxis in the guinea pig do not permit a definite conclusion regarding the role of complement.

Weigle and Dixon (1958) failed to find a correlation between the

change in C' titer and the development or severity of serum sickness in rabbits produced by the injection of soluble complexes. Rhyne and Germuth (1961) found that the daily administration of unrelated immune complexes 48 hours after the specific antigen had little effect on the titer of hemolytic rabbit complement but suppressed the incidence and severity of the arterial lesions of accelerated serum sickness. This inhibition was seemingly independent of the C' titer. The glomerular lesions were not suppressed.

It is of interest that Ovary (1960) and Bier and Siqueira (1959), who participated in the studies implicating C' in PCA in the rat (Bier *et al.*, 1955; Osler *et al.*, 1957), have not found the same circumstantial evidence under other circumstances. Ovary (1960), using the guinea pig, was able to produce PCA with human autoantibody against thyroid and reversed passive cutaneous anaphylaxis (RPCA) with hen antirabbit γ -globulin even though neither system fixed C' significantly *in vitro*. As mentioned in Section III, Ovary and Benacerraf (1962), Ovary *et al.* (1963), and Bloch *et al.* (1963) have recently observed that there are at least two populations of guinea pig 7S antibodies; one fixes C' but fails to elicit the PCA reaction, whereas the other produces PCA reaction but does not fix C'. Bier and Siqueira (1959) found no correlation between an immunologically reduced C' titer and the ability of a second, unrelated immune system to elicit the passive Arthus reaction in guinea pigs. Suppression of the reaction by the first immune system was not reversed by the injection of fresh serum.

Silverstein (1960) has studied a human in whom the C'2 titer and whole C' titer are less than 10% of normal. This individual experienced typical hay fever allergy to grass pollen and gave a positive wheal and flare reaction on skin test.

The studies concerned with the role of C' fixation in the permeability producing capacity of antigen-antibody complexes or aggregated γ -globulin are also at best only circumstantial. In their earlier studies, Ishizaka and co-workers (1959) and Ishizaka and Ishizaka (1959) observed an apparent parallelism between complement fixation and the ability of soluble complexes or aggregated γ -globulin to produce local increased capillary permeability in the skin of the guinea pig. Later Ishizaka and associates (1961a) studied antigen-coprecipitating antibody systems and found that C' fixation was maximal at equivalence, whereas skin reactivity was optimal in moderate antigen excess. On reconsidering their earlier studies, it was apparent that the Ag_3Ab_2 complex, which was as skin reactive as any, fixed C' poorly (Ishizaka *et al.*, 1959).

The injection into guinea pigs of rabbit antiserum prepared against

sheep cells (Forssman antiserum) produces death due to a hemorrhagic pulmonary edema (Redfern, 1926; Hyde, 1927; Spear, 1955), and the acute emphysema characteristic of active systemic anaphylaxis is only of secondary prominence. The presence of Forssman antigen in the guinea pig tissue (Tanaka and Leduc, 1956) suggests that this reverse passive Forssman anaphylaxis may be the systemic counterpart to *in vitro* immune hemolysis of sheep cells. The systemic reaction is not associated with histamine release (Humphrey and Mota, 1959b), and *in vitro* studies have demonstrated that Forssman antiserum does not release histamine from perfused guinea pig lung (Mongar, 1958) or perfused, chopped, guinea pig lung (Austen and Rapp, 1960), does not contract the isolated guinea pig ileum (Humphrey and Mota, 1959b) or uterus (Redfern, 1926), and does not degranulate guinea pig mast cells (Humphrey and Mota, 1959b). Spear (1955) has shown that repeated injection of Forssman antiserum simultaneously renders the animals resistant to the effect of the antiserum and depresses the C' titer to a very low level. Hyde (1932) studied a strain of guinea pigs with a low C' titer because of a deficiency of C'3 and demonstrated that these animals were resistant to Forssman shock but susceptible to active systemic anaphylaxis. Injection of heated serum to supply C'3 did not transfer the capacity to react to Forssman antiserum, and the implication that this reaction is really the systemic counterpart of immune hemolysis of the Forssman sensitized sheep erythrocyte remains to be rigorously proven.

B. ANAPHYLOTOXIN

Friedberger (1909) incubated washed, preformed, immune precipitates with fresh, normal, guinea pig serum and, after removing the precipitate, injected the supernatant (anaphylotoxin) into normal guinea pigs. The recipients presented the clinical and post-mortem findings of acute bronchiolar constriction, characteristic of anaphylaxis in this species. It was assumed that the antigen-antibody aggregates activated a serum protease which in turn digested the immune complex generating a toxic principle, anaphylotoxin; complement was implicated because heating the serum at 56°C. destroyed its capacity to elaborate anaphylotoxin. With the recognition that serum produced a toxic principle indistinguishable from anaphylotoxin after treatment with a variety of macromolecules, some of which were not nitrogenous (Bordet and Zunz, 1914; Bronfenbrenner, 1915; Jobling *et al.*, 1915), it was concluded that anaphylotoxin arose owing to digestion of recipient serum proteins. The evolution of this concept is well reviewed by Rocha e Silva (1955) and

by Burdon (1956). Rocha e Silva (1955) has observed agar-induced anaphylotoxin production in rat serum without bradykinin formation and feels that proteolytic enzymes are *not* involved in the elaboration of serum anaphylotoxin. Furthermore, guinea pig ileum desensitized to anaphylotoxin responds maximally to bradykinin, and bradykinin, in contrast to anaphylotoxin, does not release histamine (Rocha e Silva, 1955). The chemical nature of anaphylotoxin is as yet unknown.

The pertinence of anaphylotoxin formation *in vitro* to anaphylaxis *in vivo* is not yet established, although the clinicopathological similarity of anaphylactic and anaphylotoxin shock in the guinea pig has been known since 1909. Osler and associates (1959b) demonstrated that anaphylotoxin, formed by exposing rat or guinea pig serum to preformed immune aggregates, contracted the guinea pig ileum and altered skin permeability. Most of the other pharmacological data come from studies with anaphylotoxin produced by other means: Hahn and Oberdoff (1950) observed that antihistamines protected guinea pigs against the systemic effects of anaphylotoxin prepared by incubating normal guinea pig serum with inulin; Rocha e Silva *et al.* (1951) found that anaphylotoxin produced by the interaction of guinea pig serum and agar released large quantities of histamine from isolated, perfused guinea pig lung; and Mota (1957, 1959b) showed that the anaphylotoxin produced by exposing rat serum to agar caused degranulation of the mast cells in the isolated guinea pig mesentery. Although rat serum is the foremost source of serum anaphylotoxin (Novy and De Kruif, 1917; Rocha e Silva, 1955), the rat is insensitive to its action (Mota, 1959b). Even adrenalectomized rats show no systemic reaction and anaphylotoxin fails to degranulate rat mast cells *in vivo* or *in vitro*. The dog and cat are not susceptible to its action (Rocha e Silva, 1955), and Humphrey and Jaques (1955) found no evidence to implicate anaphylotoxin formation in the release of histamine from rabbit platelets by antigen-antibody interaction in the presence of plasma. Its pharmacological significance to date is limited to the guinea pig.

The possibility that serum complement might be critical to the production of anaphylotoxin has been supported by the investigations of Osler and co-workers (1959a,b). The treatment of guinea pig or rat serum with immune aggregates, agar, inulin, zymosan, and certain dextrans resulted in the elaboration of anaphylotoxin concomitant with a loss of hemolytic titer. The whole complement sequence was implicated because the loss in titer without C'3 utilization, which occurs when serum is exposed to the materials just mentioned at 0°C., was not associated with anaphylotoxin production. Further support for the view

that the whole C' sequence participates comes from studies showing that EDTA or phlorizin prevent anaphylotoxin production (Osler, 1959; Osler *et al.*, 1959b). In view of Nelson's studies (1958) showing that zymosan, as used in the properdin assay, is really reacting with an antibody in serum, it would not be too surprising if other anaphylotoxin-producing macromolecules were either reacting with a serum antibody or capable of aggregating γ -globulin.

The inability of 10 mM of DFP to prevent agar-induced or immune aggregate-induced (Becker, 1959b, 1962) anaphylotoxin formation in rat serum must be explained before the role of C' in this reaction is secure. Another organophosphorus esterase inhibitor, *o,p*-nitrophenyl-*o*-ethylphenylphosphonate, prevents anaphylotoxin formation in a concentration of 1 mM (Becker, 1962). The activity of the phenylphosphonate suggests that activation of an esterase is necessary for anaphylotoxin formation. The negative data with DFP could mean that C'1 esterase is not involved but may mean that DFP is rapidly inactivated or sidetracked by this particular test system which involved neat serum.

Mota (1959b) has used inhibitors to compare the effect of agar-induced rat anaphylotoxin on guinea pig mesentery mast cells or ileum with the *in vitro* anaphylactic reaction in these same tissues. The degranulation of guinea pig mast cells by anaphylotoxin was prevented by sulfhydryl inhibitors or by phenol. Tissue washed free from phenol prior to anaphylotoxin addition responded with the usual mast cell degranulation. Mesenteries exposed to anaphylotoxin either at 15°C. for 15 minutes or in the presence of phenol at 37°C. showed no degranulation, but yet these same tissues were desensitized to a second dose of anaphylotoxin after conditions had been restored to normal. Antihistamines in a reasonable dose suppressed the rapid phase of the anaphylotoxin-induced ileum contraction and in high dose abolished the entire response. The inhibition studies with phenol and iodoacetate, the demonstration of desensitization, and the data with mepyramine are entirely consistent with the information on *in vitro* anaphylaxis (Hawkins and Rosa, 1956; Mongar and Schild, 1957a; Mota, 1959a). In contrast to *in vitro* anaphylaxis (Mota, 1959a), neither EDTA nor heating the mesentery at 45°C. prevented anaphylotoxin-induced mast cell degranulation. Heating the sensitized ileum at 45°C. abolished the response to antigen but did not suppress the response to anaphylotoxin (Mota, 1959b). These discrepancies can be explained by assuming that anaphylotoxin participates in anaphylactic histamine release at some point beyond the calcium requiring, heat-labile step or steps. This is not unreasonable since the formation of serum anaphylotoxin per se requires

calcium and a heat-labile factor (Osler *et al.*, 1959b). On the other hand, the finding that guinea pig ileum which has been desensitized to serum anaphylotoxin responds maximally to appropriate antigen, confirming the earlier work of Dale and Kellaway (1922), is hard to reconcile with the view that serum anaphylotoxin formation is a step in the sequence leading to the anaphylactic contraction of the guinea pig ileum. In view of this and the established fact that anaphylaxis takes place *in vitro* in well-washed guinea pig tissue in the absence of an apparent serum requirement, it seems reasonable to assume that serum anaphylotoxin, at best, offers an alternative pathway to histamine release.

C. SERUM ENZYMES

Serum proteolytic activity has been implicated in anaphylaxis almost since the discovery of the anaphylactic phenomenon. As noted in the preceding portion of this section, early workers attributed anaphylotoxin formation to the digestion of serum proteins by a protease. The participation of a serum protease in histamine release independent of anaphylotoxin formation was first suggested by Rocha e Silva (1938) and was supported by the observation that trypsin released histamine from guinea pig lung (Rocha e Silva, 1938), heparinized rabbit blood (Dragstedt and Rocha e Silva, 1941), or perfused dog liver (Rocha e Silva and Grana, 1946a). This view was strengthened by the finding that anaphylactic or peptone shock in the dog activated a serum fibrinolysin (Rocha e Silva *et al.*, 1946). Fibrinolytic activity during anaphylactic shock has also been noted in man (Lowell *et al.*, 1956) and guinea pig (Burdon *et al.*, 1961).

Perhaps the strongest support for the causal participation of a serum fibrinolysin in anaphylaxis came from the studies of Ungar and associates (Ungar and Mist, 1949; Ungar *et al.*, 1953; Ungar, 1947, 1956). These workers reported that the addition of antigen to tissue suspensions or serum of sensitized guinea pigs increased the fibrinolytic activity of the tissue or serum. The subsequent realization that the fibrinogen used for assay contained plasminogen, suggested that the addition of antigen was actually releasing a plasminogen activator (Ungar, 1956). These workers also observed inhibition of anaphylactic histamine release with soybean trypsin inhibitor (Ungar, 1956), and enhancement when fresh serum was added to shocked perfused guinea pig lung (Ungar and Damgaard, 1955).

The contention that a fibrinolytic serum protease which is inhibitable by the trypsin inhibitor from soybeans (SBI) plays a role in anaphylactic

histamine release has not received support from other workers. McIntire *et al.* (1950) reported that SBI did not diminish the anaphylactic release of histamine from rabbit platelets even in a concentration sufficient to prevent trypsin-induced histamine release. It was further observed that anaphylactic histamine release from rabbit platelets was not accompanied by demonstrable fibrinolytic activity, whereas streptokinase-activated serum exhibiting significant fibrinolytic activity failed to release histamine (McIntire *et al.*, 1952). In man, neither streptokinase-induced fibrinolytic activity (Tillet *et al.*, 1955) nor that appearing spontaneously in relation to disease (Ratnoff, 1949; Clifton, 1952; Scott *et al.*, 1954; Meyers *et al.*, 1957) is associated with any signs or symptoms of clinical allergy. Burdon and associates (1961) observed that anoxic death in guinea pigs due to suffocation induced almost the same degree of spontaneous clot lysis as acute anaphylactic death. Since the latter is characterized by acute obstructive emphysema, the fibrinolysis accompanying anaphylaxis may well be secondary to the anoxia. Furthermore, fatal trypsin shock, which produces a marked fibrinolytic state in the guinea pig, is not even accompanied by significant obstructive emphysema (McGovern *et al.*, 1961).

Using the perfused guinea pig lung, Rocha e Silva (1955) was unable to release histamine with fibrinolytic preparations, and Brocklehurst (1960) did not find that serum in any way augmented the anaphylactic release of histamine from this preparation. Austen and Brocklehurst (1960a, 1961a) did not observe inhibition of antigen-induced histamine release in perfused, chopped guinea pig lung with SBI or competitive trypsin substrates and failed to release histamine from this preparation with trypsin in a concentration up to 1.6 mg./ml. of reaction mixture. Ungar (1956) heated guinea pig lung tissue and noted a parallelism between proteolytic activity and histamine release even at 60°C.; however, Mongar and Schild (1957b) found that heating lung tissue to 45°C. could irreversibly inhibit antigen-induced histamine release. It seems likely that the association of thermal histamine release with proteolysis is nonspecific. Austen *et al.* (1959), using the synthetic substrate, TAME, were unable to confirm the contention of Ungar and associates (1949, 1953) that the addition of antigen to immune serum activated a plasminlike enzyme. Furthermore, neither guinea pig nor human plasminogen was activated or removed by preformed immune precipitates (Austen *et al.*, 1959; Austen, 1960). It is evident that the studies with guinea pig lung (Brocklehurst, 1960; Austen and Brocklehurst, 1961a) or serum (Austen *et al.*, 1959) and those with rabbit platelets (McIntire *et al.*, 1950, 1952) do not support the view that ana-

phylactic histamine release is mediated by a fibrinolytic enzyme which is inhibitable by SBI (Ungar, 1956).

This conclusion in no way excludes the possibility that other types of serum enzyme activities may be involved in anaphylaxis. Serum and glandular kallikrein, which produce the plasma kinins and may, therefore, be implicated in the appearance of these kinins during anaphylaxis in certain species (Beraldo, 1950; Brocklehurst and Lahiri, 1962), are relatively poor fibrinolysins in comparison to plasmin but readily split arginine esters such as TAME and benzoyl-L-arginine methyl ester (BAME). Becker *et al.* (1959) found that peptone was involved in the activation of three, nonfibrinolytic, guinea pig serum TAME esterases, and that one of these was also activated by preformed immune precipitates or sensitized sheep red cells. Although this esterase exhibited the properties attributed to guinea pig C'1 (Becker, 1959b), its removal from serum by immune precipitates was not well correlated with the loss of C'1 hemolytic activity. Austen (1960) subsequently observed that the esterase activity taken up from human serum by preformed immune precipitates and that induced in human serum by chloroform or peptone were similar. The chloroform, peptone, and antigen-antibody inducible esterase activities were readily distinguishable from plasmin and fulfilled the criteria for activated human C'1 established by Lepow and associates (1956). The significance of this activity for anaphylaxis is unknown. If the antigen-antibody inducible esterase activity of guinea pig or human serum has a role in anaphylaxis, it could either participate as a component of complement or independently.

Ungar *et al.* (1961) have attempted to associate their earlier studies implicating a fibrinolytic protease with more recent work on the antigen-antibody activated esterase activity of serum (Lepow *et al.*, 1956; Becker *et al.*, 1959) or tissue (Austen and Brocklehurst, 1961a) by suggesting that the discrepancies are based on a confusion in terminology. Although it is undoubtedly true that these esterases have natural substrates and may some day be termed proteases or peptidases, this does not imply activity on fibrinogen, fibrin, or casein. Indeed, it has already been shown that the antigen-antibody activated serum esterase does not split the common protein substrates (Ratnoff and Lepow, 1957; Lepow, 1959; Becker *et al.*, 1959). To avoid further confusion it is essential to speak of activities in terms of the substrate specificity, to distinguish tissue from serum activities, and to show as well as possible that a given activity is directly related to the anaphylactic release of some pharmacological material. Although it must be noted that the denial of the fibrinolytic hypothesis is based on contradictory evidence

rather than terminology, Ungar's championship of the inherent plausibility of an enzymatic mechanism of anaphylactic reactions has undoubtedly been an important stimulus.

VI. Concluding Comments

Table V summarizes the inhibition data obtained with the more thoroughly studied *in vitro* anaphylactic systems; comparable data on immune hemolysis are also included.

TABLE V
THE ACTION OF INHIBITORS ON VARIOUS FORMS OF *in Vitro* ANAPHYLAXIS^a

Inhibitor	Lung (guinea pig)	Mast cell (rat)	White cell (human)	Platelet (rabbit)	Immune hemolysis
Lack of serum/ plasma	—	—	—	+	+
EDTA	+	+	+	+	+
Heat	+(45°C.)	+(45°C.)	+(45°C.)	+(64°C.)	+(56°C.)
Ionic strength	+	—	—		+
Salicylaldehyde	+	+	+		+
DFP	+	+	+	+	+
L-Tyrosine ethyl ester	+	r			+
L-Lysine ethyl ester	—				+
CBZ ^b -glutamyl-L- tyrosine	—				+
Nicotinamide	+	+			
Phenol	+	+	+	+	
Indole	+	+			
Phenylacetic acid	+				—
Thiols	—				+
Iodoacetate	+	+	+		—
KCN	+	+	—		—
N ₂ (in Tyrode's solution)	—	—			
CO	—				
Monobasic fatty acids	+	—			
Soybean trypsin inhibitor	—			—	

^a +, inhibition, —, non inhibition, r, spontaneous histamine release.

^b CBZ, carbobenzoxy.

Anaphylaxis takes place readily in well-washed guinea pig lung (Brocklehurst, 1960), rat mast cell (Humphrey *et al.*, 1963), or human white cell preparations (Middleton and Sherman, 1960), and there is no evidence that these systems have an absolute requirement for free serum factors. The fact that the release of histamine from rabbit platelets by

antigen and antibody requires serum or plasma factors is not proof that complement is involved; Barbaro (1961a,b) has carried out studies of heat inactivation, chemical inactivation, and complement fixation which point to important differences between immune hemolysis and histamine release in rabbit platelets. EDTA inhibits each system (Mongar and Schild, 1958; Mota and Ishii, 1960; Austen and Humphrey, 1962; Humphrey and Jaques, 1955; Middleton and Sherman, 1960) and its action is reversed by calcium addition; adequate studies on magnesium have not been feasible except for immune hemolysis (Levine *et al.*, 1953). Each of the systems that functions without added serum is inhibited irreversibly if the tissue or cell is *heated* to 45°C. (Mongar and Schild, 1957b; Mota and Ishii, 1960; Middleton *et al.*, 1960); complement, of course, is inactivated at 56°C. in the fluid phase, whereas some of the plasma factor needed for histamine release from rabbit platelets survives even after heating at 64°C. for 30 minutes (Barbaro, 1961b).

Both immune hemolysis (Mayer *et al.*, 1946; Becker and Wirtz, 1959) and *in vitro* anaphylaxis in guinea pig lung (Austen and Brocklehurst, 1961c) are very susceptible to small change in the *ionic strength* of the containing medium; however, these same manipulations in ionic strength have virtually no effect on reversed anaphylaxis of the rat peritoneal mast cell (Humphrey *et al.*, 1963) or on the release of histamine from the human white cell (Mathews, 1962b). Salicylaldehyde inhibits immune hemolysis (Mills and Levine, 1959) and the anaphylactic release of histamine from guinea pig lung (Austen and Brocklehurst, 1961c), rat mast cell (Austen and Humphrey, 1962), or human white cell (Mathews, 1962b). *Diisopropylfluorophosphate* inhibits in each system (Austen and Brocklehurst, 1961a; Austen and Humphrey, 1962; Barbaro, 1962a; Lichtenstein and Osler, 1962); however, its effectiveness relative to other organophosphorus esterase inhibitors differs for each system and one cannot conclude as yet that the same esterase is involved in each (Becker, 1962).

The ester substrate, *L-tyrosine ethyl ester*, inhibits both immune hemolysis (Cushman *et al.*, 1957) and anaphylaxis in guinea pig lung (Austen and Brocklehurst, 1961a). However, certain *basic esters* (trypsin substrates) and certain *peptides* (carboxypeptidase substrates) are inhibitors of immune hemolysis (Cushman *et al.*, 1957) but not of the guinea pig system (Austen and Brocklehurst, 1961a). *Nicotinamide*, *phenol*, and *indole* inhibit both the guinea pig lung (Mongar and Schild, 1957a; Austen and Brocklehurst, 1961a) and the rat mesentery (Mota and Ishii, 1960; Mota *et al.*, 1960) or mast cell (Humphrey *et al.*, 1963);

they are grouped because they are all monofunctional chymotrypsin inhibitors but there is no proof that this is the mechanism of their action. *Thiols* (Cushman *et al.*, 1957) inhibit immune hemolysis but not *in vitro* anaphylaxis in guinea pig lung (Austen and Brocklehurst, 1961a), whereas *sulphydryl inhibitors* block histamine release in each of the nonserum-requiring systems (Mongar and Schild, 1957a; Mota and Ishii, 1960; Middleton *et al.*, 1960) but do not inhibit immune hemolysis (Yenson, 1945). *Cyanide* does not prevent immune hemolysis (Yenson, 1945) or the release of histamine from human white cells (Middleton *et al.*, 1960); it is inhibitory in the rat or guinea pig system (Mongar and Schild, 1957a; Hogberg and Uvnas, 1960), but there is no convincing evidence that this inhibition arises from an effect of cytochrome-mediated aerobic metabolism. The findings with a nitrogen atmosphere depend on the concentration of glucose in the containing media (Provoust-Danon and Moussatche, 1961; Diamant, 1962a,b); if the glycolytic cycle is intact, a nitrogen atmosphere is not inhibitory. The *monobasic fatty acids* inhibit the guinea pig lung (Austen and Brocklehurst, 1961b) but not reversed anaphylaxis of the rat mast cell (Austen and Humphrey, 1962). The *trypsin inhibitor* from soybeans has been negative where tested (McIntire *et al.*, 1950; Austen and Brocklehurst, 1961a).

The dibasic acids, succinic and maleic, enhance *in vitro* anaphylaxis in guinea pig lung (Austen and Brocklehurst, 1961b) and have a similar effect on the human white cell (Noah, 1963). Succinic acid does not enhance reverse anaphylaxis of the rat peritoneal mast cell (Humphrey *et al.*, 1963).

The information available to date on the three systems that function without added serum reveals many striking similarities. There are a few significant differences, i.e., the failure of monobasic and dibasic acids to influence reversed anaphylaxis of the rat mast cell and the insensitivity of the human white cell and rat mast cell system (reversed anaphylaxis) to changes in ionic strength. When one considers that there is variation in both species and organ, it seems likely that there is a common theme to the mechanism of anaphylactic histamine release. It is not possible to speculate critically as to whether or not complement is the common thread. The fact that three of these *in vitro* systems do not require gross serum factors does not exclude the possibility that complement is already present on the tissue. A comparison of *in vitro* anaphylaxis in guinea pig lung with immune hemolysis reveals at least six similarities and six differences. Despite the differences, it can still be reasoned that complement participates merely at one stage in the *in vitro* anaphylactic re-

action, and that the remaining steps differ in the two phenomena. The final decision as to whether or not complement is involved must await further progress in the understanding of the *in vitro* anaphylactic reaction and its dissection into individual steps, as has been done with immune hemolysis.

As one looks over the data on the release of the various pharmacological materials implicated in the anaphylactic reaction, the irrelevance of the old argument about cellular and humoral factors becomes very apparent. The anaphylactic release of histamine and SRS from guinea pig lung requires no gross serum factors and therefore can properly be termed "cellular." The anaphylactic release of kinin-forming enzyme from guinea pig lung and its subsequent action on the plasma proteins to form a physiologically active polypeptide can certainly be termed "cellular-humoral." The antigen-antibody or agar-induced serum factor, anaphylotoxin, releases histamine from guinea pig tissue and this sequence is "humoral-cellular." The cytotoxic antibody, complement-requiring systems might also be placed under this heading. Finally, the immune aggregates that produce mechanical obstruction of the pulmonary capillaries in the rabbit with coincidental release of pharmacological materials might be termed "humoral"—just to round out the foursome.

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The Role of Humoral Antibody in the Homograft Reaction

CHANDLER A. STETSON

Department of Pathology, New York University School of Medicine, New York, New York

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I. Introduction

A major problem in immunopathology has been an appraisal of the respective roles played by humoral antibody and by delayed hypersensitivity in the production of various forms of tissue damage. The classical Arthus reaction, for example, was once thought to be simply a consequence of antigen-antibody interaction *in vivo*: the characteristic local hemorrhagic necrotic reaction seen on intradermal injection of antigen into actively immunized animals could be closely approximated in animals which had been passively sensitized by the injection of specific antisera, and the effectiveness of antiserum in passively inducing Arthus reactivity was shown to be quantitatively related to its precipitating antibody content (Benacerraf and Kabat, 1950). However, it has now been well established that the histopathologic findings in a classical Arthus reaction are not quite duplicated by those produced by an injection of antigen in a passively immunized animal. In the latter situation there is an acute inflammatory and vasculonecrotic reaction from the interaction of antigen with humoral antibody; this occurs also in the classical Arthus reaction, but here it is accompanied and followed by a cellular response exhibiting all the characteristics of a delayed hypersensitivity reaction of the tuberculin type. Actively immunized animals thus seem to possess delayed hypersensitivity in addition to

humoral antibody, and it is indeed only through transfer of either lymphoid cells or serum from such an animal to normal recipients that the respective contributions of delayed hypersensitivity and of humoral antibody to the histopathology of the classical Arthus reaction can be more or less clearly defined (Gell and Hinde, 1954; Gell, 1958).

The difficulties involved in interpreting certain other immunopathologic events have been even greater. The pathogenetic mechanisms involved in the production of experimental allergic encephalomyelitis and allergic thyroiditis, for example, are still not clear after many years of experimental analysis. In these disease models, the production of tissue damage following immunization is associated with the appearance of both specific antibodies and delayed hypersensitivity to antigens of the tissue involved. Since the amount of humoral antibody found is often small and not well correlated with the severity of disease, and since passive transfer of these diseases has not yet been accomplished by serum containing high titers of these antibodies, it has frequently if not generally been concluded that in these diseases humoral antibodies play no significant role in the production of tissue damage. Instead, the predominance of lymphoid cells in the tissue lesions and the development of similar lesions in animals receiving lymph node cells transferred from immunized donors have made it seem more likely that the delayed type of hypersensitivity is involved. Several investigators have suggested that tissue damage of this sort is, in fact, mediated not by antibody but by specifically sensitized lymphoid cells which invade and somehow destroy tissue containing the appropriate antigen. There are some good reasons, however, for reserving judgment on this matter. Sera of animals with allergic encephalomyelitis have recently been shown to be cytotoxic *in vitro* for cells from brain tissue (Bornstein and Appel, 1961), and analogous cytotoxins for thyroid cells have been found in the sera of patients with Hashimoto's thyroiditis (Pulvertaft *et al.*, 1961). These antibodies may or may not have similar cytotoxic capacity *in vivo*, but surely deserve more study from the point of view of their potential pathogenetic significance. The possibility that autoimmune tissue damage is caused by antibody manufactured in the locality of the tissue lesions by lymphoid or plasma cells also requires closer investigation, since the local concentration of antibody in such circumstances might not be accurately reflected by the level in the systemic circulation. Finally, the transfer of these experimental diseases by lymphoid cells from immunized donors neither proves nor implies that the lesions in target organs are produced directly by such "immune" cells; it is known that transferred antibody-forming cells continue to produce antibody in the recipient

(Dixon *et al.*, 1957, 1959), so that no effective dissociation between "cellular" and "humoral" factors is achieved by this experimental manipulation.

This preamble is intended only to indicate some of the difficulties involved in attempts to define the mechanism whereby tissue damage is produced on an immunological basis. It is abundantly clear that direct cytotoxicity, opsonization for phagocytosis, or tissue damage of the Arthus type can be produced under a variety of circumstances by the interaction of antigen with humoral antibody or "immunoglobulin." It is equally clear that a qualitatively different form of tissue injury can be produced by the injection of antigen into animals with delayed hypersensitivity of the tuberculin type (Lawrence, 1956), in which extracellular or intracellular immunoglobulin is not demonstrably involved (Gell and Benacerraf, 1961). The present review will attempt to evaluate the available evidence suggesting that the specific immunological rejection of tissue homografts is mediated by humoral antibody. Evidence supporting the alternative proposition, that rejection is accomplished by a delayed hypersensitivity reaction of the tuberculin type, has been reviewed elsewhere (Gorer, 1956; Medawar, 1958a,c; Snell, 1957a; Brent, 1958; Lawrence, 1959; Hašek *et al.*, 1961) and will not be treated here in any comprehensive or detailed fashion. Since most of the reliable and convenient means for the detection and measurement of humoral antibodies directed against graft antigens have become available relatively recently, much of the older literature bearing on transplantation immunology is primarily of historical interest and will not be cited.

II. The Isoantigens That Determine Histocompatibility

It is now well known that transplants of living cells from one adult vertebrate to another of the same species do not usually survive and function indefinitely, but rather succumb after a few days or weeks to the "homograft reaction." Subsequent transplantation of cells or tissues from the same donor to the same recipient are generally rejected much more promptly. The specificity of this "transplantation immunity," the kinetics of its development, the histopathologic findings in and near transplanted tissue undergoing rejection, the effect of agents known to suppress the immune response, and many other considerations have led to the now well-established concept that the rejection of homografts is accomplished by an immunological reaction of the recipient against antigens of the graft. These have variously been termed "isoantigens," "transplantation antigens," or "histocompatibility antigens."

A. IN THE MOUSE

The mouse has been the animal of choice in the study of transplantation antigens for a number of reasons, chief among which is the existence in this species of a large number of inbred homogeneous lines, each differing antigenically from the others. This has made the genetic analysis of histocompatibility in this species a relatively straightforward matter. Several loci have been found to be involved in the inheritance of histocompatibility antigens. Chief among these is the H-2 locus, at which numerous alleles or pseudoalleles are determined. Antigenic differences determined at this locus are generally considered as constituting "strong" histocompatibility barriers because of the relatively short survival time of transplants exchanged between mice of different H-2 genotypes; differences at other loci are generally found to constitute relatively weaker barriers to transplantation. Amos (1962a) and Gorer (1961) have recently given more adequate treatment to this aspect of transplantation biology.

1. *Methods of Detection and Measurement*

The H-2 isoantigens in mouse tissues can be detected in various ways. Cells or fractions of cells possessing these antigens induce transplantation immunity upon injection in suitable quantities into animals of appropriate genotype, as judged by the short survival of subsequent test skin homografts. Although this "immunogenic" property of histocompatibility antigens has been widely used as a means of detection and assay, it is time-consuming and of little use in quantitative work because of a relatively flat dose-response curve and because of the difficulty in determining precise graft survival times. Another method of demonstrating isoantigens in mouse tissues involves the demonstration that in response to homotransplantation there are produced specific humoral isoantibodies capable of agglutinating erythrocytes of the donor strain (Gorer, 1937). This has made possible the serological demonstration of H-2 isoantigens, either by their capacity to induce isoantibody formation *in vivo* or by their reaction with isoantibodies *in vitro*.

A number of serological techniques have been devised for the detection of isoantigens, most being empirically based on interactions between isoantibody in immune serum and test cells containing isoantigen on their surfaces. The demonstration of the hemagglutinating capacity of H-2 isoantibodies is frequently dependent on the use in the hemagglutination test of dextran and human serum or other reagents (Gorer and Mikulska, 1954) which are required to stabilize the test cell suspension and to enhance the relatively weak agglutination produced

by mouse isoantibody. Leucocyte agglutination (Amos, 1953) provides another method for detection of isoantigens. Both leucocyte agglutination and hemagglutination reactions have been useful in serological work with H-2 antigens; it is not yet clear whether they will be as generally useful in work with the relatively weaker isoantigens determined at other loci, but there are encouraging examples of such extensions (Amos, 1962a).

The addition of complement to a mixture of target cells and specific isoantibody can result in lysis of the cells. The first clear indication that H-2 isoantigens and their respective isoantibodies could interact with this consequence was found by Gorer and O'Gorman (1956), who used lymphoid cells as target cells. Hildemann (1957) showed that mouse erythrocytes are hemolyzed by H-2 isoantibody and complement, and Hildemann and Medawar (1959) used this reaction as well as hemagglutination in a study of the humoral antibody response to isoimmunization with various fractions of lymphoid tissue. Many modifications and adaptations of the "cytotoxic" test have been used with nucleated cells of one variety or another as the target-cell type. Most of these test systems (Reif and Norris, 1960; Stetson and Jensen, 1960; Terasaki *et al.*, 1961a,b; Boyse *et al.*, 1962a,b) involve the use of dyes such as trypan blue, which serve as an indicator of cytotoxicity by staining damaged cells while leaving normal cells unstained. Normal and neoplastic cells of lymphoid origin were originally thought to be peculiarly sensitive to this cytotoxic or cytolytic reaction, but many other cell types have now also been shown to be susceptible and there is at present no mouse-cell type known to be completely resistant or unsusceptible to damage of this sort. There is, however, a wide range of relative susceptibility, some cells being far more sensitive to the cytotoxic effect than others (Boyse *et al.*, 1962b,c). E. and G. Möller (1962) believe the degree of sensitivity to be related to the amount of isoantigen on the cell surface.

In such tests, complement is usually added in excess to a previously incubated mixture of target cells and isoantibody. There has been shown to be a direct relationship between the quantity of isoantibody used and the number of cells killed (Reif and Norris, 1960; Stetson and Jensen, 1960). When the number of target cells is held constant, varying the antiserum concentration permits accurate titration of isoantibody. When the concentration of isoantibody is limited, as in the case of weak isoantisera, the use of very small numbers of target cells may permit the demonstration of cytotoxic effects not observable with heavier cell suspensions (Boyse *et al.*, 1962b).

The isoantigens present on cell surfaces or in homogenates or

extracts of cells can also be demonstrated indirectly, by their capacity to absorb or neutralize the antibodies in a standard immune serum. For example, appropriately diluted isoantisera can be tested for hemagglutinating or cytotoxic activity before and after the addition of a cell fraction suspected of containing isoantigen. If isoantigen is present, it binds specific isoantibody which is then not available to agglutinate the test erythrocytes or to sensitize the target nucleated cells for lysis by complement. When the material being tested in this way for isoantigen activity is particulate in nature and is removed (with any bound antibody) by centrifugation before the test cells are added, the procedure is usually referred to as an "absorbtion." When the antigenic material is soluble or for other reasons is not removed prior to addition of test cells, it is probably more appropriate to use the terms "inhibition" or "neutralization." Various absorbtion and neutralization techniques have recently been used to great advantage in the study of H-2 isoantigens (Pizzaro *et al.*, 1961; Herzenberg and Herzenberg, 1961; Basch and Stetson, 1961, 1962; Brent *et al.*, 1961; Davies and Hutchison, 1961; Castermans, 1962). These assay techniques can be made very sensitive and have permitted reasonably quantitative work.

Since it has not yet been possible to obtain H-2 or other mouse isoantigens in pure form in true solution, most of the other powerful tools of immunochemical analysis have not yet been applied to their study. G. Möller (1961b) has had some success, however, in the qualitative demonstration of the distribution or localization of H-2 isoantigens by immunofluorescence.

2. *Distribution in Tissues and Cells*

The existence of quantitative differences in isoantigen content among various tissues was long suspected (Loeb, 1945) because of the wide differences in survival time of homografts of these tissues. For example, transplants of spleen were found to be rejected much sooner than transplants of skeletal muscle, and this was thought to indicate that splenic tissue contained a higher concentration of transplantation antigens. Direct and precise information on this point has only recently become available. The H-2 isoantigen content of several organs and tissues of the mouse has been studied by hemagglutination inhibition (Pizzaro *et al.*, 1961) and by cytotoxin inhibition (Basch and Stetson, 1961, 1962) with quite comparable findings. Spleen, thymus, liver, lymph nodes, and bone marrow have relatively high isoantigen activity per milligram of tissue, whereas kidney and muscle have considerably less, and erythrocytes, brain, and placenta yield extremely low values. In

normal mice, the concentration of H-2 isoantigen in each tissue studied seemed to be a relatively constant characteristic of that tissue. Stimulation of the reticulo-endothelial system by B.C.G. inoculation, however, was found to cause a manyfold increase in the isoantigen activity of spleen, liver, heart, and kidney (Basch and Stetson, 1963), and it is of some interest to note in passing that animals treated in this way show an increased capacity to reject skin homografts (Balner *et al.*, 1962). Neither of these latter findings has been adequately explained, and it is not known whether they are related. Variation in H-2 isoantigen content of several tissues has also been noted during maturation. The erythrocytes, spleen, and liver of mice contain only small quantities of isoantigen at birth, but there is a steady rise in isoantigen content during the next 2 or 3 weeks (Pizzaro *et al.*, 1961; Möller, 1961a; Basch and Stetson, 1963).

The location and distribution of H-2 isoantigens on and within cells is still an unsettled problem. The agglutinability of erythrocytes by isoantibody is, of course, *prima-facie* evidence for the presence of these isoantigens on the surfaces of these cells, and the lysis of nucleated cells by isoantibody and complement (presumably by the mechanism described by Green and Goldberg, 1960) argues for the presence of isoantigens on the surface membranes of these cells as well. In addition, however, it appears that a substantial amount of isoantigen may be present within cells, perhaps associated with the membrane material of subcellular organelles. To answer this and other related questions, several laboratories have recently been engaged in the separation and characterization of various subcellular fractions derived from homogenates of mouse liver and other tissues (Kandutsch and Reinert-Wenck, 1957; Kandutsch, 1960; Herzenberg and Herzenberg, 1961; Brent *et al.*, 1961; Davies, 1962a,b,c; Manson *et al.*, 1962; Castermans, 1962; Basch and Stetson, 1962, 1963). Because of substantial differences in starting material, suspending media, methods of homogenization and extraction and methods of separation of fractions, it is difficult to reach more than a few general conclusions from these studies at this time. Nearly all investigators now seem to agree that the H-2 isoantigens survive mechanical homogenization and are thrown down on high-speed centrifugation. Early work had suggested that the antigens were associated with "nuclear" rather than with "cytoplasmic" fractions (Billingham *et al.*, 1956), but most recent studies indicate that a large part of the antigenic activity sediments with smaller cytoplasmic organelles. An association with cell membrane material has been suggested (Herzenberg and Herzenberg, 1961), whereas work in our own laboratory indicates that

although "nuclear," "mitochondrial," and "microsomal" fractions all possess isoantigen activity, the highest specific activity is found in a fraction sedimenting with a density between that of microsomes and mitochondria. This fraction ordinarily contains most of the mouse liver lysosomes, and some appreciable proportion of the total H-2 isoantigen content of mouse liver appears to be closely associated with these organelles. Obviously, interpretation of such results would be facilitated by knowledge of the distribution of cell surface membrane material in these various fractions, but adequate information on this point is not yet at hand.

3. *Chemical Nature and Properties*

Several of the studies mentioned above have yielded information as to the chemical nature of the H-2 isoantigens. They are rapidly inactivated by protein denaturants such as aqueous acetone and alcohol, by exposure to temperatures of 56°C. or higher, and by exposure to extremes of pH. Inactivation of the antigens is also rapidly accomplished by exposure to periodate ion, but other common oxidizing agents produce little or no effect. As already described, these antigens appear to be integrally associated with insoluble sedimentable cell fragments, and it has been extremely difficult to get any appreciable proportion of the starting activity into true solution. Attempts at purification have therefore been generally disappointing. There were early suggestions that these antigens might be associated with nucleic acid or nucleoprotein (Billingham *et al.*, 1956) or might be aminopolysaccharide in nature (Medawar, 1958b), but these were based on work with relatively crude preparations. Analysis of the best products currently available seems to indicate that the H-2 isoantigens are protein or lipoprotein in nature.

Much of the recent productive work in this field has clearly been the consequence of the substitution of serological for biological assay methods, with attendant savings in time and improvements in ability to measure isoantigen activity quantitatively. It seems likely that a similar approach will be useful in the studies of other isoantigens of the mouse and other species. It remains to be seen whether other histocompatibility isoantigens resemble the H-2 antigens in being proteins or lipoproteins of cell membrane material. The major blood group antigens of man, of course, are complex polysaccharides but it is not yet clear whether they are also histocompatibility antigens.

4. *Relationship to Hemagglutinogens*

One problem long at issue has been the relation between the histocompatibility antigens and the hemagglutinogens determined at the H-2

locus. Two main possibilities have been considered. The first was that a single class of isoantigens are determined at H-2 and that these antigens are shared by erythrocytes and nucleated cells, manifesting themselves in serological work as blood group antigens and in transplantation work as histocompatibility antigens. This "one gene, one antigen" hypothesis is economical, orthodox, and a priori somewhat more attractive than the alternative: that two classes of isoantigens are determined at H-2, one possessing the properties of hemagglutinogens and the other constituting the histocompatibility or transplantation antigens. Until recently, however, this latter hypothesis had been more generally favored, in view of evidence suggesting that "H antigens" (hemagglutinogens) and "T antigens" (transplantation antigens) had different tissue distribution, different physical and chemical properties, and qualitatively different immunogenicity (Medawar, 1959). Thus, the H antigens were thought to be present on erythrocyte membranes as well as other cell membranes, to be relatively more stable to such procedures as lyophilization, to induce isohemagglutinin production, and to produce "enhancement" of graft survival rather than transplantation immunity. The T antigens, in contrast, were thought to be absent from erythrocytes but present in association with the nuclear fraction of lymphoid and other nucleated cells, extremely labile to lyophilization, and capable of inducing transplantation immunity without inducing humoral hemagglutinating antibody formation (Medawar, 1958a,c; Brent, 1958; Hildemann and Medawar, 1959).

As more quantitative data have become available, these apparently distinguishing characteristics have disappeared. The tissue distribution and the physical and chemical properties of the isoantigens responsible for the induction of transplantation immunity and of circulating isoantibodies have been shown to be the same and there seems at present no good reason for continuing to suppose the existence of two separate classes of H-2 isoantigens (Brent *et al.*, 1961; Basch and Stetson, 1962). In particular, Lejeune-Ledant (1962) has shown that the antigenicity of various mouse cell extracts as measured by capacity to induce transplantation immunity corresponds precisely with antigenicity as measured by capacity to react *in vitro* with isoantibody in a hemagglutination-inhibition test.

As far as the H-2 isoantigens are concerned, then, it is clear that the same antigens that induce homograft immunity induce humoral antibody formation, and the humoral antibodies formed after homografting are actually directed against the transplantation antigens. This point is worth emphasizing, since the contrary view was widely held until quite

recently. Little is known of the isoantigens determined at H-1, H-3, H-4, and other histocompatibility loci in the mouse, as regards their tissue distribution or chemical nature; some of them, at least, appear to be present on erythrocytes but to have a tissue distribution quite different from that of the H-2 antigens (Amos, 1962a).

B. IN OTHER SPECIES

Comparatively little is known as yet of the isoantigens of other species, but the availability of inbred lines of rats, chickens, and other animals has already permitted a considerable amount of work patterned after that done in mice. Histocompatibility antigens showing dominant inheritance and considerable polymorphism have been demonstrated in several species but there are as yet no systematic data on the tissue distribution or chemical nature of any of these isoantigens. In the rat it appears that there may exist major blood group isoantigens that do not function as histocompatibility determinants. The converse also appears to be true: that certain rat histocompatibility antigens are present on nucleated cells but absent from erythrocytes (Bogden and Aptekman, 1962). Jaffe and McDermid (1962) found that the B locus in chickens is involved in the determination of histocompatibility, and in studies of the blood group antigens determined at this locus, Schierman and Nordskog (1962) have shown that antigens of the A, D, and L blood group systems are found on erythrocytes only, whereas those of the B and C systems are found on leucocytes as well as erythrocytes. Thus, although the concept of T and H antigens has turned out to be invalid for the H-2 isoantigen system of the mouse, it does seem to apply in other species. In our own laboratory, recent work with the rabbit indicates that the major hemagglutinin system (Hg) involves isoantigens which are certainly not major histocompatibility determinants, whereas certain other isoantigens present in epidermal cells and leucocytes induce formation of humoral isoantibodies which are cytotoxic for donor lymphocytes but do not agglutinate donor erythrocytes (Kapitchnikov *et al.*, 1962). In man, none of the "blood group" isoantigens has been identified as a histocompatibility antigen and it seems quite clear that none is as "strong" a determinant as the H-2 antigens of the mouse. The occurrence of erythroblastosis fetalis in cases of Rh or ABO incompatibility may, of course, represent a special manifestation of histocompatibility, but it remains to be determined whether these antigens constitute a barrier to skin grafting or other tissue transplantations.

In summary, then, it appears that blood group antigens may or may not also serve as histocompatibility antigens. In the mouse, those iso-

antigens determined at the H-2 locus happen to be both the major blood group antigens and the major histocompatibility antigens. In other species, it is by no means yet clear that the major blood group antigens have anything whatever to do with histocompatibility.

III. Isoantibodies

As the isoantigens involved in histocompatibility are better defined in the mouse than in other species, so the antibody response of the mouse to isoimmunization has been more extensively studied, and by the same token it is the isoantibodies directed against the H-2 isoantigens that have been most carefully investigated. It is now becoming quite generally recognized that circulating isoantibody production is probably an invariable consequence of single or repeated antigenic stimulation by transplantation of skin or other normal tissues, by transplantation of neoplastic tissue, by injection of erythrocytes, or by isoimmunization by any of the methods currently available. The most sensitive and widely used techniques for isoantibody demonstration are those that employ target cells (erythrocytes, leucocytes, or ascites tumor cells) bearing the appropriate isoantigens. Agglutination of mouse cells by mouse isoantibody is usually weak and there have been difficulties with prozones and with incomplete or "blocking" antibodies. The cytotoxic tests already described are somewhat more satisfactory, although prozoning is common here too. It should be emphasized that all the serological techniques currently available for the study of murine isoantibodies still leave much to be desired, especially in their sensitivity. Although they are generally satisfactory for use with hyperimmune sera, they are barely adequate for the detection of the humoral antibody response to single homografts in some mouse strain combinations and quite inadequate for others. Better technology is urgently needed to support a definitive study of the time of appearance of isoantibodies during the response to skin homografts, for example. Gorer *et al.* (1959) have found evidence for the presence of isoantibody in serum taken on the third or fourth day after grafting. Terasaki (1959) has carried out some kinetic studies on the appearance of lymphagglutinins in chickens, and Jensen and Stetson (1961) made a comparative study of the primary and "booster" antibody responses in mice, but immunization schedules for the production of potent isoantisera are still largely empirical.

The primary humoral antibody response following the first exposure to isoantigen is typically weak, detectable within a few days, and reaching peak titer during the second or third week. On subsequent exposures to antigen, animals commonly exhibit booster or secondary responses,

with prompt rises in humoral antibody titers to levels that are often two or three orders of magnitude higher than those attained during the primary response. Application of skin grafts, injection of whole blood or erythrocytes, transplantation of tumor fragments or tumor cell suspensions, and injection of tissue homogenates or homogenate fractions are among the methods in general use for the stimulation of isoantibody production. Much work remains to be done in evaluation of the effect of Freund's and other adjuvants, the effects of administration of antigen by different routes, and the effect of booster doses of antigen.

Only meager information is available concerning the chemical nature of the H-2 isoantibodies and particularly of those antibodies arising during the primary response to homotransplantation. Antibody obtained after booster doses of antigen appears to be 7S γ -globulin (Stetson and Jensen, 1960), but it is possible, and indeed probable, that the isoantibody formed during the primary response is of the 19S variety. Because of the remarkable differences which exist between the biological properties of 7 and 19S γ -globulins, a thorough investigation of this aspect of the isoantibody response is much needed. Some attention has been paid to the possibility that there may be several classes of isoantibody against a given H-2 isoantigen, one variety mediating hemagglutination and another variety exhibiting the capacity to sensitize donor cells for complement lysis. Jensen and Stetson (1961) were unable to find evidence that such a situation exists for the H-2 isoantibodies of mice but, as mentioned in an earlier section, there do appear to be isohemagglutinins in other species which are not cytotoxic for nucleated cells. Apparently pertinent to this problem are recent observations that antibodies can be subfractionated into two immunoelectrophoretically distinct classes, only one of which shows fixation of complement after interaction with antigen (Benacerraf *et al.*, 1963; Ovary *et al.*, 1963; Bloch *et al.*, 1963). The possibilities for interference or competition between such classes of isoantibodies are interesting and deserve further study.

In retrospect, it is unfortunate, indeed, that earlier experiments attempting to demonstrate a humoral hemagglutinating antibody (Medawar, 1946) or cytotoxic antibody (Medawar, 1948; Allgöwer *et al.*, 1952) in response to skin homografts in rabbits were negative, as these reports have been widely cited to support the notion that a humoral antibody response to skin homografting is not a usual or regular event. Skin homografts in rabbits do induce a humoral antibody response (Terasaki *et al.*, 1961a; Kapitchnikov *et al.*, 1962; Zotikov, 1956; Hancock and Mulan, 1962) and isoantibodies occurring after isoimmunization with homo-

grafts of normal or neoplastic tissue or blood have been detected in the rat (Palm, 1962), guinea pig (Walford *et al.*, 1962a), man (Walford *et al.*, 1962b), dog (Swisher *et al.*, 1962), trout and salmon (Ridgway, 1962), sardine (Sprague and Vrooman, 1962), amphibians and reptiles (Hildemann, 1962), dove (Shaw, 1962), chicken (Gilmour, 1962), pig (Andresen, 1962), mink (Saison and Ingram, 1962), horse (Franks, 1962), sheep (Rasmusen, 1962), cattle (Stormont, 1962), aurochs (Gasparski and Dubiski, 1962), elk (Braend, 1962), rabbits (Cohen, 1962), mouse (Amos *et al.*, 1954), and indeed wherever they have been sought intensively. In some of these cases it is clear that the isoantigens involved are histocompatibility antigens, but in most cases this has not been demonstrated.

IV. Participation of Isoantibodies in Homograft Rejection

If, as appears to be the case, tissue homotransplantation and other modes of isoimmunization regularly result in the production of humoral isoantibody cytotoxic for donor cells, then the possible role of such antibodies in the mediation of graft destruction clearly requires careful consideration. Until recently, most of the evidence derived from a variety of experimental approaches to this problem seemed to indicate that humoral antibodies are not involved in homograft rejection. In this section, some of these lines of evidence will be re-examined.

A. EVIDENCE FROM DIFFUSION-CHAMBER EXPERIMENTS

One experimental approach to the problem of defining the role of humoral factors in transplantation immunity has been through the use of "millipore" membranes of a porosity chosen to permit passage of antibody and other molecules but not of cells. It was reasoned that homografts enclosed in chambers constructed of such material and inserted into subcutaneous tissues or into the peritoneal cavity of an incompatible host should succumb to a homograft reaction if humoral factors were sufficient to mediate graft destruction but should survive if the direct participation of host cells were required. In early experiments, graft survival did appear to be prolonged in such chambers, even when the recipient animals had been previously immunized to donor isoantigens (Algire, 1954; Algire *et al.*, 1954, 1957). These early results were widely accepted as providing strong evidence against the role of humoral antibodies in homograft immunity (Medawar, 1958a; Brent, 1958; Lawrence, 1959; Hasek *et al.*, 1961).

However, a number of investigators have now obtained diametrically opposite results. In a series of careful analyses (Amos and Wakefield,

1958, 1959; Wakefield and Amos, 1958) it was shown that although humoral isoantibodies and complement penetrate such chambers, they do so with some difficulty; when antibody and complement levels were appropriate, many homologous tissue grafts were destroyed in such chambers. Gorer and Boyse (1959) confirmed this finding with a mouse leukemia graft, and Cabourel (1961) reported that incompatible mouse fibroblasts were destroyed inside diffusion chambers in actively and passively immunized hosts. In his last published work, Algire (1959) reported similar observations and ascribed earlier negative results to technical inadequacies. It seems that not all graft cells are equally susceptible to destruction in these chambers. Thus, Algire (1959) showed that neoplastic graft cells of the plasma cell series were destroyed while some nonneoplastic donor cells survived; Gorer and Boyse (1959) mention similar findings with another tumor. Such observations quite probably reflect quantitative rather than qualitative differences in susceptibility of the various cell types to the cytotoxic effect of isoantibody and complement, as has been observed *in vitro*.

Another kind of evidence has been obtained recently by placing immune lymphoid cells *inside* a diffusion chamber and observing whether test skin homografts on the body surface are destroyed. In extensive experiments on mice (Najarian and Feldman, 1962a,b) and rats (Kretschmer and Perez-Tamayo, 1962), the results clearly indicate that diffusible material is released by the immune cells, makes its way through the cell-impenetrable membrane of the chamber wall, and reaches the target homograft where it leads to accelerated graft destruction. This diffusible material has not as yet been shown to be isoantibody, but this is certainly the most reasonable possibility.

Thus, although the first work with diffusion chambers yielded results suggesting that host cells and not host antibodies were the agents responsible for homograft rejection, all of the more recent work makes it quite clear that host cells are *not* required to come into intimate contact with the graft and that humoral factors are both necessary and sufficient to accomplish graft destruction. One is inclined to agree with Amos (1962b) in deploring the misconceptions introduced by the widespread and uncritical acceptance of the early negative experimental findings with this technique.

B. EVIDENCE FROM FETAL LAMBS

The situation in fetal lambs is worth reviewing briefly, not because the findings provide positive evidence for the role of antibody in homograft rejection but because they have been erroneously interpreted as

providing evidence against such a role. Schinkel and Ferguson (1953) made the interesting observation that fetal lambs are capable of rejecting skin homografts *in utero* many days or weeks before birth. Since normal fetal lambs, calves and other ungulates are known to be agammaglobulinemic, it was concluded by the authors and by Medawar (1958a), Brent (1958), and others that their capacity to reject skin grafts must reside in some kind of immune response other than the formation of conventional antibody. It is obvious, however, that the agammaglobulinemic status of the fetal lamb could as well be a reflection of the efficiency with which it is protected against antigenic stimulation as an indication of immunological incompetence. When this matter was explored, it was found (Fennestad and Borg-Petersen, 1957, 1962) that fetal calves exposed *in utero* to microbial antigens develop plasma cells and humoral antibodies to these antigens, and it has recently been shown that fetal lambs immunized *in utero* form large amounts of γ -globulin and specific antibody (Silverstein *et al.*, 1963). Thus, although it has not yet been shown that fetal lambs do in fact make humoral isoantibody during the process of rejecting skin homografts, there is no reason to think that they do not do so, and there exists clear evidence that they do possess the requisite immunological competence. As soon as one or more of the histocompatibility isoantigens of the sheep have been positively identified, it will be of interest to search for the corresponding isoantibody in this experimental model.

C. EVIDENCE FROM PASSIVE SERUM TRANSFER EXPERIMENTS

The establishment of the essential role of humoral factors in other immune phenomena, such as Arthus reactivity and antibacterial immunity, has classically rested largely on the procedure of passive immunization with antiserum. Until quite recently, it has been generally believed that passive immunization against homografts cannot be accomplished with serum; that homograft survival either is not affected by or is actually prolonged by the administration of immune serum; that some grafts of neoplastic or lymphoid tissue may, indeed, be destroyed *in vivo* or *in vitro* by immune serum, but that this sensitivity is peculiar to a few cell types and is not characteristic of the majority of tissues; and that the weight of evidence from such experiments argues against the causal role of isoantibody in homograft rejection (Medawar, 1958a; Brent, 1958; Snell, 1957a,b; Lawrence, 1959; Gorer, 1956). This view is probably erroneous, and has been seriously challenged recently by reports from several laboratories of the successful passive transfer of homograft immunity with serum. These can best be reviewed according to the nature of the test homograft.

1. Immunity to Ascites Tumors

Largely as a result of the work summarized by Gorer (1956) and Gorer and Kaliss (1959), transplantable neoplasms of mice have been considered to fall into three groups: (a) those completely sensitive *in vivo* and *in vitro* to the cytotoxic effect of isoantibody, (b) those completely resistant to isoantibody *in vivo* and *in vitro*, and (c) those of intermediate sensitivity (Snell, 1957b). For example, Gorer and Kaliss (1959) showed that the C57BL/6 leukemia EL4, highly sensitive *in vitro* to cytotoxic isoantibody and complement, failed to grow in passively immunized recipients; the A-strain sarcoma I, thought to be completely resistant *in vitro* to cytotoxic antibody, showed "enhancement" rather than inhibition of growth in passively immunized recipients; and the C3H sarcoma BP8, thought to consist of a mixture of "antibody-sensitive" and "antibody-resistant" cells, showed enhanced growth at low serum dosage and inhibition of growth at high serum dosage in passive immunization experiments. Thus, within certain quantitative limits, it appeared possible to judge or predict whether a given tumor would show growth inhibition or growth enhancement in a passive serum transfer experiment, based on the *in vitro* sensitivity of its cells to cytotoxic isoantibody and complement (Gorer, 1961).

The development of more sensitive and simplified techniques for determining susceptibility *in vitro* to cytotoxic antibody (Boyse *et al.*, 1962a) has permitted the demonstration that each of these tumors consist in fact of antibody-sensitive cells, sarcoma I differing in being only quantitatively less susceptible to immune lysis than BP8 which in turn is less susceptible than EL4. With the demonstration of successful passive transfer with serum of immunity to sarcoma I (Chouroulinkov *et al.*, 1962; Phillips and Stetson, 1962), the behavior of all these tumors *in vivo* has also finally been shown to be qualitatively similar: all show enhancement at low serum dosage and immunity at high serum dosage, differing only in the range of antibody doses required to produce these effects (Boyse *et al.*, 1962b,c). As far as grafts of dissociated cells of neoplastic origin are concerned, then, it seems clear from both serum transfer and diffusion-chamber experiments that conventional humoral isoantibodies, presumably acting with the participation of complement, are after all sufficient to account for the homograft rejection phenomena. It is interesting to note that Boyse *et al.* (1962d) found transplantation immunity in newborn mice born to immunized mothers. The immunity in the newborn may be presumed to have been produced by passive transfer of antibodies from the mother via colostrum and milk, although no serological findings were reported.

a. *Enhancement and Synergism.* It was first clearly shown by Kaliss and Kandutsch (1956) that injection of isoantibodies could, under certain conditions, result in "enhancement" of growth of an incompatible tumor such that the host, rather than the tumor, was overcome. An excellent review of the literature on immunological enhancement is given by Kaliss (1958), who has also recently summarized various concepts of its mechanism (Kaliss, 1962). The powerful suppressive effect of passively administered antibody on the active production of antibody (Uhr and Baumann, 1961) is probably involved, as there are clear indications that active antibody production to H-2 antigens is, indeed, suppressed during immunological enhancement (Snell *et al.*, 1960). Large doses of antibody, enough to kill the entire inoculum of tumor cells, would be expected to produce passive immunity with complete inhibition of growth. Smaller doses of antibody, inadequate to kill the entire tumor inoculum outright, could in this manner inhibit the host's own immune response with consequent enhancement of graft survival. If this hypothesis were correct, it might be predicted that the period of enhanced growth of a tumor inoculum might be preceded by a short period of growth inhibition, reflecting the fact that some proportion of the tumor inoculum had been killed by antibody; such is indeed a common observation, and this hypothesis generally seems to account well for the phenomenology of enhancement.

Another possibility, however, is that there may exist a special class of blocking isoantibodies, capable of binding to graft cells without harmful results and effectively preventing the subsequent binding of conventional complement-fixing cytotoxic antibody. In consequence of this, the graft could survive and show enhanced growth in the face of an active isoantibody response. Gorer (1961) has dealt with this general subject in more detail, as have Boyse *et al.* (1962a,b,c) and Brent and Medawar (1961).

At a time when it was still erroneously believed that some or all of the cells of certain tumors (for example, sarcoma I and BP8) were partly or completely resistant to isoantibody, it was reasoned that the rejection of these tumors *in vivo* must be accomplished by some cellular mechanism, acting alone or in cooperation with antibody. Experiments interpreted as demonstrating a "synergic" action of cellular and humoral factors have been reported by Batchelor *et al.* (1960), whereas Batchelor and Silverman (1962) have found that humoral isoantibody may antagonize the inhibition of tumor growth caused by "sensitized" or immune cells. Now that it is clear that the tumor (BP8) used in many of these experiments is fully susceptible to cytotoxic antibody (Boyse

et al., 1962b), it seems more reasonable to ascribe the synergism to a simple additive effect of antibody in immune serum plus antibody derived from or produced by the regional lymph node cells which are used as sensitized cells.

b. *Other Considerations.* Most of the critical work on passive immunity to tumors has been done with ascites tumors in which the tumor cells grow singly or in small clumps in free suspension. Among the advantages of such a system are the facts that careful measurements can be made of the number of viable cells inoculated, and that such cell suspensions are particularly suitable sources of target cells for serological studies. The design of passive transfer experiments with such cells has been to mix cells and serum before inoculation, or to inoculate serum separately at about the same time that the tumor inoculum is given. The animals are then examined periodically for presence or absence of subsequent tumor growth. What such experiments really detect is the ability of humoral antibodies to prevent the "take" of transplanted tumors, and the antiserum is being presented to a relatively small number of dispersed cells under highly favorable circumstances. This may or may not, of course, have relevance to the mechanism by which the animal normally rejects a large established solid homograft of tumor which has been growing for a week or more before being effectively attacked. There are no convincing reports in the literature of successful immunotherapy of "cure" of such established tumors by antiserum. That is, once a homografted tumor has grown into a palpable nodule, the administration of immune serum either has no effect or actually enhances the growth of the tumor.

While it is clear, then, that humoral isoantibodies can effectively prevent the take of dissociated tumor cell grafts and, in this sense at least, can immunize against such a homograft, it seems equally clear that such antibodies are usually ineffective against solid or orthotopic tumor grafts. This may be merely a matter of dosage, of course, although this does not seem likely in view of considerations to be discussed later. Another obvious possibility is that dissociated cells are more easily reached by antibody and complement, and a third is that the cells of solid tumors are simply not susceptible to these humoral factors. In order to test the latter point, Boyse (1960) developed a method for the production of viable dissociated cell suspensions from solid tumors, and reported that each of the six solid mammary tumors tested yielded cells sensitive *in vitro* to cytotoxic antibody and complement. It seems likely, in view of the subsequent experience of Boyse *et al.* (1962a,b,c), that all of the cells of a solid tumor are sensitive to antibody. The differ-

ence between the response of leukemic or dissociated tumor cell grafts and that of solid tumors, as far as response to isoantibody is concerned, may then be more a matter of accessibility of antibody and complement to tumor cells than anything else (Wissler, 1962).

Clearly relevant is the finding by Amos (1955) that graft cells outside the circulation cannot effectively absorb passively transferred humoral antibody. Using the highly antigenic and antibody-sensitive tumor EL4, it was found that when antibody was given intraperitoneally before an intravenous dose of tumor cells, there was clear serological evidence of binding of antibody to tumor cells as judged by the disappearance of antibody from the circulation. When, however, the dose of serum was not given until 18 hours after the dose of tumor cells, there was no evidence of *in vivo* absorption of antibody. In other experiments, animals with actively growing subcutaneous tumors were given injections of isoantibody and it was found that the titers of antibody in their serum reached and remained at the levels shown by control animals, again indicating failure of the antibody to reach and fix to tumor cells. Direct evidence for the existence of effective "blood-tissue" barriers has recently been provided by Mancini *et al.* (1962), who found that fluorescein-labeled rat globulin reinfused intravenously into the rat does not cross the basement membranes in most tissues including skin. Although quantitative data are not yet available on this point, there seem good reasons to suspect that the relative invulnerability of solid tumors to passively administered antibody involves the logistics of delivery of antibody to graft, rather than any intrinsic invulnerability or insusceptibility of the cells of the tumor.

Amos (1960, 1962b), recognizing that humoral isoantibody and complement suffice in many situations to destroy homografts, has properly insisted that phagocytosis by host cells plays a striking and obvious role in other systems (Journey and Amos, 1962) and has suggested that release of enzymes from host cells in contact with graft cells may result in damage to both cell types. There is no direct evidence to support this latter view, but the phenomenon of adhesion of host histiocytes to graft cells, with or without subsequent phagocytosis, has attracted the attention of many workers. In a recent review (Amos, 1962b) is given a detailed account of the various mechanisms by which isoantibody may mediate *in vivo* destruction of allogenic cells.

2. Immunity to Other Dissociated Cell Grafts

Happily, this is a matter about which there seems to be little dispute. Harris *et al.* (1958, 1961) have shown that antibody-producing lymph

node cells of the rabbit cease functioning shortly after transfer to other rabbits; that this is in all likelihood a manifestation of a homograft response of the recipient against these allogeneic cells; and that the immunity can be passively transferred with serum. Siskind and Thomas (1959) showed that isoantibody could effectively passively immunize newborn mice against adult mouse lymphoid cells, preventing the "runt disease" that would otherwise result. Gorer and Boyse (1959) demonstrated that allogeneic bone marrow grafts were destroyed in irradiated mice by passively administered isoantibody. Several other workers have obtained similar results (Garver and Cole, 1961; Loutit and Micklen, 1961), and there seems no question that immunity to grafts of dissociated nonneoplastic cells can be passively transferred with isoantisera as readily and as reproducibly as can immunity against the dissociated tumor cell grafts described in the preceding section. Again, the results of the *in vivo* passive transfer experiments quite closely mirror the *in vitro* cytotoxic effect of isoantibody and complement on these cells. As Winn (1960) points out, sera which show a cytotoxic effect against a given cell type will usually passively immunize against grafts of that cell type, subject to obvious quantitative considerations.

Again, however, the problem of the relative invulnerability of established cell grafts must be mentioned. It appears that it is either very difficult or quite impossible to abolish tolerance by the injection of immune serum (Brent and Medawar, 1961). If tolerance results from the continued presence of donor lymphoid and hematopoietic cells in the chimeric host and if these cells are intrinsically sensitive to isoantibody, it should be possible to destroy them and "break" tolerance by the injection of potent antisera. The failure to observe such an outcome in the majority of animals so treated may be a matter of dose of antibody, of diminished isoantigenicity of donor cells after long residence in an animal of foreign genotype, or of sequestration of some fraction of the donor cells in some compartment relatively inaccessible to the passively administered antibody.

3. Immunity to Skin Grafts

Billingham and Sparrow (1954) were able to show that rabbit epidermal cells are intrinsically susceptible to some damaging effect of isoantibody. Exposure of dissociated epidermal cells to relatively large amounts of immune serum affected the cells in such a way that they failed to grow when seeded back onto an appropriate bed in the donor. In extensive experiments in mice, however, Billingham *et al.* (1954) were completely unable to achieve passive immunization against ortho-

topic skin grafts by use of serum or whole blood. In their experiments, mice of the CBA line were immunized with an A strain skin homograft and bled 11 days later. Large doses of serum obtained in this way did not passively immunize CBA hosts against A skin grafts transplanted on the same day. No other strain combination was employed and no serological studies were performed on the sera.

Voisin and Maurer (1956, 1957) observed shorter survival of guinea pig skin grafts in recipients that had received serum from other guinea pigs previously grafted from the same donor. Lymphoid cells from the immune animals were somewhat more effective, and there was a suggestion that immune cells plus immune serum gave better passive immunity than either alone. The authors regarded their data as confirming earlier studies on the "adoptive" transfer of graft immunity with lymphoid cells (Mitchison, 1954, 1955; Billingham *et al.*, 1954) but also suggested that their serum transfer experiments provided tentative evidence for the role of isoantibody in graft rejection. No attempts to confirm these results have been reported.

Billingham and Brent (1956) made another even more extensive effort to passively immunize mice with serum against skin grafts and were again unsuccessful. Here too, a single strain combination was used, but in these experiments the authors gave additional repeated intraperitoneal and subcutaneous injections of spleen and kidney cells from the donor strain over a period of 6 weeks. Serum was obtained 8 days after the final immunizing injection, at a time when it might be expected that the humoral isoantibody level was far higher than after a single graft. Although interpreting their results as negative, the authors described "outward signs of vascular dilatation and congestion" in the test skin grafts when they were examined on the sixth postoperative day. This effect, which was described as being "transient and clearly of minor importance," may of course have been a specific phenomenon.

Stetson and Demopoulos (1958) described severe damaging effects of immune sera on test skin homografts in rabbits and mice. The sera were prepared by a single immunization of rabbits or mice against allogeneic splenic tissue suspended in Freund's adjuvant. Sera were obtained from 2 to 3 weeks later and were pooled without benefit of serological investigation. Some of these pools were active in the passive transfer of "white graft" reactivity and in causing accelerated breakdown of established test homografts (Stetson, 1959a,b). This latter effect was specific and was obtained only when serum was injected locally around test and control grafts. These experimental findings could not be reproduced in Medawar's laboratory (Brent *et al.*, 1959; Brent and Medawar,

1961) but have been confirmed and extended in Hašek's laboratory. Chutna (1960) used dissociated allogeneic epidermal cell suspensions, implanted subcutaneously, as a target for passively transferred isoantibody and found accelerated destruction of these cells. In further experiments, conventional established orthotopic skin homografts were also destroyed by local injections of isoantibody; the author demonstrated complement-fixing antibodies in the sera used. In still other studies in the same laboratory (Chutna, 1961; Chutna and Pokorna, 1961) it was concluded that humoral antibodies are probably involved in the Arthus-like reaction which characterizes the "white-graft" reaction.

Stetson and Jensen (1960) found that accelerated skin graft destruction could be achieved by parenteral injection of immune serum, provided steps were taken to produce local increases in vascular permeability in the graft. High-titered isoantisera were injected intravenously into animals bearing established skin grafts, and a portion of the grafts was then treated with histamine or with agents known to produce local histamine release. The treated portions of the grafts showed severe hemorrhagic necrosis, and it was suggested that the local increase in vascular permeability had permitted humoral antibody to leave the vascular bed in this area and make effective contact with the graft antigens.

A careful and thorough series of passive transfer experiments in rabbits were next reported by Kretschmer and Perez-Tamayo (1961). For recipients, these workers used either normal young rabbits or rabbits that had been "conditioned" by cortisone treatment so as to prolong the period of test homograft retention. Antisera were obtained from skin graft immune animals 5, 10, and 15 days after grafting and were pooled without further study. Recipient animals received test and control skin homografts and were then given either local or systemic injections of pooled immune serum at the time of grafting. In both the normal and conditioned rabbits, survival of the test skin homografts was considerably shortened, and the authors concluded that their data support the concept that humoral antibodies play a definite role in the rejection of rabbit skin homografts.

Perhaps the most impressive of the successful attempts to passively transfer skin homograft immunity with serum was reported by Steinmuller (1962), using grafts exchanged between the Lewis and BN strains of rats. Serum obtained from BN rats at about the time of maximal rejection of first-set Lewis skin homografts was consistently effective in producing accelerated rejection of test Lewis grafts, whereas sera harvested a few days before or later were much less effective. Estab-

lished grafts on "tolerant" animals were not affected by the injection of even large doses of serum, nor was the passive transfer of immunity possible with other donor-recipient pairs, including the reverse (BN to Lewis) combination. Surprisingly, the cytotoxic and hemagglutinating antibody levels in the sera tested had no obvious relationship to their *in vivo* activity. That is, sera harvested at 6, 10, and 15 days after grafting had little or no antibody activity as judged by serological testing *in vitro* but were effective in producing accelerated destruction of skin grafts after parenteral injection; sera obtained after 20 to 60 days, on the other hand, had appreciable serological activity against Lewis lymphocytes and erythrocytes but little action *in vivo* on test Lewis skin grafts.

It is interesting to note that virtually all the successful experiments along these lines have used serum obtained relatively early during a primary response to homologous tissue. This would be surprising if only quantitative considerations were involved, since antibody titers have been shown to rise considerably following hyperimmunization by booster injections. The data rather suggest that there is something qualitatively different about "early" isoantibody. It has already been suggested that most of the antibody produced during a first-set homograft reaction may be high molecular weight material, whereas that occurring after repeated antigenic stimulation may be largely 7S γ -globulin. No data on this point have yet appeared. Another possibility is that early antibody, produced during the immune response to a first-set skin homograft, may have a more "cytophilic" character than antibody produced later. Such antibody, binding as it does to lymphoid and perhaps other cells (Boyden and Sorkin, 1960, 1961), may be carried into contact with graft antigen where it can interact with target graft cells in a manner analogous to that described recently by Koprowski and Fernandes (1962).

An exciting observation of clear relevance to this problem has recently been made by Bennett *et al.* (1963), who have observed that the phagocytosis of tumor cells by "immune" peritoneal macrophages is abolished by washing the immune cells. Furthermore, normal macrophages were shown to be converted to immune cells, capable of phagocytizing target tumor cells, by exposure to immune serum or to the washings from peritoneal macrophages from immune animals. This is the first demonstration of a specific antigraft action of histiocytes in the presence of humoral isoantibody, the specificity of the interaction between host cell and graft cell being determined by the antibody rather than by an intrinsic alteration in the host cell. These observations may well provide an adequate explanation for the phagocytic phenomena described by

Baker *et al.* (1962) and a similar mechanism may well be involved in other *in vitro* host cell versus tumor cell interactions, such as those described by Rosenau and Moon (1961, 1962).

4. Immunity to Other Orthotopic Grafts

Relatively little work has been done in this field as yet. Terasaki *et al.* (1962) have reported that antisera produced by hyperimmunizing A-strain mice with minced kidney tissue from C57BL mice produced renal damage when infused into the renal arteries of mice of the donor strain. Normal sera were usually ineffective, as were antisera which had been absorbed *in vitro* with donor kidney tissue. Crude γ -globulin fractions of the immune sera showed activity, and the authors concluded that "it seems likely that the specific effect of antibodies played some role in producing injury." The experimental model was not quite satisfactory, however, as corresponding renal lesions were sometimes produced simply by injection of saline or normal serum, or even by needling the renal artery.

B. Altman (quoted by Murray *et al.*, 1962, and by Dammin *et al.*, 1962) has evidently observed a cytotoxic effect of immune dog serum, consisting of cessation of renal function immediately following the injection of immune serum into the renal artery of a homografted kidney. It is not clear, from the brief accounts given, whether the agent in the immune serum was isoantibody.

Experiments of this sort, involving whole organs in which donor and recipient vasculature are surgically anastomosed, provide a valuable model for the study of the direct effects of passively administered antibody on graft blood vessels, as there can be no question here of humoral antibody failing to make contact with graft antigens. It is to be hoped that this approach will be more adequately exploited in the future.

D. OTHER EVIDENCE

Gorer (1956, 1958) drew attention to the histological observation that homografts of certain tissues are rejected by some process that clearly does not involve the direct participation of host cells. In this sort of reaction, destruction of intramuscular or subcutaneous grafts of leukotic tissue can be seen histologically long before host lymphocytes or histiocytes appear on the scene. Gorer interpreted these observations as indicating that this sort of graft rejection, at least, was largely owing to extravasated isoantibody. Gorer *et al.* (1959) have also emphasized that histological and serological evidence indicates that in all types of homograft response it is possible to detect humoral antibody before

there is any macroscopic or microscopic sign of graft destruction and that the earliest histological sign of the homograft reaction is frequently a vascular reaction with leakage of plasma into the graft. Some of Gorer's observations can certainly best be explained by invoking the early and active participation of humoral isoantibody.

The long survival of homografts in humans suffering from agammaglobulinemia (Good, 1959; Good *et al.*, 1962) is certainly one of the strong points in favor of the requirement for antibody in effecting the homograft reaction. In the hereditary form of this disease, the patients seem to suffer from a single genetically determined defect: the inability to respond to antigenic stimulation with the production of differentiated plasma cells and specific antibody. For some unaccountable reason, this evidence has not been generally accepted at face value. Similar caution in the evaluation and interpretation of cell-transfer and diffusion-chamber experiments would have been valuable and welcome, as these are extremely complex situations which require careful analysis. In contrast, the patient with congenital agammaglobulinemia has provided a relatively simple and direct means of "dissociating the capacity to develop delayed allergy from the capacity to produce circulating antibody" (Good, 1959). These patients have been shown (1) to express and sustain delayed hypersensitivity to streptococcal antigens following injection of leucocytes from sensitized donors, (2) to develop regularly delayed allergy to a bacterial antigen on sensitization by antigen-antibody complex, (3) to develop regularly delayed hypersensitivity of the contact type to 2,4-dinitrofluorobenzene, and (4) to develop and express delayed hypersensitivity of the tuberculin type to tuberculin following infection with attenuated tubercle bacilli. In contrast, these patients generally failed completely to show in their circulation (1) "natural" antibodies to any of a large variety of bacterial or viral antigens, (2) natural isohemagglutinins, (3) any antibacterial or antiviral antibody response to single or multiple immunizing injections of potent vaccines, or (4) any immune isohemagglutinin response to single or repeated injection of human blood cells of different Landsteiner groups (Good, 1959). At least one of these patients is still bearing a skin homograft, applied 8 years ago (Good, 1963), and it is difficult to escape the conclusion that his inability to reject it is a consequence of his inability to form humoral isoantibodies.

V. General Discussion

It is necessary, in order to put the preceding observations in their proper perspective, to recall and insist upon the distinction between

those immunological reactions that are mediated by antibody and those that are not. Although much more is known of the former than of the latter, they can and must be rigorously distinguished as far as possible, for only confusion can result when one man's "antibody" is another man's "sensitized cell." Antibodies, or specifically configured immunoglobulins synthesized and secreted by lymphoid or plasma cells, can be detected and studied by a variety of highly sophisticated approaches, and the elucidation of the structural basis for their biological activity is currently the object of one of the most exciting studies in molecular biology. Anaphylactic hypersensitivity and antibacterial and antitoxic immunity belong to the antibody-mediated class of immune reactions, as they are clearly associated with demonstrable circulating antibody and can be passively transferred with antibody-containing serum. On the other hand, there exist many immunological reactions that appear not to be mediated by antibody. That is, they occur "in the absence of demonstrable antibody of the conventional type" (Gell and Benacerraf, 1961) and "appear to have no relation to serum antibody, precipitable or otherwise. To date, no antibody has been found to mediate or parallel the highly specific inflammatory response to the presence of antigen characterizing the delayed sensitive state" (Lawrence, 1956). These reactions include delayed hypersensitivity of the tuberculin type to various bacterial, viral, and fungal antigens, delayed hypersensitivity to simple protein antigens, and "contact" sensitivity to poison oak, and to various simple chemical compounds such as 2,4-dinitrofluorobenzene. The evidence that antibodies are not involved in such reactions includes (1) the existence of delayed hypersensitivity in patients with congenital agammaglobulinemia and the transfer of the hypersensitivity with the cells of such patients, (2) the fact that cells and extracts of cells used to transfer delayed hypersensitivity do not contain demonstrable antibody, and (3) the fact that recipients of such cell transfers do not exhibit antibody in their serum. This has led to the concept of the existence of specifically sensitized cells that are considered to mediate the reactions of delayed hypersensitivity without the participation of conventional immunoglobulin or antibody (Lawrence, 1956, 1959; Gell and Benacerraf, 1961). The lymphocyte seems by general consent to be the best candidate for the mediator of such reactions, although just how it functions is still a matter for conjecture. Brent and Medawar (1961) have suggested that "a 'sensitive' phase in the reaction of the cells of the host is superseded by a relatively insensitive phase in which the cells are preoccupied by the manufacture of humoral antibodies"; this assumes "that the immunological response of cells occurs in two

stages: first, a stage in which the cells become 'sensitive' and can act as the effectors of hypersensitivity reactions of the delayed type; and secondly, a stage in which the same cells (or their descendants) manufacture humoral antibodies and in so doing pass out of the sensitive state."

It is to this class of delayed hypersensitivity reactions that most authors have relegated the homograft reaction (Burnet and Fenner, 1950; Medawar, 1958a; Brent, 1958; Lawrence, 1956, 1959; Mitchison, 1954; Snell, 1957a; Hasek *et al.*, 1961) for reasons which at the time seemed compelling. From the evidence cited in the present review, however, it would seem that this view should now be abandoned. Antibodies to transplantation antigens exist during transplantation immunity and can be shown to damage graft cells under a wide variety of circumstances. These and other considerations, including the abandonment of the distinction between T and H antigens, the abandonment of the concept that sensitivity to antibodies is a special property of lymphoid cells and their derivatives, and the replacement of earlier negative evidence from diffusion-chamber and passive serum transfer experiments, leave little to support the view that homograft immunity depends on some specific sensitization of cells distinct from the production or transport of conventional antibody.

There is some question, indeed, as to whether delayed hypersensitivity, in fact, exists at all as a facet of the immune response to transplantation antigens. The "direct" skin test reactions described by Brent *et al.* (1958) in guinea pigs were interpreted as providing an indication that such a state does exist, at least in that species. This conclusion was based entirely on the gross and histologic features of the skin reactions, however, and Gell and Benacerraf (1961) have emphasized that the "macroscopic characteristics of the delayed reaction, and still less the histologic features, do not constitute a satisfactory criterion for defining a type of reactivity" (see also Gell, 1958). The "transfer" reaction seen by Brent *et al.* (1958) after injection into a homograft donor of regional lymph node cells from a sensitized recipient was also construed by these authors as a manifestation of delayed hypersensitivity, although an alternative interpretation, that these skin reactions resulted from the local production of antibodies against antigens of the donor skin, was considered by the authors and would seem equally justified. Certainly neither of these reactions fits the rigorous definition of "delayed hypersensitivity" as given by Gell and Benacerraf (1961). Of course, even if it were to be shown unequivocally that animals rejecting skin homografts do develop delayed hypersensitivity, this would not of itself

implicate that hypersensitivity in the graft rejection process; as we have seen, diffusion chamber and serum transfer experiments indicate that humoral factors alone are quite sufficient to account for the breakdown of even orthotopic grafts, and there is not an experiment on record which clearly shows that "sensitized" cells have an antigraft activity other than that mediated by antibody.

The experiments of Lawrence *et al.* (1960) in man indicate that procedures known to transfer delayed hypersensitivity states also lead to accelerated skin graft breakdown. Since human leucocytes are potent sources of transplantation antigens (Friedman *et al.*, 1961), the possibility that these experiments involved active stimulation of immunity to antigens shared by the skin graft donors and leucocyte donors needs to be taken into account. Also, it has recently been shown (Baram and Mosco, 1962) that disrupted leucocyte preparations such as those used contain sizable amounts of a protein with the mobility of serum γ -globulin, and their possible isoantibody activity therefore requires investigation. If these possibilities can be ruled out, this model will provide the first clear indication that delayed hypersensitivity to homograft antigens exists and participates in the homograft reaction. The question would still remain, of course, as to whether isoantibodies or "sensitized" cells actually serve as the specific immunological agent effecting graft destruction, and experiments of this sort performed on patients with agammaglobulinemia would be an interesting approach to this problem.

Finally, the terms "cytophilic antibody" and "cell-bound antibody" require some clarification. Cytophilic antibody is the term generally used to designate that fraction of the antibody in certain immune sera which shows a particular affinity for spleen cells (Boyden and Sorkin, 1960, 1961). This antibody attaches firmly to the cells, remains attached after washing, and endows the cells with the capacity to interact specifically with antigen. The "contactual agglutination" phenomenon of Koprowski and Fernandes (1962) apparently represents another example of the conferring of immunological reactivity on normal lymphoid cells by the attachment of "cytophilic antibody" from serum. This type of antibody, although bound to cells and perhaps delivered to the very doorstep of a homograft by the cells, must still be considered humoral antibody in the sense that it exists as a serologically detectable, specific immunoglobulin in serum. In sharp contrast, the term cell-bound has most frequently been used to refer to the quite hypothetical and as yet unspecified modification of the sensitized cell of delayed hypersensitivity states (Smith, 1960; Lawrence, 1956; Lawrence *et al.*, 1960; Hašek *et al.*, 1961; Kretschmer and Perez-Tamayo, 1961; Amos, 1962b). It is most

important, then, that this latter term not be used loosely to describe antigen-cell interactions mediated by adsorbed cytophilic antibody. For example, it is not entirely clear whether Berrian and Brent (1958) in using the term *cell-bound antibody* intended to refer to conventional antibody bound (as cytophilic antibody is bound) to lymphoid cells, or whether they were thinking in terms of lymphoid cells sensitized as in delayed hypersensitivity states; their repeated use of the term "antibodylike reactivity" rather suggests that the latter interpretation was intended.

VI. Summary

Humoral isoantibody formation, once thought to be inconstant, now appears to be an ineluctable feature of the isoimmune response to tissue homografts. These antibodies have been shown to mediate cytolysis of a wide variety of cell types, both normal and neoplastic, and it is probable that no cell type is completely resistant to this form of damage. Whereas some humoral isoantibodies may be directed at isoantigens irrelevant to the homograft reaction, others are clearly directed at the histocompatibility antigens themselves. Humoral isoantibodies have now been shown to pass through diffusion chambers and mediate destruction of homografts inside without participation of host cells; humoral antibodies have also been shown to be elaborated by "immune cells" within such chambers and to effect the accelerated destruction of grafts outside on the body surface. Humoral isoantibodies reproducibly passively immunize against dissociated cell grafts of normal or neoplastic origin; they are usually unable to immunize passively against solid or orthotopic grafts, apparently because of limited accessibility, but an increasing number of successful experiments of this sort have been reported. Although host cells may participate in the destruction of homografts, as they destroy bacteria, specific isoantibody appears to be the agent that permits them to do so.

This view leaves many practical problems unsolved and many specific questions unanswered. Whether antibody reaches a graft by perfusion, whether it is delivered as "cytophilic antibody" bound to lymphoid cells, or whether it is synthesized locally by immunologically competent cells may well be determined by the nature and location of the graft. It is not yet clear whether a special class of isoantibodies exists with antigraft activity qualitatively different from that of those isoantibodies mediating immunological enhancement. The effectiveness of lymphoid cells in conferring adoptive immunity has not yet been clearly shown to be due to their production of humoral antibody, although this seems likely. These and other unsettled matters stand in

the way of making a solid case for antibody at present, but after a decade of preoccupation with the lymphocyte and delayed hypersensitivity it now seems that most of the formal demonstrations point to a central role for conventional antibody in the destruction of homografts.

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Immune Adherence

D. S. NELSON

*The Department of Experimental Pathology, John Curtin School of Medical Research,
Australian National University, Canberra, Australia**

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I. Introduction and Historical Survey

The processes whereby cells, bacteria, viruses, and soluble materials adhere to other cells have a perennial fascination for biologists. To

* Present address: Department of Pathology, University of Cambridge, Cambridge, England.

the immunologist certain of these processes are particularly interesting, either because of their usefulness in laboratory investigations or because of their possible role in the body's defensive and allergic reactions. One may cite as examples the agglutination of erythrocytes by antibody; the attachment of viruses to host cells at the time of infection or of certain viruses to erythrocytes causing hemagglutination; the attachment of bacteria to phagocytes before their ingestion; the adherence of bacteria to antibody-producing cells; or the attachment to each other, mediated by antibody, of dissimilar cells which happen to share a common antigen.

One adherence reaction of special interest and importance is "immune adherence" (IA). This term was coined in 1953 by R. A. Nelson to describe the attachment to human erythrocytes of microorganisms sensitized with antibody and complement (R. A. Nelson, 1953). In the light of subsequent work in Nelson's laboratory and elsewhere IA may be defined as the attachment of antigen-antibody-complement complexes to the surface of untreated primate erythrocytes or nonprimate platelets (the indicator particles). The rider should be added that adherence to other indicator particles may possibly be included later in this definition, provided that the mechanism can be shown to be identical with that of IA.

Nelson's was by no means the first description of an immunologically mediated adherence reaction of this type. There has been some controversy in this field and the need for some more exact definitions was highlighted recently by the work of Lamanna and Hollander (1956) and by an extensive review of adhesion reactions and IA by Lamanna (1957). These workers sought to group most or all these reactions together under the name "serologic adhesion." Much of the earlier work in this field has been reviewed by Lamanna. It seems appropriate, however, to survey it briefly here and to reconsider the interpretation of some of the findings in the light of work done after the publication of Lamanna's review. Some of this work has also been reviewed by Piéron (1959).

Probably the first descriptions of the attachment of parasites to host cells in the presence of specific antibody were those of Laveran and Mesnil (1901) and Levaditi (1901). The former described the attachment *in vivo* of *Trypanosoma lewisi* to the leucocytes of immune rats. Levaditi observed the attachment of *Vibrio cholerae* to circulating blood platelets and polymorphonuclear leucocytes of immune, but not of normal, rabbits. At the same time he showed that *V. cholerae* pretreated with immune serum and injected intravenously into normal

animals also adhered to platelets. Some years later both workers published further observations on these phenomena. Laveran and Thiroux (1911) and Levaditi and Mutermilch (1910) successfully identified strains of pathogenic trypanosomes by their adherence to guinea pig or rabbit leucocytes in the presence of specific immune serum. At about the same time Aynaud (1911) observed that addition of a variety of bacteria (e.g., *Escherichia coli*, staphylococci, anthrax bacilli) to oxalated dog or rabbit blood resulted in the formation of mixed aggregates of platelets, bacteria, and leucocytes.

Interest seems to have waned until Rieckenberg (1917) described the adherence of trypanosomes to the platelets of rats, mice, guinea pigs, and rabbits in the presence of specific antibody. Shortly afterwards Levaditi's work was confirmed by Delrez and Govaerts (1918) and extended by Govaerts (1920a,b, 1921, 1922, 1923). He suggested that the adherence of intravenously injected bacteria to the platelets of rabbits and dogs aided their clearance from the blood stream, thus playing a part in host resistance to infection. He also demonstrated the adhesion of bacteria to washed rabbit and dog platelets *in vitro* in the presence of a heat-labile serum factor and claimed that phagocytosis of bacteria was enhanced following their adherence to platelets.

Under the name of Rieckenberg, *Beladung*, or thrombocytobarian reaction, the adherence of microorganisms to platelets was extensively investigated between 1925 and 1931 by Kritschewsky, Brussin, Aristowsky, and their co-workers. [See for example Brussin (1925), Kritschewsky and Tscherikower (1925), Aristowsky and Schaechter (1928); also Kritschewsky (1927) and Kritschewsky and Brussin (1931) for brief reviews of the work of this group.] Using trypanosomes, spirochetes of relapsing fever, and leptospire, they demonstrated that antibody and complement were required for the reaction and that platelets from many species, but not from man, could serve as indicator particles. These workers comment particularly on the specificity of the reaction.

In none of these papers is there reference to the adherence of microorganisms to erythrocytes, though human erythrocytes do not seem to have been used. Leupold (1928), investigating the Rieckenberg reaction with trypanosomes, observed the occasional adherence of trypanosomes to mouse erythrocytes but Duke and Wallace (1930) were the first to describe a "red-cell adhesion" reaction which occurred consistently. They showed that trypanosomes would adhere to primate erythrocytes and guinea pig platelets and rarely to guinea pig erythrocytes in the presence of specific antibody. Wallace and Wormall (1931) in more detailed experiments confirmed most of these results but found that only

primate erythrocytes were reactive. They showed that in addition to specific antibody, a factor present in normal serum and having the properties of complement was required. On the other hand, adhesion of trypanosomes, in the presence of antibody, to rat or mouse erythrocytes was reported by Raffel (1934), and to horse and mule erythrocytes by Taliaferro and Taliaferro (1934).

There are scattered references to the adherence of microorganisms to particles other than animal cells. In a paper by Rössle (1905) there is a suggestion that mixed agglutination of different species of protozoa can occur in the presence of antibody to one of them, and there was some interest later in mixed bacterial agglutination, though this was not considered in relation to adherence phenomena (Mudd, 1933). Several workers on the adherence reactions reported that bacteria and inorganic particles such as gamboge and India ink could replace platelets or erythrocytes as indicator particles (Krantz, 1926; Davis and Brown, 1927; Kritschewsky and Tscherikower, 1928; Brussin and Kalajev, 1931; Raffel, 1934; Lamanna and Hollander, 1956). Mostly adherence was not consistently demonstrable, though Brown and Davis (1927), Brown (1935), and Lamanna and Hollander (1956) considered it to be sufficiently so for use as a diagnostic test.

Interest in adhesion reactions waned during and after the Second World War, apart from some work by Houlihan and Copley on the adhesion of bacteria to platelets (Houlihan and Copley, 1946; Houlihan, 1947a,b). R. A. Nelson's work on the disappearance of *Treponema pallidum* from mixtures of this organism with antibody, complement and erythrocytes (R. A. Nelson, 1952a,b) led to his description of immune adherence (R. A. Nelson, 1953) and a revival of interest in this and related phenomena. In his initial report Nelson described the attachment to human erythrocytes of microorganisms in the presence of antibody and complement. Human platelets were found not to participate in this reaction though in later work (R. A. Nelson, 1956; R. A. and D. S. Nelson, 1959) it was found that other primate erythrocytes and nonprimate (rabbit and guinea pig) platelets could replace human erythrocytes in this reaction. Thus there are similarities to both the Rieckenberg reaction and the red-cell adhesion test of Duke and Wallace. Washed antigen-antibody-complement complexes adhered as strongly as did microorganisms in the presence of serum containing antibody and complement. In the form described by Nelson IA was shown to occur with a great variety of antigens including many bacteria, other particulate antigens such as starch granules, rickettsiae, and viruses, and soluble antigens such as ovalbumin. The observation that

IA could enhance the phagocytosis of microorganism *in vitro* and the demonstration of its occurrence in the intact animal strongly suggested that it was a reaction of some importance in the resistance of the host to invading microorganisms. In addition to this biological importance, IA has found diverse applications in the laboratory. For example, antibodies in syphilis and leptospirosis may be detected rapidly and easily; the measurement of complement activity in human serum may be made much more accurate and sensitive; and the detection of plant viruses may be simplified and expedited.

Since the time of R. A. Nelson's description in 1953, he and others have not only sought applications of the reaction but have investigated more closely the conditions in which it occurs, the nature of the reagents, and the nature of the bond between sensitized antigen and indicator particle. A further advance was made when Nelson and Nelson, stimulated by the apparent conflict between this description and the findings of Lamanna and Hollander (1956) and of earlier workers as reviewed by Lamanna (1957), described two other reactions which could closely mimic IA (see D. S. and R. A. Nelson, 1959; R. A. and D. S. Nelson, 1959). These are (1) acid-adhesion—the adherence of bacteria to erythrocytes which occurs only at low pH; and (2) complement-dependent mixed aggregation—the union of two antigen-antibody complexes in the presence of complement.

To facilitate discussion, those reactions described by earlier workers that appear to conform to the definition of IA will be discussed in the following as examples of IA. A more detailed discussion of the reasons for their acceptance as such and for the rejection of others is given in Section IV,H.

II. The Nature of Immune Adherence and the Reagents Involved

A. ANTIGENS

1. Protozoa and Metazoa

Much of the early work on platelet adhesion was concerned with trypanosomes. Most frequently adherence was observed in mixtures of citrated blood from an experimentally infected animal, e.g., rat, guinea pig, and mouse, containing live trypanosomes (*Trypanosoma brucei*, *Trypanosoma equiperdum*) and citrated plasma or blood from an immune animal, containing antibody, sometimes with the addition of normal serum or plasma as an additional source of complement. As early as 1901, Laveran and Mesnil commented on the specificity of adherence reactions, and this was amply confirmed by subsequent workers. The

reaction was quite specific for a particular strain of organism, even variants arising in the course of the disease failing to react with antiserum to the original strain (Brussin and Beletzky, 1925; Kritschewsky and Heronimus, 1927; Kritschewsky and Tscherikower, 1925, 1926a; Davis and Brown, 1927; Raffel, 1934). *Trypanosoma gambiense*, *T. brucei*, and *Trypanosoma rhodesiense* were shown by Wallace (1933) to react in the red-cell adhesion test of Duke and Wallace. Heated (45°C. for 30 minutes) trypanosomes could induce the formation in mice of the antibody responsible for the reaction, but it seemed that living organisms had to be used in the reaction mixture for its demonstration (Wormall, 1933).

Leishmania tropica was reported by Messik (1927) to participate in the Rieckenberg reaction even in the absence of immune serum, since normal mouse serum induced adhesion, presumably by means of natural antibody. E. A. Mills *et al.* (1931) reported the usefulness of the reaction in the diagnosis of oriental sore, cultured leptomonad forms of *Leishmania* being used as the antigen.

The only report dealing with Metozoa in an adhesion reaction is that of Pandit *et al.* (1929). They described adherence of microfilariae to human leucocytes in the presence of serum presumed to contain antibody. Many nonspecific reactions were observed and the relation of this phenomenon to IA is doubtful.

In view of the backward state of immunological studies with parasitic Metazoa it seems unfortunate that the possible applications of IA in this field have not been further explored.

2. Spirochetes, Leptospires, and Related Organisms

The Rieckenberg reaction was extensively used by Brussin and co-workers in investigations of relapsing fever experimentally induced in mice (Brussin, 1925; Brussin and Rogowa, 1927; Brussin and Schapiro, 1928; Brussin and Kalajev, 1931). Other workers confirmed the finding that pathogenic spirochetes adhered to platelets in the presence of antibody and complement (Krantz, 1926; Rosenholz, 1927; Jakimow, 1928; Kritschewsky and Tscherikower, 1926a). Infected mouse blood and extracts of infected Heteroptera were used as sources of antigen. Some strain specificity was observed but this was less marked than with trypanosomes (Brussin and Schapiro, 1928). Brussin and Kalajev (1931) and Jakimow (1928) claimed that killed spirochetes could be used as immunizing antigens or as antigens in the reaction. Neither Krantz (1926) nor Kritschewsky and Tscherikower (1928) were able to confirm

this, perhaps because they destroyed the reactive antigenic sites in the process of killing the organisms.

The occurrence of adhesion reactions with *Treponema pallidum* was reported by Krantz (1930) and Aristowsky and Wsorow (1931). "Particulate adhesion of the Rieckenberg type" was also reported by Lamanna and Hollander to occur with *T. pallidum*. However, more definitive and fruitful work with this organism followed R. A. Nelson's experiments on IA (R. A. Nelson, 1952a,b, 1953). Since then, IA has been extensively investigated as a diagnostic test for syphilis and its sensitivity and specificity have been repeatedly confirmed (Daguet, 1956a,b; Miller *et al.*, 1957; Moser, 1955; Piéron, 1959; Olansky *et al.*, 1954). Heat or phenol killed *T. pallidum* reacted as well as live organisms and the antigen was stable on storage. Comparisons of the results of IA tests with those of *T. pallidum* immobilization tests have shown that the antigen is a specific component of *T. pallidum*. The nature of the antigen was further investigated by Király and Porgányi (1960). They found that the antigens involved were susceptible to the action of protein-digesting enzymes, ribonucleases, hyaluronidase, and heat, and they concluded that no well-defined antigen was involved but that IA occurred if antibody and complement reacted with any surface antigen of the spirochete.

Leptospire were shown to react as antigens in the Rieckenberg reaction by Kritschewsky and Tscherikower (1926b), Kritschewsky and Lebedewa (1927), Aristowsky and Schaechter (1928), and Inoue (1930). Only live motile organisms reacted and different strains could be distinguished. Adhesion of leptospire to bacteria in the presence of antibody and complement, an example of complement-dependent mixed aggregation (discussed later), rather than of IA, was reported by Brown and Davis (1927) and Brown (1935). As with *T. pallidum*, more detailed investigations followed R. A. Nelson's description of IA. Linscott and Boak (1961) and Linscott (1961) investigated both the mechanism of IA and the distribution of antibodies to leptospire using living organisms or organisms killed by merthiolate. Species specific antigens could be detected by IA.

Spirilla were shown by Lebedewa (1928) to adhere to mouse platelets in the presence of mouse antibody.

3. Bacteria

In one of the first reports of IA to platelets Levaditi (1901) described the adhesion of *V. cholerae* to platelets in the blood stream of immune rabbits. Other organisms were shown to adhere *in vivo* to platelets

of various species; these included staphylococci, streptococci, typhoid and paratyphoid bacilli, pneumococci, and *Vibrio metschnikovii* (Govaerts, 1921; Bull and McKee, 1922; Taniguchi *et al.*, 1930). Adhesion *in vitro* of bacteria to platelets was reported with a large number of Gram-positive and Gram-negative organisms (Govaerts, 1920a, 1922; Le Fèvre de Arric, 1920; Aynaud, 1911, Roskam, 1921; Houlihan and Copley, 1946). Contrary findings with some bacteria, even in the presence of putatively immune serum, were reported by others (Richter, 1921; Kritschewsky and Tscherikower, 1926a; Lebedewa, 1928). Bacteria which have been reported to undergo IA with human erythrocytes include staphylococci, pneumococci, *Erysipelothrix rhusiopathiae*, *E. coli*, *Salmonella typhosa*, *Shigella flexneri*, *Vibrio el tor*, *Bacillus megaterium*, and *Mycobacterium tuberculosis* (Kourilsky and Piéron, 1957; Kourilsky *et al.*, 1956, 1957a,b,c; R. A. Nelson, 1953, 1956; D. S. and R. A. Nelson, 1959; R. A. and D. S. Nelson, 1959; Piéron and Barreto, 1959; Piéron and Kourilsky, 1958; Turk, 1959a). The failures reported by early workers may have been due to their use of citrated or oxalated plasma, complement activity being limited with low levels of calcium ions. The antigen involved in IA of pneumococci is the type specific capsular polysaccharide. The responsible staphylococcal antigen was found to be distinct from an agglutinogen, being resistant to the action of papain (Kourilsky *et al.*, 1956). The antigen of tubercle bacilli was heat stable, resistant to papain, present in several strains (H37Rv, BCG, bovine strains, antibiotic resistant human strains) as well as in *Mycobacterium phlei*, and according to inhibition studies was related to tuberculin and to an "antigène méthylique" (Piéron and Kourilsky, 1958). Piéron and Barreto (1959) remarked that heat, proteolytic enzymes, and bile salts had no effect on those antigens of *E. coli* and *B. megaterium* responsible for their participation in IA.

4. Other Particulate Antigens

Various inorganic particles have been observed to adhere *in vivo* and *in vitro* to platelets in reactions analogous to IA. These include India ink, colloidal silver, and quartz particles (Dudgeon and Goadby, 1931; Govaerts, 1921; Tait and Elvidge, 1926; Wright, 1927). Erythrocytes of foreign species injected into rabbits and guinea pigs adhere to circulating platelets, presumably by IA mediated by natural hemagglutinins. IA is also probably involved in the reaction described by Swisher (1956) in which canine group A erythrocytes adhere to platelets when transfused into dogs having anticanine A antibody. R. A. and D. S. Nelson (1959) and Siqueira and Nelson (1961) demonstrated

A of sensitized sheep erythrocytes to washed guinea pig platelets in the presence of complement. Other particulate antigens which can participate in IA *in vitro* are starch granules and zymosan granules (R. A. Nelson, 1956, 1958a).

Human cells can also react as antigens on IA. Brody and Finch (1961) and Brody (1962) demonstrated IA of normal and leukemic human lymphocytes in the presence of complement with rabbit antibody, Coombs-positive sera, or eluates from Coombs-positive erythrocytes. Högman (1962b) described "serological adhesion" of cultured human fetal kidney and lung cells to human erythrocytes.

5. *Rickettsiae and Viruses*

R. A. Nelson (1956) and R. A. and D. S. Nelson (1959) refer to IA occurring with *Rickettsia burneti*, vaccinia virus, and poliovirus, but give no details. An extensive study of virus IA was made by Taverne (1957) using T2 phage. In the presence of antibody and complement, the radioactivity of S^{35} -labeled phage was shown to become associated with human erythrocytes. In hemagglutination assays of IA, 5×10^6 plaque-forming units of phage gave a positive result. The hemagglutination technique, which overcomes the difficulty of detecting IA with submicroscopic antigens, has also been applied to the detection of plant viruses such as cauliflower mosaic virus by D. S. Nelson and Day (1963), either crude plant sap or purified virus being used as a source of antigen.

6. *Soluble Antigens*

The use of soluble antigens in IA has also followed the development of hemagglutination assays. Turk (1958) used polysaccharides of *Shigella flexneri* and *Salmonella typhosa*, diphtheria toxoid, and ovalbumin. The sensitivity of the reaction was emphasized in this study, as little as 0.005 μg . of ovalbumin giving a clearly positive reaction. The present writer has found even 0.001 μg . of human serum albumin to be detectable. The failure of IA with a streptococcal polysaccharide and one sample of diphtheria toxoid was attributed by Turk to deficient complement fixation. Pontecorvo (1959) reported the detection by IA of natural antibodies to various soluble antigens including bacterial polysaccharides. Soluble antigens (ovalbumin, dextrans, lipopolysaccharides of *S. typhosa*) react in IA with animal platelets in the presence of antibody and complement (R. A. and D. S. Nelson, 1959; Siqueira and Nelson, 1961).

B. ANTIBODY

In some studies no mention is made of the necessity for antibody to induce adherence of bacteria to platelets (e.g., Aynaud, 1911; Delrez and Govaerts, 1918; Houlihan and Copley, 1946). In the light of later work it seems probable that natural antibody was responsible; Govaerts (1920a) in fact suggested that "opsonins" were required. A reaction analogous to direct bacterial hemagglutination (discussed later) may also have been responsible. It was, however, shown quite early that antibody was required for the adherence of *V. cholerae* to platelets *in vivo* (Levaditi, 1901) and of trypanosomes to leucocytes (Laveran and Thiroux, 1911). Kritschewsky and others investigated the nature of the antibodies involved in the Rieckenberg reaction, which they termed "thrombocytobarins." Some of these experiments indicated the presence of thrombocytobarins in normal sera. More recent work has shown that both natural and immune antibodies can sensitize antigens for their participation in IA. The properties of immune antibodies will be considered first.

1. Immune Antibodies

The fixation of antibody to the antigen is the first step in the sensitization of that antigen for its participation in IA; neither the nature of the antibody nor the antigen-antibody bond seems to differ from those involved in other antigen-antibody reactions. The antibodies are heat stable (56°C. for 30 minutes) γ -globulins which can react with the antigens at low temperatures; they can be formed by humans, rabbits, guinea pigs, horses, rats, and mice though perhaps not by frogs (Schwartzmann, 1927). The specificity of the antibodies produced in response to infection or immunization with trypanosomes was commented on by many early workers, though cross reactions have not subsequently been investigated extensively. Linscott (1961) studied the cross reactions in IA of rabbit antisera to various species of *Leptospira* and found these to be only limited. Antibody to *L. canicola* reacted to high titer with *L. ballum* and *L. icterohaemorrhagiae* but in other cases there was a tenfold or greater difference between the titers of the antibody to the homologous and to the cross-reacting antigen. Although the antibody inducing IA of *Treponema pallidum* is found, like the immobilizing antibody, only in the sera of patients with syphilis and not in the sera of other patients (Daguet, 1956a,b; Miller *et al.*, 1957; Olansky *et al.*, 1954), its reactions with other spirochetes have not been investigated. Slightly more information is available concerning the relation of IA antibodies to other antibodies. This was the subject of

some controversy in early investigations of the Rieckenberg reaction with spirochetes of relapsing fever. The "spirochetolysins" were considered to be distinct from thrombocytobarins because of the appearance of the former in some species (rat, guinea pig) in the absence of the latter and the failure of some sera to kill spirochetes while inducing their adherence to platelets (Kritschewsky and Tscherikower, 1926a,b; Krantz, 1926). Adherence sometimes failed to occur in the presence of powerful spirochetolysins (Kritschewsky and Lebedewa, 1927). An analogous situation was held to exist with trypanosomes in that the presence of thrombocytobarins was sometimes associated with reinfection with the same strain (Brussin and Rubinstein, 1925; Kritschewsky and Heronimus, 1927). Other workers considered spirochetolysins or trypanosomes agglutinating antibodies to be identical with thrombocytobarins (Aristowsky and Schaechter, 1928; Regendanz and Jurikoff, 1930). Such contradictory reports highlight the deficiencies of many early studies on platelet adhesion. The presence of oxalate or citrate ions, which bind calcium, reduces complement activity; the quantity of antigen used was not standardized; and in the absence of quantitative data interpretation of results is difficult. In more critical experiments on IA with soluble antigens, Turk (1958) demonstrated the existence of a prozone with excess antigen or antibody, just as there is in agglutination, precipitation, or complement-fixation tests and herein might lie the explanation for the contradictory findings just mentioned. Similar criticisms may be made of the work of Kourilsky *et al.* (1956) and Kourilsky and Piéron (1957) who found no relation between staphylococcal agglutinins, antitoxins, and IA antibodies in human sera; immunization with staphylococcal vaccine and toxoid raised the antitoxin but not the IA titer. In measuring IA antibodies they used the same serum as a source of both antibody and complement, in which case the end point of the titration might well be limited by the complement available, rather than by the antibody. Other evidence, cited earlier, suggested that the agglutinin and the IA antigen were distinct. The question of the relationship between IA antibodies and other antibodies remains open. One would strongly suspect that any antibody which, in combination with antigen, fixes complement appropriately will induce IA. When intact organisms are used, it is probably necessary for the antigen(s) to be present on the surface.

The sensitivity of IA has become apparent only recently. D. S. and R. A. Nelson (1959) could detect as little as 0.005–0.01 μg . antibody nitrogen per milliliter of serum in IA tests with pneumococci and human erythrocytes. Siqueira and Nelson (1961) found that 0.02 μg . antibody

nitrogen could give positive reactions with soluble antigens and platelets. R. A. Nelson (1962b) more recently still suggested that in some assays as little as 0.0005 µg. antibody nitrogen was detectable. This is of the same order of sensitivity as passive hemagglutination (Stavitsky, 1954) and passive cutaneous anaphylaxis (Ovary, 1959) and rather more sensitive than either precipitin or complement-fixation tests. Such sensitivity is also stressed by Linscott (1961) who found much higher titers of antibody to leptospire in IA than in microscopic agglutination tests. This sensitivity also permitted the detection of antibody in rabbits as early as 48 hours after immunization with *Leptospira canicola*.

One early controversy deserves passing mention. Kritschewsky and Tscherikower (1925) comment on the absence of thrombocytobarin from serum prepared from clotted blood as opposed to plasma or serum from defibrinated blood. Mention is made of this in later papers from the same group but others did not find this deficiency (Krantz, 1926; Davis and Brown, 1927), and serum from clotted blood has been used routinely in most experiments.

2. Natural Antibodies

The absence of thrombocytobarin for trypanosomes from normal sera of many species was commented on by Cerikover and Trivus (1926) though Regendanz and Jurikoff (1930) found "nonspecific" adherence to be induced by human sera. Kritschewsky and Tscherikower (1926b) found no thrombocytobarin to *Spirocheta icterogenes* (*Leptospira icterohaemorrhagiae*) in normal rat and mouse sera, nor did Lebedewa (1928) find such activity against various bacilli, cocci, spirilla, or vibrios in normal mouse serum. The relatively crude *in vitro* techniques employed would preclude the detection of small amounts of antibody.

The investigations by Pillemer and his group of the "properdin system" (Pillemer *et al.*, 1954) stimulated interest in natural antibodies. The finding by R. A. Nelson that normal serum induced IA of zymosan granules was an initial step in his reinterpretation of properdin activity as being due to low levels of natural antibody acting in conjunction with complement (R. A. Nelson, 1958a). By means of IA, the sera of unimmunized and uninfected animals have been shown to contain antibodies to many particulate and soluble antigens, for example, staphylococci, *Escherichia coli*, *B. megaterium*, *S. typhosa*, *Erysipelothrix rhusiopathiae*, *M. tuberculosis*, zymosan and starch granules, leptospire, and bacterial lipopolysaccharides (Kourilsky *et al.*, 1956; Linscott, 1961; Piéron and Barreto, 1959; Piéron and Kourilsky, 1958; Turk, 1959a). Turk (1959a,b) has investigated the nature of the serum factors in-

volved. The antibodylike activity to starch granules and several bacteria was associated with Cohn Fraction III_{1,2,3}+I, comprised largely of β -globulins, a finding confirmed by the writer (D. S. Nelson, 1957). Ultracentrifugally the globulins had a sedimentation coefficient of S_{20} 6.5. The activity was in most cases destroyed by heating to 56°C. for 20 minutes or by cysteine treatment. These characteristics may be contrasted with those of immune antibodies which are heat-stable γ -globulins found in Cohn Fraction II and having sedimentation coefficients of S_{20} 7 or S_{20} 19 (Fahey, 1960). Turk also attempted to determine the specificity of these antibodylike activities in normal human, rabbit, and guinea pig sera, using as antigens *Salmonella typhosa*, *Shigella flexneri*, *Escherichia coli*, *Vibrio el tor*, *Staphylococcus aureus*, *Erysipelothrix rhusiopathiae*, and starch granules. The IA antibody activity of the sera was measured before and after (a) heating to 56°C. for 20 minutes; and (b) two absorptions of 0°C. with 100 mg. zymosan per milliliter of serum. With the exception of *E. rhusiopathiae* (guinea pig) and *S. flexneri* and starch (rabbit) the titers were greatly reduced by both these treatments. The activity of human serum was greatly reduced by heating but only slightly by zymosan absorption. The activity of human and guinea pig serum was found in both euglobulin and pseudoglobulin fractions but only that in the euglobulin was removed by heating. Absorption of the sera with the bacteria or with polysaccharides reduced their activity against all the other bacteria except *E. rhusiopathiae*. Linscott (1961) also noted the instability to heat of natural antibodies to leptospire. In both these series of experiments, the heat lability was not due to loss of complement activity, since an additional source of complement (free of antibody) was present in the reaction mixtures. Turk interpreted his results as indicating the presence of both a specific or "classical" heat-stable antibody present in the euglobulin fraction and a nonspecific heat-labile "properdin-like" substance present in the pseudoglobulin fraction. It seems unnecessary to postulate a different mechanism for the non-specific activity, particularly in view of the reinterpretation of the properdin system by R. A. Nelson (1958a) supported on the basis of other data by Muschel and his co-workers (Muschel *et al.*, 1958; Muschel, 1960; Osawa and Muschel, 1960). Such activity may be interpreted as due to complement-fixing antibody either of very broad specificity or reacting with antigenic determinants common to many bacterial and other polysaccharides. Its physical characteristics, such as heat lability and electrophoretic mobility, may be merely a reflection of the lack of a high degree of specific folding of the globulin molecule in response to particular antigens.

Natural antibodies to *Candida albicans* were found by Brody and Finch (1960) to be present in the sera of most humans, rising from low titers in infants to high titers (1:4096) in adults. The titers were unaltered in patients with lymphomas before or after X-ray therapy or treatment with cytotoxic drugs. These findings illustrate both the high level of activity of such antibodies and their continued formation despite considerable insults to the antibody-producing system.

Blood group isoantibodies may be considered as a special group of natural antibodies. Högman (1962b) and Fjellström and Högman (1962) showed that such antibodies, together with human complement, could sensitize cultured cells of human fetal lung and kidney for adherence to human erythrocytes. Rarely, sensitization could be effected by thermostable serum substances lacking blood group specificity. Högman and Killander (1962) further showed that anti-A antibody could be separated chromatographically into a 7 and a 19 S fraction and that either could sensitize cultured human cells.

3. *Miscellany*

Antibodies involved in IA reactions have been shown to be absent from newborn mice (Messik, 1927) but to be present in newborn humans (Piéron and Kourilsky, 1958), newborn calves, and suckling rabbits (Turk, 1959a,d) and in mouse milk or colostrum (Inoue, 1930). They have also been shown to manifest their activity after passive transfer of serum to nonimmune animals (Jakimow, 1928; Kritschewsky and Brussin, 1931).

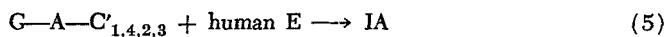
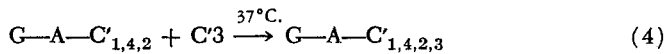
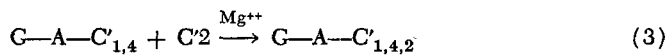
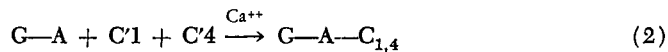
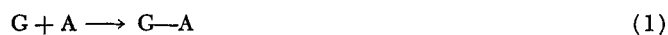
C. COMPLEMENT

Kritschewsky and Tscherikower (1925) showed that in addition to antibody a heat-labile factor present in normal mouse or guinea pig serum was essential for the Rieckenberg reaction. This was confirmed by Jakimow (1928), Inoue (1930), and Brussin and Kalajev (1931) and the factor was considered to be complement. The red-cell adhesion described by Duke and Wallace (1930) was shown by Wallace and Wormall (1931) and by Brown and Broom (1938) also to require a heat-labile factor present in normal serum and susceptible to the action of ammonia, carbon dioxide, cobra venom, and filtration through a Berkefield candle; this was again presumed to be complement. Definite evidence that complement is essential for IA to primate erythrocytes or nonprimate platelets came from the work of R. A. Nelson (1953), R. A. and D. S. Nelson (1959), and Siqueira and Nelson (1961), showing that serum from which complement had been removed by absorption with a hetero-

logous antigen-antibody complex would not induce IA of antibody-sensitized antigens.

IA has been shown to be a very sensitive measure of complement activity. Although different numerical titers for complement activity are obtained in different assay systems based on IA, there is agreement on certain basic findings. Guinea pig complement is active in IA with human erythrocytes at greater dilutions than in hemolytic assays. Human complement is as reactive as guinea pig complement in IA; this contrasts markedly with the relative reactivity of these two sera in hemolytic assays. Greater latitude is thus allowed for the measurement of changes in activity in disease states (see Section VII, B) (R. A. and D. S. Nelson, 1959; Woodworth, 1962a,b). Rabbit, horse, and pig sera are also very active, though mouse serum is not (R. A. Nelson, 1953; Turk, 1959c).

The components of complement involved in IA have also been investigated. Initial studies by R. A. Nelson (1956) suggested that the fixation of complement (C') followed a sequence similar to that proposed by Mayer *et al.* (1954) for the fixation of complement to sensitized sheep erythrocytes (E):



Subsequent experience (R. A. and D. S. Nelson, 1959; Siqueira and Nelson, 1961; Woodworth, 1962b) seemed to bear out this contention, as least for the first three stages. Calcium and magnesium ions were shown to be required for the sensitization of antigen-antibody complexes by complement for IA, whereas heat (56°C. for 30 minutes) treatment, which destroys C'1 and C'2, and ammonia treatment, which destroys C'4, both inhibited the complement activity of serum in IA. The low activity of mouse serum has been attributed to a deficiency of C'2 (Turk, 1959c) and the failure of some soluble antigens to participate in IA to the failure of their complexes with antibody to fix adequate amounts of C'2 (Turk, 1958). The requirement for C'2 of human complement has recently been questioned by Fjellström and Högman (1962). The component preparations were made by a new method of gel filtration, and it is not at the moment possible to assess

their relationship to classical complement components. Their work also suggested that the 11 S component described by Müller-Eberhard and Kunkel (1961) was required whereas the " β_1C " component of Müller-Eberhard and Nilsson (1960) was not.

A requirement for C'3 seemed to be indicated by the finding that only lysed sheep erythrocytes adhered to platelets in the presence of complement and that inhibitors of C'3 fixation, such as formaldehyde and salicylaldehyde, both inhibited the activity of complement in IA (S. E. Mills and Levine, 1959; Siqueira and Nelson, 1961). On the other hand, treatment of antigen-antibody complexes with complement at 0°C. (D. S. Nelson, 1957) or at 1-5°C. (Linscott and Boak, 1961), followed by washing at 0°C. to remove serum, yielded complexes which theoretically contained no C'3 but which nevertheless reacted in IA. More light has recently been thrown on this aspect of complement fixation by the work of R. A. Nelson, Linscott, and Nishioka (R. A. Nelson, 1962b; Linscott, 1962). They have found that C'3 is composed of three subcomponents—one heat-labile (C'3a) and two stable at 56°C. (C'3b and C'3c). All three are essential for immune hemolysis but only C'3c is essential for IA. Guinea pig C'3c is fixed to G—A—C'_{1,4,2} complexes at 0°C. but human C'3c is inactive at this temperature. In both human and guinea pig sera C'3a and C'3b are fixed almost simultaneously at 37°C.¹ In addition to explaining the apparent aforementioned contradictions, these findings have important implications for the study of complement by classical hemolytic techniques. For example, the standard methods for the preparation of EAC'_{1,4} and EAC'_{1,4,2} result in a high proportion of reactive sites being in the state EAC'_{1,4,2,3c}. The relationship of C'3a, C'3b, and C'3c of Nelson, Linscott, and Nishioka to C'3a and C'3b of Amiraian *et al.* (1958) and Rapp *et al.* (1959) and the two C'3 components of Hawkins (1960) and Hawkins and Haurowitz (1962) is not at the moment clear.

Turk (1959c) investigating complement components required for IA found that a combination of zymosan treated (?R3) and ammonia treated (R4) serum was ineffective as a source of complement. Somewhat similar results had been reported earlier by Brown and Broom (1938). Turk postulated the existence of a further hydrazine or ammonia sensitive cofactor required for IA, resembling a factor described by

¹ More recently, Nishioka and Linscott suggest the presence of a fourth subcomponent of guinea pig C'3, namely C'3d. The complex reacting in IA is believed to be G—A—C'_{1,4,2,3c}, whereas the sheep erythrocyte undergoing immune hemolysis is EAC'_{1,4,2,3c,b,a,d} (E* of Mayer *et al.*, 1954; Nishioka, 1962; Nishioka and Linscott, 1963; Linscott and Nishioka, 1963).

Pensky *et al.* (1958) as being involved in the inactivation of C'3 by the properdin system. This may be related to C'3c.

Finally, in regard to the involvement of complement in IA, it is interesting to note that bovine conglutinin inhibits the reaction regardless of the species of complement used (Turk, 1959c).

D. INDICATOR PARTICLES

True IA has so far been shown to occur only with primate erythrocytes and nonprimate platelets, though it seems likely that other cells may also be shown to react. Certain reports of adhesion of sensitized antigens to other particles are open to the criticism that the sera used probably contained antibody to the particles, thus allowing the occurrence of complement-dependent mixed aggregation (discussed later).

1. Erythrocytes

Wallace and Wormall (1931) showed that primate (human, monkey, and baboon) erythrocytes reacted in the red-cell adhesion test of Duke and Wallace and could not be replaced by erythrocytes of calf, goat, hen, guinea pig, lizard, or mouse. R. A. Nelson (1953) found rabbit and sheep erythrocytes to be unreactive in IA. Subsequent work has confirmed the nonreactivity of erythrocytes of rabbit, fetal rabbit, guinea pig, horse, cow, sheep, dog, and chicken (Kourilsky *et al.*, 1957 a,b,c; D. S. and R. A. Nelson, 1959; Pautrizel *et al.*, 1957). On the other hand, Högman (1962b) claimed that some rabbit and guinea pig erythrocytes were "weakly reactive" with cultured human cells sensitized with antibody and complement. The lysis of erythrocytes by water does not alter their reactivity or nonreactivity (D. S. and R. A. Nelson, 1959). The reactivity of lysed nonprimate erythrocytes reported by Lamanna and Hollander (1956) was probably due to complement-dependent mixed aggregation between the sensitized antigen and erythrocytes lysed by natural antibody and complement in the sera present in the reaction mixture. Human erythrocytes of any blood group are reactive. Some investigators have found all normal human erythrocytes to be reactive (Kourilsky and Piéron, 1957; D. S. Nelson, 1957) but Brown and Broom (1938) reported some to be unreactive—the reason for this is not clear, but may be related to technical deficiencies in their method of carrying out the reaction. Turk (1962) found two of over one hundred samples of human erythrocytes to be unreactive. No defect in the pattern of surface antigens was detected, and the family of one subject all had reactive erythrocytes. Reactivity is retained on storage of blood for at least 1 month (Daguet, 1956a). Erythrocytes from patients with certain

diseases have been reported to be unreactive. These diseases include hemolytic anemia with autoantibodies (Kourilsky and Piéron, 1957), staphylococcal septicemia (R. A. Nelson, 1962a), and some cases of rheumatic fever and tuberculosis (Brown and Broom, 1938). The reactivity of normal human erythrocytes may be abolished by treatment with trypsin, tannic acid, papain, chymotrypsin, formaldehyde, and crude staphylococcal α -toxin, but not with receptor destroying enzyme of *Vibrio cholerae* or with periodate (Kourilsky *et al.*, 1955; D. S. and R. A. Nelson, 1959; R. A. Nelson, 1962b).

2. Platelets

Platelets from many species were shown to participate in the Reickenberg reaction. These included mouse, rat, guinea pig, rabbit, and dog platelets (Inoue, 1930; Kritschewsky and Tscherikower, 1925, 1928; Rieckenberg, 1917). Aristowsky and Schaechter (1928) and Dudgeon and Goadby (1931) found human platelets to be unreactive. The adhesion of sensitized antigens to human platelets, described by Lamanna and Hollander, was probably due to complement-dependent mixed aggregation. The mixed agglutination of human platelets and bacteria described by Houlihan (1947a) appears to have been due to the non-specific entrapment of bacteria among spontaneously agglutinated platelets. The reactivity of guinea pig and rabbit platelets and the non-reactivity of primate platelets in IA was confirmed by R. A. and D. S. Nelson (1959) and Siqueira and Nelson (1961) in repeated assays. IA to guinea pig platelets was detectable with smaller quantities of antigen and antibody than IA to rabbit platelets. Heat (56°C. for 15 minutes) or trypsin treatment abolished the reactivity of guinea pig platelets. Roskam (1921), however, claimed that the reactivity of rabbit platelets persisted after heating to 56°C. for 1 hour as well as after treatment with distilled water or 1% sodium fluoride.

3. Other Body Cells

Platelets could be replaced in the Rieckenberg reaction by megakaryocytes of mice or guinea pigs or by spindle-shaped cells ("thrombocytes") of fowls and other animals not possessing true blood platelets (Kritschewsky and Tscherikower, 1925; Grünbaum, 1928). Lymphocytes of guinea pig, rabbit, and dog were found to be unreactive in IA (Siqueira and Nelson, 1961). Sensitized trypanosomes were reported by Levaditi and Mutermilch (1910) not to adhere to liver and kidney cells, but to adhere to bone marrow, spleen, and lymph node cells of unspecified type.

The nature of the adherence of sensitized antigens to polymorphonuclear or mononuclear phagocytes is still unclear. This has been described by Laveran and Mesnil (1901), Laveran and Thiroux (1911), Levaditi and Mutermilch (1910), Manwaring and Coe (1916), Regendanz and Jurikoff (1930), Davis and Brown (1927), and Raffel (1934). *In vitro*, adherence occurred in the presence of heated serum, suggesting that complement was not involved and that the reaction was therefore not true IA. However, enough fresh serum may have been present in the leucocyte suspensions or in the tissue to provide the requisite complement. Cells killed by heat or cold reacted as well as live cells. When live cells were used, sensitized trypanosomes were ingested after adherence. It seems very likely that adherence of an antigen to a phagocyte should precede its ingestion. Bessis (1961) has produced evidence from electron microscopy that this is so, whereas Vaughan and Boyden (1962) have observed the adherence of antibody-sensitized erythrocytes to guinea pig macrophages cultured *in vitro*. This type of adherence may well be mediated by a mechanism similar to that of IA to erythrocytes and platelets. Indeed, R. A. Nelson (1962b) refers, but without detail, to experiments suggesting that phagocytes possess a substance similar to the postulated IA receptor of human erythrocytes. Further circumstantial evidence of a relationship between IA and the clearance of antigens by the reticulo-endothelial system (RES) *in vivo* is considered later (Section VI, C).

Adherence to other tissue components, dependent on the presence of antibody, is suggested by the work of Rich (1933) and Rich and McKee (1934). They found that Type I pneumococci injected intradermally into immune rabbits were localized for some time to the site of injection. When sections were examined microscopically, the bacteria seemed to be adherent to the tissues. This did not occur in the absence of antibody. *In vitro*, pneumococci adhered to fibrils of connective tissue only in the presence of antibody. The role of complement was not studied. The possible significance of these observations is further considered below.

4. Other Indicator Particles

The adherence of antigens to bacteria in the presence of antibody and complement has been demonstrated frequently although not consistently (Davis and Brown, 1927; Kritschewsky and Tscherikower, 1928; Inoue, 1930; Brussin and Kalajev, 1931; Lamanna and Hollander, 1956). These reactions were shown by R. A. and D. S. Nelson (1959) to be examples, not of IA, but of complement-dependent mixed aggregation, occurring only in conditions in which antibody to the indicator

particles would be present, as well as antibody to the antigen in question. Similar considerations probably apply to adhesion to inorganic particles such as silica and quartz (Lamanna, 1957).

E. CONDITIONS FOR THE REACTION

The conditions governing the occurrence of IA can best be studied by the use of washed antigen-antibody-complement complexes, which adhere to indicator particles in the absence of serum (e.g., in saline suspensions). The formation of the bond between antigen-antibody-complement complexes and human erythrocytes requires no cofactors, adherence occurring efficiently between washed complexes and erythrocytes suspended in physiological sodium chloride solution. The adherence of preformed complexes to platelets in the presence of serum is inhibited by ethylenediamine-tetraacetate (EDTA) (Siqueira and Nelson, 1961). However, experiments on IA to washed platelets have been carried out only in media containing calcium and magnesium ions and it is not known whether such inhibition is due to the chelation of divalent cations or to some other action of EDTA. The effect of EDTA on the adherence of preformed complexes to erythrocytes has not been investigated. The question remains open whether IA to primate erythrocytes and IA to nonprimate platelets differ in this regard. It should be remembered that the nonspecific "stickiness" of platelets requires calcium and magnesium ions (Garvin, 1961).

It has been shown that IA of washed complexes to human erythrocytes occurs almost instantaneously at 37°C., more slowly at room temperature, and very slowly and incompletely at 0°C. (D. S. and R. A. Nelson, 1959; Linscott and Boak, 1961). Similar results were reported for erythrocytes by Taverne and for platelets by Siqueira and Nelson (1961), both of whom used unwashed complexes in the presence of serum. Washed complexes are stable, adhering to erythrocytes or platelets even after being heated to 56°C. for 30 minutes (Govaerts, 1920a; Linscott and Boak, 1961; Siqueira and Nelson, 1961). The adherence of washed complexes to human erythrocytes is partly inhibited in media of high ionic strength, for example 1.5 M sodium chloride (D. S. and R. A. Nelson, 1959) though the agglutination of platelets by IA of soluble complexes in serum is inhibited at lower ionic strengths (Roskam, 1922a; Siqueira and Nelson, 1961). This inhibition may be due either to some interference with the formation of the bond or, more likely, to the partial dissociation of the complexes at high ionic strengths.

An unconfirmed finding of Linscott and Boak (1961) suggests the

presence in some sera of a substance which can inhibit the attachment of washed complexes to erythrocytes at low temperatures (1–5°C.) but not at 37°C. This substance was found to be present in low concentration in fresh or heated guinea pig serum and in heated rabbit serum. Its nature has not been further investigated but it may conceivably be related to conglutinin (cf. Turk, 1959c), to the heat-stable factor in guinea pig serum which inhibits the hemolytic action of complement (Hawkins, 1961), or to the nonspecific inhibitor of complement postulated by Volkert (1960).

III. Methods for Detecting Immune Adherence

A. DIRECT MICROSCOPIC OBSERVATION

Immune adherence of antigens to erythrocytes is very easily detected by direct microscopic observation of a sample of the reaction mixture. This technique was used by D. S. and R. A. Nelson (1959) and by Woodworth (1962a,b) to detect and measure IA. Dark-field examination is most convenient when bacteria or other small particulate antigens are used but light microscopy is adequate for larger antigens such as starch granules. This is illustrated in Fig. 1, a dark-field photomicrograph showing a chain of pneumococci adherent to a normal human erythrocyte in the presence of antibody and complement. For the titration of antibody or complement an excess of erythrocytes over antigen particles is necessary for maximum sensitivity, the adherence of 50% of the antigen particles being taken as the end point. The error of counting with this technique is 6%. In investigating the reactivity of erythrocytes after various treatments, an excess of antigen particles over erythrocytes is necessary for valid results; in these conditions all normal erythrocytes in the reaction mixture have been found to have antigen particles adherent.

B. HEMAGGLUTINATION

Sensitized antigen particles can become adherent to two or more erythrocytes causing hemagglutination (Fig. 2). In a similar way soluble antigen-antibody complexes can induce hemagglutination. This is detectable by the formation of distinctive patterns after the erythrocytes have settled to the bottom of the tube, as in viral hemagglutination. This is the most convenient technique for the measurement of IA with viruses or soluble antigens (Taverne, 1957; Turk, 1958; D. S. Nelson and Day, 1963) but it is equally applicable when particulate antigens are used (Daguet, 1956b; D. S. and R. A. Nelson, 1959). It is a particularly sensitive technique, as has been pointed out previously

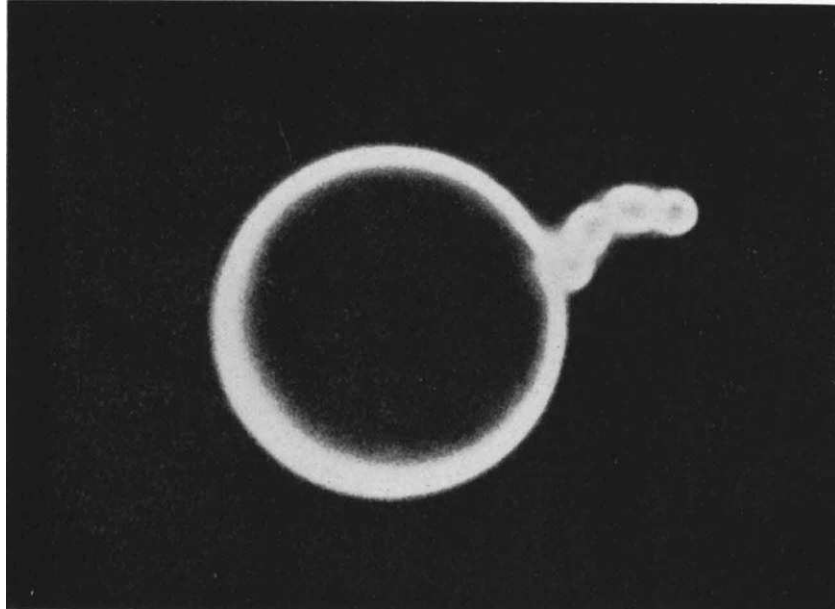


FIG. 1. A chain of *Diplococcus pneumoniae*, sensitized with antibody and complement, adherent to a human erythrocyte. Dark field. [From R. A. Nelson (1953); reprinted by permission of *Science*.]

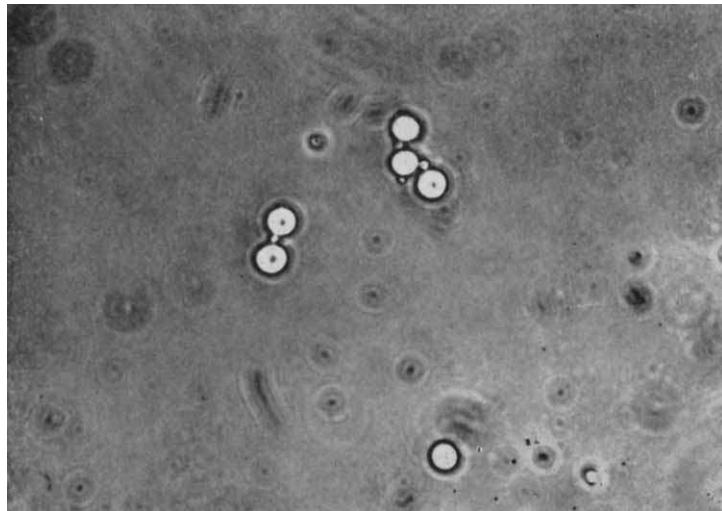


FIG. 2. Human erythrocytes agglutinated by immune adherence of starch granules sensitized with antibody and complement. Phase contrast.

(Sections II, A and B). The chief disadvantage is the instability of the patterns formed when soluble antigens or viruses are used; this has been noted by both Turk (1958) and D. S. Nelson and Day (1963). When low dilutions of heterologous serum are used as sources of antibody and complement, nonspecific hemagglutination may occur due to natural hemagglutinins in these sera; this is detectable in control mixtures lacking antigen.

R. A. Nelson (1962a) has pointed out that excellent hemagglutination patterns are produced by IA when the reaction is carried out in small plastic hemagglutination trays, with very small quantities of reagents. This technique is very convenient for the "screening" of large numbers of sera (e.g., to detect antibodies or to measure complement activity).

C. PLATELET AGGLUTINATION

This was first noticed by Govaerts (1920b) and later by Houlihan and Copley (1946) to occur *in vitro* as the result of adherence of sensitized bacteria. Macroscopically visible platelet agglutination due to IA has been investigated by Siqueira and Nelson (1961). It was found to occur when antigen, antibody, complement, and a washed platelet suspension were rotated in tubes at 8 r.p.m. for 60 minutes at 37°C. The best suspending medium was veronal buffer containing calcium and magnesium ions and gelatin. Clear and consistent results were obtained in the titration of antigen, antibody, and complement with either rabbit or guinea pig platelets. As with hemagglutination tests, nonspecific reactions may occur owing to the presence of natural anti-platelet antibodies in the sera used; these also are detectable in control mixtures lacking antigen.

D. CLEARANCE TECHNIQUES

With small particulate antigens that sediment much more slowly than erythrocytes this technique is of considerable value. In experiments which led to the recognition of IA, R. A. Nelson (1952a,b; 1953) first noted that light centrifugation of mixtures of erythrocytes, *Treponema pallidum*, antibody, and complement removed not only erythrocytes but also *T. pallidum* which had undergone IA. Counts of treponemes remaining in the supernatant fluid after centrifugation of test and control mixtures permit quantitative measurements of IA. The degree of IA is usually calculated as

$$\% \text{ adherence} = \frac{\text{count in control} - \text{count in test}}{\text{count in control}} \times 100$$

This clearance reaction has been used in the serological diagnosis of syphilis by Olansky *et al.* (1954) and Miller *et al.* (1957) and with leptospire by Linscott (1961) and Linscott and Boak (1961). For maximum sensitivity a high ratio of erythrocytes to antigen particles is necessary; Linscott and Boak used 150 erythrocytes per leptospire. The commonest pitfall inherent in this technique is the disappearance on centrifugation of agglutinated organisms which sediment rapidly in the absence of IA; this is detectable in control mixtures lacking complement.

Daguet (1956a) has used a variant of this technique, described as "adherence-disappearance," disappearance being caused by the phagocytosis of *T. pallidum* by polymorphonuclear leucocytes after IA to human erythrocytes. This technique seems unnecessarily complicated.

E. THE USE OF LABELED REAGENTS

When submicroscopic antigens such as viruses or soluble proteins are used in IA, the reaction may be detected by the use of labeled reagents as well as by hemagglutination. For example, Taverne (1957) studied IA with T2 bacteriophage labeled with S³⁵. In the presence of antibody and complement the phage adhered to human erythrocytes and after light centrifugation the radioactivity was found to be associated with the sedimented red cells. This is analogous to the clearance reactions except that the presence of the antigen in the sediment is measured rather than its absence from the supernatant. Again, non-specific reactions may occur because of the rapid sedimentation of agglutinated virus particles. No other studies have been made using this technique but it is applicable in theory to IA with other small antigens, using either labeled antigen or labeled antibody. Other labels than radioactivity could be used; for example a colored protein such as hemocyanin could be used as an antigen.

IV. The Differentiation of Immune Adherence from Other Adherence and Hemagglutination Reactions

A. ACID ADHESION

Both acid adhesion and complement-dependent mixed aggregation were described by Nelson and Nelson (see D. S. and R. A. Nelson, 1959; R. A. and D. S. Nelson, 1959) following investigations of the discrepancies between recent results with IA and the claims of Lamanna and Hollander (1956) and Lamanna (1957) that a very wide variety of indicator particles could be used to detect "serologic adhesion."

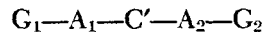
IA of washed antigen-antibody-complement complexes occurs constantly in media between pH 4 and 9, but below pH 5 to 5.5 both sensitized and unsensitized antigens adhere to a wide variety of indicator particles including erythrocytes of several nonprimate species, human and rabbit platelets, monkey kidney cells, and *Escherichia coli*. In further investigations of acid adhesion, with chicken erythrocytes as the indicator particle, it was found that different bacteria adhered to erythrocytes over different pH ranges. In some cases, for example with Type XXXI pneumococci, presensitization with rabbit antibody allowed adhesion at a higher pH, and with both antibody and guinea pig complement, at a higher pH still. In certain conditions, therefore, the reaction could mimic IA. In contrast with IA, adhesion occurred as rapidly as 0°C. as at 37°C. and was very easily inhibited in media of high ionic strength. Treatment of the erythrocytes with trypsin or tannic acid failed to prevent this type of adherence. The evidence favored the interpretation of this reaction as being due solely or mainly to an electrostatic bond between oppositely charged particles, the low isoelectric point of erythrocytes allowing them to remain negatively charged at pH's at which sensitized or unsensitized bacteria were positively charged.

A somewhat similar phenomenon was described by Broom *et al.* (1936). They found that the electric charge of trypanosomes varied from positive to negative and that positively charged forms adhered to erythrocytes in saline-glucose suspensions.

B. COMPLEMENT-DEPENDENT MIXED AGGREGATION

One divergence between the techniques used by R. A. Nelson (1953, 1956) and by D. S. and R. A. Nelson (1959) and those used by others, particularly Lamanna and Hollander (1956), was the use by the former of reaction mixtures freed of antibody to the putative indicator particle. This was done by (a) preliminary absorption with the particle in question of the sera used as sources of antibody and complement; or (b) dilution of such sera beyond the point at which natural antibody was active; or (c) the use of washed antigen-antibody-complement complexes. This suggested that some other forms of serological adhesion could depend on the presence of antibody to the indicator particle, a suggestion confirmed in several experiments (R. A. and D. S. Nelson, 1959). These revealed that (a) antibody-sensitized antigens (*Salmonella typhosa*, *T. pallidum*, a precipitate of human serum albumin with its rabbit antibody) did not adhere either to intact sheep erythrocytes or to sheep erythrocytes lysed by distilled water in the presence of comple-

ment free of antibody to the sheep cells; (b) such sensitized antigens adhered to antibody-sensitized sheep erythrocytes in the presence of complement, which also lysed the erythrocytes; (c) similar adherence occurred when sheep erythrocytes were sensitized by natural antibody present in unabsorbed guinea pig serum used as a source of complement, sensitization being revealed by lysis of the erythrocytes; (d) adherence did not occur when complement activity was removed or inhibited by absorption of serum with a heterologous antigen-antibody precipitate, by heating, or by the use of a chelating agent. These experiments showed that two antigen-antibody complexes can be united by complement. When both complexes are particulate a phenomenon very similar to IA is observed. This implies that complement or some component thereof is bivalent or polyvalent, i.e., a single molecule or group of molecules can be attached to two antibody-sensitized antigen sites. This may be analogous to the manner in which complement influences precipitin reactions (Maurer and Talmage, 1953a,b; Morton and Deutsch, 1956). Complement titers in this complement-dependent mixed aggregation are low, activity being lost in dilutions of human or guinea pig serum greater than from 1:8 to 1:16, in marked contrast with the high activity of these sera in true IA. The components of complement involved were not investigated, but the lack of reactivity of serum in the presence of EDTA or of heated serum suggested that at least C'1, C'4, and C'2 were involved. The hypothetical complex may be crudely represented as

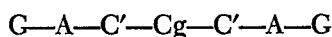


The reaction may also be demonstrated in mixtures of guinea pig serum and two bacteria to which natural antibody is present in that serum, for example *E. coli* and *Staphylococcus aureus*, in which case mixed agglutination occurs. This reaction is of some interest in connection with the old controversy over the occurrence of mixed agglutination by anti-sera (Mudd, 1933).

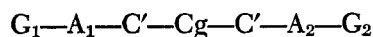
C. CONGLUTINATION

Conglutinin (Cg), the substance responsible for this reaction, was first detected in bovine serum by Bordet and Streng (1909). It was described as a protein which is resistant to heating for half an hour at 56°C. and which reacts with antigen-antibody-complement complexes to cause clumping or "conglutination" of the reactants. It has more recently been extensively investigated by Coombs and co-workers (see, for example, R. R. A. Coombs *et al.*, 1950; Blomfield, 1952; A. M. and

R. R. A. Coombs, 1953; A. M. Coombs, 1954; Marks and Coombs, 1957). The complex may be represented as



This appears to have no relation to IA in which antibody to the indicator particle plays no part. In fact, Turk (1959c) has noted that conglutinin actually inhibits IA. There are, however, obvious similarities to complement-dependent mixed aggregation. C'1, C'4, and C'2 are required for the reaction of conglutinin with sensitized antigens, though a requirement for C'3 has not been demonstrated. The low levels of conglutinin activity in guinea pig and human sera are similar to the low levels of activity of these sera in complement-dependent mixed aggregation. The latter reaction may in fact be due to conglutinin, in which case the complex may be represented as



Further evidence on this possible relationship is lacking.

D. MIXED AGGLUTINATION AND MIXED ANTIGLOBULIN REACTIONS

These reactions, too, have been described and investigated by Coombs and his co-workers. Mixed agglutination occurs when two cells sharing a common antigen are mixed with serum containing antibody to that antigen. For example, a group A human erythrocyte and another cell, such as a leucocyte, with blood group A substance at its surface can be caused to adhere to each other by human anti-A antibody. The resultant adherence resembles IA but the reaction differs from both IA and complement-dependent mixed aggregation in that complement is not required (R. R. A. Coombs and Bedford, 1955) as well as in more obvious ways.

The mixed antiglobulin reaction (R. R. A. Coombs *et al.*, 1956; Chalmers *et al.*, 1959) occurs when cells such as leucocytes, HeLa cells, or platelets coated with human incomplete antibody are mixed with similarly treated human erythrocytes in the presence of antihuman γ -globulin antibody. Again, the end result is adherence of the red cell to the other cell as in IA but the mechanism is different and complement is not required. The possibility that IA is occurring should, however, be borne in mind when such reactions are observed with primate erythrocytes or nonprimate platelets.

E. BACTERIAL ADHERENCE

Reiss *et al.* (1950), Moeschlin and Demiral (1952), and Hayes and Dougherty (1954) noted that certain lymphoid cells removed from

rabbits at the height of the immune response to a killed bacterial antigen would collect bacteria on their surface when incubated with that antigen. This phenomenon was also observed by Nossal (1959) with live motile bacteria and rat lymph node cells. Mäkelä and Nossal (1961) presented evidence that "bacterial adherence" occurred only with plasma cells producing antibody and was produced by a reaction between bacteria and cell-associated antibody. It is distinguished from IA by its occurrence with plasma cells only and its lack of requirement for complement.

F. OTHER HEMAGGLUTINATION REACTIONS

Hemagglutination patterns similar to those produced by IA may be produced in a variety of ways, all distinguished by a lack of requirement for complement. These include: the direct agglutination of erythrocytes by iso- and heterohemagglutinins and by plant, bacterial, and viral hemagglutinins (see review by Neter, 1956); the agglutination by antibacterial antibodies of erythrocytes directly sensitized with antigens of certain bacteria such as the antigens of tubercle bacillus (Middlebrook and Dubos, 1948; Boyden and Andersen, 1955) or *E. coli* (Neter, 1956); and the agglutination by antiprotein antibodies of erythrocytes to which protein antigens are coupled by tannic acid (Boyden, 1951), by diazotization (Stavitsky and Arquilla, 1955), or by combination with incomplete Forssman antibody (R. R. A. Coombs and Fiset, 1954).

There have been suggestions that the agglutination of sheep erythrocytes sensitized with incomplete antibody by the factor present in the serum of patients with rheumatoid arthritis (rheumatoid factor) may involve complement but such suggestions have received little support (Vaughan, 1959). In any case, the necessity for the pretreatment of sheep erythrocytes, or other indicator particles, with antibody or other γ -globulin distinguishes this reaction from IA, while suggesting a possible tenuous relationship with complement-dependent mixed aggregation.

G. THE AGGLUTINATION OF PLATELETS BY ANTIGEN-ANTIBODY COMPLEXES

Miescher and Cooper (1960) have described the agglutination of rabbit platelets *in vitro* in the presence of soluble complexes of ovalbumin and rabbit antiovalbumin antibody. This reaction seems, superficially, to resemble that described by Siqueira and Nelson (1961), which is due to IA. The reaction described by Miescher and Cooper, however, did not require complement and was only observed when large amounts of antibody were used (3.9 mg. antibody protein in a reaction mixture). The relationship between the two reactions is not clear, though they appear to be distinct.

H. THE RELATIONSHIP OF IMMUNE ADHERENCE TO OTHER FORMS OF SEROLOGICAL ADHESION

In the preceding sections, IA has been differentiated from several other serological reactions which can produce similar end results. It remains to point out in more detail the distinction between IA and the varieties of serological adhesion described by Lamanna and Hollander in which complement is also required. Both the Rieckenberg reaction and the red-cell adhesion of Duke and Wallace are clearly examples of IA, since they occur with nonprimate platelets and primate erythrocytes, respectively, in the absence of antibody to the indicator particle and in the presence of antibody to the particular antigen and of complement. It seems most likely that adhesion to primate platelets, nonprimate erythrocytes, bacteria, and inorganic particles is caused by complement-dependent mixed aggregation. Close examination of the experimental protocols of Lamanna and Hollander (1956) and earlier workers reveals that such adhesion occurred only when the presence of natural antibody to the indicator particle was not eliminated by one of the methods already described (Section IV, B). Natural antibodies to bacteria are widespread and often present to high titer in many animal sera (cf. Turk, 1959a; R. A. Nelson, 1958a) as also are natural antibodies to erythrocytes or platelets of other species. Adherence of sensitized treponemes to human platelets and to lysed sheep erythrocytes was detected by Lamanna and Hollander in the presence of guinea pig serum, the lysis apparently being due to natural antibody and complement in that serum. The high concentration of guinea pig serum required for this type of serological adhesion (activity was lost on dilution of the serum 1:10) is in marked contrast with the small amount of complement required for IA and is similar to that required for complement-dependent mixed aggregation. The loss of activity at low dilutions may also have been owing in part to the loss of natural antibody activity by dilution. The failure of some workers (Inoue, 1930; Pandit *et al.*, 1929; Brussin and Kalajev, 1931) to induce this type of adhesion to bacteria may be attributable either to the absence of natural antibody to these bacteria or to the low complement activity to be expected when calcium-binding agents such as oxalate or citrate are included in the reaction mixture. The adherence of trypanosomes to erythrocytes of rats, rabbits, mice, horses, and mules, noted by Raffel (1934) and Taliaferro and Taliaferro (1934) occurred only when the reaction mixture contained blood or serum of another species.² Serological adhesion to inorganic particles

² On the basis of finding weak reactivity of some rabbit and guinea pig erythrocytes toward washed, sensitized human cells in culture, Högman (1962a,b)

may also be explained on this basis. Lamanna (1957) comments on the presence in guinea pig serum of an agglutinin for silica particles. Suggestive evidence that other inorganic particles such as bentonite and cellulose react with serum components (P antibody) to fix complement has come from studies on chemotaxis (Boyden, 1962a,b,c) and phagocytosis (Potter and Stollerman, 1961). The fixation of complement by aggregated γ -globulin has been demonstrated by Ishizaka *et al.* (1961). Even substances that adsorb γ -globulin nonspecifically may aggregate it and fix complement, thus becoming suitable for participation in complement-dependent mixed aggregation.

Lamanna and Hollander (1956) suggested that serological adhesion of this type might be used to detect antibody to specific antigens. This has occasionally been done in clinical laboratory practice (for example, Brown, 1935) but it should be emphasized that such techniques are much less suitable for this purpose than IA. Natural antibody to the indicator may not be present in all sera, though immune antibody might be added to the reaction mixture. The chief disadvantage appears to be the requirement for a high concentration of the serum used as a source of complement. False positive reactions are therefore liable to occur owing to the presence in such sera of natural antibody to the antigen in question.

The relationship between IA and the adherence of sensitized antigens to phagocytes is discussed elsewhere (Sections IV, D and VI, C).

It can be concluded that immune adherence is a specific reaction, clearly distinguishable from several other adherence reactions giving similar results.

V. The Nature of the Bond

There was surprisingly little experimentation or even speculation by early writers on this aspect of adherence reactions. Rieckenberg considered that platelets from immune animals possessed the property of "stickiness" directed at the specific organism to which they were immune. This view soon became untenable in the light of the work of Govaerts, Kritschewsky, Brussin, and others showing that platelets from normal animals reacted as well as those from immune animals. Roskam (1922b) suggested that adherence was due to a layer of plasma at the surface of washed platelets rather than to a specific property of the platelets themselves, whereas Regendanz and Jurikoff (1930) considered that a

considers that the term serological adhesion should be retained for such reactions whose relationship to IA, as defined here, is not at present clear.

"tektin" or sticky substance produced by the interaction of antibody and antigen caused the adherence of trypanosomes and platelets.

Little or no evidence was produced for any of these concepts. More recent work has provided data bearing on this question but the nature of the bond between sensitized antigen and indicator particle is still obscure. The studies of D. S. and R. A. Nelson (1959) quoted in the foregoing clearly differentiated IA from acid adhesion and indicated that the bond was not, primarily at least, electrostatic. Govaerts (1923) had previously noted that adherence of bacteria to platelets occurred despite the similar charge of the two reactants.

R. A. and D. S. Nelson (1959) considered the possibility that small amounts of autoantibody or other antibody on the surface of human erythrocytes might allow them to react as antigen-antibody complexes as do other indicator particles in complement-dependent mixed aggregation. Chaplin and Cassell (1960) have, in fact, shown that injection into rabbits of eluates from normal human erythrocytes gives rise to weak but definite immune responses, the antibody being an antiglobulin. Nelson and Nelson treated erythrocytes with 15% sodium chloride, which would be expected to dissociate this hypothetical antibody. After washing, the erythrocytes reacted in IA. This finding supports the other evidence that the two reactions are not related. Högman (1962b) also excluded the sensitization of human erythrocytes by cold autoantibodies as a factor in IA.

The fact that IA occurs very rapidly at 37°C., slowly at room temperature, and slowly and incompletely at 0°C. suggests a mechanism of attachment analogous to enzyme action. If this were the case one might expect that the bond would break on further incubation. The bond, once formed, is, however, extremely stable. Sensitized particles remain adherent to erythrocytes (D. S. Nelson, 1957) or to platelets (Krantz, 1926) for at least 24 hours at room temperature or 4°C. Krantz reported that adhesion of relapsing fever spirochetes broke down after 24 hours leaving free motile spirochetes which could no longer adhere to fresh platelets. Since this reaction was carried out in mixtures containing whole blood it is possible that the bond was broken by enzymes present in the plasma or released from dead cells, rather than breaking spontaneously. It is also possible that the union between antigen, antibody, and complement was disrupted. The attachment of sensitized *Leptospira canicola* to human erythrocytes was found by Linscott and Boak (1961) to resist at least four cycles of vigorous manual shaking and centrifugation of the reaction mixture, though some weakening of attachment was evident after further cycles. Nishioka (1962) observed

that after IA of vaccinia virus to human erythrocytes in the presence of serum containing antibody and complement, storage of the reaction mixture at 2–4°C. for 48 hours resulted in lysis of the erythrocytes. This finding has not been confirmed or extended and it is not clear whether lysis was due to IA alone or to additional serum factors acting on cells conditioned in some way by IA. It could perhaps be construed as evidence that an enzymatic mechanism operates in IA.

Whether the bond itself is due to an enzymatic action or not, it seems necessary to postulate the existence of a specific receptor on the surface of primate erythrocytes and nonprimate platelets but not on other cells. This receptor on human erythrocytes appears to be susceptible to the action of trypsin (0.01–0.5%), chymotrypsin (3 µg./ml.), papain, crude staphylococcal α -toxin, formaldehyde (0.12%), and tannic acid but not to that of 0.001% trypsin, periodate ions, or receptor-destroying enzyme of *Vibrio cholerae* and influenza virus (Kourilsky *et al.*, 1955; D. S. and R. A. Nelson, 1959; R. A. Nelson, 1962b). Trypsin (2 µg./ml.) treatment of platelets also destroys their reactivity in IA (Siqueira and Nelson, 1961). These results suggest that the receptor is protein in nature. It would seem likely that the receptor on nonprimate platelets and that on primate erythrocytes are identical. This implies the possibility that these two cell types share a common antigen, though there are no data relevant to this question. The failure of reactivity of erythrocytes from some patients with hemolytic anemia associated with autoantibody (Kourilsky and Piéron, 1957) may be a result either of combination of the antibody with the receptor itself or of something akin to steric hindrance, the IA receptor being passively covered by antibody molecules attached at other sites. Although the action of blood group antibodies interferes with the detection of IA by hemagglutination, treatment of cells with natural isoantibodies does not interfere with the actual adherence reaction. It seems possible, therefore, that autoantibodies in some cases of hemolytic anemia are directed at the IA receptor.

Further studies of the nature of the receptor are required and would perhaps permit a better understanding of the nature of the bond. Inhibition studies with purified extracts of red cells, analogous to the method used to assay blood group substances in solution (Kabat, 1956) would offer a useful avenue of approach.

The nature of the reactive site on the antigen–antibody–complement complex is also unknown. It could, for example, be a portion of one of the components of complement, or perhaps a new molecular group resulting from the reaction of complement with the antigen–antibody complex. Again, relevant experimental data seem to be lacking.

VI. Biological Consequences and Implications

A. PROMOTION OF PHAGOCYTOSIS

Early workers on leucocyte adherence (Laveran and Mesnil, 1901; Mesnil and Brimont, 1909; Levaditi and Mutermilch, 1910) showed that, *in vitro* or *in vivo*, attachment of trypanosomes to polymorphonuclear or mononuclear phagocytes preceded their ingestion and digestion and that this was much more rapid with sensitized than with unsensitized organisms. The relationship of this phase of phagocytosis to IA has been considered. Later workers (Delrez and Govaerts, 1918; Govaerts, 1921; Wright, 1927; Dudgeon and Goadby, 1931; Kritschewsky and Brussin, 1931) noted an association between adherence of sensitized organisms to platelets *in vivo*, their rapid clearance from the circulation and phagocytosis by both polymorphonuclear and mononuclear phagocytes. R. A. Nelson was the first to demonstrate clearly *in vitro* that IA of sensitized organisms to erythrocytes enhanced their phagocytosis by polymorphonuclear leucocytes. Mixtures of pneumococci, rabbit antibody and complement, human or rabbit erythrocytes, and rabbit polymorphs were incubated in siliconized tubes rotated at 37°C. for 30 minutes. The percentage of leucocytes containing pneumococci was counted on stained smears. The occurrence of IA was confirmed by the disappearance of organisms from the fluid phase after centrifugation of mixtures containing no polymorphs. In the complete reaction mixtures, in which IA occurred, there was a four- to fivefold increase in phagocytosis compared with controls in which IA had not occurred (for example, mixtures free of antibody or complement or containing rabbit instead of human erythrocytes) (R. A. Nelson, 1953, 1956, 1958b). Lamanna (1957) criticized these experiments on the grounds that enhancement of phagocytosis could occur because of the greater density of the reaction mixtures containing erythrocytes, especially during centrifugation. The facts that samples were removed before centrifugation and that enhancement did not occur in mixtures containing rabbit erythrocytes invalidate this criticism. A cinematographic study of phagocytosis and IA was made by Robineaux and Nelson (1955; see also Robineaux and Pinet, 1960), and the film clearly demonstrated the removal by polymorphs of bacteria from the surface of erythrocytes. The polymorphs seemed to scour the surface of the erythrocytes, often producing a marked deformity but ultimately removing the organism, thus breaking the bond formed by IA, and leaving the erythrocyte intact. The mechanism of enhancement of phagocytosis is not known though R. A. Nelson (1953) put forward two possible explanations that (1) this may be similar to the enhance-

ment of phagocytosis by the entrapment of bacteria against certain surfaces, as shown by Wood (1951) and (2) substances from the surface of the erythrocyte coat the bacteria and render them more susceptible to phagocytosis.

Quantitative studies of the effect of IA to platelets on phagocytosis *in vitro* have not been made.

B. OCCURRENCE OF IMMUNE ADHERENCE *in Vivo*

Reports of the adherence of sensitized organisms to circulating platelets have been noted in the foregoing. IA to primate erythrocytes *in vivo* was demonstrated by R. A. Nelson (1956). Type I pneumococci presensitized with antibody were injected intravenously into a normal monkey and blood samples were removed a few minutes later. The erythrocytes were separated by light centrifugation and essentially 100% of the bacteria were found to be associated with them, the plasma being almost completely cleared. Unsensitized organisms injected into a monkey and sensitized organisms injected into a rabbit were found in the plasma.

C. POSSIBLE ROLE IN RESISTANCE TO INFECTION

The early demonstration of IA to platelets and the occurrence of this reaction in the intact animal led to speculation concerning the role of platelets in resistance to infection. Govaerts (1921) considered that an "antixenic"³ function could be attributed to platelets. He and other workers cited here were impressed by the speed with which bacteria undergoing adherence to platelets were cleared from the blood after intravenous injection and by an apparent increase in the rate at which such organisms were phagocytosed after sequestration in lungs, spleen, and liver, as judged by microscopic examination. Bull and McKee (1922) cast considerable doubt on the validity of this concept by showing that the clearance of organisms was equally rapid in rabbits depleted of circulating platelets by the injection of an antiplatelet serum. Further slight doubt arose from the work of Adant (1929), Brussin and Rubinstein (1925), Kritschewsky and Awtonomoff (1925), and Kritschewski and Brussin (1927) who showed that the presence of "thrombocytobarin" in the serum of mice did not necessarily confer on them immunity to the homologous strain of trypanosomes. Even in the presence of other antibodies, however, immunity to many parasitic and bacterial infections is rarely absolute. The demonstration that platelets are not vital for the clearance of bacteria in a particular experimental

³ His expression, "fonction antixénique," does not seem to be translatable precisely into English but implies a defensive reaction to foreign substances.

situation, when another mechanism also operates, does not necessarily imply that the reaction is of no value to the organism in its resistance to infection in other circumstances. For example, when small numbers of bacteria enter the blood stream, IA to platelets (in animals whose platelets are reactive) might well be a valuable first line of defense, immobilizing the bacteria and promoting their phagocytosis by polymorphs without recourse to the RES. Furthermore, a mechanism analogous to IA might well operate in the clearance of bacteria by the RES in the absence of platelets. Granulocytopenic animals have some resistance to infection, but no one would deny the role of these cells in resistance to infection because of this fact.

A further role of platelets in resistance to infection, perhaps related to their participation in IA is suggested by other work. Govaerts (1921) cites the work of Gruber and Futaki (1907) and Werbitzki (1911) suggesting that platelets contain bactericidal substances. This suggestion was originally made by Metchnikoff and has recently been confirmed by Hirsch (1960) and Jago and Jacox (1961). Amano *et al.* (1952, 1953) extracted an antibacterial substance ("plakin") from horse platelets, whereas Tocantins (1938) cited evidence that the "nonspecific antibody" activity of animal sera was raised when platelets were destroyed by antiplatelet serum. This writer also suggested that platelets were lysed after their attachment to intravenously injected bacteria. This may be a misinterpretation of the sequestration of platelet-bacteria agglutinates by the RES. Other direct evidence on this point is lacking. Neither R. A. and D. S. Nelson (1959) nor Siqueira and Nelson (1961) comment on the stability of platelets after IA *in vitro*. However, Taniguchi *et al.* (1930) described a process of pepticytosis or digestion by platelets of erythrocytes or bacteria which had undergone adherence *in vivo*. This report is unconfirmed but it seems justifiable to speculate whether IA of bacteria to platelets results in the release of bactericidal substances in a manner somewhat analogous to the release of histamine (see discussion following). It is particularly pertinent to note that substances bactericidal for *Bacillus subtilis* were absent from human platelets, which do not react in IA, but present in rat and rabbit platelets, which do react (Jago and Jacox, 1961). Further investigation seems indicated.

The role of IA to primate erythrocytes in resistance to infection is even more difficult to assess. Its occurrence *in vivo* and the enhancement of phagocytosis shown *in vitro* together suggest that it may aid the immobilization and clearance of organisms from the blood stream. Erythrocytes entering areas of inflammation by diapedesis from capillaries may likewise promote phagocytosis in those areas.

The relationship of IA to phagocytosis has been briefly considered above. In addition to the reports of adherence of sensitized antigens to polymorphs or macrophages, before phagocytosis, other work suggests a parallel between the process of IA to erythrocytes or platelets and the adherence preceding phagocytosis of particles by the reticulo-endothelial system *in vivo*. Manwaring and Coe (1916) used the term "endothelial opsonin" to describe antibodies which, probably acting in concert with a normal serum component, brought about the clearance of pneumococci by perfused rabbit livers from the perfusion fluid. Microscopic examination of such livers showed that pneumococci had adhered to endothelial cells (probably cells of the RES). More recently, Howard and Wardlaw (1958), Jenkin and Rowley (1961), and Rowley and Jenkin (1962) have emphasized the importance of opsonins for the clearance of bacteria and other particles by the RES. The role of complement in opsonization of this sort has not been defined, though its role in opsonization of particles for phagocytosis by polymorphs was clearly indicated by R. A. Nelson and Lebrun (1956). A mechanism similar to IA may be operative in the phagocytosis of antigens by the RES as well as by the polymorphs.

Rich (1933) and Rich and McKee (1934) whose work was briefly described (Section IV, D), have suggested that the spread of bacteria may be hindered by their adherence to tissues at the site of infection. In rabbits, localization of intradermally injected pneumococci to the site of injection was demonstrated both by negative blood cultures and by the apparent adherence of bacteria to tissues seen on microscopic examination of sections. Antibody was required for this localization which occurred even in animals rendered leucopenic and in the absence of any inflammatory reaction. Confirmation of Rich's findings came from the work of Cannon and Pacheco (1930), Catron (1935), and Hamerschmidt (1938). Cannon (1940) reviewing his own and other work supported Rich's findings and conclusions. Adherence reactions of this sort may be of considerable value to an animal in resisting infections such as staphylococcal skin infections. Although caution is necessary in drawing conclusions from the microscopic appearance of fixed, stained tissue sections, the mechanism of such reactions and their relation to IA are worthy of further investigation.

D. POSSIBLE ROLE IN GENERALIZED SCHWARTZMAN AND ALLERGIC REACTIONS

Thrombocytopenia has long been known to result from the intravenous injection of antigens into an immune or hypersensitive animal.

It has been shown that when bacteria are injected this is produced by adherence of bacteria to platelets and the subsequent sequestration of the mixed agglutinates (Aynaud, 1911; Delrez and Govaerts, 1918; Govaerts, 1921; Dudgeon and Goadby, 1931; Tocantins, 1938). A similar association has been shown by Tait and Elvidge (1926) for quartz particles, by Dudgeon and Goadby (1931) for India ink and colloidal silver, and by Taniguchi *et al.* (1930) and Swisher (1956) for foreign and incompatible erythrocytes. Aynaud (1911) observed thrombocytopenia in rabbits following intravenous injections of peptone. Thrombocytopenia is also a feature of the Schwartzman reaction in which a complex picture of shock, hemorrhagic, and other phenomena is produced by intravenous injection of bacterial endotoxin (provoking injection) following a preparative injection of endotoxin either intravenously (generalized Schwartzman reaction) or intradermally (local Schwartzman reaction) (Stetson, 1951; McKay and Shapiro, 1958). Siqueira and Nelson (1961) attempted to implicate IA in the causation of thrombocytopenia in generalized Schwartzman or allergic reactions. Platelet clumping similar to that observed by R. A. and D. S. Nelson (1959) following IA of particulate antigens to platelets was observed *in vitro* in the presence of soluble antigen-antibody complexes and complement. This was also observed in the presence of certain macromolecules, such as dextran, known to be active in the Schwartzman phenomenon and to which natural antibody was present in serum. The hypothesis that IA may play a part in these reactions in nonprimates is attractive, but the lack of reactivity in IA of primate platelets *in vitro* seems to make it unlikely that this is a mechanism operative in these animals, as Siqueira and Nelson themselves point out. There are examples of thrombocytopenia during anaphylaxis in monkeys, of leucocyte-platelet agglutination in Schwartzman-like phenomena, and of platelet thrombi in some disease states of humans. Siqueira and Nelson have suggested two possible explanations for the nonreactivity *in vitro* of human platelets: (1) the receptor site is labile and is either lost or masked on isolation of the platelets *in vitro*; (2) there may be a cation or cofactor requirement for IA to primate platelets which is not at the moment apparent. If primate platelets are, in fact, nonreactive *in vivo* as well as *in vitro*, then in the Schwartzman reaction as well as in certain diseases in which platelet agglutination occurs, the antigen may be passively attached to the surface of platelets, agglutination resulting from the action of antibody alone, with complement acting as a lytic agent (Ackroyd, 1962; Shulman, 1958).

IA may also be involved in the formation of some of the lesions of

serum sickness. This is suggested by the binding, shown by fluorescent antibody techniques, of antigen, antibody, and complement at the same sites in the tissues (Lachmann *et al.*, 1962).

Further implications of IA in allergic reactions are speculative. The release of histamine and 5-hydroxytryptamine from rabbit platelets by antigen-antibody complexes (Humphrey and Jaques, 1955; Barbaro, 1961a, b) may involve complement or some components thereof since calcium ions are required and the cofactors present in normal plasma are wholly or partly susceptible to various procedures that inactivate components of complement, such as the action of ammonia, hydrazine, zymosan, and heating to 56°C. for 30 minutes. In addition, Humphrey and Jaques noted that suspensions of rabbit platelets in plasma were clumped when antigen and antibody were added, suggesting that IA had occurred. It would be worth while to investigate the effect of washed particulate antigen-antibody-complement complexes on histamine release from platelets. The fact that histamine is released from human platelets despite their lack of reactivity in IA would, however, suggest that other mechanisms are operative here. If IA does, in fact, occur with other tissues, the release of histamine from guinea pig lung in anaphylaxis *in vitro* (Austen and Brocklehurst, 1961) may have a similar basis. It would seem desirable to reinvestigate, in terms of the possible participation of IA, this and other *in vitro* allergic reactions such as the release of histamine and 5-hydroxytryptamine from rat mast cells in the presence of serum and dextran (Archer, 1959, 1961); the degranulation of basophiles in whole blood incubated with a specific allergen (Shelley and Juhlin, 1961); and the lysis of leucocytes in rabbit blood by antigen and antibody (Waksman, 1953).

E. EVOLUTIONARY CONSIDERATIONS

The blood platelets of nonprimates, their precursors (megakaryocytes) or their functional equivalents (spindle-shaped cells or thrombocytes) have been shown to react in IA, whereas those of primates have consistently failed to react in IA *in vitro*. On the other hand, in those species examined (man, monkey, baboon) whose platelets do not react, erythrocytes do react, whereas in nonprimates erythrocytes are unreactive. If a specific receptor is involved in IA, it would appear that during evolution the mechanism determining its development was transferred from platelet precursors to erythrocyte precursors. In this connection it is interesting to note that certain other properties of platelets and erythrocytes seem to have changed at about the same stage of evolution. (1) A bactericidal substance present in rat and

rabbit platelets, active against *B. subtilis*, is absent from human platelets (Jago and Jacox, 1961). (2) Human erythrocytes contain a substance, absent from platelets, which increases the prothrombin consumption during blood coagulation (Quick, 1961). (3) Human erythrocytes contain a substance, recently identified as adenosine diphosphate, which is necessary for the adhesion and aggregation of platelets to a wound surface or site of intimal damage (Hellem, 1960; Gaarder *et al.*, 1961). Although such considerations do not clarify the reasons for the evolutionary change, they indicate that platelets have altered in other ways during evolution and that in humans, erythrocytes have properties which supplement those of platelets.

If the speculation is correct that the adherence of sensitized antigens to phagocytes and other tissue cells occurs by a mechanism identical with that of IA, it may be more profitable to view this as a more important manifestation of the reaction. IA to primate erythrocytes or nonprimate platelets may, indeed, be only an incidental evolutionary development.

VII. Laboratory Applications

The extreme sensitivity of IA and the convenience of methods for its detection have suggested its usefulness as a standard laboratory technique both in clinical and investigational laboratory work. Reference has been made in the foregoing to some of these applications. Certain practical results and applications are considered in more detail in the following sections.

A. THE DETECTION OF ANTIGENS

The very sensitive hemagglutination technique for measuring IA is particularly suitable for detecting small quantities of soluble antigens or of viruses. In principle there are two limitations: (1) adequate complement must be fixed by the antigen-antibody system; (2) the specificity is limited by the specificity and cross reactivity of the antibody. Taverne (1957) studied IA with T2 phage and found that 5×10^6 plaque-forming units gave a positive hemagglutination reaction with rabbit antiserum, guinea pig complement, and human erythrocytes. The technique has been applied by D. S. Nelson and Day (1963) to the detection of cauliflower mosaic virus (CIMV) both in purified preparations and in crude turnip sap. An antiserum prepared in a rabbit against purified CIMV was used with human complement and erythrocytes. In purified preparations of CIMV, antigenic activity was detectable in 0.1-ml. quantities diluted 500 times. This represents a sensitivity approxi-

mately 10 to 100 times that of the agar gel diffusion or microprecipitin tests previously used to detect antigenic activity. The much lower concentrations of virus present in crude sap were also detectable. In tests with crude sap the rabbit antiserum reacted at low dilutions with components of normal uninfected sap but at higher dilutions only with infected sap. By titrating the activity of the antiserum against undiluted sap, it was possible to determine whether virus was present or not. In a series of saps examined without foreknowledge of their virus content, the presence of virus was determined or excluded with complete accuracy. The technique appears to be of considerable potential value in plant virus serology and further studies are being made of its possible use with other plant virus diseases. No other studies of this application of IA have been published. It might be useful for the early detection of viruses growing in tissue culture, when the sensitivity of IA might allow earlier detection and typing of viruses than is now possible.

An attempt was made by Kourilsky *et al.* (1957c) to apply IA to the determination of the pathogenic character of staphylococci. They found that with a variety of human sera, 97% of pathogenic staphylococci reacted positively in IA but only 35% of nonpathogenic organisms. It is difficult to assess the significance of these results.

B. THE DETECTION AND MEASUREMENT OF ANTIBODIES

1. Humoral Antibodies

The use of the Rieckenberg reaction and the red-cell adhesion test of Duke and Wallace in the detection of antibodies has already been noted here. The techniques used were fairly crude—particular criticism might be made of the use of citrated or oxalated plasma and of the use of whole blood as a source of trypanosomes or spirochetes. Even so, Wallace (1933) and Brown and Broom (1938) found the red-cell adhesion test in trypanosomiasis to be reproducible and specific. Since the work of R. A. Nelson (1953), refined IA techniques have been applied to the detection and measurement of antibodies, particularly in syphilis. The high incidence of biological false positive reactions in the standard serological tests for syphilis such as the Wasserman reaction renders them much inferior to the highly specific *Treponema pallidum* immobilization (TPI) test (R. A. Nelson and Mayer, 1949). Comparisons between TPI and the *Treponema pallidum* immune adherence (TPIA) test have shown the latter to be equally specific. Most workers have used clearance techniques. R. A. Nelson (1953) examined 385 human sera in this way and found correlation between the results of TPI and TPIA tests in all

but 5 instances. Olansky *et al.* (1954) found less correlation but Moser (1955) and Miller *et al.* (1957) found from 90 to 100% correlation. Fegeler and Knauer (1955) and Rein and Kelcec (1957) found TPIA to give "generally excellent" correlation with TPI tests and considered it to be of practical value in the serodiagnosis of syphilis. Vaisman and Hamelin (1958), however, considered TPIA to have considerable advantages but to be rather difficult to read. Daguet (1956a) used an adherence-disappearance reaction based on phagocytosis of *T. pallidum* after IA. Of 382 TPI-negative sera only 1 was positive in this reaction and of 307 TPI-positive sera, 7 were negative. Daguet (1956b) also used a hemagglutination technique which gave results exactly comparable with the adherence-disappearance reaction. The advantages of TPIA over TPI noted by all these workers included its greater ease and speed of performance, its safety and cheapness, and the stability of the antigen (killed treponemes can be used and are stable on storage in the cold). False positive tests may result from sensitization of *T. pallidum* by rabbit antibody during their growth in rabbit testicular syphilomata, and false negatives from "anticomplementary" effects of the test serum. Miller *et al.* (1957) and Rein and Kelcec (1957) have published comprehensive descriptions of the technique of TPIA including controls to detect the occurrence of false positives and negatives. Only rarely were difficulties encountered in determining whether a test was positive on the basis of specific disappearance of treponemes. Piéron (1959) also cites references to the work of other groups, in France, Holland, and Poland, who have had satisfactory results from the use of TPIA tests. The titers of antibodies to *T. pallidum* were noted by R. A. Nelson (1953) to be about 1:25 in 0.1 ml. of serum but other workers have not titrated antibodies in TPIA. The absence from normal sera of natural antibodies to specific antigens of *T. pallidum* is a great advantage in both TPIA and TPI tests.

The application of IA to the detection of antibodies to other organisms has been described but in these cases natural antibodies are always detectable. For example, in the work of Kourilsky and Piéron (1957) on human antibodies to staphylococci there was no correlation between the IA activity of sera and the presence of staphylococcal infection. Piéron and Kourilsky (1958) likewise found antibodies to *Mycobacterium tuberculosis* present in nearly all sera drawn from a French population. There was no correlation between positive skin reactions to tuberculin and the presence of antibodies. Both these investigations may be criticized for their failure to distinguish between antibody and complement activity in the sera examined; the end point of the titration

might well have been reached because of a limited supply of complement rather than of antibody.

An interesting clinical application of IA has been described by Brody and Finch (1961) and Brody (1962). They used this technique to study antibodies in acquired hemolytic anemia associated with lymphomas and leukemias. Using guinea pig platelets as indicator particles they showed that factors in the sera of these patients could sensitize erythrocytes or lymphocytes for their participation in IA. Eluates from Coombs-positive erythrocytes were capable of sensitizing either the patients' leukemic lymphocytes or, occasionally, normal lymphocytes so that they reacted in IA with normal human erythrocytes or guinea pig platelets. These findings suggested that the abnormal lymphocytes in these disease states could provide an antigenic stimulus resulting in the production of antibodies responsible for Coombs-positive hemolytic anemia.

The further application of IA to the detection and measurement of antibody in the clinical laboratory might be fruitful provided that investigators bear in mind such pitfalls as natural antibody activity and the necessity for the provision of adequate complement in reaction mixtures. It is, of course, characteristic of all sensitive serological tests that normal sera in low dilutions show positive reactions because of the presence of natural antibodies.

2. *Cell-Bound Antibodies*

R. A. Nelson (1962c) has described the use of IA in detecting cell-bound antibody postulated to be responsible for the homograft reaction. He proposed that a close antigenic relationship exists between tissues of different individuals of the same species and that a homograft stimulates the production of antibodies that not only react with the donor antigens but also cross react with antigens in the host's tissues. Because of this cross reaction such antibodies would become bound to host cells including mobile leucocytes. Graft rejection would occur when such cells invaded the graft and antibody dissociated from them to recombine with donor cells. In testing this hypothesis Nelson examined lymph node cells from normal guinea pigs and from guinea pigs which had received autografts, homografts, heterografts, or injections of vaccinia virus or pollens in the area drained by the lymph nodes. The cells were washed and incubated with washed human erythrocytes. In all cases a small number of cells (3-8%) adhered to the erythrocytes. When complement was added a much higher proportion (28-40%) of cells from homografted animals adhered, though there was no increase in the proportion of adherent cells from other groups of animals. Although

other interpretations are possible, these findings were taken to indicate the presence of the postulated cell-bound antibody which fixed complement *in vitro* to react as an antigen-antibody-complement complex in IA. This work is clearly important in the study of the mechanism of the homograft reaction but certain questions arise: for example, whether complement is fixed *in vivo* to antibody-carrying host cells and if so, why the host cell is not destroyed by the combination; whether the "cytophilic" antibody described by Boyden and Sorokin (1960, 1961), produced in response to antigens such as human albumin, is related to this antibody or may, indeed, be responsible for the reaction; and in what way this antibody is related to hemagglutinating and enhancing antibodies described in connection with homografts.⁴ Nelson adduces further support for this concept from studies on the transfer of hemolytic antibody from "mismatched" to "matched" erythrocytes but further studies and confirmation of his findings with IA will be awaited with interest.

C. THE DETECTION AND MEASUREMENT OF COMPLEMENT

In the classical hemolytic assay for complement, guinea pig serum is highly reactive but sera of other species, notably man, are much less reactive. Furthermore, the hemolytic activity of human complement is not proportional to the concentration of serum in the reaction mixture. In IA, on the other hand, human, rabbit and guinea pig sera are equally active as sources of complement and the activity of human complement is proportional to the serum concentration. Numerically high titers of human complement may be obtained though they vary, depending on the antigen-antibody system used. The titers are determined from the quantity of serum required to give 50% IA or an arbitrarily determined degree (2 plus) of hemagglutination in a standardized system. For example, Woodworth (1962a, b) obtained titers of 400-600 units per milliliter using starch granules and natural antibody. Nishioka (1963) studied in detail the measurement of human complement by hemagglutination due to IA. He used rabbit antibody and a variety of antigens including soluble proteins, bacteriophage, bacteria, and sheep erythrocytes. Complement titers varied with variation in the relative proportions of antigen molecules or particles, human erythrocytes, and antibody. In optimal conditions they were usually of the order of 4000 units per milliliter. Nishioka also found it possible to measure a subcomponent of human C'3 by this technique. The speed, simplicity, reproducibility (10%), and greater allowance for variation of IA gives

⁴ This last question is discussed briefly by D. S. Nelson (1962).

it a clear advantage over hemolytic techniques (for example, Laurell and Grubb, 1958; Ellis and Felix-Davies, 1959) in the measurement of variations of human complement activity in disease.

VIII. Conclusions

It is hoped that this survey of immune adherence has given a clear picture of (1) its nature, so far as this is understood; (2) its relation to other similar reactions; (3) its specificity and sensitivity as an immunological technique; and (4) its possible biological significance.

Further investigation of certain aspects of IA is necessary. These include (1) the nature of the receptor on reactive cells, (2) the nature of the bond between the immune complex and the reactive cell, (3) the relationship between IA and the attachment of antigens to polymorphonuclear and mononuclear phagocytes before ingestion, (4) the relationship between IA and the immobilization of bacteria in the tissues of an immune animal at the site of entry, and (5) whether pharmacologically active substances are released from erythrocytes, platelets, or other cells after their participation in IA.

Immune adherence has widespread potential application as a standard immunological tool in clinical and research laboratories.

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Reaginic Antibodies

D. R. STANWORTH

*Department of Experimental Pathology, The Medical School,
University of Birmingham, Birmingham, England*

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I. Introduction

A. SCOPE

Reagin still represents a nebulous concept to many immunologists, some doubting the legitimacy of its classification as an antibody. Various factors have contributed to this situation, foremost among these being the difficulty of studying reagins *in vitro*. This has led to conflicting findings and, in some cases, misinterpretation of results.

This review is an attempt to assess critically the important data available on the biological and physicochemical properties of reaginic antibodies, with the object of providing a clearer indication of their nature in relation to our rapidly increasing knowledge of immune antibodies. The results of investigations on artificially induced skin-sensitizing antibodies are also considered, particularly where these throw light on the character of the spontaneously occurring reagins. Finally, an immunochemical theory of reagin formation is presented which, if substantiated, could lead to an explanation of many of the peculiarities of immediate-type hypersensitivity reactions.

B. TERMINOLOGY

Application of the term "reagin" is restricted to the spontaneously produced skin-sensitizing antibodies found in allergic human sera. The corresponding antigen is referred to as "allergen," no attempt being made to distinguish between the antigen responsible for the initial sensitization process (sometimes referred to as "allergenoid") and that evoking skin reactions in actively and passively sensitized humans. The more general term "allergic" has been preferred to "atopic" in referring to individuals showing immediate hypersensitivity.

In distinguishing between the different types of "immune serum globulins," the American practice of classing all these proteins as γ -globulins has been adopted in preference to the French terminology based solely on immunoelectrophoretic properties. Hence, when necessary the three main classes of immune globulins are referred to as γ_1 or γ_2 (the subscripts referring to electrophoretically "fast" or "slow" 7 S γ -globulin, respectively), γ_{1M} [equivalent to electrophoretically "fast," 19 S γ -globulin—the " β_{2M} " component of Grabar and Burtin (1960)], and γ_{1A} (equivalent to the electrophoretically "fast," predominantly 7 S γ component, isolated by Heremans *et al.* in 1959 and originally termed " β_{2A} -globulin"). Although this nomenclature is not ideal, it does signify the immunological relationship demonstrated between these three classes of globulin (Franklin and Kunkel, 1957; Heremans *et al.*, 1959; Franklin and Stanworth, 1961) besides giving an indication of their physicochemical properties. The antigenic relationships between the various types of γ -globulin have been discussed in detail in a recent review by Fahey (1962) in this series.

To avoid confusion, Porter's (1959) original nomenclature has been applied to the papain digestion pieces of 7 S γ -globulin (rabbit or human). Thus, the pieces containing an antibody-combining site are referred to as "I" and "II" (also termed "A" and "C" by Franklin, 1960;

and "S," for "slow component," by Edelman *et al.*, 1960) and the piece containing the principal antigenic determinants is termed "III" (equivalent to the piece "B" of Franklin and the "F," i.e., "fast component," of Edelman and his associates). Until the structure of 7S γ -globulin has been elucidated, it will be necessary to adopt this arbitrary form of nomenclature when referring to the separate parts of the molecule.¹

Finally, there is the problem of selecting a suitable terminology to differentiate between the different classes of protein-carbohydrate conjugates. As a rule those conjugates comprising predominantly protein are referred to as "glycoproteins," whereas those consisting largely of carbohydrate are termed "mucopolysaccharides" or "glycopeptides" according to the preference of their investigators. This practice has limitations, however, as do all the other classification systems which have been employed (referred to by Winzler, 1960).

II. Passive Transfer of Immediate Sensitivity

A. HISTORICAL BACKGROUND

Although Ramirez (1919) had demonstrated accidentally the transfer of immediate sensitivity between humans, by transfusing a nonallergic recipient with the blood of a horse-sensitive donor, the classical experiment of Prausnitz and Küstner (1921) is recognized as the starting point in the study of reagins. By passively sensitizing sites on his forearm to fish by means of the intradermal injection of serum from his allergic patient Küstner, Prausnitz demonstrated that the substance(s) responsible for this transfer of hypersensitivity could be localized in the skin where it could be caused to evoke a typical wheal and erythema reaction on challenge with the specific allergen to which the donor was sensitive. This observation was soon followed by similar demonstrations of the transfer of hypersensitivity to other materials, such as horse serum (De Besche, 1923) and grass pollens (Freeman, 1924). Here, then, was a method of studying the reaction between allergic serum and allergen without submitting the normal recipient to any systemic hypersensitive response. Although the "P-K test" (as it has come to be known) has proved useful in diagnosis in certain cases as an alternative to the direct skin test (Walzer and Bowman, 1931), its main value has been in the assay of reagins where it still forms the basis of all available methods for measuring sensitizing activity.

¹ The specific immunological activities associated with the different types of polypeptide chain (e.g., "A" and "B") isolated recently from 7S γ -globulins (Porter, 1962) have not been considered in this article.

B. BIOLOGICAL PROPERTIES OF THE SKIN-SENSITIZING FACTOR

The extensive studies of Coca and Grove (1925) provided the first indication of the nature of the skin-sensitizing factor passively transferable in the sera of hay-fever patients. It was these workers who coined the term "atopic reagin" to designate this substance, because of its association with an hereditary (atopic) condition in humans and because of their uncertainty of attributing its unusual properties to those of an antibody. As will be indicated, however, subsequent investigations have tended to suggest that Coca and Grove were over cautious, although their findings, which have been largely substantiated by later studies employing more refined techniques, did reveal that this was *no ordinary antibody*. The reagins appear to possess the unusual property of becoming rapidly and permanently attached to the skin of normal recipients, local passive sensitization being demonstrable by challenge with allergen as soon as the transfer injection swelling and irritation has subsided (in 1-2 hours) and persisting for at least 4 weeks. Unlike the anaphylactic-type antibodies, however, the reagins are incapable of passively sensitizing guinea pig skin (J. P. Fisher *et al.*, 1956). This was confirmed independently by Augustin (1955) studying reagins to grass pollen and by Stanworth (1961) studying reagins to horse dandruff, both with the aid of Dr. Ovary's skill in performing his passive cutaneous anaphylaxis (PCA) test in guinea pigs (Ovary, 1952). It is important, therefore, that the property of reagins of binding to human skin should not be confused with the passive cutaneous reaction induced by transfer of anaphylactic-type (nonreaginic) antibodies in the guinea pig which, according to the macroscopic and microscopic observations of Fisher and Cooke (1957), is a manifestation of an interaction between a *passively* fixed antibody and circulating antigen. This applies particularly to studies of the antibodies in the sera of humans undergoing an attack of serum sickness where, as will be discussed later, both reaginic and anaphylactic types of antibody are present together at certain times.

Reagins also differ from the more usual type of antibody in their failure to form precipitate, or to fix complement, on mixing with the specific antigen (allergen) *in vitro*; nor do they appear to inhibit complement fixation in a ragweed pollen antigen-rabbit antibody system (Portnoy and Sherman, 1954). Another distinctive property is their deactivation by heat treatment although, as W. B. Sherman (1958) points out, this property is not unique as some human precipitins are also destroyed by heat. Coca and Grove (1925) showed that heating allergic serum at 56°C. for 30 minutes causes a "distinct weakening of its sensitizing power." Stanworth and Kuhns (1963b) have recently em-

ployed quantitative P-K testing in different recipients to show that 75-90% of the skin-sensitizing activity of serum from a horse dandruff sensitive individual was destroyed by heating in a water bath for 30 minutes at 56°C., whereas heating at the same temperature for a further 30 minutes was sufficient to eliminate entirely the residual activity. Addition of fresh, nonallergic serum from the recipient of the P-K tests failed to restore this activity. On the other hand, Loveless (1940) had to heat sera from ragweed-sensitive individuals for 2 to 5 hours at 56°C. or for 30 to 60 minutes at 60°C. in order to destroy their skin-sensitizing activity. She made use of this effect to remove the reagin from the sera of hyposensitized ragweed-sensitive individuals prior to estimation of the allergen-neutralizing antibody ("blocking antibody"), which was produced as a result of the "desensitization" treatment (Cooke *et al.*, 1935) and which Loveless showed to be heat stable at 56°C. (For a full comparison of the biological properties of blocking antibody with those of reagins see Kabat and Mayer, 1961.)

Of the other biological properties of reagins, that revealed by studies of the transmission of skin-sensitizing activity across the placentas of hypersensitive mothers is probably the most striking. Unlike blocking antibodies and other more usual types of antibody, reagins do not appear to cross the human placenta. This was demonstrated by Bell and Eriksson (1931), who failed to detect skin-sensitizing activity in the undiluted cord blood of ten allergic mothers whose sera had shown positive P-K tests at relatively high dilutions. These findings were confirmed by Sherman *et al.* (1940), who compared the P-K titers of the sera from a group of twelve hypersensitive mothers and their infants (3-6 months of age) with the blocking antibody and hemagglutination titers. The cord sera from four of the cases were shown to contain the same type of isoagglutinin as the maternal sera but at reduced titers. This suggestion of a certain degree of selectivity with regard to placental transmission of isoagglutinins gained further support from the results of studies by Wiener and Sonn (1946), who found that incomplete isoagglutinins traversed the placenta far more readily than complete isoagglutinins in two cases of hemolytic disease of the newborn due to A and B sensitization. These findings led to the concept that ABO isoagglutinins, like Rh agglutinins, are larger molecules than incomplete ABO (or Rh) antibodies and would, therefore, be expected to traverse the placenta less readily. Ultracentrifugal studies provided evidence in support of this idea—both the ABO isoagglutinins (Pederson, 1945) and the complete Rh antibodies were associated with 19 S components. In contrast, Moore *et al.* (1949) showed that 7 S γ -globulin and a number of antibodies

associated with this fraction readily cross the placenta so that the antibody titer of the cord sera is of the same order of magnitude as the titer of the maternal sera. Similarly, the typhoid H agglutinin (7 S) is found to reach equal levels in both maternal and fetal circulation, whereas the typhoid O agglutinin (19 S) fails to cross the placenta. Franklin and Kunkel (1958) employed a quantitative precipitin technique to show that the level of 19 S γ -globulin in the serum of the newborn is much lower (1/10–1/20) than that in adult serum and interpreted their results as a demonstration of the ability of the fetus to synthesize 19 S γ -globulin as well as 7 S γ -globulin but, unlike the low molecular weight globulin, the 19 S γ -globulin is not derived from the mother by placental transmission. In keeping with these findings is the observation of Den Oudsten *et al.* (1958) that lupus erythematosus (L.E.) factor (7 S) crossed the placenta, whereas rheumatoid factor (19 S) did not pass from the circulation of a mother with rheumatoid arthritis, who contained both factors in her serum.

Thus, in man, the failure of proteins (particularly antibodies) to cross the placenta has come to be equated with a relatively large molecular size. Inevitably, this concept has been applied in reverse in speculation as to the size of the reagin molecule, its nonplacental transmission being associated with a 19 S class of antibody. Such speculation requires the dubious assumption that reagins do not bind onto placental tissue, nor do they form complexes with high molecular weight serum constituents that fail to cross the placenta. In other words, an ideal filtration process through an inert "sieve" is envisaged. The elegant studies of Hemmings and Brambell (1961) and associates suggest that this is far from the case, not only in the rabbit but also in the human (Brambell, 1961). It appears that the selective transmission of proteins through the cells of the placental membranes is extremely precise and dependent upon very small antigenic differences. In fact, the response to foreign proteins is graded according to the species of origin (Batty *et al.*, 1954) and is independent of molecular size. For instance, 7 S equine diphtheria antitoxin reaches concentrations in the rabbit's fetal circulation of not more than 2% of those achieved by either 7 or 19 S homologous antibody (Brambell *et al.*, 1951). Moreover, a reduction in the molecular size of rabbit antibody γ -globulin (7 S) by means of the Porter (1959) papain digestion technique resulted in a *decrease* in the rate of placental transmission of the fragments (I and II) containing the antibody-combining sites; such fragments showing only about one-tenth the rate of transmission of the parent molecule in spite of an approximately threefold reduction in molecular size (Brambell *et al.*, 1960). Only the

digestion Fragment III (mol. wt. 80,000) enters the fetal circulation at the same rate as the whole molecule.

It should be clear from these observations that any attempt to assign a relatively high molecular weight (e.g., equivalent to a sedimentation coefficient of 19 S) to reagins as a result of their failure to appear in the cord blood of the offsprings of allergic mothers is not justifiable. Of more value, in our attempts to explain distinctive biological properties of the reagin molecule, would be a study designed to establish whether the absence of placental transmission of reagins is the result of an immunological exclusion process. In this respect, it would be interesting to know whether reagins will cross the placentas of nonallergic mothers transfused inadvertently with blood from hypersensitive donors.

Sherman *et al.* (1940) observed that two out of six colostras of allergic mothers showed a small amount of skin-sensitizing activity (1/100–1/1000 of P-K activity of the serum) suggesting that small quantities of reagin can cross some cell membranes. Comparative gel-diffusion precipitin and immunoelectrophoretic analyses (Hanson, 1961) have shown that human colostrum taken within 1 day of parturition contains antibodies closely related immunologically to immune serum globulins (γ , γ_{1M} , and γ_{1A}), but showing differences which may reflect a modification of serum antibodies in the mammary gland or the synthesis of the milk antibodies in the gland itself. Hence, it is not easy to draw any conclusions about the nature of reagins from present knowledge of the passage of serum γ -globulins across the mammary membrane.

C. MEASUREMENT OF SKIN-SENSITIZING ACTIVITY

1. *Methods Based on the Prausnitz-Küstner Reaction*

Various modifications of the P-K test have been employed in the assay of reagins. These include a reverse procedure, in which passive transfer of allergen (horse serum) was performed at the optimum time of 12 hours before intradermal injection of serum from an allergic (horse-sensitive) individual (Wright and Hopkins, 1941). A quantitative comparison of results obtained by this method with those given by conventional P-K tests performed in parallel suggested that the reverse technique was from four to eight times more sensitive. In spite of this, it has not found a wide application, although Loveless *et al.* (1955) have used it to demonstrate the absence of reactive allergen in skin sites of normal recipients previously sensitized with reagin-allergen mixtures. Other variations of the P-K test have involved administration of the challenging allergen, after intradermal injection of the allergic serum, by alternative routes, such as by the introduction into the nostrils

of a powder from a hand blower (Cohen *et al.*, 1930) or by ingestion in the case of studies on food sensitivities (Woringer, 1933). These procedures have obvious disadvantages for the recipients, however.

The two most widely used methods of measuring skin-sensitizing activity, the dilution and neutralization procedures, stemmed from the first attempts of Levine and Coca (1926b) to quantitate the P-K test. Although the technique of these workers could be criticized on the grounds that they appeared to have tested each solution only once, their extensive studies (in which they recorded results of P-K tests with sera from thirty-three hay-fever patients by tracing the areas of wheals and erythema elicited) showed that the P-K activity could be correlated approximately with the skin reactivity of the donors (revealed by direct testing). In other studies, the same investigators (Levine and Coca, 1926a) confirmed that allergen "neutralizes" the reagin when the two are preincubated together for 24 hours *in vitro* (at room temperature or in an icebox) prior to passive skin sensitization with the mixture and subsequent challenge with allergen alone. In contrast, the reagin was found *not* to neutralize the allergen, whose capacity to evoke a reaction at a passively sensitized skin site was not impaired by admixture with specific reagin. The neutralization procedure has been employed as an alternative to the dilution method, with which it gives a comparable measure of reagin concentration, in an effort to obtain more definite end points (Lippard and Schmidt, 1937). It has the advantage that reagin concentrations can be expressed in terms of the smallest amount of allergen nitrogen which inhibits skin sensitivity. Patterson and Correa (1959) have used the technique to demonstrate a constant quantitative relationship between reagin and allergen (ragweed) at equivalence. Thus if an extract contains sufficient allergen to neutralize the reagin in serum, any dilution of that extract will neutralize an equivalent dilution of the allergic serum. This is analogous to the situation obtaining at equivalence in precipitating antibody-antigen systems. There does not appear to be any point of optimal proportions, however, in the neutralization titration of reagin with allergen. Nevertheless, recent studies of *in vitro* release of histamine from the leucocytes of allergic individuals (to be discussed later) suggest that the use of a large excess of allergen, for challenge in P-K tests, might exert some inhibitory effect on the wheal and erythema response. On the other hand, the results obtained by Patterson and Correa, and others, indicate the importance of using a sufficient amount of challenging allergen to react with all the available reagin at the passive transfer site.

The widest application of the neutralization technique has been, of

course, in the measurement of blocking antibody. This resulted from the observation of Cooke and his associates (1935) that, in contrast to pretreatment allergic sera, sera from "desensitized" patients showed a discrepancy between P-K titers measured by the neutralization as compared to the dilution method because of the inactivation of a certain amount of allergen (in the neutralization tests) by the blocking antibody present. Measurement of this antibody has been simplified by Loveless (1940), who adopted the practice of first deactivating (by heat) the reagin also present in the sera from hyposensitized individuals. Nevertheless, there is still uncertainty about the mechanism of neutralization of allergen in these tests. There is no firm evidence from tests with concentrated allergic sera to suggest that an *in vitro* combination of allergen and reagin occurs during their mixing prior to passive transfer into normal skin sites. Loveless (1940) and others have observed, however, in the course of neutralization tests, that within 30 minutes of introducing the mixture into the skin a reaction is produced, even before any further challenge with antigen. An immediate erythematous reaction was also observed by Wright and Hopkins (1941) to occur 3-5 minutes after the intradermal injection of allergic serum into skin sites sensitized 24-48 hours previously with the allergen (horse serum). The occurrence of these immediate effects suggests that the *deactivation* of reagin by allergen in neutralization tests could occur *in vivo*, in the interval between the initial injection of the reagin-allergen mixture and subsequent challenge with allergen, rather than in the test tube before injection into human skin.

E. L. Becker (1948) has made a thorough quantitative study of the direct skin test (scratch method) in allergic individuals using an "all or none" response. It is somewhat surprising, however, that the accuracy of the indirect skin test techniques employed in the assay of reagin concentration has never been ascertained; nor has there been any attempt to study how various factors affect this accuracy. As a consequence results obtained in studies concerned with the characterization of reagins have sometimes been weakened through uncontrolled errors in the semiquantitative methods adopted in the measurement of skin-sensitizing activity. Recently Stanworth and Kuhns (1963a) have carried out a critical study of the factors influencing the accuracy of the P-K test as a result of which a quantitative testing procedure, designed to achieve maximum accuracy and reliability, has been evolved. In this work the prick method, first used by Lewis (1924) in his skin test studies with histamine, has been used to introduce the challenging allergen in passive transfer tests as this possesses several advantages (Harley, 1953) over

the more common practice of introducing a small volume (e.g., 0.01 ml.) of allergen solution by an intradermal injection into the sensitized skin site. The quantal responses of a group of normal recipients to passive sensitization with horse dandruff reagin have been studied and a statistical analysis of the results carried out. A variation in the reactivities of normal recipients was confirmed by this procedure. In the most suitable subjects—presumably among the 84% of nonallergic individuals who were found by Coca and Grove (1925) to be receptive to passive sensitization—an analysis of the results obtained from P-K testing various dilutions of allergic serum on randomly arranged skin sites on the back revealed a coefficient of variation of the order of 20% for the wheal area given by a single test (and sometimes, in tests with undiluted allergic serum, as low as 10%). This value compares favorably with the coefficient of variation (16%) of a single determination of the size of wheal elicited by the *direct* prick testing of histamine in the forearms of normal individuals (Squire, 1950). The type of quantal relationships shown by different recipients is illustrated by the log dose-response curves plotted in Fig. 1. The flattening out of most of these curves at relatively high serum concentrations (above 20% of the concentration of undiluted allergic serum) could be indicative of saturation of the recipient's

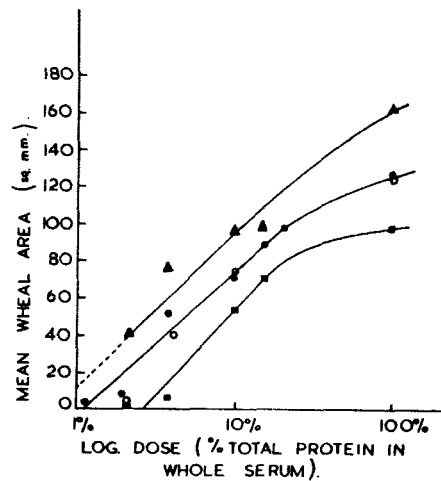


FIG. 1. Log dose-response curves obtained by P-K testing four different recipients (▲ = "H.G.," ● = "G.W.," ○ = "R.D.," and ■ = "J.F.") with varying dilutions of the same allergic serum, from a horse-dandruff-sensitive individual. No curve has been drawn through the points (○), referring to the responses of recipient "R.D.," for the sake of clarity. (To be published in detail by Stanworth and Kuhns, 1963a.)

skin sites with reagin, the excess having diffused away. On the other hand, a relative absence of this effect (as for instance in the log dose-response curve of recipient H.S. in Fig. 1) suggests that the recipient's skin is binding all the reagin presented to it, even in undiluted allergic serum. The approximately parallel slopes of the log dose-response curves, however, indicate that the same tissue-destructive mechanism probably operates in all recipients.

It seems that this quantitative P-K test procedure (which, in suitable recipients, estimates skin-sensitizing antibodies as accurately as many other biological tests estimate other types of antibody) could be profitably employed more frequently in the study of reagins. This applies particularly to the measurement of the reagin content of allergic serum fractions, where the dilution and neutralization procedures are less practicable owing to the relatively large number of tests required on any one solution. As a routine procedure it is suggested that tests be made in a preselected suitable recipient with three or four dilutions of the parent allergic serum, to provide a standard log dose-response curve from which the reagin concentrations of the serum fractions (tested in quadruplet in randomly arranged sites in the same recipient at the same time) can be read. In the unlikely event of the skin-sensitizing activities of the serum fractions proving so high as to fall off the linear range of the log dose-response curve for the recipient, a preliminary dilution of the test solution would, of course, have to be made. As the back of an individual of average size will not accommodate ideally more than thirty-six P-K test sites (at least 5 cm. apart in every direction), this means that the reagin concentration of six allergic serum fractions could be evaluated simultaneously in the same recipient.

Stanworth and Kuhns (1963b) have adopted this method of quantitative P-K testing in studies of the stability of reagins under various conditions, some of which are encountered in fractionation procedures. In addition, in studies of the effect of the time and position of the allergen challenge of passively sensitized skin sites, these authors have provided evidence in support of the firm and rapid binding of reagin to normal human skin. For instance, wheals elicited in sensitized sites in three different recipients, 1-2 hours after transfer, were 76, 87, and 93% ($\pm 20\%$) the size of wheals elicited in the same recipients 31-53 hours after transfer. Moreover, in other tests, passively sensitized skin sites were discharged step by step by successively pricking in allergen at multiple points on their peripheries. Challenging in the same manner at different times at a point 5 mm. outside the peripheries of the transfer sites gave an indication of the rate of outward diffusion of unbound

reagin. These findings also emphasized the importance of accuracy in the siting of the challenging allergen injection in P-K testing.

The observations of Bowman and Walzer (1953) that a period of unresponsiveness of skin sites, gradually diminishing over a period of 4 weeks, follows the discharge of reaginic and histaminic wheals offer a warning against the too frequent use of the same recipient in P-K testing. This could introduce a complicating factor into the neutralization technique, where the transfer of untreated allergic serum-allergen mixtures containing high reagin concentrations is followed by an immediate response (preceding the reaction which is evoked by allergen challenge).

2. *Alternative in Vivo Procedures*

Although there have been reports of the passive sensitization of various species of animal with human reagins, the replacement of man as a recipient for P-K testing has not been achieved. In the case of some species the physical unsuitability of the skin is obviously a disadvantage but, apart from this, there is the suspicion that immunological specificity presents an additional obstacle. The danger of transmitting infective hepatitis virus to the recipient of P-K tests has naturally provided the impetus to much of this work.

As a result of an early attempt to sensitize passively ring-tailed monkeys and chimpanzees with the sera of humans sensitive to ragweed and other allergens, Grove (1928) observed that the interpretation of the resultant reactions was not as clear-cut as in humans owing to the difference in the texture of the skin of lower animals and the tendency of the injected fluid to spread out. Later studies appear to support these conclusions. Caulfield *et al.* (1936) demonstrated that the monkey (*Macaca rhesus*) reacted to P-K testing with the serum of a ragweed-sensitive human by producing a wheal with marked pseudopodia and no surrounding flare, in contrast to the "buttonlike wheal" with surrounding flare elicited by passive sensitization of a human recipient with the same allergic serum. A similar finding resulted from the more extensive studies of Straus (1937) of the passive transfer of seven human allergic sera into the skin of rhesus monkeys, in that the response elicited by challenge with allergen comprised almost entirely an ill-defined edema at the transfer sites. The complication of an inhibition of wheal formation by physical struggle and excitement, as discussed by Layton *et al.* (1962), suggests that there are other reasons for preferring the well-behaved human recipients in P-K testing. A report of the passive sensitization of bovine skin with human allergic serum (Reddin, 1945), however, raises hope that an even more placid recipient may yet be found.

Although there have been occasional reports of the homologous local transfer of the immediate-type sensitivity found in certain animals (Wittich, 1949), this line of investigation has not been actively pursued in relation to its possible contribution to a better understanding of the nature of human reagins. Recently, however, Patterson and Sparks (1962) have demonstrated the transfer to normal dogs of wheal and erythema sensitivity by means of a heat-labile factor in the serum of a dog with spontaneous ragweed pollen sensitivity. The skin-sensitizing antibody moves as a β -globulin on zone-electrophoresis and is associated—but not exclusively—with a 7 S ultracentrifugal component (Patterson *et al.*, 1963). The discovery of reagin-like antibodies in untreated cows and of blocking antibodies in cows treated with ragweed pollen extract has also been reported (Weil and Reddin, 1943).

It is likely that the risk of transmitting infective hepatitis in P-K testing will ultimately be avoided by other approaches. Loveless (1952) attempted to achieve this by irradiating the blood of pollen- and dander-sensitive individuals with ultraviolet (UV) light in the manner used at the time in the United States for the commercial processing of plasma. Unfortunately, although she failed to detect any lowering of skin-sensitizing activity as revealed by the dilution or neutralization P-K procedures in most of the tests performed, this method of treating sera in order to eliminate the hepatitis virus did not fulfil its early promise and is not used any longer (James, 1954). More drastic UV treatment inhibits the P-K activity of allergic human sera (Battisto *et al.*, 1953). Harter (1961) has endeavored to overcome this difficulty by storing Seitz-filtered reaginic sera from ragweed-sensitive individuals at 32°C. for 6 months, by which time any contaminating hepatitis virus would be expected to have been destroyed (Sayman *et al.*, 1958). Such treatment had surprisingly little effect on the P-K titer of the sera, but it is improbable that irreversible protein interactions had not occurred, which would complicate any subsequent attempts to isolate the reagin. A possible solution to this problem might be achieved by differential ultracentrifugation of human reaginic sera in a density gradient. If the infective hepatitis virus proves to be of similar size to tissue-cultivated virus, which has been found to have a sedimentation coefficient of 80–100 S (Boggs *et al.*, 1961), it could possibly be separated from the more slowly sedimenting reagin by a careful slicing of the tube at the end of the ultracentrifugation process. This method proved unpromising, however, in an attempt (Walton and Flewett, 1961) to achieve a clear-cut fractionation of ECHO virus from a total lipoprotein fraction of human plasma to which it had been added.

3. *Attempted in Vitro Assay of Reagins*

In general, the various techniques employed in efforts to fulfil the urgent need of an alternative *in vitro* procedure of measuring reagin concentration have proved disappointing. There are promising signs now, however, that the reliable measurement of reagins in the test tube will soon be accomplished. The attempted use of red cell-linked-antigen agglutination techniques for this purpose has been reviewed in some detail by Augustin (1955). Coombs and his associates (Coombs, 1955; Britton and Coombs, 1955) employed red cells coated with incomplete antibody to which diazotized pollen antigen had been conjugated, whereas Stavitsky and Arquilla (1955) preferred to link their antigen direct to red cells via bisdiazotized benzidine. Such procedures, as well as the tanned cell technique of Boyden (1951), have been employed, also, by other groups (Orlans *et al.*, 1953; Feinberg *et al.*, 1956; Gordon *et al.*, 1958; Frick *et al.*, 1960; Arbesman *et al.*, 1960b) in the detection of hemagglutinating antibodies in the sera of hypersensitive individuals. There is no convincing evidence from any of these studies, however, that reagins are being measured. On the other hand, it seems likely that blocking antibodies are contributing, although probably not exclusively, to the high hemagglutination titers of the sera of hyposensitized individuals. Attempts to measure reagins by the specific agglutination of collodion particles coated with grass pollen or wheat flour antigens (Kallós and Kallós-Deffner, 1951) and with ragweed pollen antigen (Swineford and Houlihan, 1947) were equally unsuccessful. There is no evidence, too, that reagin was being measured in the attempts of Campbell and Sussdorf (1961) to develop an indirect test involving the detection of the specific binding of human antibody on to ragweed pollen grains by means of a fluorescein-tagged rabbit antihuman γ -globulin.

Techniques involving the agglutination of other formed elements of the blood have proved just as unsuccessful as the red-cell agglutination procedures. Storck *et al.* (1955) claimed to have demonstrated the *in vitro* agglutination of platelets on the addition of allergen to a suspension of the platelets in nonallergic serum, if the dialyzate of a reaginic serum is also present. Moreover, it was claimed by Hoigné *et al.* (1955) that the essential reactants in this system could also be measured by a turbidometric procedure, of potential value in the diagnosis of drug and food sensitivity. The failure of other investigations (Kleine *et al.*, 1957; A. B. Taylor *et al.*, 1958) to confirm these findings has avoided the difficulty (at least for the present) of having to equate reaginic activity

with a specific factor that is dialyzable through cellophane membranes, as the observations of Storck and his associates implied.

Studies designed to simulate in the test tube the *in vivo* interaction between reagin and allergen and to measure the main product of the ensuing tissue destruction have met with more success. These were encouraged by the observations of Waksman (1953), who demonstrated specific white-cell lysis by the addition of homologous antigen to the fresh heparinized blood of certain rabbits immunized with ovalbumin, which correlated moderately well with the ability of the sera to give passively transferred urticarial reactions in human or guinea pig skin (but not with the ability to cause passive anaphylaxis in guinea pigs). In line with these findings is the demonstration by Humphrey and Jaques (1955) of the release of histamine and 5-hydroxytryptamine on the addition of pneumococcal type III polysaccharide or ovalbumin to rabbit platelets suspended in heparinized rabbit plasma containing specific rabbit antibody.² An earlier observation by Katz and Cohen (1941) had shown that histamine was released from the blood of ragweed-sensitive individuals into the plasma on incubation with the specific allergen *in vitro*. Moreover, the effect could not be induced in the blood of a nonallergic individual, when this was incubated with allergen in a similar manner. This important observation, surprisingly ignored for many years, has now been convincingly substantiated by other groups (Noah, 1954; Noah and Brand, 1955; and VanArsdel *et al.*, 1958), whose work has indicated that histamine release from the leucocytes in the blood of individuals sensitive to a wide range of materials (including pollens, moulds, danders, foods, and dusts) can be evoked on incubation with allergen, providing that the appropriate skin-sensitizing antibody is present. Moreover, the amount of histamine released in such systems has been measured quantitatively by a sensitive spectrophotometric assay procedure developed by Lowry *et al.* (1954). The limitation of this method as a suitable *in vitro* technique for the measurement of reagin has been, nevertheless, its sensitivity. Even though the technique involved the concentration of histamine by adsorption on columns and by solvent extraction procedures, a 25 ml. volume of allergic serum was required for an assay. This problem seems to have been overcome by Noah and Brand (1961), who have applied a more sensitive, fluorometric procedure (devised by Shore *et al.*, 1959) to the measurement of histamine release in as little as 2 ml. aliquots of allergic

² Such reactions are discussed in greater detail in the article by Austen and Humphrey in this volume.

blood. This technique would seem to offer the most promising alternative to the P-K test for the measurement of reagins *in vitro*, although its results have yet to be shown to correlate with those obtained by parallel P-K tests on the same samples of allergic serum. It is obviously not simulating the skin test completely in that leucocytes from the donor of the reaginic serum appear to be essential for the *in vitro* procedure. Nevertheless, leucocytes have been shown to retain their capacity for the allergic release of histamine after being washed three times and resuspended in the washing fluid (Middleton and Sherman, 1960). This is interpreted as evidence of the firm binding of reagin to leucocytes, particularly as these cells taken from allergic individuals are claimed to have the ability to sensitize normal human skin to *immediate* hypersensitivity (Walzer *et al.*, 1957; Walzer and Bowman, 1960). The wheal reactions obtained in such studies were often relatively small, however, and prolonged inflammatory responses (lasting for at least 6 days) following the transfer injection of leucocyte suspensions were a complicating factor. This latter difficulty has been avoided, in studying the transfer of ragweed sensitivity, by employing leucocyte extracts rather than intact cells for the transfer. Substantiation of these findings, by means of experiments in which the effect of nonspecifically adsorbed reagin could be satisfactorily eliminated, would raise the need for revision of the currently held concept that delayed sensitivity is distinct from immediate sensitivity in that it is transferable by peripheral blood leucocytes.

The studies of Humphrey and Mota (1959) on the mechanism of mast cell damage during anaphylaxis in the guinea pig,³ which is thought to be produced by an antigen-antibody interaction with one of the reactants being reversibly adsorbed at the mast cell surface, might help in an attempted understanding of the role of the human leucocytes in the investigations just discussed and might also provide a guide as to the use of alternative cell types in the *in vitro* measurement of reagin-allergen combination. In this respect, the ingenious test of Shelley and Juhlin (1961), based on the basophile leucocyte degranulation response, would seem to be the most promising. This "miniature *in vitro* anaphylaxis system," as it is termed by the originators of the test, was used initially to demonstrate penicillin sensitivity by means of the explosive degranulation evoked on addition of the specific antigen to a sensitive individual's basophiles suspended in his own fresh heparinized blood. More recently, however, Shelley (1962) has reported the important development of an

³ See also the article by Austen and Humphrey in this volume.

indirect technique whereby it is possible to use rabbit's basophiles and the allergic patient's serum. Apparently the test has proved useful in diagnosing not only immediate hypersensitivity to a whole range of allergens, including foods, drugs, and inhalants, but also contact dermatitis and ulcerative colitis. It remains to be seen, however, whether it can be developed into a quantitative and specific *in vitro* alternative to the P-K test for the measurement of reagin. Cruickshank *et al.* (1962) have found that a positive basophile reaction occurs with the cells of allergic subjects, but not in all cases of immediate sensitivity.

It is possible, of course, that other types of cell such as the eosinophile, which is suspected to be implicated in some way in immediate sensitivity reactions, will also prove a useful indicator of reagin-allergen combination in a suitably designed *in vitro* system. Alternatively, the extremely sensitive "immune adherence" technique developed by Nelson (1953) might prove useful in the assay of reagins. In assays with sheep erythrocytes, Nelson (1962) has found that as little as 0.0005 μg . of antibody nitrogen will induce immune adherence agglutination, an effect which has proved of value in attempts to demonstrate antibody on the cells of recipients following homotransplantation, and in the serum following heterotransplantation. The need of complement for the occurrence of this phenomenon might not disqualify it, since the allergic release of histamine from leucocytes or platelets *in vitro* appears to show a similar requirement—Ca ions being found essential (Audia and Noah, 1960; Noah and Brand, 1959). The contrary conclusion of Middleton and Sherman (1960), that no measurable consumption of complement results from the addition of specific allergen to the plasma-suspended leucocytes of allergic individuals, has been criticized by Osler (1961), on the grounds that unfavorable experimental conditions for detecting complement activity were employed.

The finding that allergic human sera have a lower capacity than normal sera to inactivate histamine *in vitro*, apparently because of their deficiency of a histamine-binding γ -globulin (Parrot, 1958), deserves further attention. The observation by VanArsdel *et al.* (1958) of an inhibition of histamine release, from leucocytes suspended in allergic plasma *in vitro*, by high concentrations of allergen is also important as it could lead to spurious results from neutralization P-K tests (in which a large excess of challenging allergen is usually employed).

Of the potentially useful techniques that do not make use of cellular agglutination or destruction in the measurement of antibody concentration, the isotopically labeled antigen techniques, such as have been used in the estimation of insulin-binding antibody (Burrows *et al.*, 1957),

would appear to be the most promising. In this connection Stanworth (1961) failed to demonstrate any selective binding of I^{131} -labeled crude allergen by γ -globulin in the serum from a horse dandruff-sensitive individual, as revealed by the method of "overtaking electrophoresis" (Lang, 1955) on cellulose acetate paper, which was afterward autoradiographed; or by zone-centrifugation in a density gradient followed by a comparison of the radioactivity levels in fractions separated by slicing the tube. It is possible, however, that such an approach would have been more rewarding if highly pure reactants had been used. The availability of pure I^{131} -labeled allergen might lead to the adaptation of the extremely sensitive immune elimination technique of Patterson *et al.* (1960) to the measurement of reagins. This is capable of detecting a plasma antibody N level of 0.0033 $\mu\text{g./ml.}$, in rabbits passively sensitized with rabbit anti- I^{131} -labeled bovine serum albumin or γ -globulin. Lidd and Farr (1962), on the other hand, have employed an I^{131} -labeled ragweed pollen antigen mixture in an *in vitro* study of the binding of antigenic components of the pollen with the antibodies produced in the sera of humans and rabbits as a result of immunization with pollen extract. It was concluded, from selective ammonium sulfate precipitation, that binding by specific antibody was occurring. Pruzansky *et al.* (1962) have gone a step farther in demonstrating significant binding of I^{131} -labeled purified ragweed antigen by sera from untreated ragweed-sensitive individuals, but not by the sera of untreated nonallergic individuals. Sera from treated allergic individuals showed a higher maximum binding, attributed to more than one antigen being present in the purified ragweed preparation used. It will be necessary, however, to exclude the possibility that the same nonreaginic antibody is responsible for the binding by sera from both treated and untreated individuals.

Currently available physicochemical techniques would appear to be far too insensitive to measure reagins, although it is possible that the depolarization of fluorescence procedure might ultimately prove useful if its sensitivity can be increased. Dandliker and Feigen (1961) have applied this technique to the measurement of nonprecipitating rabbit antihapten antibody.

4. Calculation of Reagin Level in Human Allergic Sera

What is the concentration of reaginic antibody in the sera of hypersensitive individuals? It is obvious from the previous section that an accurate answer to this question cannot be provided at present. Nevertheless, it is possible to get an approximate idea of its order of magnitude, mainly from results obtained by the quantitative skin testing of anaphy-

lactic-type antibodies in animals and of skin-sensitizing animal antibodies in humans.

For instance, Ovary (1958) obtained strong PCA reactions in guinea pig skin with as little as 0.0032 μg . of antibody N in tests in which both antibody and challenging antigen (5 μg . N) were administered intradermally, as in the P-K test in humans. In the more commonly used technique, involving intravenous injection of antigen following intradermal sensitization with antibody, 0.003 μg . of antibody N was sufficient to provoke a reaction in most guinea pigs when using the rabbit anti-ovalbumin-ovalbumin system, provided the antigen was in great excess.

This provides some indication of the threshold quantities of reactants involved in the elicitation of P-K reactions in human skin, although it is not intended to imply that the same mechanism obtains in both the PCA and P-K reactions. Apart from the evidence for differences which have already been mentioned (in Section II, B), Ovary (1958), in drawing attention to the rapid disappearance of homologous and heterologous antibody from the guinea pig skin in contrast to the persistence of reagins in passively sensitized human skin, has suggested that perhaps the anaphylactic-type antibody does not fix itself to the cells of the guinea pig as readily as the reagins bind to human skin cells. As this is likely, the P-K test might be expected, perhaps, to be even more sensitive than the PCA procedure.

Ovary and Biozzi (1954) have shown that threshold PCA reactions in guinea pigs could be provoked with 0.13 μg . of antibody N of the non-precipitating diphtheria toxin-neutralizing antibody found by Kuhns and Pappenheimer (1952) in certain Schick-negative individuals' sera, which were devoid of precipitating antitoxin and which produced a wheal and erythema reaction on passive transfer into normal recipients later challenged with diphtheria toxoid. Kuhns and Pappenheimer (1952) and Kuhns (1955a) found that marked P-K tests could be elicited by dilutions of such sera containing 0.02-0.002 μg . of nonprecipitating antitoxin, as estimated by an independent titration with Schick toxoid in the skin of nonallergic Schick-positive humans. They also employed the highly sensitive and specific alternative method of assaying antitoxin by titration with toxoid in rabbit skin. Such a procedure is capable of detecting amounts of antitoxin smaller than 0.01 unit/ml. (equivalent to 0.2 μg . of antitoxin). If, as Kuhns and Pappenheimer believe, the nonprecipitating antitoxic and reaginic properties of their sensitizing sera are different manifestations of the same molecule, then here is a means of not only comparing the sensitivities of the PCA and P-K tests but also of obtaining an independent measurement of a human skin-sensitizing antibody.

Unfortunately, there are drawbacks to adopting such an approach. Finger and Kabat (1958) objected to the equating of reagin with the nonprecipitating antitoxin on the grounds that the skin-sensitizing antibody could have been directed against one of the contaminants readily detectable in diphtheria toxoid preparations by gel-diffusion precipitin techniques. If this is the case, the system is still interesting as an example of immediate sensitivity, but it then has nothing special to contribute to the attempts to ascertain the sensitivity of the P-K test. It is possibly significant that a study of the wheal and erythema sensitivity developed by a group of individuals vaccinated with diphtheria toxoid (Henocq *et al.*, 1962) has shown that, whereas 73% of Schick-negative persons were found to be sensitive to crude toxoid, only 18% were sensitive to a highly purified toxoid. Schick-positive persons were not sensitized to the pure toxoid. Nevertheless, Kuhns (1962a) still maintains that skin-sensitizing diphtheria antitoxin can be induced in Schick-negative persons receiving repeated doses of toxoid, probably as a response to antigen-antibody complex formation (and assisted by the delayed reactions generally occurring prior to the onset of immediate wheal reactivity). It is significant that the schedule and mode of injection of diphtheria or tetanus toxoid proved to be important factors in the induction of skin-sensitizing antibody, as was found in studies on the induction of reagin-like antibodies in animals (discussed in Section V, B).

In skin testing humans with specific precipitating *rabbit* antisera, W. B. Wood (1940) found that about 1 μg . of antipneumococcal antibody N was needed for passive sensitization, whereas in patients treated with the rabbit antipneumococcal sera the skin test with polysaccharide became positive when antibody N levels of 0.1 $\mu\text{g}/\text{ml}$. were reached. On the other hand, passive sensitization has not been achieved with *human* antipneumococcal antibodies, or with *human* antidextran in quantities of 1-8 μg . antibody N (Kabat and Mayer, 1961). In individuals immunized with dextran fractions Kabat and Bezer (1958) have observed wheal and erythema reactions associated with a circulating antidextran level of 7-8 μg . antibody N/ml., whereas in individuals immunized with native dextrans similar skin reactions were observed when the serum antidextran level was as low as 2-3 μg . antibody N/ml. (Kabat and Berg, 1953). In comparison, Farah *et al.* (1960) found that there was about 3 μg . of anti-2,4-dinitrophenyl (DNP) antibody per milliliter in the serum of an actively sensitized human, who showed wheal and erythema reactivity on intradermal injection of DNP protein or of the hapten. There are, however, obvious differences between the antibacterial polysaccharides or anticonjugated proteins and reagins, which limit any correlation

between the threshold levels revealed in the skin testing of the artificially induced antibodies and those revealed by P-K tests with the "spontaneously occurring" antibodies.

By employing the appreciably less sensitive technique of free-resolution electrophoresis, in an apparatus incorporating a Rayleigh interference optics system, Gyenes and Sehon (1961) decided that the failure to detect any change in allergic serum before and after "specific" adsorption of its reagins (and/or blocking antibodies) onto a polystyrene-allergen conjugate was indicative that reagins were present initially in the serum below a level of 0.1 mg./ml. (i.e., the measurable limit of protein concentration). As, however, the specificity of the adsorption procedure is doubtful, and recovery of skin-sensitizing activity from the column was not possible, this method appears to be of little value in the estimation of the concentration of reagins in allergic sera.

From the immunological data considered, it would seem likely that the sera of allergic individuals showing positive P-K reactions could contain as little as 0.02 μ g. antibody N/ml. (i.e., approximately 0.13 μ g. of protein) or about 0.1 mg./liter. This estimate should be borne in mind when assessing the results of physicochemical studies (to be considered next) in which attempts have been made to apply procedures designed primarily for the fractionation of the major serum protein constituents, to the isolation of a labile trace component.

III. Physicochemical Characterization of Reagins

A. LIMITATIONS

1. *Difficulties of Correlative Investigations*

As the previous discussion has indicated, the currently used physicochemical methods of analyzing protein mixtures are at least a thousand times too insensitive for the detection of reagin in allergic human sera. Hence it is not possible to show, for instance, a quantitative difference in the γ -globulin fraction of allergic as compared to nonallergic serum; nor can the specific removal of skin-sensitizing antibody from allergic serum be demonstrated as a reduction in the area of the γ -globulin peak of its electrophoretic pattern, in an analogous manner to the demonstration of antibody in immune rabbit serum (Tiselius and Kabat, 1939).

For this reason nearly all our present findings on the physicochemical characteristics of reagins have been obtained by means of correlative processes, the distribution of skin-sensitizing activity being *associated* with the characteristics of readily definable serum protein components separated in the various fractionation procedures employed. Such an

approach assumes that the reagin behaves ideally, as a separate molecular entity, under the varying conditions adopted. It is also necessary to assume the absence of inhibitors or cofactors, unless these can be distinguished from the reagin and measured independently. It is obvious, therefore, that the value of any attempted physicochemical characterization rests largely on the reliability of the techniques employed in the analysis of the fractions separated from allergic serum. The more specific, sensitive, and accurate these are, the more reliable the assignment of a particular property to the reagin molecule.

Apart from these limitations, there is also the problem of obtaining an accurate measurement of the sensitizing antibody in the isolated reagin-containing fractions. As already mentioned, the lack of a suitable *in vitro* method of assay and the variability of the P-K test in human recipients are two serious handicaps, although the latter can be minimized by quantitating the P-K test along the lines outlined in Section II, C. Another difficulty is the instability shown by isolated reagin-containing fractions, resulting in considerable losses in activity during dialysis and concentration procedures necessary in most characterization studies.

In view of these difficulties it seems reasonable to attribute the apparent divergence between the results obtained in early physicochemical studies of reagins to limitations of both the fractionation procedures employed and the techniques then available for ascertaining the composition of the resultant fractions. As the methods of analyzing proteins have become more refined and the resolution of fractionation procedures sharpened by the introduction of zone separation techniques, so has also the reliability of reagin characterization studies increased. This is reflected to some extent by the greater accord of the data obtained from recent physicochemical studies of reaginic sera, such as those involving zone electrophoresis. Nevertheless, the difficulties associated with the isolation of trace amounts of a serum protein remain. It is most essential, therefore, that any study of a particular allergic serum should employ the maximum possible number of complementary physicochemical techniques. It is also necessary to relate the P-K activities of fractions to their protein contents, in order to permit a comparison of their "specific activities" with that of the parent allergic serum. Only results obtained from such an approach will lack ambiguity.

Finally, there is the problem of inhibitory serum constituents, such as blocking antibody. It is possible that the presence of this antibody, in the fractions of serum from an allergic individual in a doubtful state of hypersensitivity, could lead to spurious results if the neutralization P-K test is used for the assay of reagin activity. It is most important, therefore, to

know whether physicochemical studies have been carried out on serum from treated or untreated patients. For this reason such information, where available, has been included in Tables I and II which summarize the physicochemical data on the properties of reagins. Another potential inhibitor is the nonspecific γ -globulin present in reagin-containing fractions isolated from allergic sera. It is conceivable that such a protein could inhibit P-K activity in the same way that nonspecific rabbit serum γ -globulin inhibits the passive sensitization of isolated guinea pig ileum by rabbit antibody (Binaghi *et al.*, 1962).

2. *Instability of Reagins in Vitro*

As is well known, allergic sera can be stored for long periods, under suitable conditions, without loss in their P-K activity. For instance, it has been possible to preserve the activity of serum from a horse dandruff-sensitive individual for at least a year by storage under sterile conditions at -20°C . or in freeze-dried form under nitrogen in sealed ampoules at 4°C . (Stanworth, 1961). There have been many other reports of the reagin activity of whole serum withstanding storage in the frozen state and, sometimes, at 4°C . In contrast, however, considerable losses of P-K activity have been found to occur under conditions obtaining during many serum fractionation procedures. Thus, Cooke and his associates (1960) have shown that once reagin is submitted to conditions outside its pH range of relative stability (pH 6–11), as are encountered in some electrophoresis-convection techniques, appreciable losses of P-K activity can occur. On the other hand, losses of a similar order of magnitude have been frequently experienced under conditions within this pH range, when procedures involving fractionation in solid media have been employed. In particular, starch electrophoresis has proved deleterious, as also have chromatographic techniques, although these sometimes involve the use of eluting solvents with pH values outside the stability range. It is difficult, however, to establish at which stage loss in P-K activity occurred. Nevertheless, there is plenty of evidence to suggest that, once isolated from other serum proteins, the reagin is readily susceptible to surface deactivation. This can take the form of irreversible adsorption to the supporting medium employed in the fractionation procedure or denaturation during subsequent manipulations in which the isolated reagin molecules come into contact with a variety of surfaces such as cellophane dialysis tubing, cellulose nitrate ultrafilters, and cellulose acetate Seitz filters. Furthermore, isolated reagin fractions do not retain their P-K activity when stored under conditions that preserve the activity of whole serum (Sehon *et al.*, 1956). Activity is also readily lost

during refractionation procedures. Efforts to reduce losses in P-K activity during fractionation of allergic sera by means of additives such as sucrose, human serum, and albumin have not proved very successful, although the activity of column eluates can be preserved to some extent by collecting them in tubes containing small aliquots of serum or serum protein solutions. This is important when dealing with dilute protein eluates, which are particularly susceptible to denaturation. Use of P-K test recipients' sera or fractions is to be recommended in such practices, as this avoids the risk of transferring infective hepatitis. It is essential, of course, to subject all fractions of allergic sera to the same conditions during concentration and sterilization procedures, if a true comparison of their skin-sensitizing activities is to be made. Even then, the relatively big differences in the protein concentrations of solutions of serum fractions isolated in a procedure such as DEAE (diethylaminoethyl) cellulose chromatography can lead to artifacts, on account of greater losses in P-K activity occurring in the dilute protein solutions. Such solutions are also more likely to deposit insoluble material, which can adsorb reagins irreversibly.

B. SALT AND ETHANOL FRACTIONATION STUDIES

It is not surprising that the degree of resolution of serum proteins effected by ammonium sulfate or sodium sulfate fractionation (Scherrer, 1930; Stull *et al.*, 1937) proved insufficient to differentiate between reagin and immune antibodies, both precipitating in the pseudoglobulin fraction. On the other hand, the Cohn low temperature-ethanol procedure concentrated ragweed-reactive reagin in a different fraction (predominantly β -globulin) to that in which precipitating antibodies would have been found (Vaughan *et al.*, 1952). Other investigators have employed fractional precipitation techniques to demonstrate that the skin-sensitizing activity of allergic sera is associated with fractions containing α_2 -globulin as a major component (Kuhns, 1953, 1962b; Cooke *et al.*, 1960).

Heremans and Vaerman (1962) have reported the results of a preliminary study of the distribution of reagin activity among the protein fractions obtained from the sera of grass pollen-sensitive patients, by means of a small-scale simplification of the zinc sulfate precipitation procedure used by Heremans (1960) for the isolation of γ_{1A} -globulin (previously termed β_{2A} -globulin). Fractions rich in γ_{1A} -globulin (containing twice the starting serum level) showed P-K activities of the same order of magnitude as their corresponding allergic sera, whereas their γ -globulin concentrations never exceeded 1% of that in serum as

measured by a quantitative gel-diffusion precipitin technique. On the basis of these observations, it was suggested that the sensitizing activity of allergic sera is "essentially and perhaps quantitatively" carried by their γ_{1A} -globulin. This is an attractive idea in view of the lack of success of efforts to assign an antibody activity to the γ_{1A} -globulin, which has been shown by gel-diffusion precipitin analysis to be structurally related to the γ and γ_{1M} immune globulins (Heremans, 1960; Franklin and Stanworth, 1961). Besides, the γ_{1A} -globulin shares the reagin's inability to cross the placenta, and its heterogeneous ultracentrifugal composition (Heremans, 1960) could account for the apparently divergent claims as to the reagin's molecular size. Immunoelectrophoretic analyses of Heremans and Vaerman's allergic sera fractions revealed, however, variable amounts of transferrin, haptoglobin, 3.5 S α_1 -glycoprotein, and albumin, in addition to a strong γ_{1A} line. It will be important to establish whether P-K activity can be associated exclusively with immunoelectrophoretically and ultracentrifugally homogeneous γ_{1A} -globulin fractions, before accepting that this protein is the skin-sensitizing antibody. The apparent tendency of γ_{1A} -globulin to form complexes (Heremans, 1960) with other proteins also deserves further attention in relation to its potential reaginic properties. This could, of course, explain the localization of reagin in γ_{1A} -globulin fractions of allergic serum. It would be interesting to see whether the γ_{1A} -globulin level in the sera of hypogammaglobulinemic patients can be correlated with any clinical symptoms of hay fever (and with corresponding P-K activity).

A recent observation by Fireman *et al.* (1963) that precipitation with a specific sheep antihuman γ_{1A} -globulin serum quantitatively removed the P-K activity from the sera of three ragweed-sensitive individuals, would seem to support the suggestion that reagin can be classified as a γ_{1A} -globulin.

C. ELECTROPHORETIC CHARACTERISTICS

As the data given in Table I indicate, free-solution electrophoresis procedures have proved as inadequate as fractional precipitation techniques in the isolation of reagin. Moreover, the considerable overlapping of the components of boundary electrophoresis fractions prevents any definitive conclusion as to the reagin's mobility.

The development of zone-separation procedures (in supporting media) has extended the scope of the electrophoresis technique, however, both as a preparative and as an analytical tool. This is due largely to the enhanced boundary stabilization achieved during separations in solid media, which has not only permitted a high degree of *absolute*

TABLE I
ELECTROPHORETIC CHARACTERISTICS OF REAGINS

No. of sera studied	State of donors	Method of fractionating allergic serum	Method of characterizing fractions	P-K procedure used for assay of skin-sensitizing activity	Allergen against which reagin is directed	Electrophoretic characteristics of reagin	References
3 (+1 pooled)	?	Free-solution electrophoresis	—	Qualitative	Ragweed pollen Rabbit P dander	γ -Globulin	Newell <i>et al.</i> , 1939
1		Electrophoresis convection	Free-solution electrophoresis	Dilution test	Egg white	α_2 -Globulin (pre-dominantly)	Campbell <i>et al.</i> , 1950
1	?				Cottonseed	α_1 - and γ -Globulin	
Several	? Hormone-treated	Free-solution electrophoresis	Free-solution electrophoresis	Dilution test	Ragweed pollen Cat and dog dander	Not γ -globulin (predominantly)	Cooke <i>et al.</i> , 1951
1	?	Free-solution electrophoresis	Free-solution electrophoresis	Dilution test	Ragweed pollen	? α - and γ -Globulin	Menzel <i>et al.</i> , 1952
1	Treated	Electrophoresis convection (Combined with salt precipitation)	Free-solution electrophoresis	Dilution and neutralization tests	Insulin	β -Globulin	Loveless and Cann, 1953
1				Dilution and neutralization tests	Ragweed pollen	β -(γ)-Globulin	
7	Untreated	Electrophoresis convection	Free-solution electrophoresis	Dilution test (average of results from 4 recipients)	Ragweed pollen	α_2 -Globulin (pre-dominantly)	Campbell <i>et al.</i> , 1954
					Ovalbumin Cottonseed	α - and γ -Globulin	

TABLE I (Continued)

No. of sera studied	State of donors	Method of fractionating allergic serum	Method of characterizing fractions	P-K procedure used for assay of skin-sensitizing activity	Allergen against which reagin is directed	Electrophoretic characteristics of reagin	References
1	Untreated	Electrophoresis convection	Free-solution electrophoresis	Dilution and neutralization tests	Cottonseed	β -Globulin (pre-dominantly)	Cann and Loveless, 1957
8 (at least)	Untreated	Free-solution electrophoresis "Beaker train" electrophoresis Starch block electrophoresis	Free-solution electrophoresis Paper electrophoresis	Dilution test (fraction end titer referred to serum end titer)	Ragweed pollen	β - and γ -Globulin γ_1 -Globulin (pre-dominantly) ?	Cooke <i>et al.</i> , 1960
9	Treated	Starch block electrophoresis	—	Dilution and neutralization tests	Diphtheria toxoid	γ_1 -Globulin	Kuhns, 1954a
1	? Untreated	Continuous, starch electrophoresis	By analogy with electrophoretic properties of normal serum fractions	Direct, semi-quantitative estimate of wheal size	Cottonseed	β_2 - and γ_1 -Globulin	Brattsten <i>et al.</i> , 1955
10	? Untreated	Starch block electrophoresis	Free-solution electrophoresis Paper electrophoresis	Direct, semi-quantitative estimate of wheal size	Ragweed pollen	β - and γ -Globulin	Sehon <i>et al.</i> , 1955

TABLE I (Continued)

No. of sera studied	State of donors	Method of fractionating allergic serum	Method of characterizing fractions	P-K procedure used for assay of skin-sensitizing activity	Allergen against which reagin is directed	Electrophoretic characteristics of reagin	References
18	10/17 Untreated 7/17 Treated	Starch block electrophoresis	Free-solution electrophoresis Paper electrophoresis	Wheat size compared visually with sizes of wheals elicited by serial dilutions of serum	Ragweed pollen	γ_1 - and γ_2 -Globulin	Sehon <i>et al.</i> , 1956
8	1/8 Treated	DEAE cellulose chromatography	Paper electrophoresis	Direct measurement of wheal diameters	Grass pollens Cat and dog dander Horse serum	γ_1 -Globulin (predominantly) γ_1 - and γ_2 -Globulin	Humphrey and Porter, 1957
1	Untreated with specific allergen	DEAE cellulose chromatography	Paper electrophoresis Immunoelectrophoresis	Direct measurement of wheal area (converted to diameter)	Horse dander	γ_1 -Globulin (predominantly)	Stanworth, 1959, 1960b

resolution but has also facilitated the detection of minor components missed by the free-solution procedures. Taking into consideration these basic differences in technique, it is interesting to compare (in Table I) the characteristics of reagin revealed by means of zone-electrophoresis studies with those revealed by the earlier moving boundary fractionations of allergic sera. Although there is some divergence between results obtained from zone electrophoresis in the hands of different investigators, maximum reagin activity is generally found in the "fast" γ -globulin region. This is supported by the results of electrophoretic analyses of fractions obtained by the chromatographic fractionation of allergic sera on substituted cellulose ion exchangers, according to the method devised by Sober and his associates (1956). Humphrey and Porter (1957) showed, for example, that the reagin content of sera from individuals hypersensitive to grass pollens or to other allergens was confined largely to a γ_1 -globulin chromatographic fraction, comprising 10% of the total serum γ -globulin and distinct from the major γ_2 -globulin fraction. This was confirmed by Stanworth (1959) in the chromatographic analysis of serum from a horse dandruff-sensitive individual.

What is the reason for the apparent difference in the electrophoretic characteristics of reagins revealed by zone-separation procedures as opposed to those indicated by free-solution electrophoresis studies? The lower resolving power of the earlier methods and the relative insensitivity of the technique used to obtain the electrophoretic compositions of the separated fractions are no doubt partially responsible. After allowing for the contribution of these factors, however, it seems that the reagin molecules have a tendency to move faster during free-solution electrophoresis than during electrophoresis in solid media. An obvious explanation of such behavior would be an interaction between the reagin molecules and the material of the supporting medium, resulting in a reduction in electrophoretic mobility. In discussing such a possibility, Cann and Loveless (1957) have cited as an analogous example the property of C-reactive protein, which was found by H. F. Wood *et al.* (1954) to move as a β -globulin during free-solution electrophoresis but as a fast γ -globulin during starch electrophoresis. If reagin shows a similar type of binding to the carbohydrate of the separation medium it would be expected to behave differently during zone electrophoresis in a synthetic medium such as polyvinyl chloride. Stanworth and Kuhns (1963b), however, have failed to observe any difference between the electrophoretic characteristics of horse dandruff reagin when fractionated in such a medium as compared with its behavior during electrophoresis in potato starch. Unfortunately, the results were difficult to interpret

because of the appreciable losses of skin-sensitizing activity encountered in both cases. In contrast, admixture of allergic sera with potato starch or polyvinyl chloride *without* application of an electric field had little effect on P-K activity. On the other hand, Cooke *et al.* (1960) have observed that losses of P-K activity of at least 50% were incurred by merely passing allergic sera through potato starch columns, although the eluates apparently contained all of the original serum proteins. Passage through filter paper columns caused even greater losses in P-K activity, amounting to as much as 60–90%. Losses of P-K activity of a similar order of magnitude were noted after zone electrophoresis of allergic sera in potato starch, recoveries greater than 15% never being achieved. There seems little evidence, therefore, to support the recommendation of starch electrophoresis as useful for the fractionation of allergic sera on account of its mildness toward biologically active materials (Sehon *et al.*, 1955, 1956). Sehon and his associates base such a recommendation on their failure to observe any significant change in the electrophoretic pattern of recombined eluates obtained from a starch electrophoretic fractionation of allergic serum, after Seitz-filtration and concentration by pervaporation through Visking tubing. Stanworth (1959) has shown, however, that reconcentration of an allergic serum, diluted fifty times with saline, to its original volume by ultrafiltration through Visking tubing leads to a 50% loss in P-K activity; although no significant *detectable* changes in the serum ultracentrifugal or electrophoretic patterns were observed. Furthermore, Sehon and his associates (1955, 1956) themselves have reported complete losses of the skin-sensitizing activity of several sera from ragweed-sensitive individuals, as a result of starch electrophoresis.

Considerable losses in reagin activity are also frequently encountered during the chromatographic fractionation of allergic sera on DEAE cellulose. For example, even in the most successful fractionations of allergic serum from a horse dandruff-sensitive individual only 60% of the total activity applied to the column was recovered. This paralleled closely the recovery of total protein. Perelmutter *et al.* (1961) reported even greater losses in skin-sensitizing activity, amounting to 50–70% of the original titer, as a result of fractionating sera from ragweed-sensitive individuals on commercial DEAE cellulose.

An attempt to overcome reagin deactivation arising from this irreversible adsorption on DEAE cellulose columns was made by application of a batch chromatographic technique (Stanworth, 1960a) designed for the rapid isolation of pure γ -globulin. The two-stage process adopted involved arranging the conditions so that all serum proteins except the

total γ -globulin (γ_1 , including the reagins, + γ_2), were adsorbed on to DEAE cellulose. Conditions were then altered so that all the γ_2 -globulins were adsorbed onto CM (carboxymethyl) cellulose with the aim of leaving behind a purified reagin preparation supposedly of high activity, because it had at no stage been specifically adsorbed onto the ion exchangers. Immunoelectrophoretic analysis showed that a pure γ_1 -globulin component had been isolated, but it proved inactive on P-K testing. This loss of activity could have been caused by nonspecific irreversible adsorption, although there is another explanation as will be discussed.

It would appear that free-solution techniques are to be preferred for the potentially greater recovery of biological activity they afford, whereas zone-separation procedures have the advantage of providing greater resolution of serum protein components. This superior resolution could be responsible for the frequently observed instability of isolated reagin fractions. Indeed, the author has formed the impression, from the results of a large number of chromatographic fractionations of allergic serum on DEAE cellulose, that the greater the degree of resolution achieved, the lower the recovery of P-K activity. This was noticeable from the results of two-stage batch chromatographic fractionations already described and also in fractionations by means of a modified column procedure, involving subfractionation of the γ_1 -globulin as a result of slight changes in ionic strength at a constant pH of 7.5 (Stanworth, 1960b). A minor, highly pure, γ_1 -globulin subfraction thus obtained, comprising only about 4% of the total serum globulin, showed little P-K activity after concentration by ultrafiltration. On the other hand, premixing of the fraction with about 2% (w/w) of pure inactive human serum α_2 -glycoprotein, resulted in the detection of appreciable P-K activity (comparable to the activity of whole serum) in the concentrated γ_1 -globulin fraction. This strengthened the suspicion that traces of α_2 -macroglobulin contaminant had been protecting previous less purified, but P-K active, γ_1 -globulin fractions obtained by DEAE cellulose batch chromatography. It is possibly significant, too, that Augustin and Hayward (1960) have found that even their active γ -globulin chromatographic fractions, obtained from sera of grass pollen-sensitive individuals, contained traces of α_2 -macroglobulin. Kuhns (1962b) also has provided evidence that a serum α_2 -globulin component stabilizes reagin activity in ethanol-precipitated fractions of diphtheria antitoxic sera. If the serum α_2 -macroglobulin does exert a protective effect on reagin activity, it is conceivable that other processes such as zone electrophoresis, which usually accomplish a clear-cut separation of the α_2 - and γ -globulins, will lead to losses of P-K activity. On the other

hand, the free-solution techniques do not effect the same degree of separation of these components and, therefore, provide better recoveries of P-K activity. Of interest, in this connection, is Kuhn's (1954b) observation of an alteration of the α_2 -globulin component, associated with a relative preponderance of slow migrating protein-bound polysaccharide, in paper electrophoretic patterns of skin-sensitizing diphtheria antitoxic sera from subjects showing marked eosinophilia. Such alterations, distinguished by a relatively large β -globulin component and a small or indistinct α_2 -globulin (or sometimes by a fusion of both) were not observed in sera taken from the same individuals before or after their period of eosinophilia.

Summarizing the results obtained from electrophoretic studies of allergic sera, it seems likely that reagins are γ_1 -globulins, readily susceptible to deactivation when separated from other serum protein constituents by starch electrophoresis or DEAE cellulose chromatographic fractionation procedures. There is a possibility that some form of combination with an α_2 -globulin component occurs under certain conditions, which stabilizes the trace reagin component during free-solution electrophoresis of allergic sera.

In contrast to reagin, blocking antibody has been found, by electrophoresis-connection studies (Loveless and Cann, 1955) and by starch electrophoresis (Sehon *et al.*, 1957) of allergic sera (from treated hay-fever patients) to move in a "slower" γ -globulin fraction (often referred to as γ_2 -globulin).

D. SEDIMENTATION CHARACTERISTICS

Failure to detect skin-sensitizing antibodies in cord blood or in the blood of infants of ragweed-sensitive mothers led to the speculation that reagins are probably 19S type antibodies like the isoagglutinins, Rh agglutinins, and typhoid O agglutinins, which all show a similar inability to cross the placenta. Sehon (1958, 1959) and his associates have subscribed to this idea, in their claims to have shown that ragweed reagins are 19S antibodies. Their evidence (Gyenes *et al.*, 1961), however, is based entirely on the results of sedimentation studies carried out in separation cells with fixed (Tiselius *et al.*, 1937) or moving (Yphantis and Waugh, 1956) partitions. Ideally, such a technique has the advantage of providing the sedimentation coefficient of a biologically active trace component of an impure preparation, merely by measurement of the rate of transport of its activity across the partition. There are, however, limitations to this approach (Schachman, 1959), which could restrict its application to the study of the sedimentation charac-

teristics of reagins. This is obvious from the variation in the results obtained by the Sehon group—the sedimentation coefficient of reagin determined from repeat runs on the same allergic serum being found to range from 14.1 to 22.5 S. It is unfortunate that these findings were not checked by an examination of the protein compositions of the contents of the two compartments in the partition cell after sedimentation. This could have been achieved by application of the sensitive, quantitative gel-diffusion precipitin technique, rather than by relying solely on the appreciably less sensitive Schlieren optics system of the analytical ultracentrifuge.

Ultracentrifugal studies by other investigators have failed to substantiate the findings of the Sehon group (see Table II). For example, Heimlich *et al.* (1960) have employed preparative ultracentrifugation to show that the skin-sensitizing activity of sera from individuals with sensitivities to various allergens could not be associated exclusively with a 19 S component. This was indicated by the demonstration that repeated preparative ultracentrifugation of allergic sera, with separation of the pellet which formed each time, led to complete removal of 19 S protein as revealed by analytical ultracentrifugation, whereas the supernatants showed P-K activity comparable with that of the whole serum. The results obtained from analytical studies, in which a partition cell was used as a preparative tool, were entirely consistent with these findings. Heimlich and his associates have suggested that the finding of some skin-sensitizing activity in the pellet material could be explained if the reagins have an intermediary sedimentation coefficient between 7 and 19 S. As an alternative explanation it was also suggested that skin-sensitizing activity might be associated with antibodies of different molecular weights. Another reason might be, however, that reagin becomes occluded in the pellet material, as is quite possible in the type of preparative ultracentrifugation procedure adopted. Moreover, ultracentrifugal analysis of a solution of the final pellet material did appear to reveal some contaminating 7 S component. The results of the rather complicated preparative ultracentrifugation studies of Augustin and Hayward (1960), on fractions of sera from grass pollen and mold-sensitive individuals, tend to support this suggestion. By repeated cycling of DEAE cellulose chromatographic fractions, depleted of 19 S γ -globulin, these investigators demonstrated that the activity of the pellet material could be progressively reduced at a rate compatible with it being associated with 7 S γ -globulin contaminant, as measured by gel-diffusion precipitin techniques.

These sedimentation studies employed ultracentrifugal methods

TABLE II
SEDIMENTATION CHARACTERISTICS OF REAGINS

No. of sera studied	State of donors	Method of fractionating allergic serum	Method of characterizing fractions	P-K procedure used for assay of skin-sensitizing activity	Allergen against which reagin is directed	Sedimentation characteristics of reagin	References
2	Untreated	Ultracentrifugation in separation cells	Analytical ultracentrifugation		Ragweed pollen	16.5 S	Sehon, 1958, 1959
15	?	Preparative ultracentrifugation	—	—	? Pollen	Not 19 S	Campbell, 1959
Small number	Treated	? Starch electrophoresis	Analytical ultracentrifugation	—	Diphtheria toxoid	Same properties as precipitating antitoxin (i.e., 7 S)	Kuhns, 1959
1	Untreated with specific allergen	DEAE cellulose chromatography Zone centrifugation in buffered sucrose gradients	Analytical ultracentrifugation Quantitative gel-diffusion precipitin analysis	Direct measurement of wheal area	Horse dandruff	7 S	Stanworth, 1959
12	6 Untreated	Preparative ultracentrifugation	Analytical ultracentrifugation Free-solution electrophoresis	Direct, semi-quantitative estimate of wheal size	Grass, weed, epidermal and mould extracts	Not 19 S	Heimlich <i>et al.</i> , 1960

TABLE II (Continued)

No. of sera studied	State of donors	Method of fractionating allergic serum	Method of characterizing fractions	P-K procedure used for assay of skin-sensitizing activity	Allergen against which reagin is directed	Sedimentation characteristics of reagin	References
3	Untreated	Preparative ultracentrifugation of DEAE-cellulose chromatographic fractions	Immunoelectrophoresis Gel-diffusion precipitin analysis	Direct, semi-quantitative, comparison of wheal size	Grass pollen	Probably 7 S	Augustin and Hayward, 1960
12	Untreated	Ultracentrifugation in separation cells	Analytical ultracentrifugation	Dilution test	Ragweed pollen	12.4-22.5 S	Gyenes <i>et al.</i> , 1961
1	Treated	Zone centrifugation in buffered sucrose gradients	Comparison with sedimentation characteristics of isohemagglutinins	Direct, semi-quantitative estimate of wheal size	Mammalian glucagon (insulin preparation)	8-11 S	Rockey and Kunkel, 1962

which share the disadvantages of free-solution electrophoretic fractionations in that they achieve a *boundary* separation of *overlapping* zones, in contrast to zone-separation procedures such as starch electrophoresis where separation into discrete zones is accomplished. It is surprising, therefore, that the technique of zone centrifugation in a density gradient has not been employed more widely, particularly as this procedure avoids the deleterious irreversible adsorption effects of a solid supporting medium by incorporating a *liquid* buffered sucrose or salt gradient to stabilize against convection and other disturbances. It also has the advantage of avoiding pellet formation by the fast sedimenting proteins. Stanworth (1959) has successfully applied this simple and direct technique to the fractionation of allergic serum from a horse dandruff-sensitive individual. The serum was zone centrifuged in buffered sucrose gradients, in lusteroid tubes in a swingout rotor. Afterward the tubes were sliced with a mechanical slicer and the P-K activities of the fractions were estimated directly after dialysis and Seitz filtration. There was a good correlation between the distribution of reagins in the four fractions recovered and the distribution of 7 S γ -globulin (but not 19 S γ -globulin) as estimated both by analytical ultracentrifugation and by the much more sensitive, quantitative gel-diffusion precipitin technique devised by Gell (1957). Moreover, the recoveries of P-K activity were consistently as high as 90% or more in contrast to the low recoveries usually obtained by zone electrophoresis or column chromatographic procedures. This could probably be attributed to the presence of other protective serum proteins in the heterogeneous fractions or to the stabilization afforded by the high viscosity sucrose solutions employed in the centrifugation medium. A refinement of the technique involved the use of purified 4.5, 7, and 19 S serum proteins labeled with different colored dyes to act as markers, in a reference mixture run in parallel (Stanworth *et al.*, 1961). In this way a direct comparison can be made between the location of maximal P-K activity and the principal serum protein zones. Moreover, by slicing the tube into many fractions (at least 13) an ultracentrifugal pattern, analogous to the protein distribution pattern obtained from analysis of zone-electrophoresis fractions, can be constructed by plotting the optical density at 280 m μ against the distance of the center of the zone from the meniscus. Determination of the P-K activities of the multiple fractions permits a detailed demonstration of reagin distribution throughout the centrifuge tube. By this approach (Stanworth, 1961) maximal reagin activity was located slightly ahead of the 7 S zone during fractionations of similar allergic serum to that used in the previous zone-centrifugation

studies, where relatively small differences in sedimentation rate would not have been differentiated. On the assumption that there were only two sedimenting classes of antibody (i.e., 7 and 19 S), these results were thought to reflect some form of alteration in the size of a 7 S reagin molecule (possibly as a result of combination with another serum component).

It is of particular interest, therefore, that Rockey and Kunkel (1962) have used an essentially similar zone-centrifugation procedure to demonstrate that the glucagon-reactive skin-sensitizing antibody, in the serum of an allergic diabetic patient, possessed a sedimentation coefficient in the range 8–11 S. Furthermore, these workers have provided evidence that certain isohemagglutinins sediment at similar rates, thereby constituting a *third sedimenting class* (9–15 S) of antibodies; all of which appear to be deactivated by treatment with 0.1 M mercaptoethanol. Obviously, it will be most important to establish whether the naturally occurring reagins referred to in Table II show similar characteristics, as the methods employed in their study would not have distinguished between 7 S and 8–11 S sedimenting classes. On the other hand, 19 S type antibodies should not have been confused with the 8–11 S class.

The observations of Rockey and Kunkel (1962) would seem to support the findings of other investigators (discussed earlier) which suggested that the skin-sensitizing activity of sera from ragweed-sensitive individuals was associated with the γ_{1A} -globulin component. As already mentioned, however, the observed intermediate sedimentation rate of certain γ_{1A} myeloma proteins (Laurell, 1961) might result from the tendency of these globulins to combine readily with other serum proteins. A similar combination between 7 S reagins and an α_2 -globulin component, such as was proposed previously to explain the divergent free-resolution electrophoresis results, might have complicated the results obtained in some procedures applied to the investigation of the sedimentation characteristics of skin-sensitizing antibodies. Augustin and Hayward (1960) have suggested that their preparative ultracentrifugation results might be due to this form of combination between 7 S reagin and α_2 -macroglobulin.

The application of alternative techniques is required to establish firmly the size of reaginic antibodies. In this connection it is interesting to note that a close correlation was found between skin-sensitizing activity and 7 S γ_1 -globulin concentration in the DEAE cellulose chromatographic fractions of the serum of a horse dandruff-sensitive individual (Stanworth, 1959). No 19 S γ -globulin was detectable (either by ultra-

centrifugal or immunological estimation) in the fractions containing maximum P-K activity; nor could 8–11 S component be detected, but this could have been present in concentrations below the lower limit of detection of the Schlieren optical system of the analytical ultracentrifuge. It is significant, however, that Rockey and Kunkel (1962) found that the major activity of intermediately sedimenting antibodies (isohemagglutinins and the glucagon-reacting skin-sensitizing antibody) was eluted from DEAE cellulose chromatography columns at a later stage than the usual position of elution of the principal 7 S γ_1 -globulin fraction, in which spontaneously occurring reagins have been mainly located (Humphrey and Porter, 1957; Stanworth, 1959). Moreover, the intermediately sedimenting antibodies showed a wide distribution of activity among the chromatographic eluates, which resembles the wide distribution of electrophoretic mobility shown by the skin-sensitizing antibodies induced in animals by prolonged treatment (which will be discussed later).

The newly developed technique involving selective filtration through cross-linked dextran gels, manufactured under the trade name of Sephadex (Porath and Flodin, 1959), should prove useful as an alternative method to ultracentrifugation in the elucidation of the true size of the reagin molecule. Preliminary results obtained by this approach are not inconsistent with horse dandruff-reactive reagin having a sedimentation coefficient in the 8–11 S range. Fireman *et al.* (1963) have recently reported a similar finding, from studies of the distribution of skin-sensitizing activity in fractions obtained by gel-filtration (on Sephadex G-200) of sera from ragweed-sensitive patients.

It is possible, of course, that different allergens evoke the production of antibodies of different sizes. Alternatively, reagin molecules of different molecular size may be produced against the same allergen by different individuals, or by the same individual at different times. Ion-exchange analyses of human sera (Abelson and Rawson, 1959; Fahey and Morrison, 1960) have demonstrated an association of isohemagglutinin activity with both 7 and 19 S classes of γ -globulin. Of possibly greater relevance, however, are the results of studies of the various molecular forms of antibody produced by infants and adults at different stages after immunization. For instance, Lospalluto and his collaborators (1962) have shown that antibodies of different sizes are formed at different stages during the immunization of normal and diseased humans with typhoid antigens. If a similar response is shown by predisposed humans, as a result of inhalation of allergenic materials, one might expect to find 7 S reagin in the sera of all but the newly sensitized in-

dividuals. Nevertheless, the finding of the Lospalluto group, that the change in antibody form from 19 to 7S type following typhoid immunization requires a considerable time in adults and is rarely complete, suggests that traces of 19S reagin might also be detectable. Before all these possibilities are invoked, however, to account for any apparent discrepancies in the results of sedimentation studies of reagins, it will be necessary to ascertain that divergencies have not arisen as a result of the limitations of the techniques employed in the determination of antibody molecular size. If substantiated, the existence of yet a third class of antibodies with sedimentation rates intermediate between the 7 and 19S types will complicate the issue and demand repeat studies by improved methods of determining molecular size.

IV. Tissue Studies

A. REAGIN-BINDING SITES

Results obtained from the passive sensitization of normal humans with blood of hypersensitive donors (Mills and Schiff, 1926; Garver, 1939) support the findings of local transfer studies in demonstrating a rapid attachment of reagin to the recipient's tissues. Loveless (1941) has shown by means of controlled tests in the skin, conjunctiva, and nasal membranes that the ragweed-sensitizing antibody from transferred blood was taken up by all such tissues, but most readily by the skin. Walzer and his associates have also demonstrated the passive local sensitization of ophthalmic (Walzer *et al.*, 1935) and nasal (H. Sherman *et al.*, 1937) mucous membranes. There is evidence, too, that reagins attach to other tissues in man and monkeys such as the linings of the stomach and intestine (Walzer *et al.*, 1938; Gray *et al.*, 1940; Walzer, 1941). Moreover, the demonstration (Schild *et al.*, 1951) that a chain of bronchiole rings, taken from the lung of a pollen-sensitive asthmatic, will respond to contact with specific allergen in a Schultz-Dale experiment indicates an attachment of reagin to bronchiole tissue.

Little is known, however, about the mechanism of this reaction or about the nature of the reagin-binding sites. Few *in vitro* studies with human tissue have been reported, although Samsøe-Jensen and Hauge-Kristensen (1960) claim to have probably demonstrated the *in vitro* fixation of grass pollen-reactive reagins to freshly excised normal skin but not to mesenchymal tissue. It is always difficult to be certain that the possibility of nonspecific adsorption or deactivation has been excluded in such experiments, but it is perhaps of some significance in this respect that no fixation to heated (30 minutes at 60°C.) skin was detected. W. B. Sherman and Seebohm (1950) had employed a similar

procedure to demonstrate the removal of passive skin-sensitizing activity from the serum of a cold-sensitive individual, by *in vitro* absorption at 4°C. for 24 hours with a human skin cell preparation (but not by absorption with type O red cells). This apparent fixation of "cold" reagins to skin *in vitro* has been since confirmed by Samsøe-Jensen (1955), who used small pieces of skin excised from different nonallergic individuals. If one postulates that a tissue antigen (allergen) is probably implicated in cold-sensitization, it might be expected that the "cold" reagins would fix more readily to skin *in vitro* than would reagins directed against external foreign substances. Attempts of this kind to show the fixation of reagins to skin taken from *allergic* individuals do not appear to have been made.

Rappaport (1960) has endeavored, however, to study reagin-allergen combinations in skin from allergic individuals, which had been challenged with antigen either *in vivo* before biopsy or afterward *in vitro*, by means of the "direct staining technique" with fluorescein-tagged antibody (Coons and Kaplan, 1950). Specific staining of sections of skin taken from an egg albumin-challenged site in an egg-sensitive patient was claimed to have been achieved with crude reagin serum globulin fractions (prepared by ethanol or salt fractionation), and more easily with unfractionated serum, conjugated with lissamine rhodamine B 200 or with fluorescein isothiocyanate. The epithelial cells of the sweat glands, hair follicles, and sebaceous glands and also macrocytes and pericytes were all specifically stained by this procedure as well as by similarly conjugated *rabbit antihuman globulins*. Moreover, similar effects were observed in skin tissues which had been coated with specific antigen *in vitro*, from which it was inferred that the skin of hypersensitive individuals contains specific reagins in all epidermal cells in unchallenged as well as in challenged areas. Although control staining of unchallenged and histamine-challenged human skin sections failed to reveal any reaction, it is not certain that *specific* staining with conjugated *reagin* has been achieved in these interesting studies. Even although the P-K activities of the reagin preparations used apparently withstood the prolonged fluorescent labeling process, there is some doubt as to whether reagin-bound antigen was being revealed. It is possible, for instance, that a precipitating antibody (directed against ovalbumin or some other minor egg white constituent) was conjugated in the crude reagin globulin preparation isolated from the egg-sensitive individual's serum. This would reveal antigen which had been localized nonspecifically *in vivo*, as a result of the mobilization of macrophages. It is perhaps significant, in this respect, that a greater number of stained

macrophages were observed (especially about the blood vessels) in the *in vivo* reactions, than was seen *in vitro*.

Whatever the correct interpretation of Rappaport's findings, however, they are obviously important if only because they have drawn attention to the antibody detected in the epidermal cells at a greater concentration than in any other skin structure. Further investigations are needed, preferably with conjugated purified reagin directed against inhalant allergen.

B. SITE OF REAGIN FORMATION

Identification of the cellular site of reagin formation is hampered by the unavoidable restriction on the study of human tissue processes. Hence, although it is possible to investigate reagin-allergen reactions in human skin obtained by biopsy it is not possible to acquire readily other tissue which is likely to prove active in reagin synthesis. Nevertheless, an investigation of the site of formation of the reagin-like antibodies induced spontaneously or artificially in animals might contribute to an understanding of reagin production in hypersensitive humans. It should be possible to adopt methods such as the indirect fluorescent antibody technique of Coons *et al.* (1955), which has been applied effectively by White (1958) to the study of antibody production to different antigens in single cells of rabbit lymph nodes.

There is available, at present, only circumstantial evidence as to the site of formation of reagins. If substantiated, the claims of Walzer and his associates (see Walzer *et al.*, 1957; Walzer and Bowman, 1960) to have passively transferred immediate sensitivity by means of washed leucocytes from ragweed-sensitive patients would raise the possibility of such cells being involved in reagin synthesis. The simpler explanation, however, would be that they are capable of fixing the skin-sensitizing antibody. In this connection, an observation of Berdal (1952) is of interest. This investigator found reagin to be accumulated in the edema fluid of some cases of nasal polyps, taken from hypersensitive patients, in concentrations considerably higher (1600-fold in one case) than the levels of reagin in the patients' sera. As nasal polyp fluid is known to be a rich source of plasma cells and, on occasions, eosinophiles, it is tempting to associate their presence with an increased humoral reagin level, particularly as plasma cells (the abundant evidence implicating this cell is reviewed by Good, 1957) and possibly eosinophiles (Speirs, 1958) are thought to be involved in antibody production (as also are macrophages). Raffel (1961) has pointed out that the only immunological occasion when more than occasional plasma cells are

found in the bloodstream is during serum sickness (Markoff, 1937; Gormsen and Heinzelman, 1941; Good, 1957). It is on such occasions that transitory skin-sensitizing antibodies are often detectable in the serum of patients convalescing from the sickness, in addition to anaphylactic (precipitating) antibodies (Karelitz and Glorig, 1943). On the other hand, patients with seasonal hay fever as their only allergic manifestations show a blood eosinophilia, but only in summer months when symptoms are present and not during the winter (Frankland, 1963). Many eosinophilic cells are found also in the nasal polyps of asthmatics, in cases where an offending allergen has not been identified.

An examination of evidence of immediate hypersensitivity in hypogammaglobulinemic patients suggests that plasma cells are involved in reagin formation, for the same reason that Gitlin (1956) and Good (1957) associated these cells with the production of the usual type of antibody. Although the skin testing of some cases of hypogammaglobulinemia with a wide range of allergens has failed to reveal any immediate sensitivity (Gitlin *et al.*, 1959) there have been various reports of hypogammaglobulinemic patients with the symptoms of allergic rhinitis and asthma and who usually show skin reactivities to common allergens (Brem and Morton, 1955; Fischer, 1955; Zbar *et al.*, 1956; Freedman *et al.*, 1958; Kallós, 1958). Gitlin and his associates (1959) are of the opinion that reagins can be synthesized by patients with acquired or with congenital hypogammaglobulinemia, but the numbers tested are too small to make any useful comparison. It is important to know, of course, whether such individuals possess transferable reagins in their sera as this is a more reliable guide to the diagnosis of immediate hypersensitivity in children than is the direct skin test. There appear, however, to have been only two cases reported of elicitation of a positive P-K reaction with serum from a hypogammaglobulinemic patient. Crowder *et al.* (1959) demonstrated passive sensitization to "room" dust with the serum of a patient with acquired hypogammaglobulinemia (and pernicious anemia) who gave immediate reactions to a number of foods as well as to dust and also a delayed reaction to tuberculin. The P-K activity of this serum was compatible with its γ -globulin level of 28 mg.% (w/v). A puzzling observation, however, was that of the development of skin reactivities to a number of allergens such as feathers, silk, and wool *after* the patient had received a course of treatment with immune globulin. This could have been produced by, as Crowder and his associates suggest, the presence of reagins in the γ -globulin used in the therapy. The positive P-K reaction was achieved, however, with serum taken *before* treatment commenced. The other passive transfer of

skin sensitivity, also to house dust, was achieved by Vaughan (1958) with the serum from a patient with hypogammaglobulinemia who had clinical manifestations of nonseasonal rhinitis and who possessed a γ -globulin level of 23 mg.% (w/v).

Unfortunately, in the studies of hypersensitivity phenomena shown by hypogammaglobulinemic patients most attention has been given to manifestations of delayed sensitivity. A thorough investigation of the immediate sensitivities demonstrated by these patients is urgently needed. If, as a result of such a study, a low incidence of passively transferable sensitivity in the sera of hypogammaglobulinemic patients (preferably of the congenital type) was found as compared with the incidence of hay-fever type sensitivity in the rest of the population, there would be reason to suppose that the reagins are synthesized at similar sites to those at which the more usual antibodies are produced. Raffel (1961), on the other hand, has drawn the opposite conclusion on the basis that any *occurrence* of immediate sensitivity in hypogammaglobulinemic patients must mean that reagin is synthesized by a different type of cell to that responsible for the production of normal antibody.

Good and his associates (1959) have made the interesting observation that wheal and erythema sensitivity cannot be induced in hypogammaglobulinemic children in response to infection with *Ascaris* antigens by the technique of Kailin's group (1947, 1950), in spite of this procedure having proved capable of inducing an immediate type of sensitivity in over 90% of other individuals who had failed to show an initial reaction to an intracutaneous test with *Ascaris* extract. These findings, however, only reflect the inability of the hypogammaglobulinemic patient to develop a wheal and erythema type of response to artificial sensitization. Of greater significance is the recent report by Good and his collaborators (1962) of an adult, who prior to onset of her agammaglobulinemia was troubled with urticarial reactions to certain foods, but who lost this reactivity completely after she began to experience the "sequence of infections which signalled the onset of a profound hypogammaglobulinemia." It would seem, then, that wheal and erythema sensitivity may be lost with the development of hypogammaglobulinemia.

All evidence available at present is consistent with reagins (like immune 7S antibodies) being formed in the plasma cells.

V. Artificial Induction of Reagin-like Antibodies

A. SKIN-SENSITIZING ANTIBODIES INDUCED IN HUMANS

1. Response to Heterologous Serum and Protein Fractions

Although there is uncertainty as to their etiological role (if any), and their relationship to concomitant antibodies has yet to be established, the skin-sensitizing antibodies detectable in the sera of patients convalescing after serum sickness are of interest in any discussion of reagins. Early investigators failed to dissociate the P-K activity of serum disease patients' sera from the precipitating (anaphylactic-type) antibody also present which—in the form of soluble complexes with free antigen—is assumed to play a dominant part in evoking the symptoms of the sickness. More recently Arbesman and his associates (1960a) have applied sensitive hemagglutination techniques, employing tanned red cells coated with horse serum and horse dandruff proteins, in the measurement of antibody in the sera of individuals recovering from serum sickness as a result of prophylactic tetanus antitoxin. There appeared to be no relationship between hemagglutination titers and precipitin contents—eleven out of twenty-two sera with high titers failing to show any precipitin formation. The hemagglutination titers of sera from patients who had developed serum sickness, however, were appreciably greater than the corresponding presickness titers. In another systematic study (of two sera from patients with acute symptoms) the Arbesman group (Reisman *et al.*, 1961a) showed that P-K activity appeared concomitantly with the onset of the clinical symptoms and seemed to precede the rise in hemagglutination titer, which correlated well with precipitin formation and complement-fixation activities. Moreover, the skin-sensitizing activity disappeared rapidly in contrast to a lingering high hemagglutinating antibody level.

Similar results were obtained from the study of a case of serum disease following administration of bovine tetanus antitoxin. Immuno-electrophoretic analysis of bovine serum (but not horse serum), employing serum from the patient as antiserum, revealed a single precipitin line in the α -globulin position. It was shown likewise (Rose *et al.*, 1962) that the sera of patients developing primary serum sickness following injection of equine tetanus antitoxin precipitated an α -globulin of horse serum, as well as a component migrating as a γ -globulin. Furthermore, both antigenic (or haptenic) components were present in commercially available "purified" antitoxins. Hence, it would seem likely that patients developing serum diseases are reacting primarily to components *other than* the active antitoxin and that one of these migrates electrophoretically as an α -globulin component.

It will be important to establish whether such minor antigenic components are actively engaged in the induction of reagin-like antibody during serum sickness. Alternatively, it is possible, of course, that precipitating antibody, alone or as soluble complexes with antigen, is capable of effecting passive wheal and erythema reactions in normal humans. In their studies of the sera of patients developing serum disease after receiving horse antitoxin, Reisman and his associates (1961a) found that hemagglutinating, precipitating, and anaphylactic activities resided in the same agar-electrophoresis fractions (i.e., mainly γ and β , with traces in the α region); whereas the P-K activity was located predominantly in the γ_1 -globulin fraction. Free-solution electrophoresis and salt precipitation findings of Cooke and his associates (1956) pointed to an inverse relationship between the skin-sensitizing activity of fractions of sera from patients with serum sickness and their α_2 -globulin contents, in contrast to the relatively widespread distribution of P-K activity among the electrophoretic fractions (e.g., γ_1 , β , and α_2) of sera from patients with hay fever. This raises the question as to whether the skin-binding groups of the reagin-like antibodies appearing in human serum during an attack of serum sickness can be blocked by an antigenic determinant carried by a human serum α_2 -globulin component. (A mechanism whereby such a process could occur is discussed later, in this and the next section.)

Intensive physicochemical and immunological studies of the skin-sensitizing antibodies induced during serum sickness are required, as such investigations could lead to better understanding of the mode of production of spontaneously occurring reagins in predisposed individuals. At the moment, however, any detailed comparison of the properties of the artificially induced and naturally occurring antibodies (such as was attempted by Cooke *et al.*, 1956) seems somewhat premature. Admittedly there is evidence to suggest that the induced skin-sensitizing antibodies are less thermolabile than reagins, and their transitory appearance in the circulation contrasts with the persistence of reagin. Nevertheless, any differentiation of the two types of skin-sensitizing antibody on the basis of differences in immunological specificity, as was made by the Cooke group, is hardly justifiable at present when highly pure antigens are not yet available.

This question of specificity is important in trying to explain the severe responses shown by some horse-dandruff-sensitive individuals injected with horse antitoxin. Squire (1950) suggested that a serological relationship between horse dandruff allergen and horse serum albumin, contaminating the antitoxin γ -globulin, might be responsible for this reaction. More recently, however, Stanworth (1957a) employed the

sensitive and specific Ouchterlony gel-diffusion technique to demonstrate traces of horse serum proteins in horse dandruff extract. It seemed, therefore, that horse-dandruff-sensitive individuals might also develop a hypersensitivity to the trace amounts of horse serum proteins, inhaled along with the dandruff particles.

Further studies (Stanworth, 1963) of the horse serum antigens present in horse dandruff have, however, provided results which could lead to the formulation of an immunochemical basis for the formation of skin-sensitizing antibodies in serum sickness. Some antisera from rabbits hyperimmunized with horse dandruff extract show a precipitin formation with *human* serum, even after absorption with homologous dandruff antigens. Moreover, immunoelectrophoretic analyses have revealed that the rabbit antibody responsible for this effect is directed toward the human serum α_2 -macroglobulin component; whereas gel-diffusion analysis showed that the antibody was incapable of differentiating this glycoprotein from horse serum α_2 -macroglobulin—a precipitin reaction of identity occurs between the two when analyzed in adjacent wells in the agar plate. It appears, therefore, that prolonged immunization of certain rabbits with horse dandruff extracts produces a precipitating antihorse serum α_2 -macroglobulin antibody, presumably against the serum α_2 component present in extremely small amounts (undetectable by gel-diffusion precipitin analysis) in horse dandruff extract. The cross reactivity of this antibody with human α_2 -macroglobulin is consistent with the findings of von Scheiffarth *et al.* (1957), who in an immunoelectrophoretic study of the cross reactivities of various mammalian sera with antihuman serum observed a frequent precipitin line formation with an α_2 -globulin component. It is considered highly significant that similar analyses of the serum of patients recovering from serum sickness have demonstrated precipitin formation with an α -globulin constituent of horse serum (Rose *et al.*, 1962) and of bovine serum (Reisman *et al.*, 1961b). Moreover, as there is evidence that glycoproteins similar to the serum glycoproteins occur in the tissues and are probably released into the bloodstream as a result of tissue alteration (Shetlar, 1961), it is proposed that there are now grounds for an explanation of the formation of skin-sensitizing antibodies in serum sickness. The wider implications of this concept in relation to the formation of reagins will be discussed in the next section, in a proposed immunochemical mechanism of immediate-sensitivity reactions.

2. Response to Bacterial Polysaccharides

The reaction of humans to bacterial polysaccharides, whether accidentally introduced through infection or deliberately by immunization

with pneumococcal polysaccharides, dextrans, etc., is of interest to any discussion of the induction of reagin-like antibodies in man. An important advantage in studying this type of reaction is the availability of highly purified, well-defined antigens. For instance, specific capsular polysaccharide can be used to elicit immediate wheal and erythema reactions in patients convalescing from pneumococcal lobar pneumonia (Tillett and Francis, 1929), and in normal individuals who have received as few as 3 intradermal injections of 10 μ g. of purified polysaccharide at weekly intervals (Francis, 1934). Similar reaction can also be evoked in normal humans by immunization with dextrans (Kabat and Berg, 1953). As already mentioned in Section II, C, the skin sensitivity in all these cases can be correlated with the amount of precipitating antipolysaccharide antibody in the serum. Unfortunately, however, such sensitivity does not appear to be passively transferable to the skin of normal recipients. The demonstration of passive sensitization with sera from pneumonia patients, previously treated with specific rabbit antipneumococcal serum, can be attributed to the presence of excess rabbit antibody in the treated patients' sera. Similarly, an immediate wheal and erythema reaction can be produced in human skin by injecting locally at the same site rabbit antiserum followed by homologous capsular polysaccharide. Finland and Sutliff (1931) did demonstrate, however, the elicitation of a positive skin test in a patient suffering from pneumonia (but who gave negative skin tests and contained no demonstrable circulating antibody) within 1 hour after receiving 130 ml. of serum from a patient convalescing from the same type of pneumonia. Kabat (1958) has suggested that a similar passive sensitization of normal human skin could be accomplished with large amounts of human antidextran serum. Nevertheless, the human antibacterial polysaccharide antibodies, which are produced under different conditions, appear to lack the reagin's ability to bind readily to human skin. The explanation of this difference in behavior on passive transfer might lead to a better understanding of the unique biological properties of reagin. It could, of course, arise from the production of large amounts of *blocking antibody* in response to the bacterial polysaccharides. Hence, it will be important to establish the part played by the protein-free polysaccharide structures in determining the type of antibody produced. In this connection of considerable interest is the observation of Kabat and his associates (1957) that the incidence of reactivity to straight-chain dextrans is less than the incidence to dextrans with more complex branched structures, among the high proportion of the population showing skin reactivity to dextran in the absence of deliberate contact with the polysaccharide. The considerable reduction in the number of allergic reactions to infusion dextran by use of straight-

chain dextrans (Kabat and Bezer, 1958) is of similar significance. The development of skin sensitivity to heterologous pneumococcal polysaccharides in patients convalescing from pneumonia as a result of repeated intradermal tests with the type-specific polysaccharide (Francis and Tillett, 1930) is also of interest in the study of the cross reactivities of induced skin-sensitizing antibodies. Finally, the observation of W. B. Wood (1940) that a patient's skin may lose its ability to react to capsular polysaccharide during the course of severe pneumonia despite the presence of large amounts of antibody in the blood deserves further attention, as this could shed some light on the nature of reagin-binding sites in human skin. Here again, however, the effect might be owing to the production of blocking antibodies during the disease process.

3. Response to Extracts of *Ascaris lumbricoides*

The immediate sensitivity induced in humans by extracts of *Ascaris lumbricoides*, sometimes by a single intradermal injection (Brunner, 1934), and the demonstration that such sensitivity can be passively transferred to normal recipients (Rackemann and Stevens, 1927) has attracted attention as a means of studying the factors influencing the formation of reagin-like antibodies. The investigations of Walzer and his associates (Davidson *et al.*, 1947; Kailin *et al.*, 1947) have shown that active sensitization to *Ascaris lumbricoides* extracts can be induced in over 90% of negative reactors, most of whom showed no hereditary predisposition to allergy, by repeated intracutaneous injections. Wheal and erythema sensitivity appeared to be induced at a more rapid rate in adults of the Negro race than in white people and positive direct skin tests were demonstrable in more than half the cases tested after 6 months. In a more prolonged study (Kailin *et al.*, 1950), however, artificially induced sensitivity to *Ascaris lumbricoides* extract was found to have disappeared in 79% of the thirty-three subjects tested 2 years after initial sensitization. A repeat series of sensitizing injections led to an enhanced rate of appearance of immediate positive skin reactions in all but one of the negative reactors. This has been interpreted as an amnestic response involving the skin-sensitizing antibody. It should be emphasized, however, that the serum level of *skin-sensitizing antibody* in these patients was not measured (by P-K testing).

Another significant finding is the impairment of the capacity to develop cutaneous sensitivity to *Ascaris* shown by non-*Ascaris* sensitive cancer patients, receiving weekly intradermal injections of aqueous extract of the worm (Fisherman, 1962). Sensitization can eventually be accomplished in such patients after 8-24 weeks of immunization, as compared with 2-4 weeks in allergic individuals and 4-7 weeks in non-

cancerous and nonallergic individuals. This finding is of considerable interest in relation to the reports of an impaired capacity of Hodgkin's disease patients for development of hypersensitivity of the classical delayed or tuberculin type (Schier, 1954; Kelly *et al.*, 1958).

There is obviously scope for further immunological investigations of the mechanism of induction of immediate sensitivity to intact round worms and to their extracts. Surprisingly little appears to be known about the character of the antigenic component, which possesses the unique ability to induce immediate sensitivity in the majority of normal individuals. It is of interest, however, that Gazzinelli and his associates (1961) have shown that the toxic factor in extracts of *Ascaris lumbricoides* (from hogs), which is responsible for inducing anaphylactic-like reactions in guinea pigs, is a protein-carbohydrate conjugate (containing 42% reducing sugar and 3.4-4.3% nitrogen).

It will also be important to see how closely the skin-sensitizing antibodies induced by *Ascaris lumbricoides* are related to reagins, and to determine whether they afford any protection against the (parasite) infection as do the skin-sensitizing rabbit antipneumococcal polysaccharide antibodies when injected into pneumonia patients.

4. Response to Allergens Administered in Hyposensitization Procedures

The formation of the allergen-neutralizing blocking antibodies in hypersensitive individuals, in response to a course of "desensitizing" injections with allergenic extracts, is a well-known phenomenon to which reference has already been made. Of main interest to the present discussion is the difference in the response of hypersensitive individuals to native allergens as compared to therapeutic allergenic preparations. Nonallergic individuals produced blocking antibodies, of similar nature to those induced in hay-fever patients, as a result of injection with massive doses of pollen extract (Cooke *et al.*, 1937; Fitzgerald and Sherman, 1949). A concomitant production of skin-sensitizing antibodies did not appear to occur, however. On the other hand, as a result of the difference in response between treated and untreated ragweed-sensitive individuals to two crude fractions of ragweed pollen extract, differentiated merely on the basis of dialysis through Visking cellophane tubing, Richter *et al.* (1958) have concluded that reagins of a new specificity are formed in the sera of the untreated patients following desensitization treatment. Independent substantiation of these claims does not appear to have been reported. Nevertheless, a temporary rise in serum P-K activity following the desensitization of hay-fever patients has often been observed (Sherman, 1958).

Many of the differences in the biological and physicochemical proper-

ties of blocking antibody and reagin have already been mentioned (Sections II and III). In addition, the partial inhibition of precipitin formation between ragweed antigen and its homologous rabbit antiserum can be achieved with blocking antibody (Hampton *et al.*, 1943; Bukantz *et al.*, 1949), whereas reagin seems to show no such inhibitory effect (Miller and Campbell, 1947). Unlike reagins, blocking antibodies also specifically inhibit the fixation of complement by ragweed antigen-rabbit antiragweed serum systems (Portnoy and Sherman, 1954). These findings point to structural differences between the two types of antibody molecule. Hence an understanding of the mechanism whereby blocking antibodies, rather than reagins, are formed on allergen treatment might explain some of the distinctive properties of the sensitizing antibody. In particular, why do blocking antibodies fail to bind tissues?

It would seem most likely that the differences in response to spontaneously and artificially acquired allergens are due to variations in the mode of administration. The native allergens are inhaled in particulate form, in intact pollen grains for instance, whereas the allergen used for hyposensitization is injected intravenously in a soluble form. Moreover, the timing of the doses administered and their magnitude are different in the two situations. Results of the studies of the experimental production of skin-sensitizing antibodies in rabbits (to be described in the next subsection) are of relevance in discussing the effect of the form of administered allergen on the response evoked. As alum-precipitated antigens proved superior to soluble antigens in the induction of skin-sensitizing antibodies in animals, it seems reasonable to predict that a similar effect might be observed in humans injected with more potent antigenic preparations. It will be interesting, therefore, to observe the effects of the relatively new practice of treating hay-fever patients with emulsified pollen extracts (E. A. Brown, 1959). Eight out of thirty-five ragweed-sensitive patients treated with a single repository injection of pollen extract developed significantly increased serum P-K titers (DeLorme *et al.*, 1961). On the other hand, there was a definite increase in the hemagglutination titer of the serum of the majority of treated patients, which could not be correlated with blocking or skin-sensitizing antibody activity. Furthermore, R. J. Becker *et al.* (1961) have shown that of fifteen nonallergic individuals receiving an intramuscular injection of 0.5 ml. of emulsified pollen extract in each arm, seven developed immediate skin reactivity, nine delayed reactivity, and three both types. All were still reactive after 6 months. In a later study, the same group (Sparks *et al.*, 1962) showed that the sera of four out of five allergic individuals, who developed an immediate sensitivity to an allergen to

which they were previously insensitive, passively sensitized the skin of nonallergic recipients. The induced skin-sensitizing antibody responsible for the P-K reactivity behaved like spontaneously occurring reagin in that it was heat labile, persisted at a passive skin site for at least 2 weeks, and was "neutralized" *in vivo* by specific allergen. It also failed to form a precipitin line with allergen in gel-diffusion and did not show a positive PCA reaction. It has not yet been shown to be associated with any clinical manifestations, however. Further characterization of these induced human reagins will be awaited with interest. Fisher and Connell (1962) have demonstrated the induction of an anaphylactic-type of antibody, capable of eliciting a PCA reaction in guinea pigs, in the sera of five out of eleven patients treated with a single dose of 1 ml. of ragweed pollen extract (containing 0.1 mg. protein N.) in a mineral oil emulsion. Its relationship to the blocking and hemagglutinating antibodies also present has yet to be established, however.

Studies of the relationships between the various types of antibody produced in the sera of insulin-sensitive patients are also of value in the characterization of induced reagin-like antibodies. Here, too, the relation between sensitizing and blocking activities is of interest. Lowell (1953) has suggested that the results provided by studies of this system might be explained if the skin-sensitizing and insulin-neutralizing antibodies combine with different groupings on the insulin molecule. As in the study of other induced sensitivities, such as the immediate sensitivity to diphtheria toxoid, there is always the possibility, however, that skin-sensitizing activity might be directed against a contaminant. This, in fact, is a limitation of all studies on the antibodies involved in immediate hypersensitivity processes, which can only be overcome by working with highly purified allergens.

5. Response to Artificially Conjugated Proteins

The investigations of Farah *et al.* (1960) of the wheal and erythema response of normal humans actively or passively sensitized to the 2,4-dinitrophenyl group provide an original approach to the study of immediate hypersensitivity. They suffer from similar limitations to the studies on the antibacterial polysaccharide antibodies, however, in that passive sensitization of the skin of normal humans appears to be effected only by means of rabbit antibody. Nevertheless, this work is more valuable in as much as transfer by purified specific antibody and its papain digestion fragments has been studied. The fragments differed from the intact rabbit antibody molecule by failing to passively sensitize normal human skin sites to the 2,4-dinitrophenyl group. Moreover, in

both actively and passively sensitized individuals, wheal and erythema reactions were elicited by intradermal injection of 2,4-dinitrophenyl protein, but not by the conjugated protein mixed with low molecular weight 2,4-dinitrophenyl haptens or with the papain-digested rabbit antibody fragments. These findings are assumed to indicate that the wheal and erythema response in humans requires *mutually multivalent antigen and antibody*, complexes of at least two antigen and two antibody molecules being considered essential in the pathogenesis of the allergic response. Such a complex is remarkably similar in composition to the antigen-antibody complexes (Ag_3-Ab_2) which K. Ishizaka *et al.* (1959a,b) found to have *skin-irritative* activity in guinea pigs. This similarity would seem to indicate, however, that the skin-sensitizing anti-2,4-dinitrophenyl antibody of Farah and his associates resembles more closely anaphylactic than reagin-type antibody.

B. EXPERIMENTAL PRODUCTION OF SKIN-SENSITIZING ANTIBODIES IN ANIMALS

Wheal and erythema reactions have been elicited in the skin of normal humans (see Table III for references), by transfer of animal antisera showing high titers of precipitating (anaphylactic-type) antibodies followed later by challenge with the immunizing antigen. Nevertheless, passive sensitization is not effected with every such antiserum. Moreover, the appearance of skin-sensitizing activity, for example, in the sera of 24% of guinea pigs immunized by a single intraperitoneal injection of alum-precipitated pollen extract (W. B. Sherman *et al.*, 1939), seemed to depend upon the time of bleeding, no activity being detected in blood taken less than 2 months after the sensitizing injection. It also appears that the production of skin-sensitizing activity in animal sera depends upon the form of antigen injected and on its mode of administration. Thus, Sherman (1953) has found that P-K activity can be induced most consistently in the sera of rabbits by repeated subcutaneous injections of alum-precipitated antigen (egg albumin).

The characteristics of the artificially induced antibodies are compared in Table III with those of reagin. Obviously such a comparison is of limited value at this stage, however, when the induced antibodies have yet to be isolated and when there is still uncertainty as to the identity of the antigens responsible for their production. The latter point is emphasized by the results of quantitative precipitin studies carried out by Vaughan and Kabat (1953, 1954), which indicated that there were at least two possible antibodies in rabbit anti-egg albumin sera capable of passively sensitizing human skin and that these antibodies are directed

TABLE III
A COMPARISON OF THE CHARACTERISTICS OF SKIN-SENSITIZING ANTIBODY INDUCED IN ANIMALS AND OF REAGIN

Property	Reagin	Induced skin-sensitizing antibody	References (to animal experiments)	System used
Occurrence	Spontaneously occurring in allergic humans	Induced artificially in certain members of various animal species	Cooke and Spain, 1929 De Besche, 1931 Caulfield <i>et al.</i> , 1937 Winkenwerder <i>et al.</i> , 1939 Kulka and Hirsch, 1946	Rabbit anti-egg white Rabbit anti-horse serum Guinea pig anti-ragweed extract Rabbit anti-ragweed extract Rabbit or guinea pig anti-ragweed extract
P-K activity in human skin	Positive	Positive	Sherman, 1953 (and associates)	Rabbit anti-horse serum; -bovine serum albumin; -ovalbumin; -pollen extracts. Guinea pig anti-pollen extract; -ovalbumin. Rabbit anti-ovalbumin; -conalbumin.
Passive systemic anaphylactic activity in guinea pigs	Negative	Negative	Sherman and Coulson, 1951	Guinea pig anti-ovalbumin
Response to heating at 56°C. for 4 hours	Destroyed	Unaffected	Sherman <i>et al.</i> , 1948	Rabbit anti-horse serum
Effect of mixing with allergen <i>in vitro</i> (as determined by subsequent P-K testing in humans)	"Neutralized"	"Neutralized"	Sherman <i>et al.</i> , 1939	Rabbit anti-horse serum
Electrophoretic behavior	Maximum activity usually in γ_1 -globulin fraction	Maximum activity usually in α_2 -globulin fraction	Aladjem <i>et al.</i> , 1957	Rabbit anti-ovalbumin; -bovine plasma albumin.

against egg white impurities in crystalline egg albumin other than conalbumin, ovomucoid, and lysozyme. As in the case of the induction of skin-sensitizing antibodies during human serum sickness (discussed in Section V, A) it is conceivable that the skin-sensitizing antibodies produced in the sera of rabbits immunized with other protein preparations are also directed against impurities, particularly as prolonged courses of immunization are usually required to induce P-K activity. It is quite possible that trace contaminants would remain undetectable by gel-diffusion precipitin techniques. This means that absorption of P-K reactive rabbit antisera with the immunizing antigen preparation will probably fail to remove the skin-sensitizing antibody. Hence, any conclusion as to the nonprecipitating nature of induced skin-sensitizing antibodies based on results obtained by such absorption procedures (e.g., Sherman *et al.*, 1950) seems unacceptable.

As in studies of the reagin-like antibodies produced in humans convalescing after serum sickness, it will be important to establish whether the wheal and erythema activity of animal antisera is associated with precipitating antibody (free or in the form of antigen-antibody complexes) directed against a trace antigenic constituent in the immunizing protein preparation. Surprisingly few physicochemical studies have been undertaken in order to investigate this possibility. Aladjem and his associates (1957) have found, however, by starch block electrophoretic fractionation of rabbit antisera (against ovalbumin and bovine plasma albumin), that the serum components responsible for passively sensitizing human skin are usually associated with more than one globulin component and are distinct from precipitating antibody. Moreover, the most frequently observed distribution was one in which maximum skin-sensitizing activity (after challenge with antigen) was associated with α_2 -globulin. In contrast, the maximum skin reactivity occurring immediately on passive transfer to a normal human skin, independent of any challenge with allergen, was located in the γ -globulin fraction—which also contained the precipitating antibody. Another interesting observation of the Aladjem group was that of an apparent relationship between the length of immunization and the heterogeneity of the distribution of skin-sensitizing activity among the rabbit serum electrophoretic fractions; the longer the period of immunization, the broader the distribution. These findings appear to conflict with the results obtained by Cooke and his associates (1956), who concluded that α_2 -globulin plays no role as a carrier of skin-sensitizing activity in the sera of humans with induced hypersensitivity (in contrast to its probable involvement in such a role in spontaneous hypersensitivity in humans).

Further investigations are needed to resolve these discrepancies and to establish the true role of α_2 -globulin in induced hypersensitivity reactions. It is possible that some form of complexing between a serum component of α_2 mobility and skin-sensitizing antibodies will be found to occur, as was suggested to account for the behavior of spontaneously occurring reagins under certain conditions. It would be particularly interesting to establish whether the skin-sensitizing antibodies appearing in humans during serum sickness and in animals after prolonged immunization can be classified as intermediately sedimenting (8–11 S) antibodies, like the reagin-like antibodies induced in a diabetic patient as a result of treatment with glucagon (Rockey and Kunkel, 1962).

VI. Possible Modes of Action of Reagin

An understanding of the immunochemical basis of the formation and biological activity of reagin must await the characterization of the skin-sensitizing antibody receptor groups which, in turn, depends upon the identification of the corresponding antigenic determinants. In the meantime one can only speculate about the mechanism of immediate-sensitivity reactions, taking as a guide recent findings on the cutaneous reactivity of the more readily characterizable anaphylactic-type antibodies.

A. ANTIBODIES WITH "DUAL SPECIFICITY"

It has become customary to explain the immunological properties of reagin in terms of a "dual specificity," one receptor group being supposedly directed against the sensitizing allergen and the other against a tissue component of the allergic host (Harley, 1937; Prausnitz, 1955). There has never been, however, a convincing demonstration of the experimental production of an antibody molecule carrying two receptor groups directed against distinct antigenic determinants (which Pauling in 1940 predicted as feasible). For instance, in studies with substituted protein antigens such as *p*-aminobenzoic acid azohemocyanin (Hooker and Boyd, 1941) and other azoproteins (Augustin, 1959b), the broadening of reactivity toward a number of related chemical groups following prolonged immunization appeared to result from the production of combining groups with a wider affinity rather than to an increase in antibody "valency." Similarly, studies with polysaccharide (dextran) antigens (Kabat, 1956) have shown that each human individual produces a population of antidextran molecules ranging in complementary area from sites complementary to a trisaccharide or tetrasaccharide to sites complementary to a hexasaccharide; but here too there was no evidence that sites with more than one degree of complementarity exist on the

same antibody molecule. In view of these latter findings, it is perhaps of some significance that attempts to transfer wheal and erythema sensitivity to dextran between humans have failed, in spite of there being an apparent correlation between the level of precipitating antidextran antibody in the sera of dextran-sensitive individuals and their degree of direct skin reactivity. Nevertheless, it is possible to sensitize normal human skin to dextran by passive transfer of *rabbit* antidextran anti-serum.

B. COMPARISON WITH MODE OF ACTION OF ANAPHYLACTIC-TYPE ANTIBODIES

It seems possible, that the uniqueness of reagin lies in the nature of its tissue-binding group. Hence, it will be important to establish whether reagin becomes attached passively to epithelial tissues, in a similar manner to the supposed fixation of anaphylactic-type antibodies to guinea pig skin during PCA reactions or, alternatively, whether an active antibody-tissue antigen interaction is involved. Recent findings on the mode of fixation of anaphylactic-type antibodies to guinea pig skin are of interest in this respect.⁴ For instance, there is increasing evidence that γ -globulin binds to guinea pig skin through that part of the molecule containing the principal antigenic determinant groups, i.e., corresponding to the papain digestion piece III of Porter (1959). Ovary and Karush (1961) have demonstrated this by direct and reverse PCA testing with papain digestion pieces of rabbit γ -globulin. In contrast to piece III, pieces I and II (each containing an antibody-combining site) failed to give a positive reverse PCA test on subsequent challenge with horse antirabbit γ -globulin. Support for these findings is provided by recent observations of Ishizaka and his co-workers (1962) on the lack of skin-reactive and complement-fixing properties of soluble antigen-antibody complexes composed of antibody pieces (presumably deficient in papain piece III) obtained by either papain or peptic digestion. Furthermore, tests with the three fragments (I, II, and III) after reaggregation by coupling with bisdiazotized benzidine showed that only aggregated piece III had the ability to induce skin reactions in guinea pigs or to fix complement. This is consistent with the findings of Taranta and Franklin (1961), who showed that removal of the papain digestion piece III part from the antibody γ -globulin molecule destroyed its ability to fix complement *in vitro*, even when two antigen-combining sites are available as in 5 S pepsin-digestion pieces of rabbit antibody γ -globulin.

⁴ See the article by Austen and Humphrey in this volume for a fuller treatment of this subject.

The extensive investigations of Ishizaka and his associates on the skin reactivity of antigen-antibody complexes and of nonspecifically aggregated γ -globulin have also contributed to an understanding of the mode of attachment of anaphylactic-type antibodies to guinea pig skin. These workers (Ishizaka *et al.*, 1959a,b; Ishizaka and Campbell, 1959) have made the interesting observation that only antigen-antibody complexes (such as Ag_3 - Ab_2) in which the rabbit antibody has an altered configuration, as revealed by increases in levorotation, are capable of producing skin-irritative reactions. Similar responses were produced by aggregated rabbit or human γ -globulin (K. and T. Ishizaka, 1960), presumably by a similar mechanism. It was also found that the induction of skin reactions in guinea pigs depended upon the particular animal species from which the γ -globulin used to form the soluble antigen-antibody complexes or aggregated γ -globulin was derived. Thus, horse or chicken antibody γ -globulin proved ineffective, in agreement with their failure to induce anaphylactic reactions in guinea pigs (Ovary, 1960).

Of greater interest to this discussion, however, is the observation of Osler and Cluff (1961) that heat aggregated human γ -globulin evokes a *wheal and erythema type* of reaction *immediately* upon injection into *human* skin. Although this response is elicited by a nonspecific process, it closely resembles the manifestations of tissue damage observed in a classical P-K reaction. In contrast, the skin-irritative reactions produced in *guinea pigs* by heat aggregated human (or rabbit) γ -globulins (T. and K. Ishizaka, 1959) or by soluble antigen-antibody complexes are manifested like PCA reactions. It seems that the induction of these types of reaction in human or guinea pig skin is brought about by γ -globulin which has undergone a change in tertiary structure to a specific cell-binding configuration, as a result of the interaction between antibody (γ -globulin) molecules brought into apposition by antigen or by the nonspecific aggregation processes (K. Ishizaka and Campbell, 1959). Moreover, as indicated by the previously discussed findings on the PCA activity of pieces of precipitating antibody, the configuration of that part of the γ -globulin molecule corresponding to papain digestion piece III is a critical factor in the attachment of anaphylactic-type antibodies to guinea pig skin. This is also suggested by the results of studies by K. Ishizaka and his co-workers (1961), which showed that the cleavage of the disulfide linkages in human or rabbit antibody γ -globulin molecules was correlated with a decrease in their affinity for guinea pig tissues. It is significant, however, that the irreversible reduction process employed (mercaptoethanol treatment followed by blocking

with iodoacetate or iodoacetamide) did not affect precipitin formation or complement fixation by the antibodies. Hence, it seems likely that the cleavage of the interchain disulfide bonds within the γ -globulin molecule leads to a change in configuration in the skin-attachment site (i.e., in papain piece III), while leaving the antigen-combining groups intact. It will be important to establish whether the destruction of the P-K activity of the serum of a glucagon-sensitive individual, by treatment with mercaptoethanol (Rockey and Kunkel, 1962), is produced by a similar alteration in molecular configuration. Alternatively, however, such deactivation might prove to be the result of destruction of antigen-combining groups in the labile reagin molecule.

There is no evidence available at present to suggest that reagins become attached to human skin through a part of the molecule corresponding to the papain piece III of precipitating human (7S) antibody. On the other hand, it seems likely that reagin molecules possess a distinctive piece III, which could account for some of their other characteristic biological properties. For instance, the failure of reagins to evoke PCA reactions in guinea pigs can probably be attributed to their lack of (or to the masking of) a skin attachment site similar to that present in the papain piece III part of 7S anaphylactic-type antibodies. This is inferred from the results of PCA tests on 19S antibodies (Ovary *et al.*, 1960) where the failure to elicit either direct or reverse reactions in guinea pigs has been similarly attributed to structural differences in the part of the molecule corresponding to papain piece III (i.e., that part containing the principal antigenic determinants). A similar explanation could account for the inability of reagins, like the various 19S antibodies studied, to cross the human placenta. In this connection, Porter and Press (1962) have suggested that the failure of large molecular weight antibodies to cross the placenta may not be an effect of size but rather of a blocking of the "membrane-transmission site" present in the same part of the molecule as the "skin attaching site." This idea is consistent with the argument developed in Section II, B against a direct relationship between molecular size and placental transmission, on the basis of the extensive data provided by the studies of Brambell and his associates (reviewed by Hemmings and Brambell, 1961). Nevertheless, Kallós and Waksman (1962) have inferred that human reagins fail to evoke PCA reactions in guinea pigs because they belong to the "heavy class of antibody globulins." This conclusion is based solely on the sedimentation results of Gyenes and Sehon (1961), in spite of the considerable data (see Table II) obtained by other investigators which indicates that human reagins cannot be classified as 19S antibodies. On

the other hand, in view of the recent evidence which suggests that the P-K activity of human allergic sera might be associated with a γ_{1A} -globulin, it is possible that the reagin molecule contains a "papain piece III" which is distinct from that in 19 S antibodies (as well as that found in 7 S antibodies). This is suggested by the results of gel-diffusion precipitin studies (Heremans *et al.*, 1959; Franklin and Stanworth, 1961), which demonstrated that the "immune globulins" (7 S γ , γ_M , and γ_{1A}) are differentiated by antigenically distinctive papain piece III parts to their molecules. It is important, however, to consider the alternative possibility that the failure of γ_{1A} -globulins to cross the placenta might result from a *blocking* of the "membrane transmission site," as a consequence of the tendency of this globulin to combine readily with other serum proteins. Moreover, it is not impossible that a similar effect is responsible for the lack of placental transmission shown by reagins. For example, a reagin- α_2 -macroglobulin combination might be expected to prevent the reagin entering the fetal circulation since α_2 -macroglobulin does not appear to get across the placenta (Hitzig, 1960).

Recent findings of Ovary and Benacerraf (1962) and others on the production in guinea pigs of two types of 7 S antibodies with different electrophoretic mobilities (γ_1 and γ_2),—discussed more fully by Austen and Humphrey in this volume—are of relevance to this discussion on the nature of skin attachment sites. It was shown that, although both types of antibody formed precipitin with antigen and fixed guinea pig complement, only the faster (γ_1) fraction was capable of inducing PCA reactions. Moreover, White and his associates (1963) have found (using a different antigen) that of the two fractions, only the PCA-active one adhered strongly to mast cells in frozen sections of mouse tongue. These findings raise the question as to whether a similar relationship obtains between reagins (which appear to be γ_1 -globulins) and the nonskin-sensitizing γ_2 antibodies found in human sera. Furthermore, do all types of skin-sensitizing antibody move with the mobility of a γ_1 -globulin on electrophoresis?

C. HYPOTHETICAL REAGIN-ALLERGEN INTERACTION MODEL

As already mentioned, there is no direct indication that the reagin molecule attaches to epithelial tissues in a similar manner to the attachment of anaphylactic-type antibodies to guinea pig skin. On the contrary, the relatively rapid and persistent binding of reagins to human skin in P-K tests suggests a more active and specific attachment. On the basis of findings which indicate that the less intimately the cell participates in antigen-antibody reactions the more antigen and antibody are

required to produce tissue injury, Talmage (1957) has concluded that the cell must play an unusually intimate part in the reagin-allergen interaction. It is proposed now that in P-K reactions, the interaction of challenging allergen with two reagin molecules already attached to adjoining sites on the cell surface leads to tissue injury (as depicted in Fig. 2). This is analogous to the model proposed by Weinrach and Tal-

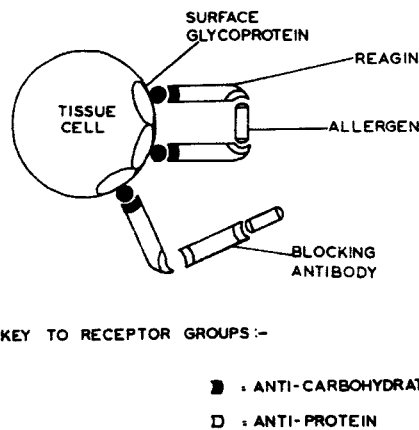


FIG. 2. Hypothetical model indicating a manner in which reagin-allergen interaction at the cell surface could lead to tissue injury. (The black circles represent carbohydrate groupings protruding from surface glycoprotein molecules.) A proposed mode of action of blocking antibody is included in the same diagram.

mage (1958) to explain the role of complement fixation in immune hemolysis. It is not intended to imply, however, that complement plays any part in the tissue damage evoked by reagin-allergen combination, as this has yet to be demonstrated (Osler, 1961). Moreover, immune hemolysis is a relatively unspecific process as compared with the reaction leading to cell injury in immediate sensitivity; although E. L. Becker (1959) has drawn attention to similarities between the mechanisms of these two types of reaction.

According to the hypothetical reaction model shown in Fig. 2, the active "reagin-allergen unit" comprises two molecules of reagin, each bound to the tissue cell by one of their antibody receptors, and one molecule of allergen (i.e., $Ag-Ab_2$). It resembles, therefore, the Ag_3-Ab_2 complexes which Ishizaka and his associates (1959a,b) found to be irritative *immediately* upon injection into guinea pig skin. There is, however, an obvious difference in the mode of action of the soluble antigen-antibody complexes in that the tissues are supposedly stimulated directly by γ -globulin which has undergone a structural alteration (in papain

piece III) to a tissue binding form as a result of *in vitro* combination with antigen (Ishizaka *et al.*, 1962). In contrast, it is postulated that the tissue damage in P-K tests is evoked by the interaction between allergen and adjacent reagin molecules *prebound* to the cell.

The question then arises as to the nature of the combination between the tissue cell and reagin. If this is an antigen-antibody reaction, how does a tissue component of an allergic individual become "autoantigenic"? In attempting to answer this question Augustin (1955) has suggested that reagin production might be a response to a tissue-allergen complex, involving a local antibody-forming mechanism which might operate in nasal polypi. There is no evidence, however, that inhalant allergens unite with epithelial mucopolysaccharides in the manner proposed by this investigator. The same objection can be leveled at a similar proposal by Najjar and Robinson (1958), who also supposed that "allergenoid" substances render the host tissue component antigenic by forming complexes with it. Moreover, although Isliker (1957) has suggested that reagin could attach itself to tissue glycoprotein or form circulating complexes with serum glycoproteins having a common configuration with tissue components, he, too, has failed to offer any satisfactory explanation as to how such a reaction occurs. On the other hand, the need to postulate a tissue component-allergen complex is avoided in the hypothesis presented here (and depicted schematically in Fig. 2). This interaction model also overcomes the difficulty of explaining why reagin can also attach to tissue that has not previously come into contact with allergen, as is the case in the passive sensitization of the skin of normal recipients in P-K tests.

D. POSSIBLE MECHANISM OF REAGIN FORMATION

According to the mechanism proposed, reagin formation results from the breakdown of the host's tolerance to a "self" tissue component, following the inhalation of antigen (allergen) of closely related structure. Thus, it is suggested that the formation of reagins in predisposed individuals is brought about by a similar mechanism to that thought by Barnett and his associates (1963) to be responsible for their induction of autoimmunity in rabbits, which develop adrenalitis as a result of injection with closely related heterologous tissue antigen. As already discussed in Section V, A, prolonged inhalation of horse dandruff by humans could lead to an autoimmune response of this type, evoked by the traces of horse serum α_2 -glycoprotein found in the dandruff. In such a process it is envisaged that the close structural relationship between the horse serum and human serum glycoproteins results in the formation

of antibodies directed against a *tissue counterpart* of the latter. A similar type of mechanism could be involved in the formation of skin-sensitizing antibodies during serum sickness (as was mentioned in Section V, A). There, it is supposed that a horse serum α_2 -glycoprotein contaminant of the antitoxin administered to the patient could lead to the breaking of tolerance to a tissue glycoprotein. Moreover, a similar response would be expected as a result of the administration of antitoxin fractions from other animal species because von Scheiffarth and his associates (1957) have shown that the cross reactivity between such sera is due mainly to the close immunological relationship of one of their α_2 -glycoprotein components. It seems significant, therefore, that the sera of humans with serum sickness following administration of horse or bovine serum antitoxin were found to precipitate an α -globulin antigen (as well as a γ -globulin) in the animal sera when the patients' sera were used as "antisera" in immunoelectrophoretic analyses (Rose *et al.*, 1962; Reisman *et al.*, 1961b). Since the serum α -globulin component appeared to play an important role in the hemagglutination by the patients' sera of tanned red cells coated with horse serum, it is perhaps surprising that there appeared to be no correlation between P-K activity and hemagglutination titer of sera examined at different stages during the disease. Nevertheless, it should be noted that cells coated with pure α -globulin antigen were not used in the hemagglutination titration. Moreover, the apparent failure to measure the skin-sensitizing activity by the tanned red-cell technique might be attributed to the practice of employing cell-bound allergen rather than cell-bound antibody, since the specific molecular configuration of the latter is considered to be a critical factor in the elicitation of tissue damage in P-K tests (on subsequent reaction with challenging allergen).

In spite of often showing a sensitivity to horse serum, which (as suggested earlier) could result from the inhalation over a long period of time of trace amounts of horse serum α_2 -glycoprotein as contaminant of dandruff, individuals sensitive to horse dandruff appear to be primarily sensitive to a horse epidermal antigen. This could explain why "horse asthmatics" can react violently to the administration of horse serum fractions, whereas patients who have had serum sickness do not become sensitive to horse dandruff. The glycoprotein allergen isolated from horse dandruff extract (Stanworth, 1953, 1957b) was found to be of considerably smaller molecular weight (i.e., 34,000) than horse serum α_2 -macroglobulin. Nevertheless, it seems significant that its carbohydrate (9% hexose) comprised similar sugar constituents to those commonly occurring in the neutral glycoproteins of human serum and tissue (Blix,

1958), namely, galactose, mannose, *N*-acetylglucosamine, fucose, and, according to recent observations, probably sialic acid. Furthermore, these sugar constituents seem to be present in the allergen in similar relative amounts to those found in human serum α_2 -macroglobulin (Schultze *et al.*, 1956). On the other hand, eight other serum glycoproteins were reported (Winzler, 1960) to possess quantitatively different sugar compositions. Recent results of Kamiyama and Schmid (1962), however, indicate that one of these—the α_1 acid glycoprotein of human serum—also possesses a similar relative sugar composition to that of the allergen.

In view of these apparent structural similarities between the carbohydrate of horse epidermal and human serum glycoproteins, it is tentatively proposed that the production of reagins to horse dandruff allergen might also result from the breaking of an individual's tolerance to an epithelial glycoprotein by means of the prolonged inhalation of a heterologous (horse) epidermal glycoprotein with a closely related carbohydrate moiety. Such a mechanism, apart from explaining the specific binding by human tissues of reagin to horse dandruff allergen, could also account for the apparent *in vitro* combination of reagin with allergic serum α_2 -macroglobulin component under certain conditions. The skin-sensitizing antibody might be expected to show stronger affinity for the tissue glycoprotein than for the serum glycoprotein, however, because of the former constituent's closer structural relationship to the offending allergen (and possibly, as will be discussed later, because of its lack of exposed protein groupings which could interfere with reagin binding). Nevertheless, a relatively weak combination of circulating reagin with serum α_2 -macroglobulin could play an important part in the stabilization of skin-sensitizing activity, by maintaining the reagin molecule in a specific molecular configuration essential for its attachment to skin cells. Furthermore, if such a mechanism is responsible for the protection of P-K activity in allergic serum, any fractionation procedure leading to a clear-cut separation of reagin from the α_2 -macroglobulin would be expected to leave the antibody readily susceptible to deactivation (as appears to be the case). Similarly, it might be expected that loss of P-K activity would result from destruction of the apparently heat-labile (R. K. Brown *et al.*, 1954; Peterkofsky *et al.*, 1956) α_2 -macroglobulin in allergic sera which are incubated at 56°C. for 1 to 4 hours.

E. NATURE OF ALLERGENIC DETERMINANTS

As implied, it is the antigenic determinants in the *carbohydrate moieties* of horse dandruff and horse serum glycoproteins that are considered to be active in the initial sensitization of predisposed individuals

by these substances. Moreover, it is not impossible that allergenic substances in general will be found to exhibit the common characteristic of possessing sugar constituents related to those occurring in human epithelial glycoproteins. The results of the limited number of chemical characterizations of purified allergens already undertaken (compared in Table IV) would seem not to negate such relationships. It is significant that in these studies allergenic activity has been shown invariably to be associated with a *protein-carbohydrate conjugate*, in the fairly narrow size range of 2.2–3.8 S, in spite of there being a wide variation in the proportion of carbohydrate to polypeptide. Nevertheless, it should be stressed that such investigations can be considered merely as exploratory, because not one purified allergen has been convincingly demonstrated to be completely homogeneous according to immunological criteria. In fact, some of the characterization studies have been carried out on preparations that did not even satisfy the physicochemical requirements of homogeneity. It is obvious that further chemical characterizations on highly purified allergens are urgently required. Attempted purifications are often hampered, however, by the difficulties involved in the fractionation of a minor component from an excess of inactive material. The high purification factors (based on increases in skin reactivity relative to that of the crude starting extract), reported in the isolation of various types of pollen allergen (listed by Augustin and Hayward, 1962), suggest that inhalant allergens frequently occur as minor components of extracts (horse dandruff allergen being an exception). A similar difficulty would be expected in attempts to identify the allergen in other systems, such as diphtheria toxoid or ovalbumin, because here the active substance appears to be present in only minute amounts. It is conceivable, therefore, that the ultimate isolation and characterization of these trace substances will reveal closer structural relationships between allergens than are apparent from present data.

F. ROLE OF EPITHELIAL GLYCOPROTEIN

It is difficult to explain the wide variation in specificity of reactivity to inhalant allergens, shown by different allergic individuals, except on the basis of an immunochemical mechanism of the type proposed. Thus allergenic substances are thought to share the common characteristic of being glycoproteins containing carbohydrate of closely related composition to that occurring in human epithelial glycoprotein. Conversely, it is predicted that the reactive tissue glycoprotein will show variations in carbohydrate composition in different individuals, those with an hereditary predisposition for allergy possessing epithelial glycoproteins with

TABLE IV
CARBOHYDRATE COMPOSITIONS AND MOLECULAR WEIGHTS OF SOME SKIN-ACTIVE COMPONENTS ISOLATED FROM ALLERGENIC EXTRACTS

Source of allergen	Carbohydrate (Hexose) (%)	Sugar constituents identified	Molecular weight	Sed. coeff. ($s_{20,w} \times 10^{13}$)	References
Horse dandruff	9	Gal, Man (1:1); N-Ac.Glu, Fuc	34,000	3.4 ^a	Stanworth, 1957b
<i>Trichophyton mentagrophytes</i>	82-88	Gal, Man (1:8-1:4)	—	2.2	Morris, 1962
Dwarf ragweed pollen	45	Arab	—	3.8 (s_{27})	Goldfarb and Callaghan, 1961; Callaghan and Goldfarb, 1962
Low ragweed pollen	16-64	Arab	32,000	—	King, 1961; King and Norman, 1962
Short ragweed pollen	Small amounts	Arab, Gal	—	3.2-3.6	Lea and Sehon, 1962; Lea <i>et al.</i> , 1962
Giant ragweed pollen	—	Arab	—	—	Goldfarb <i>et al.</i> , 1958
Timothy and cocksfoot grass pollen	—	Arab, Gal	—	—	Augustin, 1959a
Rye pollen	31-66	Man	17-21,000	2.2-2.7	Johnson and Thome, 1958
House dust	44	Arab, Gal, Xyl	22-25,000	—	Berrens and Young, 1961
Ipecacuanha	8.5	Gal, Glu, Arab, Xyl	—	3.4	Berrens and Young, 1962

Key to abbreviations: Gal = galactose; Man = mannose; N-Ac.Glu = N-acetylglucosamine; Fuc = fucose; Arab = arabinose; Xyl = xylose; Glu = glucose.

^a Unpublished.

carbohydrate more closely related to that found in potential allergens. It seemed of interest, in this connection, that Allison and Blumberg (1961) were able to demonstrate a genetically controlled serum α_2 -macroglobulin component—designated “Ag(a+)”—in the sera of a proportion of normal humans, by means of precipitin formation with serum from an anemic patient who had received approximately fifty blood transfusions. Further investigation, however, has now shown that the allotype in this case is associated with a low-density β -lipoprotein (Blumberg *et al.*, 1962). Nevertheless, hereditary groups have been demonstrated among other human serum α_2 -globulin components such as haptoglobins (Smithies, 1955), where the different electrophoretic mobilities of the phenotypes have been associated with differences in sialic acid content (Parker and Bearn, 1962), and the Gc system of Hirschfeld and his associates (1960). Similar genetic differences might be expected, therefore, among tissue counterparts of these serum α_2 -globulin components.

The theory of reagin formation proposed here offers an explanation of the puzzling observation (Frankland, 1963) that most predisposed individuals develop allergy to the same type of grass pollen, although they fail to show reactivity to a tree pollen to which they are probably exposed more frequently (it being assumed that the tree pollen granules do not possess any disability which prevents their ready contact with reagin-forming cells or their intermediaries). Conversely, an alteration in an individual's response to allergens might be expected to result from disease processes, which lead to tissue breakdown and changes in the structure of the epithelial glycoproteins. It is of possible significance, therefore, that Fisherman (1962) has recently observed that patients with cancer exhibit an impaired capacity to develop immediate cutaneous reactivity to *Ascaris lumbricoides* extract as compared to the response shown by normal individuals. It would be interesting to know whether the disease process had any effect on the ability of such patients to produce P-K reactive antibodies and also whether other immunological processes were impaired.

An impaired capacity for the development of *delayed* sensitivity to tuberculin has been frequently observed in patients with neoplastic diseases, such as Hodgkin's disease (Good *et al.*, 1962) and sarcoidosis (Friou, 1952). Such patients appeared to show a normal response to passive transfer of immediate sensitivity, however, as judged by limited qualitative P-K testing (Schier *et al.*, 1956; Sones and Israel, 1954). Nevertheless, Goulian and Fahey (1961) have made the interesting observation that the changes occurring in the serum proteins of patients

with Hodgkin's disease were associated mainly with three α -globulin components, out of which an α_2 -globulin contributed to the largest amount of hexose and also caused the elevation of total α_2 -globulin revealed by serum electrophoresis. If, as Shetlar (1961) has suggested, such changes in serum α -glycoproteins observed in neoplastic diseases could result from alterations in corresponding tissue glycoproteins, the impaired response of Hodgkin's disease patients to delayed-reacting antigens might also reflect structural changes in the epithelial glycoproteins. In other words, it is possible that epithelial glycoproteins play a key role in both immediate- and delayed-sensitivity reactions. In delayed sensitivity, cellular glycoprotein might be instrumental in firmly binding sensitizing antibody—possibly through antiprotein and anticarbohydrate groups—and thus preventing its secretion into the circulation. In contrast, reagins are thought to be more loosely bound (perhaps only through an anticarbohydrate group) to tissues and to circulating serum glycoproteins related to tissue components. This leads to a consideration of the part played by different types of antigenic determinant in the induction of hypersensitivity.

G. INFLUENCE OF NATURE OF ANTIGENIC DETERMINANT ON TYPE OF HYPERSENSITIVE RESPONSE EVOKED

In comparing the reactivities of allergic individuals to various allergens by means of direct or passive skin testing, it is important to realize that the isolated substance used for challenge is probably in a different structural form to that responsible for the initial sensitization process. Hence, it is quite possible that extraction and isolation of allergenic substances results in the unmasking of additional antigenic determinants, which play no part in spontaneous sensitization by native material. For example, an allergenic glycoprotein would be sited ideally at the surface of a pollen granule (in the ectexine), from which antigenic determinant groupings might project in the form of polysaccharide side chains. Extraction of the granule could lead to isolation of the disrupted glycoprotein, accompanied by the emergence of protein antigenic determinant groupings as a result of changes in molecular configuration. These new determinants would be prominent in the production of blocking antibodies during hyposensitization of allergic individuals. They might also be expected to play some part in the *in vivo* combination of tissue-bound reagin and allergen. Hence, differences in structure of such protein haptenic groupings might account for the cross reactivity shown by some allergic individuals in neutralization testings with a range of pollen extracts.

Of particular interest in this respect are the results of studies by Coulson and his associates (1949) on the relationship of carbohydrate to the allergenic proteins isolated from cottonseed. It was found that an increased ratio of carbohydrate to protein was associated with an increased capacity to sensitize guinea pigs, but the carbohydrate appeared not to play any role in the antigenic specificity or the "shocking capacity" of the allergenic glycoprotein. Of relevance, too, are recent studies on the role of carbohydrate in the response of sensitized humans and guinea pigs to skin testing with a glycopeptide produced in cultures of the pathogenic fungus, *Trichophyton mentagrophytes* (Barker *et al.*, 1962). Tests with glycopeptide preparations in which the carbohydrate or the protein had been specifically degraded, suggested that the carbohydrate part of the molecule evoked immediate skin reactions whereas the protein mediated a delayed response. Holborow and Loewi (1962) have obtained similar results from studies of the response of actively and passively sensitized guinea pigs to skin testing with blood-group mucopolysaccharides. Whereas the polypeptide part of the mucopolysaccharide molecule was concerned chiefly with the production of delayed hypersensitivity, the circulating antibody formed appeared to be directed against the polysaccharide moiety. These findings are consistent with the observations of Benaceraff and Gell (1959) who showed that when a picrylated protein is used as antigen in guinea pig immunizations, circulating antibody is produced against the haptenic group and delayed sensitivity against the carrier protein. Gell and Ricketts (1962) have succeeded recently, however, in eliciting a specific delayed sensitivity to a short-chain polyglucose.

The epithelial glycoprotein to which reagin is assumed to bind would be ideally situated at the surface of tissue cells (as indicated in the model in Fig. 2). Furthermore, if this glycoprotein possessed a similar type of structure to the mucin of bovine submaxillary gland, which has been characterized by Gottschalk (1960), it might be expected to project polysaccharide side chains with terminal sialic acid units from the cell surface. Weiss (1961) has adopted this type of model in explaining the changes in the strength of attachment of some mammalian cells to glass, as a result of neuraminidase treatment. If glycoprotein at the surface of reagin-binding cells is arranged similarly, it is quite possible that carbohydrate groupings (rather than protein) would be principally involved in the binding of reaginic antibodies to tissues. A similar surface arrangement of carbohydrate determinant groups could also be involved in the binding of reagins to leucocytes and, possibly, to other circulating cells. Moreover, an attachment of this type at the surface of antibody-forming cells

might interfere in some way with their function, thus contributing to the allergic individual's atypical response to allergenic antigens. In this connection, Glynn and Holborow (1959) have made the interesting suggestion that the blood group substances sited in human capillary walls and on cell surfaces may play a role in membrane permeability. It is not impossible that a tissue glycoprotein of the type which is thought to bind reagins (e.g., related to serum α_2 -glycoproteins) fulfils a similar function on the surface of antibody-forming cells or their intermediaries. Likewise, a similar glycoprotein sited on the surface of the cells of the placental membrane could influence the placental transmission of serum proteins.

To summarize briefly, a theory of reagin formation has been presented which could explain the mechanism of the firm attachment of this antibody to human epithelial tissues. It has yet to be proved that a specific antigen-antibody interaction is involved in skin binding. Alternatively, it is possible that the hereditary predisposition of allergic individuals is responsible for the synthesis of anti-allergen antibody with an abnormal papain digestion piece III, which becomes readily attachable to epithelial cells. Results of recent studies of Gm group specificity (Franklin *et al.*, 1962) have suggested that the two main parts of γ -globulin molecules (i.e., equivalent to papain digestion pieces I + II and III) are under *separate genetic control*. It is not impossible, of course, that the reagin molecule is unique in possessing a receptor group located in a part of the molecule corresponding to that part of immune γ -globulin antibodies that comprises the principal antigenic determinants (i.e., papain piece III) and from which antibody receptor groups are normally absent.

VII. Future Prospects

The frequent resort to speculation in the previous section, in the formulation of possible immunochemical mechanisms of immediate-sensitivity reactions, reflects the wide gaps in our knowledge of the nature and function of reagin antibodies. Unfortunately, it is unlikely that this situation will be improved radically until a satisfactory *in vitro* method of assaying reagins is developed, to replace the P-K test. Even then the problem of isolating sufficient purified antibody for characterization is not going to be surmounted easily, as several hundreds of liters of allergic serum would have to be fractionated in order to obtain a worthwhile yield. Moreover, this calculation assumes that the instability of the isolated antibody can be counteracted satisfactorily.

In the meantime, it is proposed that closer collaboration between

investigators working on different reagin-allergen systems should be sought, in an effort to resolve some of the apparent discrepancies in the properties of reaginic antibodies. The important need for the standardization of techniques employed in different laboratories could be fulfilled by circulation of certain well-characterized freeze-dried allergic sera (and possibly fractions) obtained from "pedigree donors."

Another profitable line of attack would seem to be a detailed investigation of the properties of the reagin-like antibodies induced artificially in some animals by prolonged immunization and in humans during serum sickness. This approach will undoubtedly lead to a better understanding of the production of skin-sensitizing antibodies, in general, but here, too, there is a need for the development of an *in vitro* method of assaying antibody activity.

The key to all such investigations lies ultimately in the identification of the antigenic determinants against which the reagin molecule's receptor groups are directed. In this connection, it seems safe to predict that the carbohydrate groupings of the glycoprotein allergens will be found to play a critical role. Substantiation of the proposal (put forward in the previous section) that the sensitizing activity of inhalant allergens is due to a close relationship between such carbohydrate determinants and similar groupings in human epithelial glycoproteins will only be achieved, however, by systematic structural analyses of the antigenic substances involved. A new method of elucidating the structure of complex glycoproteins involving sequential enzyme induction (Barker *et al.*, 1963) seems of particular potential value for this purpose, as it provides a precise indication of the arrangement of sugar residues from the analysis of only milligram samples.

More fundamental studies, on the sensitizing activity (in animals) of well-defined polysaccharides (e.g., Coulson and Stevens, 1961) and synthetic polypeptides (e.g., Maurer *et al.*, 1962; Axelrod *et al.*, 1963) are also throwing light on the nature of the receptor groups of skin-sensitizing antibodies. *In vitro* studies of reagin production (e.g., by bronchiale lymph nodes or by polypi) are also desirable, particularly as the fluorescent antibody technique would seem to offer a powerful tool in such an approach. This is suggested, for instance, by results of recent studies on the nature and incidence of organ-specific autoantibodies to gastric mucosa in pernicious anemia (K. B. Taylor *et al.*, 1962), where the fluorescent staining technique showed a greater sensitivity and specificity than the complement-fixation test. It would be interesting to employ this technique in attempts to demonstrate a similar binding of reagins to a glycoprotein constituent of gastric mucosa (and other epi-

thelial tissues). In fact, if there is any foundation to the ideas developed in the previous section, it is predicted that other methods designed to provide an indication of the nature of the antigen-antibody interaction in autoimmune conditions will also have application to the study of the mode of fixation of reagins to human tissues. Conversely, it is quite possible that the further characterization of reagins will contribute to a better understanding of the immunochemical basis of the so-called autoimmune diseases.

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Nature of Retained Antigen and Its Role in Immune Mechanisms

DAN H. CAMPBELL AND JUSTINE S. GARVEY

Division of Chemistry and Chemical Engineering, The California Institute of Technology, Pasadena, California

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I. Introduction

When foreign materials enter the tissues of intact animals, either by natural or artificial means, they may either be rapidly eliminated or retained for varying lengths of time which may include the life span of the host. In either case, foreign materials may or may not be modified chemically and they may or may not become conjugated to normal tissue constituents, e.g., normal proteins and nucleic acids. The fate and disposition of antigenic materials depends upon many factors, such as chemical, physical, and biological properties and the physiological and genetic state of the host. Many factors must be involved in antibody formation and immune mechanisms, such as the nature of the antigen and the host's reaction, but the retention and modification of foreign material may be one of the most important. Unfortunately, little attention has been given to this aspect of immunology and reviews have, in general, ignored the problem. Previous reviews which give some discussion on retention and fate of antigens have been presented by Coons (1954), Bussard (1959), and Haurowitz (1960).

The first interest in the retention of foreign material was evidenced by the work of Metchnikoff (1897) who studied the persistence of tetanus toxin in certain species of vertebrates and invertebrates. Subsequently reports were made on normal carriers of a variety of infectious organisms, including animal parasites. Experimental studies

with such infectious agents clearly showed that infectious or viable forms rapidly disappeared from tissues of immunized animals (detailed discussion by Wilson and Miles, 1946a). The persistence of bacteria, such as those involved in chronic and relapsing types of disease (e.g., syphilis, undulant fever, and relapsing fevers) or a carrier state, must play some role in the specific immune mechanism of the host. The same is true of the so-called "immunity to superinfection" which is so often encountered in diseases involving animal parasites. In most of these examples, it can be shown that a biological and antigenic change takes place in the antigen (organism) and that a synergistic relation between host and infectious agent develops. In any event, the nature of the retained organism must play an important role in the specific immune mechanism of the host as long as it or some of its antigenic components persist. Studies dealing with the retention or persistence of infectious agents have given little attention to the nature of the retained material except from a biological standpoint.

When discussing the persistence of foreign cells one must also consider the work of Owen (1945) dealing with the persistence of viable histoincompatible cells. In this instance the host was referred to as a chimera and the cells (erythrocytes) involved would normally be foreign. However, when cells were transferred *in utero* between nonidentical twins in cattle, they persisted and multiplied during the life of the host and produced a state of immunological tolerance. Except in this special case, the state of experimentally acquired immunological tolerance usually persists for only a limited time after birth.

In some instances the injection of viable incompatible cells may result in the formation of an immune reaction against the host and produce what has been referred to as "runt" disease (Billingham and Brent, 1957).

The determination of antigen retention at a molecular level presents such serious analytical problems that it received very little attention until the last decade. However, by use of rather indirect biological methods, three different earlier studies indicated that non-cellular antigens were deposited in tissues and might persist, to some extent, at least up to the time of maximal antibody formation. Sabin's classical studies (1939) showed microscopically that an azoprotein insolubilized with alum was localized in macrophages and that the dye was no longer visible a few days after antibodies were detectable. Using a reverse anaphylaxis technique, McMaster and Kruse (1951) concluded that they could detect soluble antigens in liver, spleen,

and lymph nodes as long as 101 days after injection. Felton (1949) also found that antigens such as pneumococcus polysaccharide persisted for long periods of time, perhaps the life of the animal, such as the mouse, if the amount of antigen injected was relatively large. The effect was referred to as "immune paralysis." Campbell (1942), studying eosinophilia in guinea pigs, concluded that eosinophilia resulting from parasitic infection or injection of a relatively insoluble antigen such as keratin, was due to the persistence of antigen after antibody had been formed. Haurowitz and Breinl (1932) approached the problem more directly and quantitatively using arsenic-labeled proteins.

A most important development occurred when isotopic materials became available and foreign antigenic material could be followed and detected at extremely low levels of concentration. Using radioactive labels such as I^{131} , C^{14} , and S^{35} , much valuable information has been obtained as to the rate of loss of foreign antigen from the blood stream, relation to antibody formation, localization in various tissues and cells, persistence of antigen material in tissues, and a means of identifying antigen material for isolation and subsequent characterization.

Much of this earlier work indicated that labeled soluble foreign antigens might persist for long periods of time. However, with the exception of two research groups (Haurowitz's and our own) very little attention has been given to the fate and chemical nature of antigen material that persisted in tissues long after its disappearance from the blood stream. Both of these groups feel that the persistence of antigen material in tissue cells and the nature of the retained material must play an important role in antibody formation and subsequent immune reactions.

The idea that in order to be immunogenic, a material must be susceptible to *in vivo* digestion or modified in some way is very provocative and not new. For example, Wells (1928) stated in a review, ". . . antigenic activity is in some way related to digestive proteolysis." As pointed out previously by Campbell (1957), nondigestible artificial polymers and native nondigestible materials such as gum acacia which are deposited in tissues without apparent degradation, do not induce antibody formation. Immunogenic synthetic polymers of amino acids that have bonds that would be expected to be susceptible to proteolytic enzymes have been prepared by Gill and Doty (1960). However, the susceptibility of such materials to enzymatic degradation and fate after injection have not been reported. Attempts to induce antibody formation in invertebrates (e.g., the spiny lobster) failed. It was found by specific

precipitation with rabbit antiserum that ovalbumin remained intact in practically undiminished concentration in the lobster blood for many months after injection (Campbell, 1946).

The following review is concerned chiefly with the persistence of foreign antigenic material in tissues, its chemical and biological properties, and speculations as to its role in immune mechanisms. It is important to keep in mind that although the amount of material may be small in terms of grams, it may be extremely large in terms of molecules, and particularly molecules per cell. A native antigen molecule, whether a part of a foreign cell such as *Escherichia coli* or a soluble protein molecule such as a toxin, soon changes after entering an intracellular environment. It is important, therefore, that one keep in mind the persistence or retention of "antigen material" and not the original native substance that was used for immunization. It will also be seen that the retained antigen is not only a fragment of the immunizing material but that it is combined with normal tissue components such as ribonucleic acid (RNA) and under certain conditions with its specific antibody. Very little space will be given to speculation on antibody formation since it is felt that too little is known at this time about the biosynthesis of proteins and why a foreign protein (antigen) should modify the structure of one serum protein and not another. A possible mechanism, based upon the template theory, is presented, but emphasis is essentially upon review of experimental studies.

II. Retention of Antigen

A. LOCALIZATION OF ANTIGEN IN NORMAL AND IMMUNE ANIMALS

Many different factors influence the pattern of events that lead to the *in vivo* fate of injected antigen. Because the following are the more important of the factors that are likely to influence the fate of antigen, it is useful to consider them when making a relative evaluation of different investigations: (1) whether or not the native material is soluble or insoluble, protein or polysaccharide; also (2) the dosage of antigen and (3) route of administration; (4) characteristics of the test animal, such as species and age; and especially (5) the animal's previous experience with the same antigen.

The method of assay will certainly impose certain limitations on any studies dealing with retention of antigen. A particular kind of limitation may apply more to one method than to another, such as a requirement of high concentration and little change, if any, from the original form of the antigen, e.g., precipitin reaction or infectivity.

If the form of the antigen is of no consequence in its detection, e.g., radioactivity, then corroborative techniques are needed to define the nature of the retained antigen and to make certain the label is related to the original material. Internal labels of radioactivity are subject to the normal metabolism of the experimental animal and in addition are usually of lower specific activity than the external labels. Each of the two external labels that have been most widely studied, halogen (e.g., I^{131}) and diazonium (e.g., S^{35}) has a different usefulness. The iodine label is particularly useful for studies of circulatory clearance but iodine covalently bound to protein may not be a stable linkage for assay of antigen in tissue. Tong *et al.* (1954) obtained evidence by *in vitro* assay that some tissues, particularly the liver and kidney, deiodinated diiodotyrosine, and Laws (1951) found inorganic iodine and a stable peptide as the excretory products of iodinated protein. More direct evidence should be obtained on the *in vivo* fate of iodinated antigens in tissues other than the thyroid before any definite conclusions are arrived at. The aspect of the short half-life of I^{131} is a disadvantage which may be overcome by replacement with I^{125} , which is an isotope of several times longer half-life than I^{131} . The azo linkage, used for S^{35} labeling of antigen, is foreign to the experimental animal and hence the labeled protein is rapidly removed from the circulation. The antigen-tissue relationship can be studied for very long periods of time with S^{35} -azoproteins since the label is in a haptenic group with antigenic specificity; furthermore, the half-life allows long detection and the weak β -emission permits use of radioautography.

1. Circulatory Clearance of Antigen

a. Native Antigens. Most of the early investigations on the fate of circulating antigen were concerned with living bacteria and were obviously related to practical aspects of bacteriology and immunology. Such studies were complicated by the insoluble nature of the material and the complexity in numbers and kinds of antigens in the bacterial cell. Strains of different virulence often provided the parameter for study. Following injection directly into the blood stream the numbers of organisms usually declined rapidly within a few minutes and the blood became sterile within a few hours. When conditions were duplicated, with the exception of previous sensitization, the rate of clearance was greatly accelerated. After injection into the skin or into the peritoneal cavity, both the pathway and rate of entrance into the blood stream were studied. Wilson and Miles (1946a) have compiled a lengthy list of publications and have also described lucidly the

experimental results of many of these investigations on circulatory clearance of bacteria.

As investigators began to use nonviable antigens, the fluctuation in dose caused by death and reproduction of viable cells was removed, and a precise definition of *in vivo* dosage was possible. Soluble material was generally used, but most of the early studies were with mixtures of antigens such as whole serum, still with the purpose of contributing to some particular problem in practical immunology. The precipitin reaction was applied for both qualitative and quantitative detection although much information had accumulated before the latter was developed. Uhlenhuth and Weidanz (1909) found persistence in the circulation as long as 15 days following a 5-ml. injection of horse serum intravenously into rabbits. As antibodies appeared in the circulation, the horse serum decreased below detectable limits. Longcope and Mackenzie (1920) detected, by specific precipitin reactions, horse serum injected intravenously into rabbits in a dose of 5 ml./kg. body weight as long as 3 weeks following the injection. Human serum was also examined for the presence of horse serum that had been administered for pneumonia therapy. In humans, as in rabbits, detection of the foreign serum was possible for several weeks, but the level fell very severely as antibody formation occurred. Longcope and Rackemann (1918) studied the two phenomena (appearance of serum antibody and disappearance of circulating antigen) to determine their relationship to serum sickness, with the conclusion that they were the result and not the cause of serum sickness.

Soluble specific substances (SSS) are not antigenic in rabbits and circulatory persistence is of long duration in this species (Avery and Goebel, 1933). When SSS was injected into immunized rabbits that had previously received intact organisms, disappearance was more rapid than in normal rabbits and was type specific (Downie, 1937).

Similar studies involved the circulatory clearance of heterologous diphtheria antitoxin (Glenny and Hopkins, 1923). The results indicated three phases of circulatory loss. The first, phase A, represented a 50% fall in the antitoxic content of the blood during the first 24 hours. This loss was attributed to redistribution of antitoxin from the blood into the tissues. The next, phase B, was a period of slow elimination lasting 6-7 days, during which time 25% of the amount present each day was lost by the next day. This gradual loss was assigned to metabolic processes. The last, phase C, was marked by a rapidly accelerated loss, apparently resulting from active production of antibody against the antitoxin. In cases where levels of precipitins were insufficient to

eliminate all the foreign protein, phase C was followed by a gradual loss, similar to phase B. When the subcutaneous route was used, the blood concentration after 2 hours was only one two-hundredth of that of a rabbit injected intravenously. The highest concentration occurred after 2-3 days, when it reached approximately the same value as that of rabbits injected intravenously. In sensitized (i.e., potentially immune) animals injected intravenously, phase A occurred as in a normal animal; phase B was likewise similar to that observed in the normal animal, except that the interval was shorter; phase C, however, was characterized by an extremely rapid elimination which resulted in almost total loss within 24 hours.

Opie (1923) studied the relationship of ovalbumin to antiovalbumin in the circulation of normal rabbits injected subcutaneously with ovalbumin. Antigen was demonstrated in the serum at 7-11 days following injection, persisted there for 5-9 days, and disappeared when precipitin appeared. When antigen persisted in the circulation as long as 19 days, antibody titers were lower. Antigen was not demonstrable simultaneously with antibody in the serum. Later Opie (1924) compared the fate of antigen in a sensitized animal. Again, ovalbumin was injected subcutaneously into normal rabbits and also into immunized rabbits. In the normal rabbit, ovalbumin disappeared within 3 days from the site of injection as shown by extraction and use of the abdominal tissue in tests with hyperimmune sera. As noted previously, antigen entered the circulation of the normal animal and precipitin assay showed that the urine contained antigen. In the immune animal, ovalbumin remained longer, for about 5 days at the injection site, did not enter the circulation, nor was it excreted. Horse serum, used to obtain comparative data for a complex antigen, had a fate similar to ovalbumin except the normal and the immune animals both failed to excrete the antigen.

Culbertson (1935) studied the role of circulating antibody in the removal of antigen injected intravenously into rabbits. Either horse serum or egg albumin was used as the antigen. Horse serum was more rapidly removed from the circulation of immune than normal rabbits, and this acceleration was more pronounced in rabbits concurrently producing antibody than in rabbits that showed no circulating antibody at the time of the test injection. Ovalbumin was removed so rapidly in both sensitized and normal rabbits that the difference was insignificant. Because of this, the fall in quantitative titers of circulating antibody were noted as evidence for the reaction of antigen with antibody and the subsequent removal of both from the circulation.

b. Iodoproteins. A protein labeled by means of radioactivity can be measured with a high degree of sensitivity. Such labeled proteins as the iodoproteins have been found extremely useful in immunological studies. However, the use of other techniques must accompany the radioassay in order to establish the stability of the I^{131} protein linkage. Some of the early studies with radioactive proteins, although not primarily immunological, provided some of the techniques that immunologists have found applicable to their investigations. This applies particularly to the studies of Fine and Seligman (1944), who were concerned with the rate of loss of I^{131} -labeled homologous plasma proteins from the circulation of dogs. They reported the results of one study with a heterologous protein, I^{131} -labeled bovine serum albumin (I^{131} BSA), with data through 6 hours of circulation time following injection. Little can be concluded about the fate of an antigen from such a brief study; however, both this investigation and one with a bromoprotein (Fine and Seligman, 1943) provided valuable information on the chemical stability of the halogen-protein linkage and on measurement of radioactivity.

Early immunological studies were directed toward determining whether the label had *in vitro* stability. Eisen and Keston (1949) prepared BSA tagged lightly with radioactive I^{131} either by direct iodination of the protein, resulting in an iodoprotein with about 1 iodine atom/10 protein molecules, or by coupling radioactive *p*-iodophenyldiazonium chloride to the protein to result in an average of 0.67 group per molecule of protein. When such tagged proteins were compared with native BSA in quantitative precipitation with antiserum, prepared in rabbits injected with native BSA, the results differed insignificantly.

Masouredis *et al.* (1951) hyperimmunized rabbits with human serum, then obtained an antiserum from which the globulin fraction was prepared and iodinated with I^{131} . Precipitin data were obtained with human serum albumin and the I^{131} antibody globulin, and with human serum albumin and unlabeled antibody globulin as a control. From a comparison of the two sets of results there appeared to be no loss of specificity at the level of 1.3 iodine atoms/antibody molecule and the specific activity of the antibody was constant. Pressman and Sternberger (1950) studied the relative rates of iodination of serum components, reporting the rates as follows: antibody iodinated at about one-third the rate of whole serum and at about one-half the rate of the whole globulin fraction (antibody to either beef serum albumin or ovalbumin iodinated at same rate), whereas globulin iodinated at about one-half the rate of the albumin fraction. They also reported the

effect of iodination on specific precipitation. As shown in the curves, Fig. 1, specific precipitation decreased at higher levels of iodination, but the effect was most pronounced in the region of antigen excess. Pressman and his co-workers have contributed very significantly to the chemistry of iodoproteins and also to their immunological application. The

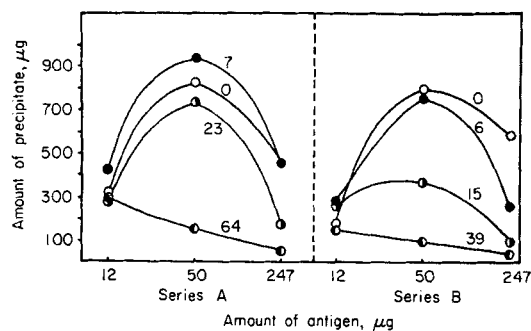


FIG. 1. Effect of iodination of whole antiovalbumin serum on specific precipitation. Two series of quantitative precipitin curves are shown with the amount of precipitate plotted against antigen added. The number above each precipitin curve indicates the average number of iodine atoms per antiserum molecule. (From Pressman and Sternberger, 1950.)

application has usually taken the form of iodination of antibody, in particular antitissue antibody, and *in vivo* study of its specific localization in tissue. Since these studies are more concerned with antibody localization per se, they will not be discussed further.

I^{131} -Labeled horse diphtheria antitoxin, containing 0.25–13.0 iodine atoms/globulin molecule was studied in precipitin reactions (Cohen, 1951). Small amounts of iodine produced little or no change in precipitable antitoxin but a distinct impairment of antibody function occurred at iodine levels between 3.5–13 atoms/molecule of globulin, the loss of reactivity being greatest in the region of antigen excess. The rate of iodination of horse antitoxic globulin was reported as 90–94% of that of average serum protein, a value higher than that given by Pressman and Sternberger (1950) for rabbit antibody. These and other results established the reliability of the label *in vitro* and stimulated the *in vivo* investigations which followed.

Knox and Endicott (1950) made one such study to determine the reliability of radioactivity measurement as a quantitative indicator of labeled antigen in the circulation. I^{131} BSA was injected intravenously into nonimmune rabbits. Serum samples were collected and used to measure antigen N both by means of I^{131} measurement and by quanti-

tative precipitation in the antibody excess zone. A moderately good estimate of circulating antigen could be obtained until the seventh day by measuring the radioactivity present in the serum (Fig. 2). At about this time, the serological detection of antigen often became negative whereas radioactivity indicated significant levels of antigen.

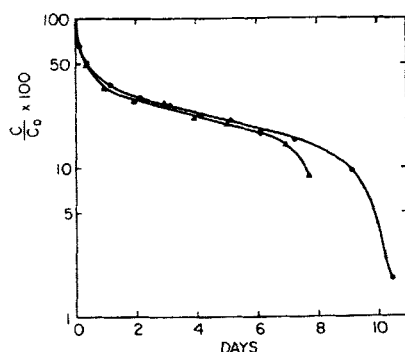


FIG. 2. A comparison of the circulatory clearance rate of bovine serum albumin (BSA) and labeled bovine serum albumin (I^{131} BSA). The data for both curves resulted from quantitative precipitation, the BSA curve (\blacktriangle) from N values and the I^{131} BSA curve (\bullet) from radioactivity values only. Antigen concentration at any time (C) was compared with antigen concentration at zero time (C_0), the latter being considered 100% in order to obtain a relative per cent as a function of time after injection. (From Knox and Endicott, 1950.)

These investigators came to the reasonable conclusion that the constant I^{131}/N ratios did not give assurance that iodine remains attached to protein. However, based on the finding that I^{131} -labeled diiodotyrosine and iodide cleared the circulation rapidly, they assumed that all split products would clear the circulation rapidly compared to intact protein.

For a study of the turnover rate of serum albumin in man, Sterling (1951) chose to use I^{131} -labeled human serum albumin. During the course of these studies, high titer antibody against I^{131} -human albumin was produced in rabbits. I^{131} -Tagged human albumin was injected intravenously into normal rabbits and detected at intervals of 2 days for a period of 14 days. Radioactivity and precipitation provided two assays for antigen in the plasma samples. There was good agreement in antigen concentration by both techniques, but there was no rate change on the semilog plot showing antigen concentration (log) versus time.

Melcher and Masouredis (1951) used as antigen I^{131} -labeled rabbit γ -globulin against human albumin, injected intravenously into guinea pigs. Samples of guinea pig serum were tested both for I^{131} content and

specific precipitation with human albumin in order to calculate the content of the injected rabbit antibody. The I^{131}/Ab N ratio remained the same in precipitates prepared with serum samples as in the original injected material until the fifth to seventh day, when circulating guinea pig antibody against the rabbit globulin was first detected; about this time the value of the ratio decreased by about one-half. At the time antibody against the injected protein was detected, there was neither a sudden decrease in serum radioactivity nor a change in the rate of excretion; these findings differed generally from those obtained in rabbits, with the possible exception of results by Sterling (1951). A half-life value for radioactivity was 107–113 hours in the serum and 117 hours in the urine and feces. The difference was attributed to the intervening influence of cellular degradation, i.e., the iodine-thyroid cycle.

Talmage *et al.* (1951) measured the circulatory clearance of I^{131} -labeled bovine γ -globulin ($I^{131}BGG$), injected intravenously into rabbits. The results were presented as a semilog plot with the per cent of antigen, i.e., protein-bound I^{131} , in the total blood volume (log scale) versus time in days (linear scale). There were changing rates of clearance which practically duplicated the observation by Glenny and Hopkins (1923) with antitoxin: (1) rapid loss for 1 day during establishment of equilibrium with extracellular pools; (2) a slower loss for the next 3 days which was similar to a normal rate of catabolism for homologous protein; (3) an increased rate of elimination observed at about the fourth day; and (4) another increase in the rate of elimination at about 5–6 days. Antibody was not observed by complement fixation until about 7 days, the reason being given that "antibody could not be detected directly until all but traces of antigen were removed from the circulation." At 7 days, 0.1% of antigen was present in the blood. Radioactivity was considered insignificant at this time in the tissues. If homologous protein were injected the exponential rate change from (1) to (2) duplicated the clearance of heterologous protein, but homologous protein continued with rate (2) unchanged during the remaining observation. Cohen *et al.* (1956) also confirmed this unchanging rate for homologous protein after initial equilibration.

Dixon *et al.* (1952) made further studies which correlated the rapid loss of circulating antigen with antibody production. Rabbits were injected intravenously with labeled bovine γ -globulin and measurements were made of the rate at which antigen disappeared from blood and also of the maximal antibody titer. Antigen was measured at various times by the determination of protein-bound radioactivity, i.e., precipitation of plasma with an equal volume of 20% trichloroacetic

acid. The average per cent of antigen found in the total blood volume was 13.9% at 4 days, 0.1% at 7 days, and 0% at 9 days. All sixteen animals showed a rapid immune clearance of antigen at 4-7 days with a maximal antibody concentration in terms of antigen N precipitated (so-called P-80 test) of 16.4 ± 10 μg . antigen N precipitated by 1 ml. antiserum.

Latta (1951) labeled bovine serum albumin and human serum albumin with radioactive iodine and injected these intravenously into a series of rabbits. The blood levels, urine excretions, and tissue concentrations were determined at intervals up to 14 days. With the exception of the thyroid, every tissue had a radioactivity concentration less than the blood. Control animals were injected with NaI^{131} in equivalent amount (per kilogram) to the protein-bound I^{131} injected into test animals. There was the indication that tissue concentrations fell almost as fast as the blood concentrations after injection of NaI^{131} and that the total excretion time for NaI^{131} was probably 1-2 days. From this it was assumed that the radioactivity remaining in the blood stream at 9-14 days after an iodoprotein injection must be largely bound.

In view of a previous finding by Dixon *et al.* (1951) that antigen is catabolized more rapidly in immune than in nonimmune rabbits, Dixon and Talmage (1951) undertook a study to compare circulatory clearance of antigen injected into animals that were either actively or passively immunized. Protein-bound and nonprotein-bound I^{131} determinations of blood and urine were made at 1, 6, 24, and 48 hours after injection of antigen. The results showed almost identical rates of I^{131}BGG catabolism in actively and passively immunized rabbits and led to the conclusion that only specific antibody, rather than cellular changes, is necessary for the rapid catabolism observed in immune compared with normal animals. Other aspects of circulatory clearance of iodoproteins are further discussed by Dixon (1953, 1954).

Laws and Wright (1952) also injected lightly iodinated bovine serum albumin and γ -globulin into normal and specifically sensitized rabbits, i.e., animals showing presence of circulating antibody. Blood was sampled at various times. At 5 hours, two-thirds of the injected radioactivity was still present in the normal animal, whereas the sensitized animals had only 10% in the circulation.

The British workers, Francis *et al.* (1957), were concerned with only the very early response of normal and immune rabbits injected with either bovine serum albumin or horse serum globulin, both trace labeled with I^{131} . In the normal rabbits, only a small loss occurred during the first 2 hours after injection, which was the time period under

study. This loss was brought about by two processes, which were graphed as the "fast" and the "slow" reaction. In the immune rabbits, a much larger proportion of the protein was removed from the blood by the fast reaction and this was attributed to *in vivo* precipitation followed by phagocytosis of the precipitate. The suggestion of the forming of a discrete precipitate also resulted from the finding that soluble antigen-antibody complexes were removed from the blood only slightly more rapidly than antigen itself. Considerable deposition of the antigen occurred in the liver, lungs, and spleen, which in case small amounts of antigen were given, might fully account for the amount lost from the blood.

Contrary to the rather slow circulatory loss of a lightly labeled I^{131} antigen, a highly iodinated γ -globulin gave rapid circulatory loss after intravenous injection into rabbits (Francis and Hawkins, 1957). The amounts of circulatory antigen were also similar for both normal and immune animals. The greater deposition in the liver and lung of immune compared to normal animals distinguished the two groups. Precipitation with trichloroacetic acid showed that both the normal and immune animals had a high percentage of nonprotein-bound radioactivity in the blood at 1 hour, whereas the tissue radioactivity was protein bound. From these findings, it was concluded that "deposition of antigens in the liver and lungs seems to be a more constant feature of the immune response than their rapid elimination from the blood."

Masouredis *et al.* (1953) studied the effect of injecting ovalbumin into mice that had received a prior injection of I^{131} -labeled rabbit anti-ovalbumin. First, the I^{131} antibody globulin was injected intravenously. The I^{131} , known to be protein bound when injected, was completely accounted for in the "globulin" space, excretions, and thyroid. At 23 hours after the injection of the antibody, some animals received antigen whereas others were controls for the antibody injection only. In the animals that received antigen, the three compartments (globulin space, excretions, and thyroid) failed to account for all the I^{131} antibody until 48 hours after the antigen was given, thus indicating transient pooling in a tissue compartment. The maximal effect of antigen on circulating antibody activity occurred at 4 hours after antigen was injected.

The sequence of administering the antigen and antibody was reversed, i.e., I^{131} -labeled human serum albumin was injected intravenously into mice and some of the same mice received antibody intraperitoneally at 24 hours after the antigen injection (Melcher *et al.*, 1953). Again transient pooling of radioactivity in a tissue compartment (liver and spleen) was responsible for a decrease in plasma I^{131} , but

this was maximal later (at 24 hours) instead of at 4 hours as in the previous investigation. The extravascular metabolism of I^{131} associated with an immune reaction was evident from the different half-life for plasma I^{131} and urine I^{131} .

Homologous proteins appeared to have an intravascular metabolism, indicated by a constant and similar exponential decay for I^{131} in both the urine and plasma, with iodide administration preventing any contribution of the thyroid (Cohen *et al.*, 1956).

c. Azoproteins. Due to the chemical reaction known as diazotization and coupling, proteins acquire a new specificity. That this specificity is closely associated with the chemical group that is introduced into the protein molecule was first demonstrated by serological tests (Landsteiner, 1936). Investigations followed to demonstrate the *in vivo* fate of these azoproteins. Pratt and Gregersen (1941) studied the rate of disappearance from the circulation of a dye protein, R-salt-azobenzidine-azo-egg albumin, which was injected intravenously into rabbits and a dog. Presence of the dye was detected spectrophotometrically. Serum samples, collected at various times, showed rapid disappearance from the plasma within the first hour (30–80%, depending upon the amount of dye protein injected) and complete removal was effected in 12–24 hours. During *in vitro* studies, 5% or less of the dye protein was taken up by cellular elements of the blood and none could be detected in the urine by either color or immunological reaction. They concluded that the rapid escape of the dye from the circulation was due neither to reaction with cellular elements of the blood nor to kidney excretion.

Studies with azoproteins have without exception shown a more brief presence in the blood stream than the native proteins from which they were prepared (Dixon *et al.*, 1951; Haurowitz and Breinl, 1932) and, to some extent at least, circulatory time could be correlated with the degree of coupling (Gitlin *et al.*, 1951; Fine and Seligman, 1943). Heavier coupling led to a reduced circulatory time. As with other antigens, sensitization also led to a faster rate of clearance (Garvey and Campbell, 1954).

The short circulatory time of this group of antigens implies a totally different catabolic mechanism, compared with those antigens already discussed, with the possible exception of heavily labeled iodoproteins. If, as has been suggested (Gitlin *et al.*, 1951), the blood only selects for degrees of foreignness, the concentration of azoprotein and heavily labeled iodoprotein would be expected to be high in tissues other than the blood. Whereas the iodine-protein linkage is subject to degradation, as discussed more fully later, the azo linkage is stable against enzymatic degradation.

2. Excretion of Antigen

If a nonantigenic material such as homologous protein is injected intravenously, intravascular metabolism may occur, with the products being directly excreted; this was postulated from the same rate of constant exponential decline of I^{131} in plasma and urine (Cohen *et al.*, 1956). However, a different half-life for albumin and globulin in this study showed that the metabolic process was reflecting unique characteristics of the protein.

Since more antigen is probably always administered than is required for effective stimulation of the antibody mechanism (Haurowitz, 1957), some molecules, if sufficiently small, may not be metabolized but may instead be excreted unchanged, as with ovalbumin (Opie, 1924).

There are several reports concerning excretion of nonantigenic material. Pneumococcus polysaccharide is such an example, being detectable as acetyl polysaccharide (SSS) in both the circulation and urine for a long time, without evidence of antibody production (Dochez and Avery, 1917). In later studies (Blake, 1918; Quigley, 1918; Pepper, 1934), SSS excretion was related to various stages of clinical pneumonia, continued excretion of antigen generally indicating unfavorable prognosis. Haurowitz *et al.* (1943) gave a comparison for arsanil-azo-globulin and arsanil-azo-gelatin both in the liver and in the urine of rabbits. According to arsenic analysis the globulin, at 1 hour after the injection, showed 34% localization in the liver and 9% in the urine. The corresponding assay on the animal injected with gelatin showed 4.4% in the liver and 66% in the urine. It was assumed that the failure of arsanil-azo-gelatin to produce antibodies is chiefly due to the fact that molecules are not deposited at sites of antibody formation. Oliver (1944-1945) also mentioned the rapid excretion of large amounts of gelatin into the urine, following intraperitoneal injection into rats.

From the investigations of circulatory clearance of iodoproteins, extensive excretion data are also available. Urinary assays usually demonstrated that the I^{131} was nonprotein bound (Latta, 1951), dialyzable (Laws, 1951), had a half-life different from that of plasma I^{131} (Melcher and Masouredis, 1951), and was excreted faster in the sensitized than in the normal animal (Dixon *et al.*, 1950). There is no direct information as to which tissues participate in these degradative changes or even whether the process is mainly accomplished intravascularly or extravascularly. When antigen was injected into mice given a prior injection of the specific antibody labeled with I^{131} , a transient pooling of I^{131} in tissues (liver and spleen) occurred (Masouredis *et al.*, 1953). The findings were similar when antibody was injected into mice

given a prior injection of the specific antigen labeled with I^{131} (Melcher *et al.*, 1953). Extravascular tissues were linked to the metabolic chain in investigations with actively sensitized animals. As already noted, deposition occurred in the liver, lungs, and spleen shortly after the injection of lightly labeled protein (Francis *et al.*, 1957) or when heavily labeled protein was injected (Francis and Hawkins, 1957).

Laws and Wright (1952) measured radioactivity in the blood, urine, and tissues of normal and immune rabbits after a test injection of the lightly iodinated specific antigen. The sera were positive by a qualitative testing procedure. Compared to the normal animals the sensitized animal showed very rapid removal of radioactivity from the blood, and the liver was particularly high for the first hour, not due to blood, but to a specific cause. By 5 hours this difference was less evident and was reversed at 24 hours with the sensitized liver lower than the control. The same differences were found for spleen although they were not so clear-cut as with liver.

The administering of iodide to the experimental animal prevented the uptake of metabolized I^{131} by the thyroid gland, and it was generally assumed that after circulatory destruction of organically bound iodine, excretion of I^{131} was probably in the form of diiodotyrosine, the radical to which specificity of iodoproteins was attributed (Wormall, 1930). Tong *et al.* (1954) studied the metabolic fate of free diiodotyrosine both in isolated tissues and in the intact animal, using paper chromatography techniques. The findings indicated that both deamination and deiodination played a role in the metabolism of added diiodotyrosine by liver and kidney tissue, whereas the thyroid was only capable of deiodination. Such investigations indicate the difficulty (also pointed out by Friedberg *et al.*, 1955) in the interpretation of I^{131} data for tissues.

Laws (1951) made the preliminary observation that rabbits excreted inorganic iodide and a stable peptide as the hydrolyzed breakdown products after an intravenous injection of I^{131} -labeled protein. In a subsequent study (Laws, 1952), qualitative chromatography confirmed the previous finding of two excretion products, one, inorganic iodide and the other, an organic constituent which was not diiodotyrosine as previously supposed. Sensitized animals eliminated a larger proportion of radioactivity in the form of inorganic iodide (rather than the organic peptide), which was attributable to a more complete breakdown of foreign protein in the tissues of the sensitized animals.

Azoprotein was similarly more rapidly excreted by a sensitized than by a normal animal (Garvey and Campbell, 1954), and this difference was obvious when the test injection was given either intravenously or

intraperitoneally. A single injection of labeled protein also demonstrated a characteristic rate of clearance for each of two azoproteins (Garvey and Campbell, 1957). Since the radioactive label was the same and linkage with amino acids was assumed similar in every respect, the significant differences in excretion may reflect unique differences for each of the native proteins. This point was not investigated further, nor is it known whether Laws (1951) confirmed his statement that "the excreted peptides may differ according to the protein used for iodination."

The following investigation provided significant information concerning the permeability of the renal glomeruli and, indirectly, some information about the fate of azoproteins prior to excretion. Smetana (1947) injected intravenously various azoproteins into different animal species. The finding of intensely stained red granules for a varying length of time in the lining cells of the proximal convoluted tubules suggested that the azoproteins had passed through the glomerular filter. A minimal amount of about 60 mg. protein/kg. body weight was necessary for the detection of granules, and the degree of storage was dependent upon the amount of azoprotein injected. No granules were found following kidney damage by uranium nitrate.

3. *Tissue Localization of Antigen*

If a material is antigenic, its circulatory fate may be only a brief prelude to a much longer cellular history. A period of tissue localization may be expected if such ideas as the following are correct. "It appears that to be an antigen a substance must have certain minimum chemical properties, but it must also be so constructed that it cannot be handled by one of the readily available mechanisms of elimination and thus be removed before it has time to exert an antigenic stimulus" (Wilson and Miles, 1946b).

Our use of the term "localization" is largely reserved for the finding of antigen in tissues after intravenous injection, which is the route of injection permitting rapid access of an antigen to all tissues, particularly when a soluble antigen is used. The period of time considered is usually very early after injection, but may extend through the rise, as well as decline, in circulating antibody titer.

Localization in the sense of antigen remaining in the site of injection into the skin or within draining lymph nodes for long periods of time will not be discussed although some recent studies are mentioned for guidance to this literature. In the following studies results were usually obtained either by fluorescein staining or by measurement of radio-

activity and with a soluble antigen (Koshland, 1957; White *et al.*, 1955; Waksman and Bocking, 1953), as well as with an emulsified antigen (Herdegen *et al.*, 1947; Freund, 1951; Talmage and Dixon, 1953). Quantitative studies of anaphylaxis have made use of I^{131} assay of soluble proteins for the Arthus phenomenon in rabbits (Korngold *et al.*, 1953) and in guinea pigs (Koutras and Schilling, 1961), as well as for fatal anaphylaxis in the guinea pig (Warren and Dixon, 1948).

a. Insoluble Antigens. There are certain organs, such as spleen, liver, lungs, and bone marrow, where reticulo-endothelial (RE) tissue is so prominent that phagocytosis promptly leads to very high local concentrations of antigen. Although localization of bacteria in tissues had been frequently observed, Sullivan *et al.* (1934) were the first investigators to give attention to a quantitative evaluation. Living bacteria were injected into normal rabbits and into rabbits previously injected with killed bacteria. Plate counts of tissue samples showed that both the liver and spleen had about the same relative concentrations, but that the level of bacteria was higher if the animal had been immunized. Kyes (1916) found that the natural immunity of pigeons to pneumococcus infection was related to rapid localization of the organisms within phagocytic cells of the spleen and liver followed by destruction of the segregated organisms within these cells. The rapid removal of circulating bacteria by the spleen was shown (Topley, 1930) by removing spleen tissue from an injected rabbit and injecting it into another rabbit (recipient). At certain times after the injection of bacteria, the spleen still gave rise to antibody in the recipient. As the time was increased for removal of the spleen, a time was reached at which an immune response could no longer be obtained in the recipient. This was assumed to mean that the antigen had been eliminated or broken down to a point where it was no longer antigenic when introduced into another animal.

The technique of fluorescent labeling of antibodies was introduced by Coons *et al.* (1942) with further modifications by Coons and Kaplan (1950). Among the early applications of the technique was the study of histochemical localization of rickettsiae and virus (Coons *et al.*, 1950). By means of specifically labeled antibody one could identify the tissue cells, as well as the locus within the cell, where antigen is fixed.

Erickson *et al.* (1953) injected tobacco mosaic virus (TMV) into the tail vein of mice, then prepared an extract of liver tissue from mice sacrificed at various times after the injection. A direct observation of the liver extract in an electron microscope revealed the presence of particles strikingly similar in size and shape to TMV through 15 days following intravenous injection. The antibody titer had risen to a peak

titer at 5–8 days but was very low at 12 days. The number of TMV rods observed in the liver had been constantly decreasing with increasing time after the injection.

Erythrocytes have been used in many immunological investigations, particularly by investigators at the University of Chicago. Only one of these investigations will be described, although others have been published during the past 40–50 years, mainly in the *Journal of Infectious Diseases*. Cary (1915) was interested in comparing the site of destruction of the rabbit's own erythrocytes (solely, the spleen) with the sites active in destruction of foreign erythrocytes. The results showed an increased activity in the spleen with additional activity by the liver after intravenous injection of beef erythrocytes. Free iron, detected by Perl's Prussian blue reaction, was a good histological indicator of the active cells. In another investigation (Luckhardt and Becht, 1911), fixation of foreign erythrocytes in the spleen was also demonstrated by removal of the spleen 24 hours after intravenous injection of the erythrocytes. Antibody production occurred in a recipient animal which received intraperitoneally the minced tissue.

Ingraham (1955a) made studies with S^{35} -sulfanilazo-sheep red-cell stromata (S^{35} A-stromata) in normal mice and rabbits for comparison with those studies which he had reported earlier with soluble S^{35} -sulfanilic acid-azo-bovine γ -globulin ($S^{35}A\gamma G$) injected into mice (Ingraham, 1951b). Blood clearance of S^{35} A-stromata was faster than of $S^{35}A\gamma G$, but gross tissue distribution of S^{35} was similar whether attached to stromata or to globulin. Insofar as the labeled stromata gave a hemolysin response in rabbits indistinguishable from that obtained with normal sheep red cells, the radioactivity was assumed to trace accurately the blood clearance and initial tissue disposition of the hemolysinogenic antigen of the stromata.

Studies (Ingraham, 1955b) of the fate of S^{35} A-stromata were continued under such special conditions as X-irradiation and splenectomy, resulting in evidence that such conditions failed to show any marked effect on concentrations of S^{35} in the tissues, although the inhibition of hemolysin response under such stress conditions, with either the labeled stromata or intact red cells, was the same.

In the investigation by Sabin (1939), tissue localization following the injection into rabbits of a dye protein in the form of an alum precipitate was extremely well described. Localization was studied following each one of four different injection routes. The antigen was described as "a suspension of purplish red particles which settled quickly on standing and were in aggregates large enough to be readily

visible." Detailed histological findings showed the involvement of both specific endothelial cells and free macrophages depending upon the injection route. After phagocytosis, the material was rendered soluble and passed into the cytoplasm where increased synthesis of globulin and modification into antibody globulin occurred. Marked reduction of the azoprotein in the cells was correlated with the appearance of serum antibody and with a marked shedding of surface films of macrophages. From these observations came a new definition of antigen, "a substance which specifically modified cytoplasm."

b. Soluble Antigens. As long as antigen is present in the vascular system, the volume of entrained blood within tissues must be evaluated for its contribution to tissue localization. This is a particularly important precaution with soluble antigens which may continue to circulate for a fairly long time after intravascular injection.

Tissue localization of toxins was assayed similarly to bacteria following injection into animals. One example is the detailed study by Wolff-Eisner (1908) involving immunization of various species (e.g., rabbits, chickens, and frogs) with tetanus toxin. The binding of toxin to various tissues was shown by the progressive steps of paralysis leading to death of recipient mice into which samples of minced tissue from the immunized donor were transferred.

Duran-Reynals (1939) studied the degree of localization of foreign sera injected intravenously in tissues of normal and tumor-bearing mice. In the assay by qualitative precipitation, some tissues gave either irregular results or spontaneous flocculation.

Gitlin (1950) made gross and microscopic examinations for the presence of dye in the tissues of mice at 1 hour after an intravenous injection of azoprotein. Tissue distribution of different azoproteins was modified by a variation in either the dye component or the protein.

Kruse and McMaster (1949) prepared brilliantly blue azoproteins and studied their behavior following a single injection into mice. Despite the soluble nature of these azoproteins, their final distribution was like that of intravenously injected, finely divided particulate matter, i.e., within RE cells in almost every organ of the body. Storage was within cytoplasm but not in the nuclei of cells. Color was equivalent to some sort of dye-to-protein-linked material, although serologically active material remained for only 2 days. These studies were continued by McMaster and Kruse (1951) with the demonstration that as antigen color disappeared from the blood it concurrently accumulated in the liver. Color was correlated with serological activity for 24-48 hours. Because of limitations in the sensitivity of serological detection, a biological test was developed which will be discussed later.

Fluorescein antibody labeling was used (Coons *et al.*, 1951) to study the fate of three soluble proteins in the following dose after intravenous injection into mice: egg albumin, 10 mg.; bovine plasma albumin, 10 mg.; and human plasma γ -globulin, 4 mg. Persistence was brief, 1, 2, and 6 days, respectively. Each of the three proteins was present in the nuclei of various cell types, often in higher concentration than in cytoplasm. [The question remains unsettled as to whether foreign material is in the nucleus (Schiller *et al.*, 1953; Coons, 1954).] The main limitations of the method are apparent in its use with soluble proteins, i.e., a fairly large local concentration is needed for detection and it is basically an antigen-antibody reaction requiring that the antigen be fairly intact for detection.

Haurowitz has continually used a chemical approach to the solution of immunological problems. This was never more true than in the investigations with arsanilate-azoprotein (Haurowitz and Breinl, 1932) which resulted in the first quantitative results of tissue localization. After intravenous injection into rabbits, the tissues were assayed at various times for their arsenic content. Within 6 hours the bulk of the antigen had been either transferred from the blood to the RE organs or had been excreted. At 10 days about 6% of the antigen still remained in the liver and 7% was present in the bone marrow. There was no attempt to determine the extent to which the antigen had been altered.

The use of radioactive isotopes has greatly facilitated the quantitative measurement of localized antigen. One of the first studies using a radioactive label to detect the *in vivo* distribution of antigen was made by Libby and Madison (1947). Mice were injected first intraperitoneally with nonradioactive TMV, then, 18 days later, intravenously with a test dose of TMV which had been biosynthetically labeled with P^{32} (i.e., the nucleic acid portion alone was labeled). The early distribution of radioactivity was mainly in the liver and spleen. The relative importance of these two tissues in this and other studies with soluble antigens (together with some quantitative data for insoluble antigen) is indicated in Table I. Due to prior immunization, the P^{32} TMV study did not represent an initial localization as did the other studies.

In the authors' laboratory quantitative data for antigen and also for circulating antibody have been obtained (Garvey and Campbell, 1954) and used to compare localization in normal and immune rabbits. Circulating antibody was measured in serum sampled just prior to a test injection of sulfanilate-azo-keyhole limpet hemocyanin (S^{35} KLH) into rabbits previously given multiple injections of the same nonradioactive antigen (SKLH). Normal control rabbits received only the test injection of S^{35} KLH. At 6 hours after the test injection of antigen, blood and other

TABLE I
LOCALIZATION OF ANTIGENS IN TISSUES OF NORMAL ANIMALS^a

Investigator	Antigen ^b	Animal	Time	% of dose in major sites			
				Liver	Spleen	Lung	Marrow
Ingraham, 1955 ^a	<i>Insoluble</i> S ³⁵ A-stromata	Rabbit Mouse	6-100 min.	73-87	~2	2-4	~1
Sullivan <i>et al.</i> , 1934	Bacteria	Rabbit	1 hr.	(Equal avg. concn. in spleen and liver) ^c			
Garvey, 1950	<i>Soluble</i> S ³⁵ AO	Rabbit	1 hr.	46	1.9	0.5	4.0
Francis and Hawkins, 1957	I ¹³¹ -iodoproteins	Rabbit	1 hr.	27	—	0.4	—
	S ³⁵ -sulfone proteins	Rabbit	1 hr.	50	—	1.4	—
Haurowitz and Breinl, 1932	AsA-serum	Rabbit	6 hr.	29	1.5	—	—
Garvey and Campbell, 1954	S ³⁵ KLH	Rabbit	6 hr.	20	0.3	0.2	—
Garvey and Campbell, 1957	S ³⁵ KLH	Rabbit	1 day	17	0.3	0.2	—
	S ³⁵ BSA	Rabbit	1 day	7	—	—	—
Ingraham, 1955 ^b	S ³⁵ A ₁ C	Mouse	1 day	14	0.4	—	—
Libby and Madison, 1947	P ³² TMV ^d	Mouse	1 day	63	3	—	—

^a From Campbell and Garvey, 1961.

^b Key to antigens: S³⁵A-stromata, sulfanilate-azo-sheep red-cell stromata; S³⁵AO, sulfanilate-azo-ovalbumin; AsA-serum, arsanilate-azo-serum; S³⁵KLH, sulfanilate-azo-keyhole limpet hemocyanin; S³⁵PBSA, sulfanilate-azo-bovine serum albumin; S³⁵A₁C, sulfanilate-azo-bovine γ -globulin; P³²TMV, P³²-labeled tobacco mosaic virus.

^c Per cent not available.

^d Prior sensitization as noted in text.

tissues were sampled and their content of radioactivity was measured. The radioactivity in blood samples was both extremely low and not significantly different in normal and immune animals. The liver and lung tissues of immune animals showed a greatly increased localization as compared with the same normal tissues. The increase in localization

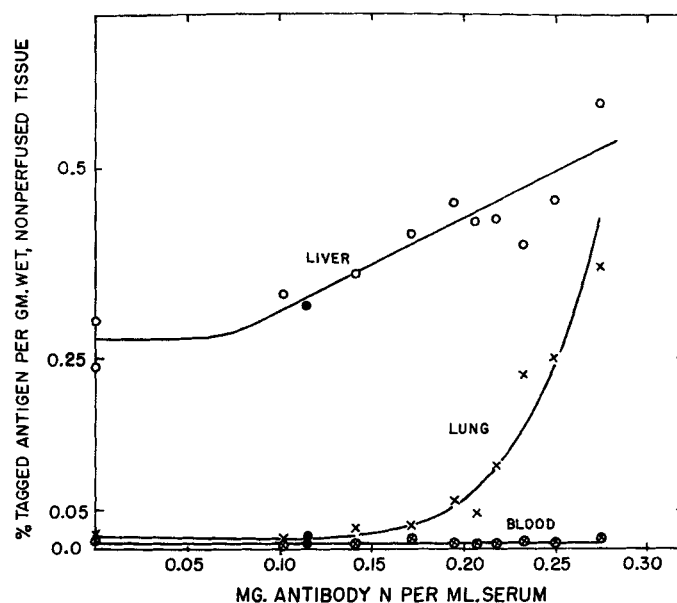


FIG. 3. Localization of antigen at 6 hours after injection of S^{35} KLH (sulfanilate-azo-keyhole limpet hemocyanin) into rabbits previously immunized with multiple injections of the nonlabeled KLH. Normal animals used as controls as well as the immunized test group were bled a few minutes prior to the injection of radioactive antigen for a determination of circulating antibody. The concentration of localized antigen in normals that had no antibody is a reference point for localized antigen in individual immune animals plotted with their corresponding circulating antibody titer. The radioactivity in the blood is at a low, barely detectable level for all animals, whereas the liver and lung tissues are not only very high but are the tissues in which localization is most affected by immunization. The solid circles represent data for a passively immunized animal. (From Garvey and Campbell, 1954.)

was found related to the amount of circulating antibody in the serum just prior to the test injection (Fig. 3). Perfusion with 1% saline removed radioactivity from the immune liver tissue to the extent that the remaining radioactivity was essentially that of normal tissue, but perfusion was less effective in reducing the radioactive content of the lung tissue (Fig. 4) which control experiments showed to be due to a hyper-

sensitivity reaction. Although the relative difference found in normal and immune liver at 6 hours was retained at 72 hours, it was no longer possible to perfuse radioactivity at the later time. Intraperitoneal injection of antigen as well as intravenous injection resulted in greater

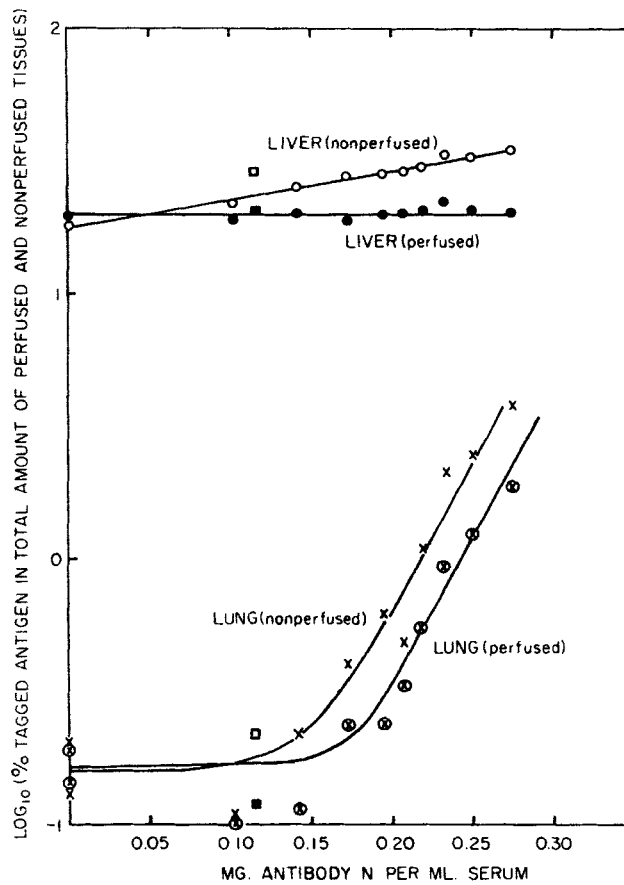


FIG. 4. A comparison of total antigen localized in nonperfused and perfused tissue from normal and immunized animals. Data for these curves were obtained from the same tissues as those shown in Fig. 3, including the passively immunized (represented by squares). (From Garvey and Campbell, 1954.)

localization in the immune than in the normal liver at 6 hours and the relative difference was still obvious at 72 hours.

Both antigen and antibody were isolated by dissociation of an insoluble complex recovered from the 6-hour perfusate of immune liver tissue. The actual isolation of antigen and antibody from the liver

perfusate is probably the first demonstration that antigen-antibody complexes form in the blood stream and are removed as insoluble complexes; likewise, results by Francis *et al.* (1957) with lightly labeled iodoproteins suggest the occurrence of an *in vitro* precipitin reaction and the quantitatively important role of the liver in removing antigen from the blood stream.

B. RETENTION OF ANTIGEN IN NORMAL AND IMMUNE ANIMALS

1. Evidence from Infectious Agents

In this section, consideration will be given particularly to the presence of antigen in the tissues after demonstration of an immune response.

There are many examples of long-lasting immunity to virus infection, e.g., smallpox, measles, and yellow fever, but an actual recovery of virus from tissues has not been demonstrated. In the absence of positive evidence Brill-Zinsser disease has been cited by Rivers and Horsfall (1959) as the best example of a balance between an immune mechanism and a persisting infectious agent. When the latter relationship is disturbed, recrudescent typhus occurs. Although Landsteiner (1945) would not exclude the persistence of active virus in such examples as the above, others, namely Burnet and Fenner (1950), invoked a complex memory mechanism to account for immunity of long duration.

An interesting example of persistence of an infectious agent during relative immunity occurred in a natural trypanosomal infection (Packchianian, 1934). In this instance, the blood from a mouse was microscopically negative as well as noninfective for rats but, despite these findings, a tissue suspension from the same mouse caused a rabbit to die of nagana infection. Trypanosomes were demonstrable in the rabbit blood, 2 drops of which produced fatal disease in a rat.

2. Evidence from Noninfectious Materials

Numerous investigations, just recounted in the previous section, have shown that not all the antigen injected is excreted but rather that very significant amounts may be localized in tissues, and predominantly in liver tissue. The fact that importance was given to the absence of data on persistence of antigen after the decline of antibody production (Burnet and Fenner, 1950) may have given impetus to studies to determine how long antigen persisted in experimental animals. However, it is just as likely that the availability of radioactive isotopes as well as the temperament of some investigators to experiment rather than to theorize led to an accumulation of considerable data related to the subject. It seems only logical that the investigators who had already

followed localization in tissues should also report on "retention," which is equivalent to prolonged localization, particularly at a time when circulating antibody has declined to low or nondetectable levels.

McMaster and Kruse (1951) were able to detect blue color microscopically for 85–120 days after injection of azoglobulin and for 36–44 days after injection of azoalbumin. (The tests to correlate antigenicity with the persisting colored material will be discussed later in terms of biological properties.)

Libby and Madison (1947) sacrificed mice at various times up to 1 month after a test injection of P^{32} TMV. The liver and spleen counts decreased rapidly from 1–12 days after injection, then slowly for the next 19 days. It was assumed that TMV was completely broken down with the initiation of a linear decrease of antibody and that the P^{32} activity in the tissues was not due to $Na_2HP^{32}O_4$, which when injected had very low distribution in the tissues. The increase in carcass radioactivity with increasing time was probably due to such a breakdown product from the labeled TMV.

Haurowitz and Crampton (1952) obtained data on the fate of an I^{131} iodoalbumin prepared with 8% iodine. The fact that the radioactivity was precipitated by trichloroacetic acid may remove some uncertainty that the I^{131} in the tissues remains combined with tyrosine groups of the antigen. Measurements at 29 days after the intravenous injection indicated that the molecular level of antigen persistence was considerable, particularly in the liver, where 2000 antigen molecules were calculated per liver cell at 29 days after injection of 86 mg. of iodoalbumin.

Ingraham (1951a, b) prepared S^{35} -sulfanilic acid-azo-bovine γ -globulin ($S^{35}A\gamma G$) for injection into mice which had been previously immunized with nonradioactive sulfanilic acid-azo-bovine γ -globulin ($SA\gamma G$). The radioactive antigen was injected similarly into normal control mice. Studies of the distribution of S^{35} in the various organs and excretions at intervals from 0.5 to 200 days following intravenous injection indicated that the retention of antigen in most of the normal tissues was either approximately the same or higher than in the immune animal; exceptions were the lymph nodes and possibly the lungs, where the reverse was true. The greatest concentration of radioactivity occurred in the liver and spleen, where 1 and 4% of the S^{35} present in the liver and spleen, respectively, at 24 hours after injection remained at 200 days. Injections of either S^{35} -sulfanilic acid or S^{35} -sulfate were rapidly eliminated, thus these could not account for gross tissue retention of S^{35} from injected azoprotein.

The retention of antigen has been under investigation in the authors' laboratory for the past 10 years. Two antigens have been used for intravenous injection into rabbits, sulfanilate-azo-keyhole limpet hemocyanin (SKLH) and sulfanilate-azo-bovine serum albumin (SBSA). The techniques of preparing the antigens with S^{35} -sulfanilate and measuring

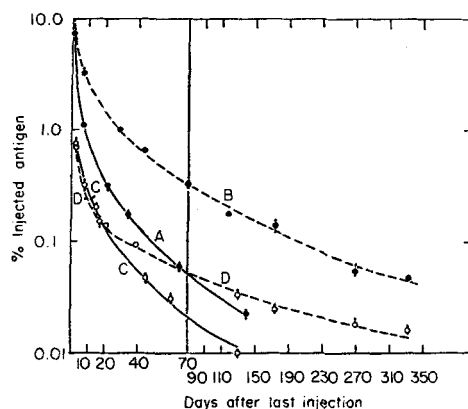


FIG. 5. Semilog plot of the retention in perfused rabbit liver tissue. Curve A, a single injection of 50 mg. of S^{35} BSA; Curve B, a single injection of 50 mg. of S^{35} KLH; Curve C, multiple injections, i.e., 9 injections of 10 mg. each of S^{35} BSA; and Curve D, multiple injections, i.e., 9 injections of 10 mg. each of S^{35} KLH. The center of the circles indicates the mean of the distribution represented by the arrows. (From Garvey and Campbell, 1957.)

radioactivity in tissues were described initially by Garvey (1950). Both are soluble antigens and, as indicated earlier, localization occurs predominantly in the liver. The aim of the research, i.e., isolation and characterization of retained antigen, placed a limit on what might be considered as significant retention. Although the specific radioactivity of spleen tissue was sometimes comparable to that of the liver, the total amount of antigen available for isolation was at least fifty times greater in the liver. Because of the high level of retention in the liver, it seemed the ideal tissue to study for technical reasons and there is no evidence that it is not representative of retention occurring elsewhere in the body.

Separate curves for antigen retention following single or multiple injection of either of the two antigens are given in Fig. 5. It is obvious from these curves that smaller amounts are retained as a result of multiple rather than single injections. The retention in terms of molecules is still impressive (Campbell and Garvey, 1958) when experimental curves are extrapolated to 3 years after injection, e.g., about 200 molecules of S^{35} BSA or 2000 molecules of S^{35} KLH per liver cell.

Results for retained S^{35} KLH and circulating antibody had indicated a rough correlation for several weeks between the amount of antigen remaining in the liver and the amount of circulating antibody (Garvey and Campbell, 1956). More recently passive hemagglutination has been used to detect longer persistence of circulating antibody (Richter and

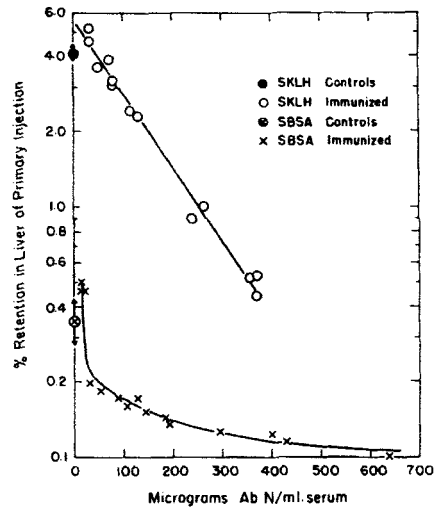


FIG. 6. A semilog plot of retention of a primary injection of antigen versus circulating antibody titer. The retention is from a primary injection of either S^{35} -labeled keyhole limpet hemocyanin (S^{35} KLH) or bovine serum albumin (S^{35} BSA) when followed by eight injections of the same but nonradioactive antigen. For controls, the center of a circle indicates the mean of the distribution which is shown by arrows. Each of the other points is for a single, immunized animal. All data were obtained at 3 days after the last injection in a series of eight nonradioactive injections. (From Garvey and Campbell, 1958a).

Haurowitz, 1960; Garvey, 1960) than had been possible by the technique of quantitative precipitation. This suggests that not only does antigen persist as long as circulating antibody can be detected but that the reverse may occur also, i.e., as long as antigen persists, antibody may be circulating, although the concentration may be so low that very sensitive methods are needed for detection.

Antigen remaining in the liver after multiple injections was less than after a single injection, both in terms of percentage as well as of the absolute quantity of the injected antigen. This finding suggested either of two possibilities: (1) that multiple injections stimulated a more rapid loss of the primary injection or (2) that subsequent injections of antigen were more rapidly destroyed. When an initial injection of

radioactive antigen was followed by injections of the same nonradioactive antigen, the loss of the initial injection was correlated inversely with the quantity of circulating antibody (Fig. 6); furthermore, the number of secondary injections was of no consequence (Garvey and Campbell, 1958a). This investigation solved the problem in favor of the first possibility but the following question still required an answer: Would antibody per se cause loss of antigen from the liver? With regard to this question, antibody, when passively administered to previously immunized animals, was found to be totally ineffective either in causing any loss of antigen from the liver or in enhancing the immune response in normal animals (Garvey and Campbell, 1958a).

On the basis of these results on antigen retention in the liver, it is obvious that antigen is retained during antibody production and seemingly for a very long period of time. Furthermore, it is obvious from such curves as in Fig. 6 that the specific loss of antigen from liver tissue is at least a consequence of antibody production. It is not possible from present data to conclude any causal relationship.

Johnson *et al.* (1955) obtained data on antigen elimination from the circulation and antibody response. Although their suggestion comes from indirect evidence, it is nonetheless interesting "that breakdown and/or removal of antigen from an antibody-forming site may be a necessary prerequisite of antibody formation."

C. RETENTION OF ANTIGEN IN UNRESPONSIVE ANIMALS

The term "unresponsive" is probably the most descriptive of all the terms that have been used for various forms of reduced immunological response. Unresponsive may be used as a general term provided certain differences are not overlooked in comparing forms of unresponsiveness known as "immunological paralysis," "immunological tolerance," and "acquired tolerance." Major differences in observations of unresponsiveness may be attributed to (1) natural, versus artificial, antigenic stimulation, (2) age (i.e., adult versus neonatal animals), and (3) nature of antigen (i.e., protein versus polysaccharide; living versus nonliving). Investigations such as the following illustrate these differences: Antigenic stimulation occurred naturally in the chimera observed by Owen (1945), rather than being introduced experimentally (e.g., Billingham *et al.*, 1953), although the neonatal period and living cells were common to both investigations. Other investigators (e.g., Hanan and Oyama, 1954) used nonliving antigens rather than living cells for injection during the neonatal period of life. The use of adult animals broadened the parameter of age and the nature of the antigen added still another variable, being

protein (e.g., Dixon and Maurer, 1955) or polysaccharide (Felton and Ottinger, 1942). A form of tolerance appears to be common to immunized adult animals in which high levels of antibody production have been observed. Antigen continues to persist at significant concentration in the tissues, but at the same time there is a failure to detect measurable quantities of antibody (Garvey and Campbell, 1956).

Unresponsiveness is a negative entity, which is recognized by an absence of delayed type hypersensitivity and an absence of serum antibody. Since antigen is a positive entity, and is furthermore the most common factor in all forms of unresponsiveness, it appears worthwhile to direct more attention to its role. With this in mind the following references on unresponsiveness were chosen because of some particular emphasis given to the matter of antigen. The limited bibliography does not attempt to cover the general subject as reviewed by Owen (1957), Brent (1958), Chase (1959), and Smith (1961), or as presented currently by conferees at meetings such as those on tissue homotransplantation sponsored by the New York Academy of Sciences.

1. *Polysaccharides*

Felton and Ottinger (1942) recognized as unusual the unresponsiveness or paralysis produced in mice by 500 μ g. of pneumococcus soluble specific substance, since no protective antibodies were produced and the animals succumbed to standard challenging doses of the organisms. Prior to the labeling of proteins with radioactivity this condition provided the best example of antigen retention. But this type of observation failed to qualify under the criterion that we established previously for retained antigen, i.e., a demonstrable antibody response. The state of unresponsiveness with polysaccharides has been studied extensively but the information should not be used for a generalization about unresponsiveness since the behavior of polysaccharides may be different from that of proteins.

It was previously observed that if enzymatic hydrolysis leads to a very rapid rate of antigen removal, this may not be conducive to antibody formation. The condition seems actually opposite in mice given a paralyzing dose of polysaccharide. One month after injection of polysaccharide, tissue extracts were prepared and used as a source of antigen in precipitin tests with antibody. From end-point analysis of precipitates, antigen was found in significant concentration in tissues (Felton, 1949). The distribution was more general than for protein antigens, with concentrations being relatively high, not only in the RE tissues but also in blood, muscle, stomach, intestine, and skin after intraperitoneal

injection. One year after injection of 0.5-mg. C¹⁴-labeled polysaccharide, Stark (1955) found radioactivity unaltered in the spleen but antigenic C¹⁴ was reduced to 1.5% of that present 2 days after injection. Polysaccharide-containing fractions isolated from the tissues of paralyzed mice were found to be effective immunizing agents against virulent pneumococcus infection (Felton *et al.*, 1955).

Felton (1949) postulated a blocking effect by the overdose of antigen so that the RE cells were no longer able to synthesize antibody to the specific pneumococcus type, and results obtained by immunofluorescence (Coons and Sercarz, 1959) tend to agree with this suggestion. Other investigations suggest an occurrence of antibody production, followed by neutralization (Kaplan *et al.*, 1950) or destruction at sites of antigen retention (Dixon *et al.*, 1955; Stark, 1955). The studies by Stark (1959) measured the loss of antigenicity in spleen extracts as influenced by a change in the *in vivo* antibody concentration. Either the use of X-rays or an injection of specific antibody caused a change in the rate of loss of antigenicity of test tissues containing the polysaccharide, a mouse protection test being used for detection. The fact that the quantity of retained tissue antigen can be altered in such experiments presents a difference from the results for protein antigen retained in liver tissue, the concentration of which could not be altered by injections of antibody (Carvey and Campbell, 1958a). One possibility to account for the difference is that the polysaccharide is retained extracellularly—an idea which is not contradicted by the fairly high concentration of SSS in the blood. As will be indicated later, retained protein antigens are fragmented. Unlike the polysaccharide antigens, which when isolated from tissues precipitate specific antibody, the retained protein fragments only show specific coprecipitation or inhibition.

There has been no direct evidence that antibody is produced during paralysis; on the contrary, the many results by various techniques indicate that the polysaccharide is persisting in a rather intact state [e.g., persistence for at least 6 months detected by fluorescein antibody labeling (Kaplan *et al.*, 1950; Hill *et al.*, 1950); haptenic properties of extracted C¹⁴-polysaccharide material demonstrated by precipitation (Jones and Howell, 1960; Jones and Carter, 1957); relative C¹⁴ content of constitutive monosaccharides in polysaccharide extracted from tissues found similar to original material (Jones *et al.*, 1962)]. Some *in vivo* changes had occurred since the polysaccharide had greater affinity for nucleic acid *in vivo* than *in vitro* (Jones *et al.*, 1962), and there was a stable association with some nitrogenous moiety (Felton *et al.*, 1955).

2. *Living Cells*

In the natural chimera, two sets of cells, one containing isoantigens that were initially foreign to the host, colonize the host tissues. Since these cells are capable of reproduction, a full complement of antigens persists. This type of chimerism may continue for the lifetime of the individual; examples are the cattle twins in Owen's (1945) original description of the phenomenon and the findings of Dunsford *et al.* (1953) in human twins, Stormont *et al.* (1953) in twin lambs, and Billingham *et al.* (1956) in twin chickens. Not only a continual supply of antigen but a high initial supply is necessary for the establishment of immunological tolerance as shown recently with a tumor virus (Rubin, 1962).

The procedure of injecting cells during early life in order to render a state of unresponsiveness attempts to duplicate the natural chimera. As a result of such work Billingham *et al.* (1953) commented "that at least some cells of the foetal inoculum survived as long as the tolerant state which they were responsible for creating." The idea that unresponsiveness depends upon persisting antigen has never been denied but too little attention has been given to the matter. However, in a study with adult CBA/Jax as recipient mice and A/Jax splenic donor cells, there was demonstrated a consistent proportionality in the skin homograft survival time and the preimmunizing dose of cells (Hildemann *et al.*, 1960).

3. *Nonliving Cells and Soluble Defined Antigens*

The state of tolerance, acquired by injections of foreign fowl erythrocytes into fowls, is normally transient but can be prolonged by repeated doses of erythrocytes (Mitchison, 1962a; Hašek, 1962). In such studies elimination of Cr⁵¹-labeled erythrocytes provided an indication of tolerance just as sensitive as skin homografts (Mitchison, 1962a). Whereas the state of tolerance could be maintained by readministration of erythrocytes, it could also be abolished by injection of antibody which eliminated the cells (Mitchison, 1962b).

That the duration of unresponsiveness is finite and is related to the amount of antigen given at birth (Table II), but that unresponsiveness may be indefinitely prolonged by repeated injections of antigen, were the conclusions of Smith and Bridges (1958). Similar studies indicated that the degree of responsiveness in chickens was dependent upon dosage at hatching (Wolfe *et al.*, 1957). The studies by Terres and Hughes (1959) with mice injected at birth with BSA demonstrated in still another species the dependence of the tolerant state on the amount of antigen given at birth. Tolerance in this species was described as a

TABLE II
INFLUENCE OF A SINGLE NEONATAL INJECTION OF BSA UPON THE IMMUNE
RESPONSE TO BSA IN RABBITS BETWEEN 87 AND 120 DAYS OF AGE

Amount BSA injected during neonatal period ^a (mg.)	Number in group	Number with detectable BSA in serum within given interval after second immunizing injection of BSA			Number with de- tectable anti-BSA in serum
		7-11	14-18	21-34	
100 at birth	24	22	22	6	2
50 at birth	12	11	11	1	0
20 at birth	12	11	9	5	1
10 at birth	14	13	9	5	3
1 at birth	7	5	1	0	6
0.8 at birth	4	1	0	0	3
0.1 at birth	3	0	0	0	3
0.01 at birth	2	0	0	0	2
0.001 at birth	4	0	0	0	4
100 at day 3	47	44	41	0	3
100 at day 9	4	3	2	2	1
100 at day 15	16	6	4	2	7
100 at day 17	16	4	4	0	12
None	18	3	1	0	16

^a Litters of rabbits were given the indicated amounts of BSA intraperitoneally on the designated day of life. After 87 to 120 days the survivors were challenged with two intravenous injections of BSA, 10 days apart, and bled weekly or more often thereafter until antigen had disappeared or antibody had appeared in the serum. (From Smith and Bridges, 1958).

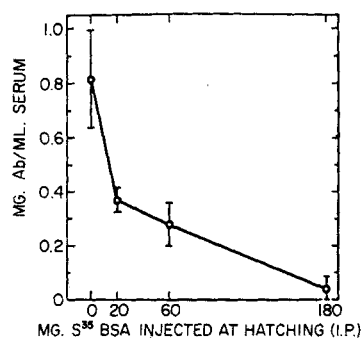


FIG. 7. The relationship between the amount of S³⁵BSA (bovine serum albumin) injected into chicks at hatching and the antibody response elicited by intravenous injection of BSA (40 mg./kg. body weight) 45 days after hatching. Vertical bars represent ± 1.0 standard error. (From Hirata *et al.*, 1960.)

lack of anaphylactic response and the failure of the serum to bind antigen in the Farr test.

In studies with labeled BSA (i.e., S^{35} BSA) injected into chicks at hatching, an inverse relationship of antigen dosage and milligrams of precipitating antibody was established (Fig. 7) and the role of retained antigen was discussed (Hirata *et al.*, 1960). When the same kind of labeled antigen was injected into neonatal rabbits (Garvey *et al.*, 1960), organ distribution of antigen was not only widespread but of considerable magnitude. It was apparent that further study might elucidate a subtle difference in the association of antigen with cellular components of neonatal, as compared with adult, tissues. Also, qualitative differences in antigen may occur as a result of neonatal rather than adult metabolism, but such studies have not been reported.

III. Characterization of Retained Antigen

A. PHYSICAL PROPERTIES

The physical properties of materials isolated from tissues often depend upon the methods used for extraction and purification. Hence, variations and discrepancies found by different laboratories may often result from some form of denaturation or complexing of antigen material with other cell constituents during *in vitro* preparation. It is important, therefore, that one take this into account before making final conclusions as to the nature of antigen retained intracellularly and its association with cytoplasmic or nuclear constituents as determined by *in vitro* physical methods. Thus, precipitation with organic solvents, such as alcohol or other reagents (e.g., trichloroacetic acid), might be expected to change the nature of many materials under consideration. For example, in our early studies we recovered as a soluble fraction, by emulsification with a Waring blender, about 50% of the total S^{35} -antigen material present in the liver. When the method was modified by freezing liver tissue and subsequently disrupting cells under high (20,000 lb.) pressure, almost 100% was extracted as a sucrose-soluble fraction. Traces of enzymes such as proteases or ribonuclease may also modify certain preparations upon storage.

It is now apparent that immunogenic materials rapidly lose much of their native molecular structure when ingested by cells. One of the first to demonstrate a rapid degradation of antigen was Erickson *et al.* (1953). Using TMV as an antigen, they found by electron microscopy that there was a persistence of rodlike structures in the liver which diminished with time after injection. Biological evidence, as noted in

the following, showed that qualitative changes occurred as rapidly as the quantitative change in structures. From our own investigations it soon became obvious that although antigen material persisted, it rapidly changed, as tested with specific antiserum, from a precipitating form to a nonprecipitating form. This latter form behaved as a specific inhibiting hapten in a precipitating system consisting of native unlabeled protein antigen and rabbit antiserum. Further studies, described later, also gave more direct evidence for the persistence of antigen in a degraded form. Downs *et al.* (1955) also found materials from tissues of typhus-infected mice that specifically inhibited serological reactions between typhus and specific antiserum.

An important practical problem regarding the physical property of retained antigen material is whether the label used to detect antigen remains associated with the original antigen or with some fragment of the original antigen. Labels might be dissociated from the original antigen and still persist in combination with some normal cell constituent; this would be particularly true of I^{131} , H^3 , N^{15} , P^{32} , and C^{14} under some conditions. Haurowitz and Walter (1955) isolated an S^{35} -labeled fraction from rabbit liver, which upon chromatography behaved like an azo dye prepared from tyrosine and diazotized sulfanilic acid. Saha *et al.* (1962) isolated a similarly degraded S^{35} fraction from the livers of rabbits immunized with S^{35} -sulfanilate bovine serum albumin. The composition of a labeled component obtained after hydrolysis of the original sucrose-soluble fraction was S^{35} -sulfanilate-azohistidyl-nucleotide. Since this component contained only a small portion of the total S^{35} in the sucrose-soluble liver fraction, one might expect that further analysis of this soluble fraction would yield a tyrosyl nucleotide. Figure 8 presents a spectrophotometric and radioactivity analysis of the sucrose-soluble antigen fraction obtained from liver tissue at 15 days after injection of S^{35} BSA. This fraction was isolated on ECTEOLA-cellulose and represented about 90% of the total radioactivity of the liver. Ingraham (1951a, b) isolated a soluble S^{35} fraction from rabbit livers after injection of S^{35} -sulfanilate bovine γ -globulin and found that it was associated with a nondialyzable component as long as 17 days after injection of the labeled antigen. Ingraham (1955a) also found, as Garvey (1950) had previously, that injection of S^{35} , diazotized and coupled to various amino acids, was rapidly excreted and not retained in the liver tissue. It would appear, therefore, that the sulfanilate group attached to a single amino acid is not retained. Indirect evidence that the S^{35} label was associated with the original antigen was given by the studies of Garvey and Campbell (1956) when S^{35} -labeled material isolated from rabbit livers a

few days after injection gave a specific precipitin reaction for the presence of antigen when tested with nonlabeled antisera. S^{35} fractions isolated after several weeks gave specific inhibition in precipitin reactions between native antigen and antisera. (Perhaps the best evidence for the association of the label with the native antigen is shown more

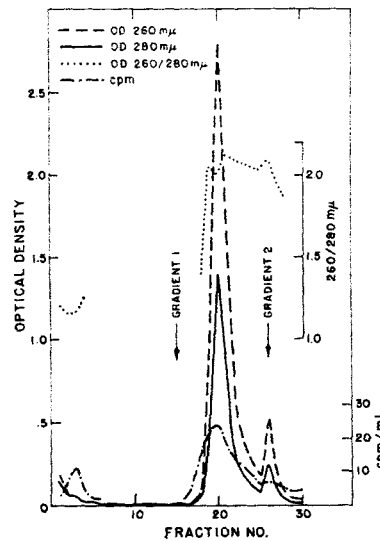


FIG. 8. Chromatography of the most radioactive fraction (about 95% of the total activity) isolated from rabbit liver 15 days after an injection of 20 mg. of S^{35} -sulfanilate-azo-BSA. (Data obtained by Dr. Anil Saha *et al.*, 1962.)

directly in the following section on biological properties of intracellularly retained antigen.)

It seems apparent at present that most of the retained antigen material is bound to intracellular constituents. The first studies to indicate this were reported by Haurowitz and Crampton (1952), Crampton and Haurowitz (1952), who found that much of an I^{131} label was associated with microsomes and mitochondria. The early studies by Garvey and Campbell (1956, 1957), Campbell and Garvey (1961), also indicated that the retained antigen material was associated with cellular constituents of liver parenchymal cells. For example, serological precipitates using retained antigen from livers of immunized animals always contained a large amount of RNA. One S^{35} -containing fraction, isolated 6 weeks after immunization, contained a high molecular weight protein which had specific antibody properties. It therefore became apparent that most retained antigen was complexed and associated with RNA and also to some extent with newly formed antibody.

Molecular weight studies of retained antigen have not been completed. Our early report (Garvey and Campbell, 1957) of sedimentation studies showed an $s_{20,w}$ value of around 4. Later work showed that this was a false value for antigen material. In spite of the high S^{35} activity, the major contributing component to the $s_{20,w}$ value of 4 was SRNA. The antigen component was too small to contribute much. Recent sedimentation values (Saha, 1962, unpublished), obtained by means of density gradient techniques, have shown differences of about $s_{20,w}$ 0.2 between SRNA-Ag ($s_{20,w}$ 3.4) and SRNA ($s_{20,w}$ 3.2) from which Ag had been removed by alkali treatment. This would indicate that antigen fragments persisting from injection of BSA have an average molecular weight of around 500. This may be a very significant figure since it would indicate a dimension about the size of an antibody-combining site.

Free-boundary electrophoresis studies of various soluble S^{35} fractions from livers of immunized rabbits have shown the antigen fragment persisting as a complex with normal cytoplasmic constituents and perhaps to some extent with newly formed antibody. Although such studies gave information as to the cellular component, they indicated very little information about the antigen fragment itself. In general, material from S^{35} BSA injections had mobilities ($\times 10^{-5}$ cm.² volt⁻¹ sec.⁻¹) of -9 to -10 , which was the value obtained for the original S^{35} BSA at early stages of immunization (3-4 days). These values increased to a range of -13 to -15 after several weeks from the beginning of immunization. Nucleoprotein from normal livers had a value of -12 to -15 . Probably the most important aspect of these studies was the indication that the nature of retained antigen and its association with cell constituents changes with time. Changes in physical properties and perhaps transfer from one cell type to another might explain the heterogeneity of antibody and the change in the nature of antibody with respect to time after antigen stimulus which has been observed by many investigators.

B. BIOLOGICAL PROPERTIES

The biological characterization of retained antigen is of fundamental importance since such data will provide a basis for the correlation of retained antigen with immune reactions. When a radioactive or other type of label is used to provide a sensitive method to detect retained antigen material, it is necessary first to determine whether the label is still associated with the native protein which was injected. Although the evidence presented in the foregoing gave good indirect evidence that S^{35} -sulfanilate labels remained attached to a portion of the injected labeled antigen, more direct evidence was provided by biological

methods. Such a method evolved from the finding (Fig. 6) that a primary injection of S^{35} -labeled antigen was lost much faster, based on S^{35} assay, from rabbit liver when subsequent secondary immunizing injections of the same, but nonlabeled, antigen were given. It was apparent that a test of whether the S^{35} was still attached to the protein carrier would be a study of the effect of injections of native protein on the retention of S^{35} deposited initially as S^{35} -azoprotein. The experiment as reported by Garvey and Campbell (1958b) was carried out by injecting intravenously one group of eighteen normal rabbits with S^{35} -sulfanilate-azo-bovine serum albumin (S^{35} BSA) and another group of eighteen normal rabbits with S^{35} -sulfanilate-azo-keyhole limpet hemocyanin (S^{35} KLH). Subsequently, a third of the animals in each of the two groups received no further injections and served as a base line for S^{35} retention. Beginning 2 days after the primary injection of S^{35} -azoprotein, another third of each group received 8 intravenous injections of the heterologous native protein on alternate days, and the remaining animals in each group received similarly 8 intravenous injections of the homologous native protein. Twenty-one days after the initial injection with S^{35} -azoprotein, all animals were sacrificed and their livers perfused and analyzed for S^{35} . The results, shown in Table III, are the range of values for retained S^{35} , with the mean averages given in parentheses.

TABLE III
PER CENT RETENTION OF S^{35} FOLLOWING IMMUNIZATION WITH HOMOLOGOUS
OR HETEROLOGOUS NATIVE PROTEIN^a

Antigen of secondary injections	Antigen of primary injection	
	S^{35} KLH	S^{35} BSA
None	0.780-0.740(0.762)	0.405-0.305(0.357)
Cross-reacting native protein	0.586-0.440(0.513)	0.263-0.122(0.182)
Noncross-reacting native protein	0.790-0.765(0.777)	0.413-0.360(0.373)

^a From Garvey and Campbell, 1958b.

These results showed conclusively that the amount of S^{35} label resulting from a primary injection of sulfanilate-azoprotein was reduced by subsequent injections of homologous unlabeled protein, which was evidence that the S^{35} was intimately associated with the original protein carrier. The loss of S^{35} was a specific effect of immunization, since secondary injections with a nonrelated antigen caused negligible change in the retention of heterologous antigen. Furthermore, the larger range of values found with the cross-reacting native protein than with the other values used for comparison was due to a spectrum of antibody titers to the native protein, the lower values of retention corresponding to higher

antibody titers. This effect of antibody production on retention has been studied (see Fig. 6) in Section II, B.

Another question with regard to the retained antigen material is whether or not it continues to function as immunogenic material. Direct proof is difficult to obtain, but the evidence, obtained by the sensitive technique of hemagglutination, shows that antibody continues to circulate at low levels (and presumably is being formed) for at least a considerable period of the time during which antigen material is present in detectable amounts in liver tissue (Garvey, 1960). Similar findings were reported by Richter and Haurowitz (1960).

Further evidence that retained antigen material has an immunogenic capacity was obtained with an isolated fraction of liver tissue studied by *in vitro* anaphylaxis. Differential centrifugation and partial purification by adsorption on a Dowex column with elution by 50% Na-salicylate was used to obtain the fraction. Physical properties such as S^{35} and RNA content were closely associated with positive results obtained by the Schultz-Dale reaction. The assay was carried out, as described by Garvey and Campbell (1957), by injecting guinea pigs intraperitoneally with the liver fraction, sacrificing the animals after 3 weeks, and using pieces of ileum tissue to test for active sensitization to the original antigen, S^{35} BSA, which had been used to immunize the rabbits from which the liver tissue had been obtained and fractionated. Parallel tests in which guinea pigs were sensitized with S^{35} BSA showed that the antigen material obtained from liver was 100–200 \times more effective in sensitization (based on S^{35} specific activity) than the original antigen. The interval of time which had elapsed between the sensitizing injection and the *in vitro* test was evidence against passive transfer of antibody in the liver fraction injected into the guinea pigs. However, the contributing role of each component (i.e., antigen fragment and nucleotide) in the immunogenic complex remains to be determined. Preliminary experiments by Garvey (1962) indicate that when separated, neither the SRNA nor the antigen fragment is immunogenic when injected intravenously.

Other investigations, directed specifically toward a biological characterization of retained antigen, have indeed been rare; however, McMaster and Kruse (1951) contributed significantly to the problem of relating a dye group to persistence of a native protein by their development and use of a biological assay for an azoprotein. This test was performed by injecting minced tissue from a mouse that had received an injection of azoprotein of an intensely blue color into a normal recipient mouse. Two days later, when the recipient mouse was injected

intravenously with specific antiserum against the native protein, an anaphylactic reaction was apparent microscopically as an ear vascular response. Positive tests were obtained only when hepatic and lymphatic tissue containing blue color (visible with the unaided eye or with a low-power microscope objective) was used as transfer material. Such tests provided strong evidence that the colored material resembled the native protein from which the azoprotein had been prepared and that such material persisted in an antigenic form for at least 14 weeks.

In further work McMaster *et al.* (1954) used the mouse transfer test to determine whether bovine γ -globulin persisted in the liver of immunized rabbits as well as in mouse liver. Using minced liver tissue, they found persistence for 8 weeks. However, when liver tissue from immunized rabbits was homogenized and only the soluble fraction tested, no antigen activity was detected in sensitized mice. It was suggested that perhaps a soluble antigen-antibody complex existed which dissociated when injected into mice. The difference in length of time during which positive results were found in the mouse and rabbit is of interest in relation to known species differences in the formation of circulating antibody. The rabbit, which forms circulating antibody better than the mouse, lost antigen more rapidly than the mouse, a finding which our own results might predict (refer to Fig. 6).

In another investigation, McMaster *et al.* (1955) used active sensitization as a biological test for the presence of immunogenic antigen material persisting in the livers of injected mice or rabbits. It was found that normal mice could be actively sensitized by transfer of liver material from animals as long as 2-3 weeks after injection of bovine γ -globulin when challenged with normal bovine γ -globulin. However, no precipitating antibodies were detected. In view of the fact that mice are poor producers of antibody, an investigation was performed by McMaster and Edwards (1957) in which rabbits were used as recipients of transferred liver from immunized rabbits. The recipient rabbits which had been sensitized previously to bovine γ -globulin later received the liver tissue from rabbits previously injected with the same antigen. It was found that enough antigen (bovine γ -globulin) was present in 60 gm. of liver tissue from the donor rabbit, sacrificed at 2 weeks following the injection of antigen, to induce an anamnestic formation of detectable precipitin in the recipient rabbit. However, similar amounts of liver taken from donors at 21-28 days after injection failed to give a similar anamnestic response. The investigators suggested that, in view of their previous demonstration of anaphylaxis with antigen persisting for longer intervals of time, perhaps "antigen as it is de-

stroyed, can pass through stages of degradation in which it loses its capacity to engender precipitating (complete) antibodies in recipient animals while it can still sensitize them anaphylactically."

The results of Erickson *et al.* (1953) with a liver extract obtained from mice injected with TMV also showed a variation in length of time during which positive results could be obtained by different biological tests. At least as long as 5 days after injection of the virus, tissue could be extracted and used to demonstrate antibody formation in rabbits, but infectivity for tobacco plants was lost after 3 hours, the latter assay demonstrating the more subtle biological property of the virus. Structures that resembled the virus particles were observed by electron-microscopy to persist in the liver for 15 days following intravenous injection. It was this morphological evidence for persisting antigen which led the investigators to study biological properties of a liver extract.

The preceding studies of biological activity are directly related to positive findings of antigen material persisting in the tissues of immunized animals. There are, on the other hand, many reactions dealing with the passive transfer of an immune mechanism without evidence of antibody or antigen which have given little or no consideration to the possibility of retained antigen in a modified form or in combination with an important factor involved in biosynthesis of protein. The possible persistence of antigen should be considered with regard to an immune response following either the transfer of cells or of cellular fractions from immunized donors to normal recipients. The problem of cellular transfer of antibody formation was the subject of a symposium several years ago (Chase and Wager, 1957; Harris and Harris, 1957; Dixon *et al.*, 1957; Stavitsky, 1957). Although the question of antigen was mentioned, its importance was considered negligible in view of the evidence available at that time. In studies of agglutinin formation in recipients which had received lymph node cells from immunized donors, Harris and Harris (1957) found that various injurious treatments, which affected the viability of the cells, both reduced and delayed the immune response in the recipients. The donor cells were subjected to such treatment as freezing and thawing several times, irradiation, or incubation at 37°C. for 24 hours. Although the investigators considered only the effect on cell viability, chemical changes such as those caused by enzymatic activity would seriously alter any persisting antigen. Ribonuclease activity, as a specific example, might alter the association of RNA-antigen fragments. In another biological system, in which *in vitro* synthesis of antibody was observed (Fishman, 1961),

ribonuclease was an inhibitor, and this finding led to the speculation of an active role for an antigen-RNA complex. Likewise, the role of antigen was not excluded in the positive findings of antibody formation recently reported by Vredevoe and Nelson (1963); liver tissue from sensitized donors was transferred in Millipore chambers to recipients, which were subsequently challenged with the original antigen. Both of these recent findings have merely predicted the active role of some form of antigen, but they indicate a renewed interest in the problem of antigen retention in all transfer studies.

The use of cell fractions for transfer of antibody formation from immunized donors to normal recipients, particularly the studies of Sterzl (1956) and Friedman (1959), emphasized the nucleoprotein nature of active fractions. In both investigations the possible presence of antigen material and the role of antigen failed to be considered principally because no antigen could be detected. Franzl (1962) recently succeeded in eliciting hemolysin formation by injecting cell fractions from animals which had received sheep red cells, particularly with a lysosome fraction from spleen, and attributed the active transfer to "activated ingested sheep red cell antigen." As regards the "transfer factor" (Lawrence, 1958), an active role for antigen has been suggested (Powell *et al.*, 1962) and the form of this appears to be associated with RNA since it is inhibited by ribonuclease. Again it should be emphasized that the direct relationship of antigen and biological activity lacks a positive detection of persisting antigen material, but the time seems to have arrived when greater attention will be given to this problem.

IV. Retained Antigen and Antibody Formation

Although the foregoing data fail to establish definitive answers about antibody formation and other immunological reactions, they do provide provocative information for reasonable speculation regarding such phenomena. Many of the answers must wait until more is known about the biosynthesis of proteins and how intracellular cathepsins handle foreign proteins and the intriguing problem of specific replication. In the meantime, one can only propose reasonable hypotheses and assumptions concerning the reactions that may be going on at a molecular and cellular level. Some of these speculations, which have been presented in previous publications (Campbell, 1957; Campbell and Garvey, 1958, 1960, 1961), will be reviewed briefly later. It will be seen that the speculations are chiefly concerned with the possible significance of the partial breakdown and retention of foreign materials such as proteins. It is assumed a priori that the presence of such ma-

terial is necessary for any immune reaction and that any proposition can be tested experimentally by chemical or biological methods.

Many factors may be involved in the immunogenicity of any given substance. Thus, most texts on immunology discuss molecular weight, rate of elimination, chemical composition, and the importance of aromatic amino acids, sulfur-containing amino acids, and perhaps carbohydrate groups. Exceptions will be found to most of these criteria except for molecular weight; there seems to be a minimal molecular weight requirement of about 5000. Small molecular weight materials (e.g., picryl chloride and dinitrophenol) which will react with tissue constituents modify normal constituents so that antibodies will be formed against the chemically reactive small molecular weight material.

The relationship of molecular weight to antigenicity has been discussed by Boyd (1956) who feels that there is a direct relationship. Molecular size may be one of many important factors which involve localization and retention, rate of excretion, and phagocytosis. However, with the newer knowledge which has accumulated during the past 10 years it seems reasonable to consider that partial breakdown of large molecular weight material is an important factor in antibody formation and that the persistence of antigen fragments plays a role in many types of immune reactions. Thus, nondigestibility would explain the lack of antibody formation against many synthetic polymers, colloidal dyes, or gum acacia. On the other hand, autologous proteins would be expected to be digested rapidly and reutilized. One would predict that certain types of synthetic polypeptides such as described by Gill and Doty (1960) would be partially broken down by intracellular proteolytic enzymes and result in antibody formation. The specificity of enzymatic activity of proteases and perhaps carbohydrases has been fairly well established (M. Dixon and Webb, 1958). Therefore, certain linkages in a large foreign molecule would be expected to present steric configurations which slow down or stop enzymatic breakdown at various positions of the molecule. Since injection of antigens that have been partially degraded *in vitro* to a molecular weight of 500-1000 does not induce antibody formation, one can assume either that such material does not reach the site of antibody formation or that the products of *in vitro* digestion tested so far differ from those produced by intracellular enzymes.

A schematic concept of the role, in antibody formation, of a foreign fragment about the size of an antigenic determinant is presented in Fig. 9. Such a concept is based on experimental evidence presented earlier and on current knowledge regarding the role of RNA and SRNA in

protein biosynthesis. It postulates (Fig. 9, A) that a normal configuration results. In Fig. 9, B, the antigen fragment modifies the specificity of RNA activity without interfering with its role in the synthesis of a protein molecule. If a large undigested foreign molecule combines with RNA (as in Fig. 9, C), the RNA would be completely blocked; the

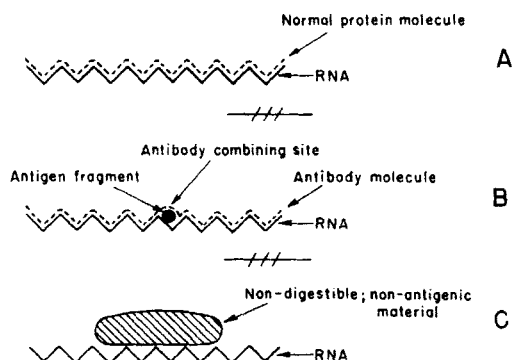


FIG. 9. A schematic concept of the role of a fragment of a foreign antigen. A represents the normal activity of RNA, B represents a molecule of RNA slightly modified by the presence of the antigen fragment which results in a slight change in the polypeptide chain, and C, the blocking of RNA activity by a large foreign molecule.

result would be no antibody formation and perhaps immune paralysis. The question arises as to why the handling of foreign material and antibody formation results in the modification only of serum globulins. Actually this problem has not been studied, so that there is the possibility that other proteins may be modified, but have different manifestations than antibody properties. Thus, chemically modified nucleotides may induce marked changes in protein biosynthesis and viruses may induce changes in the antigenic structure of host cells. It would be reasonable to speculate that only a few types of proteins have enough degrees of freedom to assume all the configurations demanded for antibody combining sites, so that only a few proteins, such as γ -globulin, can be stabilized at more than one steric configuration. Although this scheme is oversimplified and must await further information on the biosynthesis of proteins, it presents some of the facts based on experimental evidence and provides many provocative speculations and suggests possible investigations.

Assuming that large molecular weight, antigenic material breaks down into small fragments about the size of specific determinants, one might expect many of these determinants to be present only in the

internal portion of the antigen molecule. Antiserums should therefore contain antibody against internal structures of the antigen molecule which would not react with the native intact antigen molecule. Direct proof that such antibodies actually occur was given by Ishizaka *et al.* (1960). Using BSA they found that the serum of rabbits which had been immunized to BSA contained antibodies that combined only with fractions obtained from partially digested BSA. Lapresle *et al.* (1959) also found, in a more indirect manner, that antiserums to partially digested antigens contained antibodies that did not react with the native protein antigen. The presence of such internal determinants may explain why some patients fail to give skin reactions with native food allergens, but give clinical symptoms upon ingestion and subsequent digestion.

In view of the fact that most antigen material is associated with SRNA, one would like to know the role of SRNA in biosynthesis of protein and how the combination with a small foreign structure would result in the formation of a protein containing two identical steric complementary structures specific for the foreign material.

Pauling (1940) theorized that an antibody molecule should be bivalent because it consisted of a single chain and that each end contained a combining site. The bivalence of precipitating antibody was later established by Singer and Campbell (1952), but other aspects of Pauling's theory, such as the single chain structure of γ -globulin and the nature of the terminal groups, still require much study. Also, one would expect to find "heterologating" antibody molecules, i.e., bivalent antibody molecules of which one combining site would be specific for one antigenic determinant and the other specific for a different antigenic determinant. Many investigations have been made of this problem starting with those of Lanni and Campbell (1948) using quantitative precipitin techniques and later by other investigators using hapten inhibition and equilibrium dialysis techniques (reviewed by Nisonoff *et al.*, 1959). All studies reported so far fail to show that antiserums contain such antibody molecules. As a result of the work of Porter (1959) and others, e.g., Nisonoff (1961) and Dray (1962), an explanation may be possible, based on the evidence that γ -globulin molecules are composed of three components, namely, Fractions I, II, and III, which can be separated by mild proteolysis and isolated by chromatographic methods. Fractions I and II differ and carry a single antibody-combining site. Fraction III is common to all γ -globulin and carries most of the predominant antigenic determinants. Furthermore, each molecule of γ -globulin contains either I or II, but not both. Thus,

the two minor groups of any given γ -globulin molecule are identical and apparently produced by the same specific mechanism that controls the final configuration of either Fraction I or II. Therefore, *only a single template would be needed for the production of a bivalent homoligating antibody molecule and heteroligating antibodies would appear only as artifacts under abnormal conditions.*

One criticism of the template theory has been the question of the release of antibody from the template after formation, since *in vitro* studies would predict a very low dissociation constant, which is practically nonreversible ($Ag + Ab \rightleftharpoons AgAb$). However, on the basis of a small antigenic template, one would expect the template to behave as a hapten (H). Consequently, the dissociation constant would be large and would favor release of antibody ($AbH \rightleftharpoons Ab + H$). This would be particularly true during SRNA turnover and for the brief period of time when the antigen fragment is released subsequent to its incorporation into a newly synthesized SRNA molecule.

The formation of clones following secondary injections, as described by Coons (1958), may possibly be explained by fragmentation of antigen and sensitization of antibody-forming cells. One can assume that antigen will be taken up by some antibody-forming cells following a primary injection. These cells will become sensitized owing to the presence of intracellular antibody. A hypersensitivity reaction occurs upon secondary exposure to the same antigen. Injury of the sensitized cell would result in cell rupture or at least drastic changes in membrane permeability. Antigen material, which is now partially degraded, is released into the immediate environment as shown in Fig. 10. Some antigen escapes and is lost by excretion in the urine. However, much of it enters adjacent cells which in turn form antibody and the cycle can be repeated. The failure of passively transferred antibody to induce such a sensitized state results from inability of antibody molecules to enter cells, or more likely they are degraded too rapidly to retain antibody properties (e.g., passively transferred antiviral antibodies have no effect upon intracellular viruses). This aspect of immune mechanisms, involving actively and passively immunized animals, may eventually explain in part the failure to reproduce certain *in vivo* reactions by passive transfer of serum antibodies, since the reaction will depend upon whether the antibody is intracellular. Perhaps the best evidence for the injury of cells resulting from hypersensitive reactions has been presented by Miller (1940). Using dogs, it was found that after anaphylaxis there was a marked increase in urinary creatinin and uric acid, suggesting injury to hepatic cells. The loss of antigen by excretion has already been

discussed, and it was found that the appearance of S^{35} material in urine is quite characteristic of a secondary injection of specific antigen. When a primary injection of S^{35} BSA is followed several weeks later by injection of native unlabeled BSA, there is a large increase of S^{35} in the urine within a few hours (Garvey, 1962).

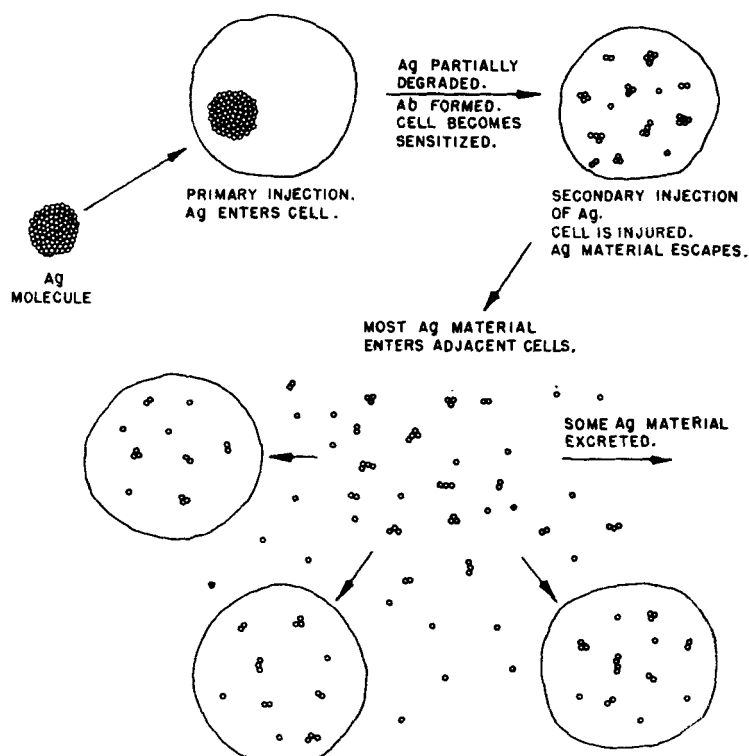


FIG. 10. A speculative schematic representation of primary and secondary reactions and formation of nonselective clones of antibody-forming cells. Ag = immunogenic molecule of antigen consisting of a mosaic of haptenic determinants that become separated upon partial intracellular digestion of the antigen molecule; Ab = antibody.

It is obvious that antigen may persist for many months or years and, as a result, antibody formation may persist at a low level, as suggested by McMaster and Kruse (1951) and shown more recently by Richter and Haurowitz (1960). This persistence of antibody formation would be very consistent with the retention of antigen and the prediction of *no antigen material, no antibody*. The proponents of the template theories of antibody formation are now faced with the para-

doxical situation of the loss of antibody formation although antigen material persists in the tissues of immunized animals. This may actually be a form of tolerance, as discussed earlier, and may involve a neutralization or blocking of antigen activity by intracellular accumulation of nondissociable antibody and stable antigen-antibody complexes.

V. Summary

It is apparent that up to the present time the role of antigen in antibody formation has been generally minimized, ever since Ehrlich first postulated that injection of antigen merely stimulated or increased the synthesis of antibodies already present in, or on, the cell. As more and more information is obtained regarding the properties of antigens, the retention of antigen molecules and their constituent components, as well as the factors involved in the biosynthesis of proteins, a clearer picture and a better experimental approach to the problem of the relation of antigen to immune mechanisms will be possible. It is obvious that the final answer will only be obtained by laboratory experimentation and by a combined knowledge of physical and biological principles and not by concepts that are unsuited for present-day methodology. One may wish to invoke Lamarckism, "memory cells," and somatic alterations to account for antibody formation. On the other hand it is reasonable that the retention of small foreign molecular structures may play an important role in the modification of protein biosynthesis by some simple mechanism.

Information related to the retention of foreign antigen material should provide a challenge to investigators of immune mechanisms. Future studies will probably revolve around investigations of chemically modified RNA in antibody-forming cells and studies of synthetic polypeptides or copolymers of various types with regard to their antigenic behavior. Studies dealing with the persistence and relation to cellular constituents of immunogenic polysaccharides will also provide valuable information.

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Blood Groups in Animals Other Than Man¹

W. H. STONE AND M. R. IRWIN

Division of Genetics, University of Wisconsin, Madison, Wisconsin

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I. Introduction

Evidence suggesting the existence of blood groups in animals other than man was published earlier than the well-known reports of Landsteiner (1900, 1901), which established the human blood group system now called ABO. For example, Bordet (1898) noted that the serum of an individual (such as a dog) injected with erythrocytes from another species (lamb) would lyse the donor cells. At the turn of the century Ehrlich and Morgenroth (1900), using isoimmune sera, demonstrated differences among the blood cells in goats.

The most important work in this field in the first 30 years of this century, though, was that done by Todd and White (1910). From their observations of cattle cells, they concluded that, "The red blood corpuscles of any individual are . . . characterized by a definite individuality of their own, and can be distinguished from those of any other individual." These men also speculated (without the benefit of genetic analysis) that the serologic properties of blood cells were probably inherited. Todd's later work (1930, 1931, 1935) on chicken blood groups strengthened this belief in the inherited individuality of red blood cells. Since then, this concept has been repeatedly verified by many studies of the blood groups in man and other animals.

Except for Todd's work, few advances were made between 1900 and 1935 on blood groups in animals, while at the same time great advances were made in both the genetics and the serology of human blood groups (see references in Wiener, 1943, in Dujarric de la Rivière and Eyquem, 1953, and in Ferguson, 1955). A primary reason for this lag was the tendency for scientists to look for systems corresponding to the naturally occurring antibodies and cellular antigens of humans. Another reason was the lack of secure linkage between the principles of genetics and immunology. In recent years, however, the alliance between genetics and immunology has been greatly strengthened, in both concept and technique, and one result has been substantial progress in the field of animal blood groups. This review, therefore, is an attempt to present the principal findings concerning animal blood groups, and will deal primarily with those studies that have used the concepts and techniques of both genetics and immunology.

II. Detection of Blood Groups

A. TECHNIQUES

The usual immunologic techniques of agglutination or lysis have been used in the studies of animal blood groups. In studying blood groups of

cattle, normal serum from rabbits (selected for the absence of naturally occurring antibodies reactive with cattle cells—*species antibodies*) is used as complement, rather than guinea pig serum, which often possesses potent species antibodies. Occasionally, however, suitably absorbed guinea pig complement may be used advantageously in the hemolytic test (see Stormont, 1962, for references).

Other specialized techniques are employed, such as the inhibition test for detecting soluble blood group substances and the use of anti- γ -globulin sera to detect incomplete agglutination (Coombs *et al.*, 1945, 1956). These special techniques for detecting various animal blood groups are fully described in the volume edited by Cohen (1962).

One of the major technical objectives is the preparation of strong and specific blood typing reagents. The keen researcher must be constantly aware of the variety of techniques available, and must use those that will produce reagents giving consistent results. Reagents may be prepared from normal sera containing naturally occurring antibodies, or from immune sera after the immunization of an individual with cells from another animal of the same species (isoimmunization), or with cells from another species (heteroimmunization). At present, isoimmunization has provided antibodies with a greater capacity for detecting individual differences *within* a species than that of antibodies produced by heteroimmunization. It should be noted, however, that a complete immunogenetic analysis of the blood group specificities often demands both kinds of antisera.

Most immune sera contain a multiplicity of antibodies (polyvalent). They must, therefore, be absorbed with blood cells of an appropriate type to make their reactions specific for a single antigenic factor. The number and kinds of absorptions necessary to produce a specific reagent depend on many variables, and a major consideration is the antigenic composition of both the donor and the recipient. With prior knowledge of their antigenic composition, immunizations can so be made that they will provide sera requiring little or no absorption to produce reagents specifically reactive with a single factor.

It is not our purpose to review the newer techniques available for demonstrating heritable differences other than in blood cells. Nevertheless, extremely significant advances have been made in studies of serum proteins. Some of these are: electrophoresis using starch and synthetic gels (Smithies, 1959; Raymond and Weintraub, 1959); the discovery of isoprecipitins in rabbits (Oudin, 1956; Dray and Young, 1958), mice (Kelus and Moor-Jankowski, 1961), and humans (Allison and Blumberg, 1961; Blumberg *et al.*, 1962); and immunoelectrophoresis (Grabar and Williams, 1955; Crowle, 1961).

B. IMMUNOLOGIC CRITERION OF A UNIT ANTIGENIC FACTOR

The immunologic criterion of unity (presumed specificity for a single blood factor) is satisfied if the antibodies in a reagent are completely removed following absorption by the cells of each reactive individual. In other words, if only reactive cells are capable of removing the antibodies from the reagent, the reagent is probably detecting a single antigenic factor or specificity. However, this does not necessarily prove that the reagent contains antibodies with only a single specificity, since test cells containing other specificities capable of reacting with the reagent may not be readily available. Frequently, a reagent which has satisfied the criterion of unity on tests of even several hundred samples of red cells, may react atypically with cells of an individual, and by absorption with such cells be fractionated. That is, these cells remove antibodies for themselves and other (atypical) cells, but not for the typically reactive cells. This is evidence that there are at least two distinct populations of antibodies in the reagent previously considered as a unit. These results (as will be illustrated later) are usually evidence of serologic subtypes. Thus, the greater the number of different reactive cells used in absorption of a reagent being tested for unity, the greater the probability that it is detecting a single specificity. It is clear, however, that the immunologic criterion can never be completely satisfied.

C. GENETIC CRITERION OF A UNIT ANTIGENIC FACTOR

Satisfying the immunologic criterion alone does not establish unity. The genetic criterion for a single factor must also be satisfied, and this means the distribution of the factor in the progeny from the various kinds of matings should be that expected of Mendelian characters. For example, the expected ratio of offspring *with* a hypothetical dominant factor to those *without* it from the three possible kinds of matings is given in Table I. Of course, these ratios are expected only if the positive

TABLE I
DOMINANT FACTOR INHERITANCE

Type of mating	Expected ratio
+ x +	3:1
+ x -	1:1
- x -	0:1

parents are heterozygous. In actual practice, the zygosity of the parents is often not known, and it would be rare indeed to find that all parents

were heterozygous. Since some of them will almost certainly be homozygous, an excess of positive offspring would be expected.

If the proportions of the offspring with and without the factor agree with those expected, it is concluded that the factor behaves as a unit in inheritance. If critical test matings can be made, direct evidence of the unitary nature of a factor may be obtained, since matings between parents of known genotypes should produce predictable ratios of offspring.

III. Genetic Associations of Blood Factors

A. TESTS FOR ALLELISM

As described above, in studying the blood groups in any species, the primary objective is to satisfy the criteria of both genetics and immunology for each blood factor. As each new factor is recognized and is found to satisfy these criteria, it must be tested further to determine if it is associated genetically with any other factors.

Let us assume that two reagents, anti-A and anti-B, are developed in the early stages of a study of blood groups in a particular species. Each factor behaves as a unit serologically and genetically. We will also assume, as is true of most blood factors studied to date, that each factor is controlled by a dominant gene. In testing several individuals, four phenotypes are found, one containing A and B, one containing only A, one containing only B, and one containing neither. A direct test of allelism of the genes producing factors A and B can be made by observing the proportion and kinds of offspring from test cross matings between parents containing both A and B and those containing neither ($AB \times -$). If A and B are produced by alleles, there should be only two kinds of offspring in equal proportions—those containing A and those containing B. Another critical mating ($AB \times AB$) should not give offspring negative for both factors. Thus, these data are evidence for a two-factor, three allele system (*A, B* and *neither*).

Very often allelism is immediately apparent from the serologic data. For example, in cattle (Stormont, 1952) there are two reagents, anti-F and anti-V, each of which detects a unit factor. When cattle of American breeds are tested with each of these reagents, they fall into three phenotypes: those with F alone, those with V alone, and those with both; the absence of a phenotype containing neither of these factors immediately shows that they behave as alternatives in inheritance; that is, the causative genes are alleles. Genetic tests have confirmed this; for example, matings between parents containing both F and V and those containing either F or V show a 1:1 segregation of the factors as expected if the FV parents were heterozygous.

Although tests on many thousands of cattle in the United States and Europe have confirmed this pattern of inheritance, Osterhoff (1962) has observed many cattle of the Afrikaner breed in South Africa whose cells were not reactive with either anti-F or anti-V. This strongly suggests that at least one additional allele is present at this locus. Recently, Stormont (1962) presented evidence for additional alleles by reporting that both F and V factors have serologic subtypes.

The relationship of F and V, as just described, will be recognized readily as similar to the relationship between the M and N factors in human blood. Such two allele systems are common in blood groups of animals and easy to recognize. Obviously, if two factors do not behave as alleles or are not otherwise associated, they must be controlled by genes at independent loci and therefore belong to different *blood group systems*.

B. SEROLOGIC SUBTYPES

Factors produced by alleles often show another kind of association—serologic subtypes. Early in the study of cattle blood groups, Ferguson (1941) noted that the factors C and E occurred in only two combinations, C and E together or C alone. Since factor E was never observed in the absence of C, a logical explanation of their association was that one gene (C_1) produced two specificities C and E together, and another gene (C_2) produced specificity C only. He recognized this as an example of serologic subtypes similar to the A_1 and A_2 subtypes of human bloods (Race and Sanger, 1958), and proposed the designation C_1 for the CE phenotype and C_2 for the C phenotype (Stormont, 1950). Examples are known also of serologic subtypes that include more than two antigenic factors (Stormont, 1962).

C. ANTIGENIC COMPLEXES (PHENOGROUPS)

Genetic associations are directly apparent from the results of test cross matings between parents containing two or more factors and those containing none of them, if two or more factors are transmitted together in groups or complexes. Such groups of factors, transmitted *en bloc* are called "phenogroups" (Stormont, 1962). For example, in the B blood group system of cattle (Stormont *et al.*, 1951) factors B and G may appear either alone or together. If individuals possessing either B or G are mated to others not possessing either, the offspring will or will not have B or G in the proportions expected of homozygous or heterozygous parents. Individuals possessing both B and G are of two kinds genetically. In matings to those not having either, one kind will produce offspring half

of which will possess B, the other half G, as would be expected if the parent with BG is heterozygous. The other kind of BG individuals will produce offspring of which either (a) all will contain BG (if homozygous) or (b) half will contain BG and the other half neither (if heterozygous), as would be expected if the two factors behave as a unit in inheritance. In other words, B and G may be alternative factors in some individuals and may behave as a unit (phenogroup) in others. Another factor, K, occurs only in combination with B and G, whereas B and G may occur alone or together. Thus, the factor K is related to B as E is related to C (described in the foregoing) and the same is true of K and G; they behave as serologic subtypes of each other, whereas the factors B and G *do not* behave as subtypes of each other.

The respective specificities of the antigenic complex or phenogroup, BGK, are, of course, detected by reagents specific for each factor, but the combination BGK has always been transmitted to the progeny as a unit.

These results clearly show a genetic association of the antigenic specificities B, G, and K. The causative genes behave as members of an allelic series: one allele produces B, another G, another BG together, another BGK, and another produces no antigenic effect yet recognized. Recently, however, a K or K-like specificity has been noted in a few individuals in combination only with B, suggesting that there is an additional allele in this allelic series (Osterhoff, 1962).

In addition to the factors B, G, and K, eighteen other factors have been reported (Stormont *et al.*, 1951) as belonging to this antigenic system, called the "B system." Additional antigenic factors belonging to this system have been demonstrated in various laboratories, but these have not been fully described to date (Stormont, 1962). If each of these twenty-one factors were independently inherited, the number of possible combinations would be 2^{21} , whereas currently over 300 phenogroups are recognized by workers in various laboratories (Stormont, 1962). On the assumption that each phenogroup is the product of an allele, more than 300 alleles are located at this particular locus—the most numerous multiple allelic system known. Several of the antigenic factors may occur singly, such as B or G, but the majority appear only in combination with one or more other factors, such as BO_1 , GY_2E_1' , BO_1Y_2D' , $BGI_1O_1T_2A'$, and $BGKO_3Y_1A'E_3'G'K'$. Ten additional antigenic systems are presently recognized in cattle, ranging in complexity from 2 to 35 phenogroups (35 alleles). This same kind of association of blood factors in phenogroups is proposed by Wiener and Wexler (1958) for the Rh system in man.

The preceding description for the antigenic systems of cattle blood

TABLE II
BLOOD GROUP SYSTEMS OF VARIOUS SPECIES OF ANIMALS

Species	No. of chromosome pairs ^a	No. of blood group systems described	Approximate No. of antigenic factors detectable	Hemolytic disease reported ^b	Erythrocyte mosaicism reported ^c
Human	23	12	60	yes	yes
Cattle	30	11	100	no	yes
Horses	33	5-7	11	yes	no
Sheep	27	7	34	no	yes
Pigs	20	10	23	yes	no
Chickens	39	7-13	23	yes	yes
Rabbits	22	5	7	yes	no
Dogs	39	7	8	yes	no
Mice	20	2-9	20-30	yes	no
Rats	21	3-5	6-10	yes	no
Rhesus monkeys	24	3	5	no	no

^a Data obtained from Spector (1956).

^b Either naturally occurring or induced.

^c As a result of fusion of embryonic blood vessels.

groups is paralleled in other species of animals. That is, an antigenic factor may be detected singly in other species, but usually two or more are associated in a larger complex, the phenogroup. Thus, in sheep, there are 50 phenogroups in one system, called B because of its relationship to the B system of cattle, and these are composed of various combinations of the twenty-eight demonstrable factors (Rasmusen, 1962). The antigenic systems of other species of animals and the number of antigenic factors presently demonstrable are listed in Table II. Detailed descriptions of these various systems in these species have recently been published (Cohen, 1962). These data give reality to the assumption that genes on many chromosomes of a species affect antigens on the red blood cells. Also, all published results to date indicate that a gene may produce multiple antigenic factors (specificities) which behave in inheritance as a unit (Stormont, 1955, 1959).

IV. The Relationship of Genes and Phenogroups

With modern advances in genetics, the concept of the gene has changed markedly, and the classical view of the gene as an indivisible unit of inheritance is no longer valid (Lewis, 1952; Benzer, 1961). It is well known today that within the gene there is "structural differentiation" (subgenetic units). One of the most challenging questions to blood group workers is the possible relationship between these subgenetic units and the individual factors of a phenogroup. Specifically, the question is whether the phenogroup (consisting of a complex array of factors) is controlled by an allelic gene (classical view) or by a cluster of subgenetic units with a one-to-one relationship between subgenes and factors.

The problem of clarifying the relationship between genes and phenogroups (or the antigenic factors of which they are composed) is complicated by the inability to define precisely antigens and antibodies. For one thing, antibodies are not absolutely specific, and for another, a single antigenic molecule may engender antibodies with multiple specificities (Landsteiner, 1945; Boyd, 1962; Owen, 1959). We can assume, therefore, that a one-to-one relationship does not hold between antibodies and antigens. Thus, in a broad sense, the problem is one of relating genes, antigens, *and* antibodies. Four models of this relationship are illustrated in Fig. 1. The interested reader should refer to Stern (1960) for a lucid discussion of this topic.

Insofar as the genes controlling the red blood cell antigens are concerned, there are no data available that clarify this relationship. There have been only a few examples of atypical genetic transmission of blood group genes (see Owen, 1960, for references), and it has not been

possible to tell whether they were due to mutation or crossing-over of linked genes (recombination). If they were mutations, they would not distinguish between the two hypotheses, but if they were recombinations, the hypothesis that the factors of a phenogroup are controlled by subgenomic units (rather than by alleles) would be favored. In the

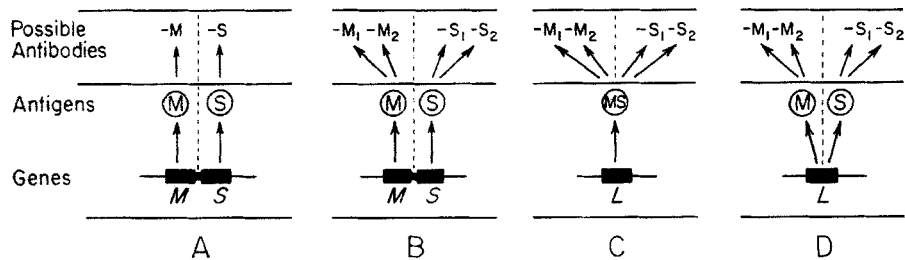


FIGURE 1. Four models of possible relationships between genes, antigens, and antibodies. (A) Two absolutely linked genes *M* and *S* produce two separate specificities (antigens) *M* and *S*, each of which may cause, in another organism, the production of one antibody, $-M$ and $-S$. (B) Gene-antigen relationship as in A, but each antigen may produce more than one antibody: $-M_1$ and $-M_2$, and $-S_1$ and $-S_2$. (C) A single gene produces a single antigen (*MS*) which may cause the production of several antibodies. (D) A single gene produces two antigens, each of which may cause the production of more than one antibody. (From Stern, 1960.)

absence of definite recombination, the phenogroups may be thought of as controlled by a series of co-dominant multiple alleles. At present, however, there is one example of definite recombination between factors of a phenogroup. This involves the H-2 erythrocyte antigens of mice (Allen, 1955; Gorer and Mikulska, 1959; Pizarro *et al.*, 1961).

The relationship between the gene and the factors within a phenogroup should be clearer when the chemistry of the blood factors *on the red cells* has been explained. Some progress along this line in human blood groups has been reported recently (Patras and Stone, 1960; Morgan, 1962). In the meantime, some information about the action of blood group genes has been deduced from the chemical structure of blood group specificities in studies of soluble blood group substances (see in following).

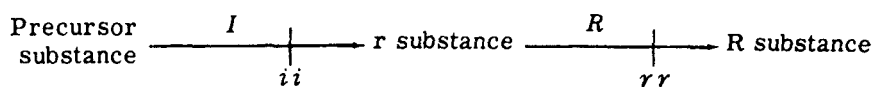
V. Cellular Antigens and Gene Interaction

As stated previously, the blood group genes usually behave as dominants. Hence, an individual will carry a blood factor on his cells *only* if one or both parents possess it. Exceptions to this generalization have been noted and show that more than one locus may act in the production of a blood factor.

A. THE R SYSTEM OF SHEEP

An antigenic character in sheep, designated R, is demonstrable by its interaction with a normally occurring antibody in the sera of some sheep which lack the R substance (T. Andersen, 1938; Kaczkowski, 1928; Ycas, 1949). Stormont (1951) reported that the cells of sheep which were not reactive with anti-R sera might react with normal sera from occasional cattle (primarily of the Hereford breed) previously absorbed with the cells of group R. He called this serum "anti-O," but we shall call it "anti-r" in accord with genetic terminology, as proposed by Rendel *et al.* (1954). The reactions of anti-R and anti-r were mutually exclusive; thus Stormont (1951) proposed that the genes for R and r were allelic, and that the gene *r* behaved as a recessive.

The observations by Rendel *et al.* (1954) and additional studies by Rendel (1957) indicated that the cells of some sheep were not reactive with either anti-R or anti-r, and it was first proposed by Rendel *et al.* (1954) that these nonreactive cells were the result of recessive inhibitor genes (*ii*) which inhibited the products of the genes for R or r. Rendel (1957) noted that saliva of both R and r individuals at high dilutions would inhibit the reaction of the r reagent with r cells. Also, Neimann-Sørensen *et al.* (1954) showed that the reaction of the r reagent with r cells could be inhibited by saliva from human secretors, but not from nonsecretors. This suggests that anti-r detects a common or related secretor substance in both humans and sheep, thus relating the dominant gene *I* of sheep to the dominant secretor gene, *Se*, of man. Rendel (1957) later proposed that no r substance is produced in *ii* individuals and that the secretor gene (*I*) converts a precursor substance into r substance. The gene *R* then acts to convert the r substance into R substance. According to this explanation, the R substance is a kind of interaction product requiring at least two genes for its production:



The foregoing explanation is almost parallel to Ceppellini's (1955) proposal of an interaction in man between the secretor gene (*Se*) and the alleles (*Ll*) for the Lewis substance (Morgan, 1960). On the other hand if the recessive gene (*i*) in sheep is an inhibitor, its action on the appearance of R or r substances corresponds to that proposed in man for the "Bombay" bloods as reported by Bhende *et al.* (1952), hypothesized by Ceppellini *et al.* (1952), and confirmed by Levine *et al.* (1955).

Very recently, E. Andresen (1962) and Saison and Ingram (1962)

reported that the A system of pigs closely parallels the R system of sheep in that the production of the a (also called O) substance may be inhibited by a recessive inhibitor gene and the A substance is a product of the interaction of two or more genes.

B. THE HYBRID SUBSTANCE

A different kind of apparent interaction between genes resulting in a cellular antigen was first noted on the cells of hybrids between two species of doves, *Streptopelia chinensis* and *Streptopelia risoria* (Irwin, 1932). The antiserum prepared in rabbits against the cells of the hybrids, after absorption with the cells of both parental species (at times even including the cells of the actual parents) was still strongly reactive with the cells of all the species hybrids. These results indicated that the cells of the hybrids carried a new antigenic product, a "hybrid substance," that was not present on the cells of either parent. This hybrid substance represents another departure from the premise that a blood factor will be present on the cells of an individual only if one or both parents possess it. However, not all kinds of species hybrids possess a hybrid substance.

The hybrids between *S. chinensis* and *S. risoria* also possess antigenic characters that are common and those that are specific to the parental species. Backcrosses of the hybrids to *S. risoria* produce the first backcross generation. If the factors specific to *S. chinensis* were heritable, one would expect a segregation in these birds. If there were one factor specific to *S. chinensis*, there would be equal numbers of offspring with and without the factor. If there were two factors, independently inherited, there would be equal numbers of four kinds of progeny—(a) those with both factors, (b) those with one, (c) those with the other, and (d) those with neither. The number of different kinds of offspring expected in this backcross generation would be 2^n , where n represents the number of factors. Actually, nine different factors specific to *S. chinensis* were demonstrated as units in the successive matings of selected backcross birds to *S. risoria* (Irwin, 1939). These are called ch-1, ch-2, . . . , ch-9, whereas previously (Irwin, 1939) the terms d-1, d-2, . . . were used.

If a bird containing only one of these nine factors, but otherwise indistinguishable from *S. risoria*, is backcrossed to *S. risoria* and produces only birds *with* and birds *without* the factor in equal numbers, the factor behaves as a unit in inheritance. Also, all birds possessing any one factor must be serologically indistinguishable. Such tests for unity and allelism have been performed and the results show that one or more genetically independent genes, presumably located on nine different chromosomes of

S. chinensis, produce blood factors that differentiate *S. chinensis* from *S. risoria*.

Inter se matings of backcross birds carrying ch-8 of *S. chinensis* have produced progeny homozygous for the gene or genes affecting ch-8. The cells of these homozygotes do not possess the hybrid substance, but all the offspring from matings of these homozygotes to *S. risoria* will be heterozygous and will carry it. That is, the hybrid substance presumably is the result of interaction of the gene controlling the ch-8 factor of *S. chinensis* and its allele in *S. risoria* (whose antigenic effect, ri-8, can be recognized by genetic and immunologic tests as a contrasting character to ch-8), or of interaction of other linked genes. The cells of these heterozygotes (*ch-8/ri-8*) have three demonstrable antigenic factors (a) ch-8 of *S. chinensis*, (b) the contrasting factor (ri-8) of *S. risoria*, and (c) the hybrid substance. In birds homozygous for ch-8, neither the hybrid substance nor the contrasting character (ri-8) of *S. risoria* can be demonstrated.

Parallel findings have been made following the transfer of factors related to ch-8 (Irwin and Cumley, 1947, and unpublished data) to *S. risoria* from other species (hu-8 of *Streptopelia humilis*, or-8 of *Streptopelia orientalis*, and se-8 of *Streptopelia senegalensis*). Any one of these three factors, if in the heterozygous condition with ri-8 of *S. risoria*, carries a hybrid substance, and some cross-reactivity has been observed among them and the *ch-8/ri-8* heterozygote. No hybrid substance has been detected by Palm (1955) or Bryan (1953) in the three homozygotes (*hu-8/hu-8*, *or-8/or-8*, and *se-8/se-8*). Parallel results relating a hybrid substance to another heterozygote have been reported by Bryan and Miller (1953), who found a hybrid substance on the cells of heterozygotes in backcross birds carrying the C factor of *Columba guinea* that had been transferred to *Columba livia*.

Further, the ch-4 factor of *S. chinensis* has always been associated with a hybrid substance in all backcross birds possessing ch-4. Also, all 57 offspring with ch-4 from *inter se* matings of backcross birds with ch-4 have carried the hybrid substance (Palm, 1955; Palm and Irwin, 1962) even though there must have been some homozygotes among these offspring (19 were expected) unless none were viable. A few of these offspring were undoubtedly homozygotes since no segregation of ch-4 has occurred in their offspring from test crosses.

Similar findings have been obtained with closely related antigenic factors from other species which have been transferred to *S. risoria*: hu-4 from *S. humilis*, or-4 from *S. orientalis*, and se-4 from *S. senegalensis* (Palm and Irwin, 1962). The backcross birds carrying any one of these

factors on their cells also carry a hybrid substance. Further, the respective homozygotes from *inter se* matings within each backcross population also possess a hybrid substance. These three factors in the backcross hybrids are indistinguishable to date from each other and from ch-4, as are the respective hybrid substances associated with each character (Palm and Irwin, 1962).

Thus, it seems certain that the cells of both homozygotes and heterozygotes in these four backcross populations carry a hybrid substance as well as the factor of system-4 (ch-4, hu-4, or-4, and se-4). The hybrid specificities presumably result from interaction between genes on nonsister (nonhomologous) chromosomes of *S. risoria* and each of the other four parental species. Hence, examples are provided of an interaction product in the factors of system-4, associated with *both* heterozygosity and homozygosity (interaction between nonalleles).

The question naturally arises whether the hybrid substance can be explained other than by assuming interaction between genes. McGibbon (1944) demonstrated that antibodies to the hybrid substance appearing on the cells of hybrids between the Muscovy and Mallard ducks could be produced by immunizing the actual parents with the hybrid cells. Miller (1956) likewise produced antibodies against the hybrid substance of the F₁ *Columba livia*/*Streptopelia risoria* by immunizing birds of the parental species, including a *C. livia* parent, with cells of the hybrids. In addition, only negative results were obtained by Miller (1956) in attempts to ascertain if the hybrid substance was *not* detectable on the cells of the parental species because it was located below the surface of the cells. Exposure of parental cells to enzyme treatment (trypsin and papain) did not render them reactive with the antiserum for the hybrid substance, nor did the injection of pooled cells of the parental species engender these antibodies.

The possibility that the hybrid substance is the result of interaction between antigenic factors, rather than between genes, cannot be eliminated entirely. In fact, it seems reasonable that the hybrid substance does not result from an interaction of genes themselves, but from products produced in the chain of reactions between the genes and the final product, the antigenic factor.

Interaction products with a new antigenic specificity may appear *within* a species as well as in hybrids between species. Cohen (1956) found a hybrid substance, I, on the cells of rabbits heterozygous for the allelic factors A and D of the Hg blood group system. Another hybrid substance, J, occurred in rabbits heterozygous for A and F of the same system (Cohen, 1960). The J hybrid substance was unusual because it

occurred as the direct product of the gene *D* (in homozygous *D/D* rabbits) as well as in *A/F* heterozygotes. Whether or not this situation is analogous to the production of the hybrid substance in birds homozygous for the *ch-4* factor is unknown.

Although examples of genic interaction are relatively rare, it may be, as Fox (1949, 1958) has suggested, that it occurs commonly but will remain undetected because of the constancy of the genetic background in vertebrates.

VI. Blood Group Systems of Various Species

Blood group systems have been found in every species studied. Table II contains a partial list of these. A great deal is known of the blood groups of cattle, sheep, pigs, and chickens, and in each of these domestic species, from seven to thirteen blood group systems have been described. Therefore, at least one-fourth of their chromosomes are presumably tagged with blood group genes. As noted in Table II, over one hundred antigenic factors are detectable in cattle, compared to sixty described in humans. In fact, of all the species studied, only cattle rank with humans in the amount of information available on their blood groups. Complex systems such as the Rh of humans and the B of cattle are known in these other species also, and as knowledge accumulates, a remarkable similarity in the complexity of the blood group systems of these species becomes more apparent.

The blood groups of laboratory animals such as rabbits, dogs, mice, and rats are reasonably well defined (Table II) and promising results are beginning to appear from blood group studies of mink, cats, goats, and various species of fish (see Cohen, 1962, for a recent collection of papers on animal blood groups). It is anticipated that similar findings will be made in other species of animals, if the basic assumption is true that genes on many chromosomes of a species affect antigens on the red blood cells. Presuming that each gene has a potentially antigenic effect, many more factors are theoretically possible in and on the red cells than are demonstrable with presently available techniques. However, since the immunologic reactions are primarily surface reactions, the number of factors detectable will undoubtedly be less than the theoretical maximum. Further, some factors are detectable only with special techniques and under certain environmental conditions. For example, in chickens, extracts of seeds (lectins or phytoagglutinins) of *Lathyrus cicera* or *Pisum arvense* agglutinate red cells of some sexually mature hens. Agglutinability of the cells appears to be controlled by an autosomal gene (*Hi*) and is conditioned by the levels of estrogen in the females.

The cells of the males carrying this gene become reactive with the lectins after injection with diethylstilbesterol (Scheinberg and Reckel, 1962).

VII. Blood Groups as an Index to Individuality

In species such as cattle, sheep, pigs, and chickens, almost complete individuality of the blood type is now determinable except in closely related and inbred individuals. In cattle, the B system alone is represented by a minimum of 300 alleles (phenogroups) allowing for 45,150 diploid combinations or genotypes [$n(n+1)/2$, where n is equal to the number of alleles]. Of course, not all these are serologically distinguishable, but Stormont (1962) estimates that there are at least 15,000 distinguishable phenotypes. The C locus of cattle contains a minimum of 35 phenogroups allowing for 630 genotypes. So, using only the B and C systems of cattle, there are $45,150 \times 630 = 28,444,500$ blood types theoretically possible. Taking into account all the possible genotypes at each of the 11 antigenic systems of cattle, there are over 25×10^{15} theoretically possible blood types. Even if only 10% of these are serologically distinguishable, the number far exceeds the estimated number of cattle in the world, about one billion (McGraw-Hill, 1958). Thus, the individuality of the blood types in this species, and probably in many others, is akin to fingerprinting in man but with the definite advantage that the inheritance of each of the components of the blood type is understood.

Because of this tremendous individuality, the blood types are very useful in identifying an animal. But a more important use of blood types is in solving cases of disputed parentage. Since almost all of the blood factors described in the various species behave as dominant traits, it follows that an individual will possess a blood factor only if one or both parents possess it. As new factors are found and their relationships to known blood group systems are determined, the efficiency of the blood test in cases of disputed parentage increases. Reagents are constantly being developed that distinguish homozygous from heterozygous genotypes ("dosage" reagents). And other reagents are being developed that delineate the genotype directly from the results of the blood test.

Identical twins are widely used in biologic research, and therefore a reliable test to differentiate identical (monozygotic) from fraternal (dizygotic) twins is valuable. In many species the blood test is the most efficient means of diagnosing the zygosity of twins. In cattle, Rendel (1958) estimated that the diagnosis of identical twins using both morphologic characters and the blood test is about 99% accurate. Twins

occur in other farm animals (sheep, horses, goats, rarely in chickens) and so the blood test will undoubtedly be used in these species to diagnose their zygosity. As the knowledge of the blood groups of litter-bearing species (pigs, mice, rats, rabbits, dogs, and cats) increases, it should be possible to determine if monozygotic twinning occurs and with what frequency.

Blood groups have become increasingly important as markers for studies of erythrocyte repopulation following homotransplantation of erythropoietic tissues in irradiated hosts. For example, Shaw and Vermund (1959, 1961) demonstrated hemagglutinins specifically reactive with the cells of the host, *in vivo*, in the circulation of pigeon-dove chimeras following lethal irradiation and transplantation of bone marrow. By using the blood factors as markers, they were able to show that the red cells of the hosts which survived this so-called "*in vivo* agglutination" were almost all donor type. Similar studies (Owen and Anderson, 1962) in rhesus monkeys are underway utilizing red cell factors as markers (Table II).

Finally, the blood groups will almost certainly become an important tool in tissue culture studies of somatic cell variation (Högman, 1960).

VIII. Erythrocyte Mosaicism

Early studies of cattle blood groups assumed, as had been found in humans, that the blood types of monozygotic twins would be identical, whereas those of dizygotic twins would be no more alike than if the twins had been born singly. However, later observations indicated that the majority of dizygotic twins had identical blood types and that their bloods reacted peculiarly. It was fortunate, indeed, that the bloods of twins resulting from superfecundation became available (Owen, 1945). A Guernsey cow had given birth to twins—a male with typical Guernsey characteristics and a female with a white face typical of the Hereford breed. The herd records showed that the cow had been bred on the same day, first by a Guernsey bull and later by a Hereford bull. Not only did the blood cells of the twins give identical reactions, but the cells of the Guernsey twin carried blood factors that could have been transmitted only by the genes of the Hereford sire, and those of the twin with the white face carried blood factors that could have been transmitted only by the genes of the Guernsey sire. Further, the blood cells of each twin reacted peculiarly in that they gave only partial lysis after treatment with certain reagents, suggesting two types of cells in each twin: those with and those without the factors under test. This was confirmed since the cells of each twin *not* lysed after treatment with reagents for factors of

the Guernsey sire were completely lysed by the reagents for factors of the Hereford sire and vice versa. Thus, the differential hemolysis tests showed clearly that the twins had two kinds of blood cells: one type was inherited from the Guernsey sire, the other from the Hereford sire.

The widely accepted explanation of these findings was proposed by Owen (1945) and is based in part upon the discovery of Lillie (1916) that about 90% of bovine twins have anastomosis *in utero* of their blood vessels. This union provides a bridge for the reciprocal migration of primordial blood cells of each twin and each kind becomes established in the hematopoietic tissues of its co-twin. Thereafter, each kind of blood-forming tissue produces its own type of cells, so that the twins have a mosaic of blood cells—those from its own blood-forming tissues and those from the tissues derived (“transplanted”) from its twin. This phenomenon of mixed blood cells is called “erythrocyte mosaicism,” and individuals with mosaicism are called “mosaics” or “chimeras” (Cotterman, 1958). Treatment of a sample of cells from a twin showing erythrocyte mosaicism with a given reagent will hemolyze the cells carrying the homologous factor leaving intact the cells of the other type not carrying the factor. The remaining cells can be typed or can be used to measure accurately the proportion of the two cell types by hematocrit technique, cell counting, or spectrophotometric determinations (Mange and Stone, 1959).

Among the many hundreds of chimeras tested in cattle, the proportion of the two types of cells in a given twin has varied widely. More often than not, the two types occur in disproportionate amounts (Rendel, 1957). Twins have been studied (for example, by Stone and Palm, 1952) in which the proportion of blood from one twin was so greatly in excess that the other type practically defied detection. Another interesting feature of chimeras is that the proportion of the two types of cells in one twin is usually the same as in the co-twin; these phenomena are not explained satisfactorily, but may be associated with the time in embryologic development when anastomosis of the blood vessels takes place.

The blood test is an extremely useful technique in identifying twins in cattle. If twins show erythrocyte mosaicism they *cannot* be identical; if their blood types are identical, with no evidence of mosaicism, they *could* but *need not* be identical. The use of the blood test for diagnosing the freemartin is of some practical significance also. A female born twin to a bull and possessing erythrocyte mosaicism will be a freemartin or nonbreeder, by virtue of the fusion of the blood vessels with her male co-twin *in utero*, but a female twin to a bull with a different blood type from the co-twin will be as fertile as if born singly (Stone *et al.*, 1952).

As shown in Table II, erythrocyte mosaicism in twins resulting from fusion of embryonic blood vessels has been reported in only three species other than cattle. Stormont *et al.* (1953) reported a case in sheep and this was accompanied with sterility of the female born co-twin to a male. There are now five cases in man (see Stone, 1962 for references), but for unknown reasons the human female chimeric twins are not sterile. Finally, one case has been reported in chickens (Billingham *et al.*, 1956). Chimeras may be characteristic of other species but they have not been observed. At present, there are no clues as to why the phenomenon is highly prevalent in cattle and rare in humans, two species in which twinning is relatively uncommon, or why it is rare in sheep, a species in which twinning is common.

Embryonic cells other than those ancestral to the blood-forming tissues must also migrate reciprocally in dizygotic cattle twins with anastomosis of their circulatory systems, because such twins will accept grafts of each other's skin, but will reject grafts from a sibling or other cattle (D. Anderson *et al.*, 1951; Billingham *et al.*, 1952). Thus, it appears that antigens or antigen-like substances from each of the tissues have established a state of "immunologic tolerance" in each twin toward antigenic substances of the co-twin. A state of tolerance must exist likewise in each twin for the hematopoietic tissue transplanted from its co-twin; otherwise two genetically and antigenically different types of cells would not coexist in the same individual.

Erythrocyte mosaicism may occur in individuals with no history of twinning, presumably as a result of somatic mutation or phenocopy production and by other means not yet understood. Examples of mosaicism resulting from presumed somatic mutations have been noted in man (Atwood and Megill, 1959; Atwood and Scheinberg, 1958; Cotterman, 1958; Furuhashi *et al.*, 1959; Atwood and Pepper, 1961). All these chimeras have highly disproportional amounts of the two types of bloods; frequently one type is as high as 99% of the total volume, so that the mosaicism is difficult to detect. The domestic animal should be excellent experimental material for studies in the area of antigenic differences *within* an individual. Gartler *et al.* (1962) reported on a human hermaphrodite with erythrocyte chimerism presumably resulting from double fertilization.

IX. Hemolytic Disease of the Newborn

One of the puzzling aspects of the interactions between mother and developing fetus in man is the relative infrequency of hemolytic disease of the newborn from incompatible matings involving the Rh system. This

suggests that antibodies may not be engendered in the majority of pregnant women, or that the antibodies even if present do not produce hemolytic disease. In rabbits, Keeler and Castle (1934a,b) observed variation in antibodies detectable in fetuses prior to birth. For example, if a doe lacking both the contrasting antigenic factors H_1 and H_2 , immunized before and during pregnancy, is mated to a male with the genes for H_1 and H_2 , she would carry either H_1 or H_2 fetuses, or both. However, only antibodies against H_2 were demonstrable in the circulation of fetuses with H_1 , and only antibodies against H_1 in fetuses with H_2 (Keeler and Castle, 1934b), suggesting that the cells of the fetuses had adsorbed their specific antibodies without damage. Heard *et al.* (1949) reported that antibodies could be detected on the cells of newborn from immunized does, with no adverse effect. However, Kellner and Hedal (1953) noted that hemolytic disease could be induced in newborn rabbits by immunization of the pregnant female, and that the concentration of antibodies was one factor involved in the incidence of the disease.

The dramatic discovery that hemolytic disease of the newborn in man (Levine and Stetson, 1939; Levine and Katzin, 1940; Levine *et al.*, 1941; see Wiener, 1954; and Race and Sanger, 1958, for additional references) was the result of isoimmunization during pregnancy also explained a similar affliction of foals and mule foals that had been known for a long time (Bruner *et al.*, 1948; Caroli and Bessis, 1947a,b; Franks, 1962; Coombs *et al.*, 1948). The antibodies formed by the mare against the cells of the fetus are transmitted to the newborn only through the milk, during the first 24–48 hours. The use of foster mothers has become an effective control measure (Cronin, 1955). It is interesting that this control measure was prescribed a century ago for mule foals by Chicoli (1861), as cited by Roberts (1959).

Hemolytic disease of the newborn has been induced in other species (see Table II) such as swine (Bruner, *et al.*, 1949; Buxton and Brooksbank, 1953; Doll and Brown, 1954) and dogs (Young *et al.*, 1951), in which the placentas are apparently not permeable to antibodies, so the antibodies reach the newborn through the mother's milk. In these species the antibodies usually result from immunization of the female with blood of the sire or similar blood. Hemolytic disease of the newly hatched chick has also been observed (Briles, 1948) and apparently results from the passage of antibodies through the egg yolk to the chick.

As shown in Table II, hemolytic disease has not been reported in cattle or sheep. Attempts to induce it in newborn calves (Braend, 1956; Kiddy *et al.*, 1958) and in sheep (Rendel, 1957) by immunization of the

mother with the sire's blood have so far been unsuccessful. It appears that the newborn calf, and presumably the lamb, are protected by the neutralization of antibodies during their passage through the alimentary canal after ingestion of mother's milk, preventing them from reaching the newborn's erythrocytes and causing their destruction. It is pertinent, however, to ask why the newborn of horses, dogs, and pigs, who also obtain antibodies through the milk, are not also protected against hemolytic disease.

Morris (1958, 1961) has induced hemolytic syndromes in young mice using antierythrocyte and antileucocyte platelet sera, and it has been induced in rats (Bessis, 1947). So it seems reasonable that hemolytic disease of the young may be produced in any species, provided antibodies of the mother reach the erythrocytes, irrespective of the route. On the other hand, the question why some antigenic factors are more often involved than others remains unanswered.

X. Soluble Blood Group Substances

Blood group substances are not restricted to the red blood cells (Kabat, 1956). Certain of them occur in other cells and fluids of the body. For example, there are soluble blood group substances of cattle (J), pigs (A), and sheep (R) that are genetically and serologically similar to each other and to the A substance of humans. Each of these substances occurs in soluble form in the serum and other body fluids or on the red cells and is detected by naturally occurring antibodies in the sera of animals lacking the corresponding substances.

A. THE J SUBSTANCE OF CATTLE

Although the J substance was first described as a cellular character (Ferguson, 1941), it was later found by inhibition tests (see Stone and Irwin, 1954, for references) that some sera were capable of inhibiting the reaction between anti-J antibodies and J-positive erythrocytes and therefore contained soluble J substance. Still later, Stormont (1949) proposed that the cellular character was only a secondary effect of the gene for J whose primary action was to produce soluble blood group substance. He observed that members of dizygotic twin pairs with erythrocyte mosaicism may differ in J, but the two kinds of cells in each twin were alike in the presence or absence of J. He concluded that in the genetically J-positive twin the cells produced by the tissues of the J-negative twin took up J from the serum and became positive, whereas in the genetically J-negative twin the cells produced by the tissues of the J-positive twin had no J substance available to them and consequently

were J-negative. This was clear evidence that the J substance was produced by tissues different from those that produced the cells and that the cells acquired J only on contact with soluble J in the serum. This was confirmed (Stormont, 1949) by showing that J-negative cells could be coated with J substance, apparently irreversibly (Patel, 1958), by incubating them in serum containing J or by injecting them into cows whose sera contained J.

The amount of J substance on the cells is partly related to the concentration of J in the serum (Stone and Irwin, 1954; Patel, 1958). Thus, the cells of cattle whose sera contain relatively high concentrations of J are usually reactive with anti-J sera (J reagents) and are classed as J^{cs} , whereas cells of cattle whose sera contain relatively low concentrations of J are not ordinarily reactive and are classed as J^s . However, J^s cells contain J substance, since they can absorb antibodies from an anti-J serum if about twenty-two times as many J^s as J^{cs} cells are used (Patel and Stone, 1957).

That the presence of J on the cells is not entirely determined by the level of J substance in the serum (Stone and Irwin, 1954; Patel, 1958) is apparent because several individuals (J^s) possess high concentrations of J in their sera (at levels typical of J^{cs} animals) but their cells fail to react; whereas there are others (J^{cs}) whose cells react but whose sera contain low concentrations of J (at levels typical of J^s animals). Pedigree examinations of these exceptional cattle suggest that some kind of a genetic mechanism operates to determine whether or not J is absorbed on to the cells from the serum. Recent genetic analyses (Conneally *et al.*, 1962) of the variations in J reactivities of cells and sera suggest that the J^{cs} and J^s phenotypes are controlled by two to four multiple alleles each producing different quantities of J substance. To our knowledge, the quantitative subgroups of the B antigens in man (Gibbs *et al.*, 1961) is the only other example in blood groups of a series of multiple alleles with different quantitative effects. Most likely the R locus of sheep and the A locus of pigs will be found to exhibit this same type of genetic variation.

Investigations (see Stone, 1962) of the capacity of serum to coat cells with J reveal that sera from adult cattle of the J^s class, irrespective of the amount of J substance they contain, are not capable of coating either j^a or other J^s cells, whereas sera from adult cattle of the J^{cs} class (except for those containing very little J substance) are capable of coating j^a or j^s cells.

The cells of newborn calves (genetically J^{cs}) are ordinarily J-negative *even though* their sera contain high concentrations of J substance

(Stormont, 1949; Stone and Irwin, 1954). The cells of these calves gradually take up J during the first weeks of life and after about one month attain a level of cellular J typical of adults. Nevertheless, the cells of newborn calves can be coated *in vitro* with J from the serum of adult cattle of the J^{cs} class, whereas sera from newborn calves containing J are incapable of coating adult cells (Stone, 1962). The results of these *in vitro* coating experiments have clearly shown that the serum and not the cells plays the major role in determining whether or not the erythrocytes take up J substance. It remains to be determined why the sera of J^s cattle and of newborn calves, containing levels of J equal to or even greater than in some J^{cs} cattle, are not capable of coating cells. A study of the changes that take place in the serum of the newborn (J^{cs}) as it matures and provides it with the capacity to coat cells should be highly informative.

There is another J phenotype, called j^a, represented by cattle whose cells and sera do not contain J substance, but whose sera may contain naturally occurring antibodies for J. Hemolytic titers of these antibodies vary widely among j^a cattle at any one time and at different times within an individual (Stormont, 1949). According to Stone (1956) the variation is correlated with the season of the year; the highest titers in Wisconsin are observed during late summer and early autumn (August to October) and the lowest during late autumn and winter (December to March). Braend (1959) confirmed these findings in Norway, and Osterhoff (1962) found the same seasonal variation in anti-J in studies of cattle in South Africa, but the months of high and low titers were reversed exactly 6 months as expected. Osterhoff also obtained highly suggestive evidence that seasonal variation was associated for the most part with changes in temperature and to some extent with natural radiation.

Seasonal variation in titer of naturally occurring antibodies of cattle other than anti-J were observed by Osterhoff (1962), but not as clearly as for J. Rendel (1957) found seasonal variation in naturally occurring anti-R of sheep, and Shaw and Stone (1958) reported that anti-A and anti-B of human sera also show seasonal variation.

It may be that all naturally occurring antibodies can show seasonal variations. However, Stone and Miller (1961) found no evidence for it with the naturally occurring anti-U antibodies of cattle, and Bednekoff (unpublished work from our laboratory) found no clear seasonal variation in naturally occurring rabbit antihuman A and rabbit antisheep R antibodies. The physiologic basis of this curious phenomenon is not known.

The J substance is ubiquitous as are the other cross-reactive sub-

stances of mammals. In addition to the cells and serum it has been found in high concentrations (Bednekoff, 1962) in milk, saliva, urine, semen, ovarian cyst fluid, gastric mucosa, intestines, and pancreas; and in low concentrations or not at all in cerebral spinal fluid, brain, heart, kidney, and liver.

Purified J blood group substance can be prepared from cattle gastric mucosa (Hayashi *et al.*, 1958) using the standard methods of preparing human blood group substances (Kabat, 1956; Morgan, 1960). It appears to be a mucopolysaccharide closely related to the ABO blood group substances of humans. J substance can be extracted also from serum (Bednekoff *et al.*, 1959). This extract behaves as a glycoprotein of the α -globulin type. The J extract prepared from serum is antigenic in rabbits whereas the preparation from abomasal mucosa is not (Bednekoff *et al.*, 1962). These preparations differ further in that J activity of serum appears associated with carbohydrates *and* protein whereas that of the gastric mucosa is associated *only* with carbohydrates. This difference may account in part for the difference in antigenicity. Although cattle cells or untreated serum are not antigenic for J in rabbits, J extracts from serum and saliva will engender anti-J (Bednekoff *et al.*, 1962) as will human A cells (Stormont and Suzuki, 1960).

As pointed out earlier, the soluble blood group substances of humans, cattle, sheep, and pigs are serologically related. Anti-J is reactive with A and AB cells of humans (Stormont, 1949), with sheep cells of type R (Neimann-Sørensen *et al.*, 1954), and with A substance of pigs (see E. Andresen, 1962, for references). There is some evidence (Hayashi *et al.*, 1958; and unpublished results from our laboratory) that these substances are also chemically related. This is expected since substances that cross-react are chemically related (Landsteiner, 1945; Boyd, 1962). To date the chemical analysis of the soluble blood group substances provides the only clues to the nature of blood group specificities (Kabat, 1956; Morgan, 1960). Whether the chemical structure of blood group substances are the same on the blood cells and in body fluids is an open question.

B. THE R SUBSTANCE OF SHEEP

The unique characteristics of the R blood group system of sheep, namely, that the r substance is controlled by a recessive gene (*r*) and that the production of both R and r substances can be blocked by a recessive gene at an independent locus has already been discussed in the foregoing (see also Rasmusen, 1962). Like J of cattle, the R and r

substances of sheep are normal constituents of the serum that are taken up by the blood cells as secondary characteristics. But, unlike J, there are no phenotypes that possess either R or r substance in the serum without also having the corresponding substance on the cells; in other words, there are no phenotypes equivalent to J^s. Either R or r substance may be present in the serum of newborn lambs depending upon the genotype, but the cells acquire the appropriate substance only after birth. According to Rendel (1957), the R substance is detectable on the cells of lambs on the average by 16 days *post partum*, whereas the r substance is not detectable until about 28 days *post partum*. The J substance, in contrast, may be detected on the cells during the first week of life (Stone and Irwin, 1954).

The results of coating sheep cells with R and r substances partially parallel the findings with J (see Rasmusen, 1962, for references). Thus, r cells can be irreversibly coated with R substance if soaked in the serum of sheep containing R substance. The coated cells are reactive with *both* anti-R and anti-r sera, a situation which does not occur in the natural heterozygous R/r cells because r behaves as a recessive. Also, i cells (containing neither R or r substance) can be coated with r substance and presumably also with R substance. Sheep of type R contain *both* R and r substances in their saliva and seminal plasma. Therefore, it is possible to coat group R cells with r substance using saliva from the same individual whose cells are being coated. Saliva from group R sheep will coat i cells with *both* R and r substance simultaneously.

Although naturally occurring antibodies for R are found in the majority of sheep of type r and rarely in sheep of type i (Ycas, 1949; Rendel *et al.*, 1954), their reactions with R cells are too variable for routine use. However, cattle anti-J sera are strongly reactive with R cells and are consequently more satisfactory. Anti-r reagents are generally obtained from normal cattle sera (Stormont, 1951; Sprague, 1958b) but may occur rarely in sheep of group i (Rendel, 1957) and in some normal goat sera (Rasmusen, 1961).

There is a relationship between the r substance of sheep and the H substance of humans (Neimann-Sørensen *et al.*, 1954) since the saliva of human secretors inhibits the reaction of anti-r with sheep r cells, whereas the saliva of nonsecretors does not. Another relationship between cattle and sheep soluble substances was demonstrated by Sprague (1958a) who found that cattle sera may possess in addition to J substance a soluble substance (O^c) capable of inhibiting the reaction of sheep anti-r. This substance may occur alone or together with the J substance.

C. THE A SUBSTANCE OF PIGS

The A system of pigs represents the second example in animal blood groups of two or more independent genes controlling a blood group substance. In this respect and in others already mentioned the A system of pigs simulates the R system of sheep (E. Andresen, 1962; Saison and Ingram, 1962). Pigs of type A contain A substance on their cells and in their sera (and presumably in other tissues and body fluids) detectable by naturally occurring anti-A which occurs in pigs lacking the A substance or by naturally occurring anti-J of cattle. Like J of cattle and R of sheep, the A of pigs is only a secondary characteristic of the blood cells. It is absorbed onto the cells of the newborn during the first week of life. The a phenotype (called O by Andresen, 1962) is noted in pigs with a substance on their cells and in their sera, and is detected by naturally occurring cattle antiserum, or by the serum of pigs lacking both the A and the a substance. These latter animals result from the inhibitory action of an independent recessive gene. Pig cells of appropriate types can be coated with either A or a substance or both. Hence, the results of the coating experiments are strikingly similar in cattle, sheep, and pigs.

XI. Histocompatibility and Blood Groups

Another convincing test for biologic individuality is the well-known rejection of grafts made between individuals of the same (homograft) or of different (heterograft) species. The genetic theory of transplantation, first presented by Little (1914) and amply confirmed since, is that transplantation antigens are controlled by dominant genes (histocompatibility genes) distributed on different chromosomes. Donor tissue possessing one or more antigens absent in the recipient elicits an immune response by the host leading ultimately to the rejection of the donor tissue.

Much of the genetics of histocompatibility has been limited to transplantation of tumor tissues in inbred strains of mice, but the findings are applicable to normal tissues and are being extended to other species (see Billingham and Silvers, 1962, for extensive references).

A. MICE

A relationship between histocompatibility genes and blood groups was first demonstrated by Gorer (1937). He found hemagglutinating antibodies in mice that had rejected a tumor homograft which were specifically reactive with the red cells of the donor of the tumor. Hence,

the tumor and the red cells of the donor strain shared an antigen. The gene responsible for this antigen, called *H-2*, was located on chromosome IX linked to easily detectable anatomical abnormalities of the tail (Allen, 1955). This *H-2* locus has been extensively studied (Snell, 1958) and contains 16–20 alleles. Each allele determines a phenogroup consisting of one or more of the thirty known antigenic factors. In this respect, the *H-2* locus is similar to the Rh locus of man, the B and C loci of cattle, and the complex blood group loci (systems) of other animals. For example, certain factors of the *H-2* system appear frequently in the same phenogroup [e.g., $H-2^a = (ACDEFHJKMNY)$, $H-2^b = (D^bEVNFK^b)$, $H-2^c = (CDE^cFHJMN)$, and $H-2^s = (CEFS)$], whereas others appear unique to one phenogroup, as for example factor "S" in the $H-2^s$ phenogroup. However, the *H-2* locus is apparently different from the complex blood group loci of other species (B of cattle, Rh of humans, etc.) because it appears to be composed of closely linked genes (pseudo-alleles), since crossovers have been detected (approximately 1%) between certain of the factors of a phenogroup (Allen, 1955; Gorer and Mikulska, 1959; Pizarro *et al.*, 1961).

Some further insight into the nature of the *H-2* locus of mice has been obtained by Pizarro *et al.* (1961) who noted that the *H-2* factors are not present in embryonic tissues or in the newborn, but appear about the third day after birth and reach adult levels at about 6 days. In adults, the *H-2* factors are not found in all tissues, but the same tissues from different animals of the same age within a given inbred strain have about equal concentrations of factors. Significantly, they observed a close correlation among different *H-2* factors with respect to age of appearance, development, and distribution in adult tissues. They interpret these data as evidence that the *H-2* locus is "one gene" because it behaves as a "physiologic unit," or a "unit of function." The data are suggestive that the "active patch" of chromosome controlling the *H-2* antigens behaves as a physiologic unit, but it is a matter of definition whether or not this is called one gene.

A similar study (Shaw and Stone, 1962) on cattle embryos of the time of appearance of the factors within various phenogroups of the B system indicates that different factors comprising a phenogroup are not developed simultaneously in ontogeny. Furthermore, the same factor develops at different times depending upon the phenogroup in which it occurs. These results suggest that insofar as age of development is concerned, the B locus of cattle *does not* behave as a physiologic unit even though recombination has not been definitely observed. There may be some significance in the observations of Stone and Miller (1955)

that certain blood factors destroyed after treatment of red cells with proteolytic enzymes are the same as those that are slow in developing embryologically.

There are three other histocompatibility loci known in mice, H-1, H-3, and H-4 (Snell and Stevens, 1961), each of which has several alleles, but only H-1 seemingly determines antigenic factors on the red cells as well as in tumor tissues.

B. RATS

A histocompatibility locus, R-1, has been described in rats by Bogden and Aptekman (1962). This locus also determines a red cell antigen B, and possibly another called C. The B factor behaves as a dominant and occurs in embryonic tissue and on red cells of R-1 rats of all ages. Another factor, also called B (see Palm, 1962) probably represents an additional histocompatibility factor in rats.

C. CHICKENS

Utilizing graft-versus-host reactions following transplantation of leucocytes (lymphocytes) from adults to chorioallantoic membranes of chick embryos, Burnet and Burnet (1961) defined 3 alleles (A, B, and C) at a single locus governing histocompatibility. However, it is not known if this locus also produces antigens on the red cells. Clear evidence of a relationship between blood type and histocompatibility in chickens has been reported recently by Schierman and Nordskog (1961). It appears that the B locus of chickens determines antigens on the red cells and in tissues, because skin grafts made between partially inbred Leghorns differing in B-locus genotypes are rejected, whereas those made between individuals with the same B genotypes are not. Gilmour (1962) discusses additional evidence for a relationship between histocompatibility and blood groups in chickens.

D. CATTLE

Unpublished results from our laboratory suggest that the J substance may act as a weak transplantation antigen since transplants between dizygotic twins with erythrocyte mosaicism and differing in J are not equally tolerated. Additional work is needed to determine if the difference in J in chimeras (which ordinarily accept each other's grafts) accounts for the "assymetry" of response noted by Billingham and Lampkin (1957) in their studies.

E. THE NATURE OF TRANSPLANTATION ANTIGENS

In 1956, Billingham, Brent, and Medawar suggested that the antigens responsible for provoking transplantation immunity were deoxyribonucleoproteins or substances of nuclear origin intimately associated with the chromatin. The main reason for this suggestion was that the antigens were destroyed by deoxyribonuclease. These same authors have since rejected this view (1958) and now believe that deoxyribonucleic acid itself is not a part of transplantation antigens. One current concept is that the determinant groups of transplantation antigens are related chemically to the human blood group substances. That is, they are *mucopolysaccharides*. However, recent immunochemical studies on the H-2 antigens of mouse liver indicate that they reside in a lipid and protein-containing fraction that consists of nuclear and cellular membranes. These preparations contained less than 1% carbohydrate and no hexosamine (Herzenberg and Herzenberg, 1961). Since these preparations were by no means pure, it is still possible that the H-2 determinant groups are carbohydrates.

XII. Association between Blood Groups and Other Characters

A. LINKAGE

An association between a blood group and some other character might be brought about by linkage. The first clear evidence of linkage between a blood group factor and a morphologic character was described by Sawin *et al.* (1944) in rabbits. The gene for the A factor was found linked with the gene on the fifth chromosome that produces brachydactyly. The recombination value was about 40%. Since then, linkages have been described (Lawler and Lawler, 1959) in humans between the Lutheran and secretor genes (7.5%); Rh and nail-patella syndrome (9.7%). Presumably different linkages between blood group genes were described in chickens by Scheinberg (1956) and by Briles (1962) with recombination frequencies of 2-5% and 40%, respectively. Two examples of blood group genes located on the sex chromosomes (sex-linked) are known. McGibbon (1946) found one of a cellular antigen of Mallard ducks and, recently, Mann *et al.* (1962) described the Xg^a factor in humans as a sex-linked character.

B. INCOMPATIBILITY BETWEEN MOTHER AND FETUS

The best-known example of this kind of association of blood groups with another character is the Rh system of humans and hemolytic disease. Also, ABO incompatibility between fetus and mother can result

in hemolytic disease and furthermore is a major cause of fetal death (for references, see Chung and Morton, 1961). At present, there is only a suggestion that incompatibilities exist in lower animals, other than those producing hemolytic disease. Morton *et al.* (1956) reported preliminary findings in cattle of a 10% deficiency of calves from incompatible matings, and Conneally *et al.* (1962) found that maternal-fetal incompatibility due to isoimmune antibodies had a significant effect on fetal mortality but only a crude estimate was obtained.

Plum (1959) suggested that the greater the number of antigenic factors by which the matings in cattle differed, the greater the increase in survival, and that the dissimilarities in antigenic factors might be used as an index of hybrid vigor. Objections to this latter proposal have been considered elsewhere (Irwin and Stone, 1961).

C. PLEIOTROPIC EFFECTS

If genes for blood groups have some pleiotropic effect (influencing the expression of a morphologic or physiologic character in addition to the antigenic factors on the cells) they might be a useful index in animal improvement. But, to date, there is no conclusive evidence of such effects. Robertson (1956) has considered various ramifications of this topic. In this connection, a significant association in cattle has been observed in different laboratories between one or more alleles at the B locus and fat percentage of milk (Neimann-Sørensen and Robertson, 1961; Rendel, 1961; Conneally, 1962).

D. HETEROSIS (HYBRID VIGOR)

A current approach to the general problem of natural selection is concerned with the possibility that the heterozygote may have a selective advantage over either homozygote, and Fisher (1930) states that a "stable" polymorphism may thereby be maintained in a population. According to workers with chickens (Allen, 1962; Briles, 1954; 1956; Briles *et al.*, 1957; Gilmour, 1954, 1958, 1959; Shultz and Briles, 1953), artificial selection in poultry flocks has favored the heterozygote in at least two of the loci affecting blood groups of chickens. The heterozygotes at one of the loci seemingly maintain an advantage over the homozygotes in hatchability of fertile eggs, body weight at 9 weeks of age, and egg production. One possible explanation of these findings might be that this locus has a major influence on a fundamental physiologic process. There is some indication that the physiologic effect might vary in different genetic combinations. Although data are relatively scarce showing definite associations between blood group genes

and other characters, it is clear that the genes controlling blood groups (at least in humans and in chickens) are not selectively neutral (for references, see Clarke, 1959; Roberts, 1959a,b; Race and Sanger, 1958). Thus, a continued search for such associations is justifiable. However, to acquire data of significance that a blood group gene exerts an influence upon a physiologically complex character is a major undertaking, requiring thousands of animals (Irwin and Stone, 1961).

XIII. Credo

We believe those who read this paper will realize that the combined approaches of genetics and immunology in the studies of blood groups reveal incredibly intricate gene effects now awaiting chemical explanation. Furthermore, a challenging problem for the future is to determine if the antigens of the blood cells and other body cells have a broader biologic significance than presently known. In the future we can expect exciting results from the newer studies of serum proteins, transplantation antigens, and antigens of bacteria which will surely contribute, along with studies on blood groups, to our ultimate understanding of the relationship among genes, antigens, and antibodies.

Although the following statement applies to humans, it expresses our view toward the studies of blood groups in animals: "Were it not for the presence of the unwashed and the half-educated, the formless, queer and incomplete, the unreasonable and absurd, . . . the horizon would not wear so wide a grin." [Frank Moore Colby, "Imaginary Obligations."]

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Heterophile Antigens and Their Significance in the Host-Parasite Relationship

C. R. JENKIN

Department of Microbiology, University of Adelaide,
Adelaide, South Australia

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I. Introduction

Heterophile phenomena concern the immunological reactions between antigens and antibody which are apparently nonspecific. For instance, the lysis of sheep erythrocytes by serum from a rabbit specifically immunized with sheep red cells is of a specific nature; however, the observation that goat erythrocytes are lysed by the same serum would appear at first sight to be nonspecific. It is now well known that the reason for this apparent nonspecificity is the existence of closely related antigens on the two types of erythrocytes, such related antigens being known as "heterophile antigens." Ehrlich and Morgenroth (1901a, b) demonstrated the existence of such cross-reacting antigens in the tissues of different and unrelated species of animals. Later Frouin (1907) and Frouin and Lisbonne (1911) showed that the serum obtained from rabbits specifically immunized with the yolk of hens' eggs lysed the erythrocytes of various species of animals. In 1911, Forssman found that saline extracts of certain organs of the guinea pig, such as the kidney,

when injected into rabbits gave rise to antibodies cross reacting with antigens on the surface of sheep erythrocytes. These sheep red-cell hemolysins could be removed by absorption of the antiserum with guinea pig kidney. For further examples of such heterophile reactions the reader is referred to Buchbinder (1935).

The present review will be confined in general to the chemistry of antigens that occur in bacteria and show some degree of immunological relationship with antigens of their potential vertebrate hosts. A discussion of the biological implications of such a relationship between host and parasite will, of course, be largely speculative since our knowledge of factors that determine susceptibility or resistance to infection by a particular parasite within a species of animal is limited. However, sufficient data exist at the present time to warrant serious consideration of the possibility that the sharing of antigens between host and parasite might play an important role in the evolution of the host-parasite association. Such an idea may also serve as a useful theoretical model, amenable to experimentation, to explain the unique susceptibility of certain species of animals, or members within a given species to infection by particular parasites.

Within the last 20 years our knowledge of the chemical structure of the surface of bacteria has increased enormously, and we can now define in chemical terms the antigenic structure of certain macromolecules occurring in the cell walls of both gram-negative and gram-positive bacteria. These advances together with those that have taken place in the field of blood group chemistry form the basis of this review. The reader may feel this approach to be somewhat narrow and overspecialized, but a deliberate attempt has been made, particularly in the first section, the chemistry of heterophile antigens, to present those experimental data which are of a precise chemical nature, and in general to omit reference to papers on the nature of heterophile antigens which give only a vague and indeterminate picture of their chemical structure and their immunological properties. In this manner it is to be hoped that some of the confusion existing in this field will be avoided.

It is unfortunate that there is a tendency among workers in this field to give a specific name to a group of antigens which cross react immunologically, e.g., Eisler-Kagaya antigen (O substance) (Iseki, 1952). This seems to have led to an oversimplified concept of the situation and has been taken to imply that such cross-reacting antigens are identical in their chemical structure but widely distributed in nature.

Most of the antigens under discussion will be polysaccharides. Largely due to the relative ease with which these macromolecules may

be purified and handled compared with proteins, and their more simple structure, considerable advances have been made in our knowledge of the chemistry of these molecules, such that we are able to define in chemical terms the units that lend antigenic specificity to these materials. Nevertheless, it should be borne in mind that shared antigens of a protein nature may have played an equally important role in determining the dynamics of the host-parasite relationship to be discussed.

II. Chemistry of Heterophile Antigens

A. CHEMISTRY OF THE FORSSMAN ANTIGEN

Perhaps the best known heterophile antigen is that described as the Forssman antigen, which is widely distributed throughout various phyla (Forssman, 1911; Buchbinder, 1935). To consider this antigen as a single chemical entity would seem to this writer to be fallacious, since in the great majority of cases the term has been applied to any antigen that, on injection into a suitable animal, will give rise to antibodies cross reacting with surface antigens of the sheep red blood cell. These antibodies are usually measured by a hemolytic assay. It would seem better to regard the Forssman antigen as a group of antigens which, although differing in their general chemical structure, contain common antigenic groupings related chemically to groups occurring on the antigens associated with the cell surface of the sheep erythrocyte. A somewhat similar idea on the nature of these antigens has been expressed by Boyd (1943). It will become apparent from the following discussion that antigens differing in their general chemical structure are capable of giving rise to antibodies cross reacting with antigens of the sheep red blood cell. Indeed, the quest for the true Forssman antigen may be as futile as the search by Diogenes for an honest man. It will be necessary, however, in reviewing the literature, to refer to the term "Forssman antigen," but the non-specificity of its use must be borne in mind in the following account concerned with the chemistry of these antigens.

The original idea that the Forssman antigen of mammalian tissues was lipoidal in nature (Forssman, 1930) was later modified due largely to the work of Landsteiner and Levine (1927) and Brunius (1936), who regarded the Forssman antigen of horse kidney to be a macromolecule consisting of a lipocarbohydrate loosely bound to protein. The whole complex was antigenic, the antigenic specificity being determined by the lipocarbohydrate portion of the molecule. Later, studies by Morgan and Partridge (1936, 1937, 1940, 1941) concerned with the chemistry of the O somatic antigen of gram-negative bacteria, showed

that this antigen derived from the cell walls of *Shigella dysenteriae* (Shiga) stimulated the production of sheep red-cell hemolysins when injected into rabbits. This property was associated with the lipocarbohydrate moiety of this antigen, which has now been shown to have the following broad structure: lipid B-protein-lipid A-polysaccharide (Westphal and Lüderitz, 1954). Similar to the heterophile antigen isolated from horse kidney, the antigenic specificity of this macromolecular complex (O somatic antigen) resides in the carbohydrate portion of the molecule. Following from the work of Bailey and Shorb (1931, 1933a,b, 1934), Goebel *et al.* (1943) isolated and characterized the Forssman antigen from a rough strain of pneumococcus type I. This antigen was shown to be a lipocarbohydrate and termed by them the "F carbohydrate." The polysaccharide portion of this complex was thought to be identical chemically with the C polysaccharide or group specific polysaccharide. The chemical similarity of these two antigens was based on certain analytical figures which unfortunately did not include data on the specific sugars of the carbohydrate chain. Both carbohydrates precipitated with an antiserum against the C polysaccharide at a dilution of $1/10^{-6}$ of the antiserum. The most striking difference between the two materials was in the amounts of carbohydrate required to inhibit the hemolytic reaction between sheep red cells and an antiserum prepared in rabbits against the rough strain of pneumococcus type I. The amount of F polysaccharide required to inhibit completely this reaction was 2 $\mu\text{g.}$, whereas the amount of C polysaccharide required was of the order of 2 mg. In contrast to the C polysaccharide, the F polysaccharide was firmly bound to lipid and it was thought that the chemically bound lipid endowed this material with its unusual chemical and immunological properties. It is difficult, in light of our present knowledge regarding the antigenic properties of closely related polysaccharides, to accept this explanation since the individual sugars of the carbohydrate chains were not known nor the types of linkages along the chain.

It is quite clear from the work on the chemical structure of the polysaccharide antigens of bacteria and blood group substances in respect to their antigenic differences and similarities that quite small differences in the chemical structure of the carbohydrates lend different antigenic specificities to these molecules (Kabat, 1956; Davies, 1960). Further work on the immunological properties of the F polysaccharide and C polysaccharide showed that although these two carbohydrates were related chemically they could be distinguished serologically (Goebel and Adams, 1943). Recently the C polysaccharide has been found to contain mannose, glucosamine, and galactose, and it would be

of considerable interest to know the sugar composition of the F polysaccharide (Smith *et al.*, 1957). At present the best that can be said is that the Forssman antigen isolated from a rough strain of pneumococcus type I is a lipocarbohydrate complex, the carbohydrate portion of which is related chemically to the group specific C polysaccharide.

Isolation of the Forssman antigen from sheep red-cell stroma gave a glycolipid containing 18% hexosamine, 18% hexose, 33% lignoceric acid, and 27% of an unidentified base. The sugars were tentatively identified as chondrosamine and galactose. It is difficult to ascertain from the immunological data given the purity of this antigen since the immunological tests were not sufficiently exhaustive (Papirmeister and Mallette, 1955).

Forssman antigens related to antigens of the sheep red cell may be found in various strains of *Salmonella* of group B (Iseki, 1952). Thus the O somatic antigens of *Salmonella paratyphi* B, *Salmonella stanley*, and *Salmonella heidelberg* produce in rabbits antibodies which cross react with the antigens of the sheep red cell. According to the Kauffmann-White (Kauffmann, 1961) scheme, the O somatic antigens of these strains may be typed as follows: *Salmonella typhimurium* and *S. paratyphi* B (O, 1.4.5.12), *S. stanley*, and *S. heidelberg* (O, 4.5.12). The numbers indicate specific antigenic sites borne on what is thought to be a single but highly branched polysaccharide chain.

Due largely to the excellent work of Westphal and his colleagues, the sugars involved in determining some of the specificities of these large molecules are known (Westphal, 1952; Pon and Staub, 1952; Westphal *et al.*, 1954; Lüderitz *et al.*, 1958; Davies *et al.*, 1958). Other strains of *Salmonella* which are antigenically related to the above strains (i.e., they possess 1.4.12 or a combination of one or more of these specificities together with others) did not show any immunological cross reactivity with sheep red-cell antigens. These experimental results suggested that the factor 5 was responsible for the Forssman specificity of the O somatic antigens of group B *Salmonella*. Further supporting evidence for this was found when a mutant strain of *S. typhimurium* lacking factor 5 would not absorb out hemolysins for the sheep red cell from a rabbit antiserum, whereas the parent strain possessing 5 did so (Iseki and Sakai, 1952). The sugar involved in determining the 5 specificity has recently been shown to be O-acetylgalactose (Kotelko *et al.*, 1961).

B. BLOOD GROUP ACTIVITY OF BACTERIAL ANTIGENS

Before the era of sulfonamides and penicillin, antiserum provided the only specific means of combating pneumococcal infection (Tonley and

Wilson, 1955). It was observed following such therapy that the injection of type XIV antipneumococcal horse serum into patients suffering from type XIV pneumococcal infection frequently led to hemolytic complications. Titration of the antiserum against human erythrocytes showed that this serum contained agglutinins against the erythrocytes of all four major blood groups. This cross reaction of blood group substances with type XIV pneumococcal polysaccharide has proved a useful tool in the study of the structure and immunological specificity of blood group substances. Mild acid hydrolysis of blood group substances although destroying the specific blood group activity increases the capacity of these materials to precipitate with the type XIV pneumococcal antiserum. This observation is of interest since it indicates that only limited degradation of these materials had taken place as a result of the acid treatment (Kabat *et al.*, 1948). Subsequent dialysis of materials subjected to mild hydrolysis showed that the greater proportion of this material was nondialyzable. From studies regarding the inhibition by simple oligosaccharides of the reaction between blood group substances and their specific antibodies it is apparent that the antigenic specificities of these materials are determined by relatively small structural units (Kabat, 1956; Morgan, 1960). The type XIV pneumococcal polysaccharide has been shown to contain galactose and *N*-acetylglucosamine units in the ratio of 3:1 (Goebel *et al.*, 1939). Recently the presence of glucose in this polysaccharide has also been demonstrated (Heidelberger *et al.*, 1954). The ability of blood group substances to react with the type XIV pneumococcus antiserum was found to be strongly inhibited by *O*- β -*D*-galactopyranosyl-(1,4)-*N*-acetylglucosamine, which has led Watkins and Morgan to suggest terminal units of this disaccharide in blood group substances. An oligosaccharide lacto-*N*-neotetraose having the structural unit *O*- β -*D*-galactosyl-(1,4)-*O*- β -*N*-acetylglucosaminoyl-(1,3)-*O*-*D*-galactosyl was an even more effective inhibitor than the previously mentioned disaccharide unit (Watkins and Morgan, 1962). These results would suggest that a unit of similar structure occurs in the type XIV pneumococcus type-specific polysaccharide. Heidelberger *et al.* (1954) have shown that most of the sugars in the type XIV pneumococcus polysaccharide chain are linked by β linkages. The terminal galactose unit is thought to be attached to galactose residues in the chain with the result that one would expect a repeating unit of 1 *N*-acetylglucosamine and 2 galactoses along the main chain with perhaps occasional units of glucose. From the studies of Kabat (1956) it is apparent that only a portion of the antibody present in the pneumococcus type XIV antiserum cross reacts with both the type-specific polysaccharide and blood group substances

These studies serve to emphasize the point that antigens, though they may differ in their general chemical structure, may yet show immunological cross reactivity because they share similar small structural units which may be composed of relatively few sugars or, in the case of proteins, amino acids.

Perhaps the most extensive study of this type has been carried out by Springer *et al.* (1961) who reported on the blood group activities of 282 Gram-negative bacteria. The blood group activity of the bacteria was assayed by a hemagglutination inhibition technique, in which serial dilutions of heat-killed bacteria were added to 4-8 minimum hemagglutinating doses of an antiserum specific for one of the blood group antigens under test. After a period of incubation the specific blood group type erythrocytes were added and the degree of inhibition of agglutination of the red cells measured. Bacterial suspensions not giving inhibition at a concentration of 2.5 mg./ml. were considered to be inactive. Nearly 50% of the strains examined showed blood group activity and in some cases two or even all three blood group activities were associated with a particular species. The most commonly occurring specificities were O(H) and B. Blood group active bacteria are capable of eliciting high titers of specific antibody against the corresponding blood group antigens (Springer *et al.*, 1959). Bacteria having specific B blood group activity were capable of absorbing completely anti-B agglutinins.

The specificity of blood group antigens appears to be determined in part by the terminal sugars of the polysaccharide. From inhibition studies using simple sugars and disaccharides it has been found that the specificity of A substance is determined by *N*-acetylgalactosamine, B substance by *D*-galactose, the terminal unit being probably *O*-*D*-galactosyl-(1,3)-*D*-galactose (Watkins and Morgan, 1962) and O(H) specificity by fucose. Thus it seems reasonable to suggest that bacteria possessing these activities also have these sugars as terminal units on some of their side chains. The mere presence of these sugars in a polysaccharide chain does not necessarily imply that the material possesses blood group activity. The position of the sugars in the chain and its relationship to other sugars, its configuration, and also the nature of the linkage all play some role in determining the specificity of the antibodies produced.

None of the *S. typhimurium* strains examined by Springer and colleagues possessed blood group A activity, whereas Iseki (1952) found that *S. typhimurium* (1.4.5.12) was capable of absorbing out completely A activity from a particular antiserum. A mutant strain lacking factor 5, produced by passaging the original 1.4.5.12 strain in a medium containing factor 5 antiserum, would not remove these agglutinins (Iseki and Sakai 1952). Other strains of *Salmonella* possessing factor 5 as part of

the antigenic structure of their O somatic antigen were also capable of absorbing out the anti-A agglutinin. It is interesting to note that Eisler (1931) observed that immunization of rabbits with *S. paratyphi* B (O, 1.4.5.12) resulted in the formation of agglutinins for erythrocytes of blood group A. Recent chemical studies have shown that the antigenic determinant of factor 5 is O-acetylgalactose (Kotelko *et al.*, 1961). In strains of *Salmonella* apparently lacking the factor 5, the terminal galactose of the chain is not acetylated. Presumably the antigenic relationship between blood group A substance and this bacterial polysaccharide resides in its possession of O-acetylgalactose or a closely related structure as a terminal sugar of one of its side chains. It is interesting that Witebsky *et al.* (1935) have also shown that the type I pneumococcus possesses blood group A activity. In this strain an important antigenic determinant of the type-specific polysaccharide is O-acetylated (Avery and Goebel, 1933).

In the studies of Springer *et al.* (1961) the bacterial strains were all heated prior to their being tested for blood group activity. Since factor 5 is labile to heating under the conditions employed by these workers, this might account for their negative findings with regard to blood group A activity compared with the results of the Japanese workers. In many instances the blood group activity of certain strains bore no relationship to the structure of its O somatic antigen according to the Kauffmann-White scheme. For instance *Escherichia coli* (O, 128), *E. coli* (O, 117), *Salmonella atlanta* (O, 13.23), *Salmonella arizona* (O, 9) all possess O(H) activity. The determinant for this specificity is fucose, a sugar that occurs in the polysaccharide moiety of the O somatic antigen of all the above strains of bacteria. The existence of this cross reaction would suggest that antigenic sites ending in terminal fucose in addition to those given by the Kauffmann-White scheme, occur in these bacterial polysaccharides. Similarly blood group B activity has been found in *E. coli* (O, 86), *S. arizona* (O, 21), and *salmonella* having the somatic antigen O, 43. The assumption is implied, however, that the blood group activity and the antigenic determinants of the O somatic antigen are structural features of the one polysaccharide molecule. In some instances this is almost certainly so, but it may not necessarily always be the case. For instance G. Bo and G. C. Nava (1958) as cited by Davies (1960) showed that from *Salmonella newington* (O, 3.15) may be obtained a polysaccharide having only the 3 specificity. The cell wall of bacteria is often regarded as a rigid structural feature showing relatively little change. From consideration of the rate of division of bacteria under normal cultural conditions it is obvious, however, that considerable cell

wall synthesis is taking place at all times. It is possible, therefore, that the polysaccharide of the O somatic antigen might be obtained from bacteria in various stages of synthetic completion. Such differences would almost certainly lend themselves to the expression of a varied antigenic structure, the stable antigenic configuration and, perhaps, the greater part of the polysaccharide being represented by the completely synthesized polysaccharide of the O somatic antigen. Differences in the rate of synthesis of polysaccharides may be determined by various environmental conditions which are difficult to control under normal conditions employed for bacterial culture. Such difference in synthetic rate and availability of certain essential nutrients might well determine the qualitative differences observed in the blood group activity of a particular strain from time to time (Springer *et al.*, 1961). In this regard it is interesting to note the observations of Iseki (1952) on changes in blood group activity of bacteria passing from the smooth to rough phases. The rough variant of a strain of bacteria is known to have a much simpler polysaccharide, in respect of the variety of sugars that it contains, than the smooth strain. In particular during the transition from the smooth to the rough phase there is a loss of the 3,6-dideoxyaldohexose sugars and methylpentose sugars with a corresponding disappearance of many of the antigenic specificities of the O somatic antigen of the smooth strain (Westphal *et al.*, 1953; Mikulaszek, 1956; Lüderitz *et al.*, 1960; Davies, 1958; Crumpton *et al.*, 1958). Various degrees of roughness have been defined with subsequent differences in the sugar constituents of the cell wall polysaccharides. The strain of *Shigella dysenteriae* studied by Iseki and Sakai (1952) possessed, in the smooth phase, antigenic groupings cross reacting with a number of blood group antigens. During the transition from the smooth to rough phases some of these activities were lost with the appearance of new specificities.

It is obvious in all these studies that unless precautions are taken to ensure that the bacterial population as a whole is in the one or the other phase, i.e., either smooth or rough, polysaccharides may be obtained having differing antigenic properties, which might erroneously be assigned to the one polysaccharide molecule.

III. Heterophile Antigens of Viruses

The two viruses of typhus fever, *Rickettsia prowazekii* and *Rickettsia mooseri*, both stimulate the production of agglutinins acting on *Proteus* OX19. These observations were first made by Weil and Felix (1916) who isolated from the urine of a patient with typhus a strain of proteus

which was agglutinated not only by that individual's serum but by the serum of a number of other typhus patients. The most highly agglutinable of them and the most specific for typhus was the *Proteus* OX19 strain (Felix, 1916). The ability of the sera from typhus patients to agglutinate this strain has been a valuable aid in the diagnosis of typhus (Weil-Felix test). In 1946, Bendich and Chargaff isolated from the proteus strain a protein lipocarbohydrate complex that reacted with both a typhus antiserum and an antiserum to the proteus strain. The carbohydrate contained galactose, glucose, mannose, and acetyl glucosamine. Glucosamine appeared to be the major sugar constituent. It would seem highly likely from these results that *R. prowazekii* contains a polysaccharide of a similar nature or at least a polysaccharide sharing some of the antigenic sites of the proteus polysaccharide. It would be interesting to know if immunization with the *Proteus* OX19 strain resulted in any significant degree of protection against *R. prowazekii*.

Another disease of man, glandular fever, which is probably due to a virus, may be characterized by the appearance in the blood of the patients of a high titer of antibodies to sheep erythrocytes (Paul and Bunnell, 1932). These heterophile antibodies may be absorbed out by ox red blood cells but not by guinea pig kidney (Barrett, 1941; Kilhane and Steigman, 1942).

IV. Heterophile Antigens and Their Significance in Host-Parasite Relationships

In order to discuss the possible advantage of the sharing of antigens between host and parasite in the initiation of infection, it will be necessary to define the type of parasite where it is felt that such a relationship would favor its residence in the host. Only those parasites that at some period in their life cycle come into intimate contact with the tissues and fluids of the host will be considered. From this group of parasites it is intended to select as examples bacteria and viruses. Many of these organisms have been shown to possess antigens cross reacting with antigens of their vertebrate hosts, and some details are known regarding the host reaction toward them. The hypothesis which is to be discussed later (Rowley and Jenkin, 1962) has also been advanced on a much broader basis by Sprent (1959) in a stimulating and provocative essay entitled "Parasitism, Immunity, and Evolution."

A. HOST IDENTIFICATION OF FOREIGNNESS

In discussing the host response to the parasite, one has to consider the possible means by which the host is able to recognize the parasite as

foreign and distinct from "self." The classical studies of Metchnikoff and his colleagues on the response of various invertebrates toward the implantation of particles in their tissues demonstrated quite clearly that a particular type of cell was involved in the host reaction against the implanted material. These cells were attracted toward the damaged area and proceeded wherever possible to ingest the introduced substances (Metchnikoff, 1893). It was quite obvious that simple animals such as the Coelenterata and Porifera could identify self from nonself. Extending these studies to the vertebrates it was shown that these animals too possessed a cellular mechanism consisting of two types of discriminating cells—the macrophage and the microphage (polymorphonuclear cell). Further experiments soon established that these phagocytic cells were not only capable of identifying injected foreign material but could also distinguish between dead and damaged nonfunctional cells. To illustrate this point further, an excellent example of this particular discriminative ability may be found in the experiments of Clark and Clark (1928). These workers observed the circulation of blood through the capillaries of the tail of a tadpole (*Hyla versicolor*). The erythrocytes were seen to contact macrophages, slide over the surface of these phagocytic cells, and continue along the capillary; at no time was ingestion of an erythrocyte observed. However, following a slight degree of trauma in the neighborhood of the capillary bed, damaged erythrocytes were seen to contact and this time adhere to the surface of the phagocytic cell. One of the most striking changes observed as a result of the induced trauma was the apparent "stickiness" of the damaged red cells. Following adhesion to the macrophage the erythrocyte was ingested.

More recently Salt (1956) has found that the eggs or larvae of an ichneumon fly (*Nemeritis canescens*) injected into adults of the stick insect (*Carausius morosus*), an abnormal host for the parasite, elicited two types of defense mechanisms. Materials thought to be melanin were deposited on the surface of the parasites and these were finally ingested with hemocytes. *Nemeritis* larvae did not live longer than 48 hours in this host. The hemocytes of this insect were also capable of ingesting to a marked degree other colloidal particles such as carbon and were also observed to congregate, but not to a marked degree, around glass rods inserted into *Carausius morosus*. These few simple examples illustrate the discriminative properties of the phagocytic cells of various animal species and serve to emphasize that this recognition mechanism is an extremely primitive property of all organized metazoa.

During the period that Metchnikoff and his colleagues were making great contributions to the study of the cellular responses of animals

to foreign particles, von Behring and Kitasato (1890) showed that the sera of animals that had received repeated injections of nonlethal doses of tetanus toxin had acquired the property of specifically neutralizing these toxins. These serum factors, later termed antibodies, are a characteristic reaction of the body to the injection of many different organic molecules. The discovery of the bactericidal properties of serum tended to split the field of study concerned with the defense mechanisms of animals into two schools of thought, one of which considered that the phagocytic cells of the body were all-important and the other that humoral factors played the major role. For the purpose of the present discussion it is not necessary to trace the development of these two concepts but to deal more specifically with the known ways in which phagocytic cells are able to recognize bacteria. In this manner it will become clear that these two systems are, in fact, interdependent, the functioning of one depending on the presence of the other.

Studies concerned with the phagocytosis of bacteria by both polymorphonuclear cells and macrophages have indicated the importance of antibody in promoting phagocytosis. In the absence of antibody little or no phagocytosis takes place (Robertson and Sia, 1927; Rowley and Whitby, 1959; Cohn and Morse, 1959; Jenkin and Rowley, 1959; Rowley, 1960; Jenkin and Benacerraf, 1960; Benacerraf *et al.*, 1959; Mackaness, 1960; Hirsch and Church, 1960). From such results it is tempting to speculate on the possibility that antibody is required for the recognition of all that is foreign and this naturally includes effete and damaged self-components. We know that the phagocytosis or elimination of antigenic material from the circulation of an animal is greatly enhanced in the presence of specific antibody (Weigle, 1961), and recent studies would suggest that even supposedly inert colloidal and synthetic particles require the presence of serum before they are phagocytized (Fenn, 1921; Nelson and Lebrun, 1956; Potter and Stollerman, 1961; Jenkin and Rowley, 1961; Murray, 1963). In the case of particles such as bentonite and carbon, the rate of elimination may depend on the adsorptive properties of the particle surface for serum proteins, including antibodies, and this may be visualized as a nonspecific combination of the particle with the serum protein rather than the combination of the particle with a serum protein specific for carbon or bentonite. Thus it would seem that in order for most particles, living or dead, to be recognized as foreign by phagocytic cells, they must combine, in general, with serum proteins of a specific nature. The ability of the animal to produce a large number of molecules each having a high degree of individual specificity for other foreign molecules with which the animal

comes into contact is clearly of great advantage to the individual, since it allows the development of a very fine discriminating mechanism which would be of potential advantage in resisting invasion by parasites. It is tempting to speculate on the possibility that the immune mechanism may have evolved in the metazoa as a system primarily concerned with the discrimination of functional self from nonfunctional self. Once this ability to identify foreignness had been established, it could clearly operate as a host-defense mechanism against the invasion of the host by parasitic organisms.

At present there is a great deal of discussion as to the possibility of phagocytic cells recognizing foreign material in the absence of specific humoral factors. This type of defense or recognition has been termed "cellular immunity" and suggests that for immune reactions, such as the rejection of a homograft, delayed-type hypersensitivity, and immunity to certain bacteria, the cells are endowed with the specific property of recognition in the absence of a humoral factor. The main reason for this suggestion is that the capacity to exhibit these immune phenomena can only be transferred from one animal to another by cells and not by serum. It is very doubtful that a specific cellular immunity mechanism devoid of antibody exists, and recent experiments would suggest that a humoral factor is involved in the homograft reaction (Stetson, 1959; Najarian and Feldman, 1962; Koprowski and Fernandes, 1962). The role of antibody in delayed-type hypersensitivity and rejection of the homograft has recently been discussed by Karush and Eisen (1962).

B. HOST SUSCEPTIBILITY TO PARASITES

If the argument is accepted for the participation of antibody in the recognition of foreignness and if we consider parasites that are capable of multiplying in the nutritional environment of several different possible hosts, then it follows that the susceptibility of an animal to infection could be attributed to one of two causes; either it is unable to make antibodies to antigens of the parasite or there is a delay in the production of antibody and perhaps an insufficient antibody response. In contrast, resistance of a particular animal to infection would lie in the ability of the host to produce antibody to antigens of the parasite and in the speed and magnitude of the response. This is especially true for parasites which during part of their life cycle are in intimate contact with the antibody-forming apparatus of the animal for sufficient time to initiate an antigenic stimulus. Into this category would fall most pathogenic bacteria and viruses.

C. POSSIBLE REASONS FOR THE INHIBITION OR DELAY OF ANTIBODY RESPONSE

Antigens that are likely to come into frequent contact with cells potentially capable of producing antibody during the embryonic development of the animal elicit no antibody response when presented to the immunologically mature animal. Into this category may be placed certain, but by no means all "self"-antigens, and antigens, particularly those of living tissue cells, that are introduced artificially at a sufficiently early period in the development of the animal. The experiments that led to the concept of immune tolerance were based on the establishment of living foreign tissue cells in the tissues of a suitable recipient animal. One might, therefore, regard these artificially implanted cells as parasites. If similar cells are grafted into an animal that is immunologically mature they are rejected after a period of time. A second injection of cells into the same donor would lead to an accelerated graft rejection. The speed of the primary rejection is correlated to some extent with the degree of antigenic disparity between the donor and the recipient of the tissue cells (Medawar, 1954; Billingham *et al.*, 1954, 1956; Gorer, 1956). The closer the antigenic resemblance between the host and the recipient animal, the longer is the graft tolerated.

Evidence has been presented in the first half of this review to show that bacterial antigens may cross react with antigens of their potential hosts. It is conceivable that in these circumstances the antibody response of the host to these antigens would be either absent or diminished.

The parasitic bacteria and viruses present a number of difficult problems when considering the importance of antigenic relationships between host and parasite and their effects on susceptibility to infection. First, if a potential host population has been isolated from a particular pathogen, it is likely that its members would be highly susceptible to the initial attack of the parasite, even though they were capable of producing antibodies. In such instances the immune response would be of a primary nature and hence there might be a lag of 4-5 days before sufficient antibody was produced to give measurable protection. Most bacteria and viruses are capable of very rapid multiplication in a susceptible host and it is conceivable that an overwhelming infection would result before the antibody was effective. There are many well authenticated examples of the severity of infection among populations on first contact with a new infectious agent. When measles was introduced into the Sandwich Islands practically all the population was infected and many thousands died (Burnet, 1959). Perhaps one of the best examples of the severity that the first impact of a parasite may

have on a susceptible animal is the relatively recent epidemic of myxomatosis among wild rabbits. Vaccination of a normal, highly susceptible population previous to exposure to the virus results in very good protection—yet in the field, the virus caused death in almost 100% of the rabbits. Obviously the wild rabbit population is not susceptible because of its inability to produce antibodies to the virus, but its susceptibility must, to some degree, be correlated with the speed of antibody response after contact with the virus, and the rate of viral replication before the antibody can function as an antidote, and so prevent further spread of the pathogen. The extremely small percentage of wild rabbits that do survive the infection are immune to further attack (Fenner, 1959). For many parasitic forms the host need only survive sufficient time to allow the parasite to reproduce, or, in some instances where a vector is required for transmission, to survive long enough to allow successful transfer to a fresh host. Once this happened it is immaterial to further generations of parasites that the initially infected host survives. Presumably there will have been very strong selective forces operating in the context of the host-parasite relationship to favor prolonged host survival, and persistence of those parasites that can carry out part of their reproductive cycle in situations where the immune reaction mediated by antibody is unable to function. Examples of such parasites are those that are capable of growing and multiplying inside cells, such as viruses, and certain bacteria such as *Mycobacterium tuberculosis* and strains of *Brucella* and *Salmonella*. All the latter strains of bacteria may persist in their hosts for very long periods of time in the face of an intense immune reaction. In such situations the balance between the host and parasite is in all probability maintained by the ease with which the bacteria can survive and multiply within cells and the rate at which they are destroyed, should they escape from their intracellular environment.

In this connection the heterogeneity of phagocytic cells with respect to their ability to kill bacteria has recently received much emphasis (Jenkin and Benacerraf, 1960; Mackaness, 1960; Pavillard and Rowley, 1962).

There can be no doubt that in most infections, certainly those with bacteria and viruses, the level of antibody at the time the parasite infects the host is of extreme importance in determining the host's susceptibility or resistance to infection. We have three clear and well-defined states which illustrate this point. First, individuals suffering from agammaglobulinemia are extremely susceptible to bacterial infection and require constant antibiotic therapy to maintain them (Good *et al.*, 1962);

second, we have the random population at large which represents individuals of high and low resistance to a particular infection and in which contact with the parasite results in infection recognized by various degrees of severity; and finally, we have the third example, a population that has been artificially immunized against the pathogen and that may be protected against the initial attack of the parasite to such an extent that it may apparently disappear from the initially susceptible population. Theoretically such a situation could occur with certain specific human pathogens.

We must now consider the state where an animal may be so highly susceptible to infection by a species of bacteria that infection with as little as from 1 to 10 organisms may lead to death of the animal, and where, in some cases, even previous immunization leads to no protection. From the latter we must exclude immunization procedures whose efficacy is in doubt (typhoid and cholera vaccines), since the fault here probably lies in the preparation of the vaccine rather than in a failure of a randomly bred population to respond to it by producing antibodies protective against the living organism. Such systems of extreme susceptibility are best examined in the laboratory, where these populations of animals to some extent may be considered experimental artifacts, since they have been constantly selected and maintained under artificial conditions (in many instances because of this peculiarity). Obviously, under natural conditions either such animals would have been eliminated by the parasites, or there would have been such selection pressure within the stock as to favor the emergence of a more resistant animal. This does not mean, however, that examples of our extremes may not exist among our randomly bred populations; indeed, there is a great deal of evidence to suggest that they do, and it is this latter group which is of particular interest to us.

From what has preceded it is obvious that antigens closely related to host antigens may not elicit an antibody response on injection into that animal. It has also been shown that bacteria may possess antigens showing some degree of relationship to antigens of their potential vertebrate host, and it is the purpose now to discuss the importance, if any, of this relationship in determining susceptibility and resistance to infection.

Some consideration must be paid to the nature of the cell surface of bacteria and the relationship of the surface antigens, the one to the other. We know little or nothing regarding the fine chemical structure of the cell wall, which is a complex mixture of antigens. We do know, however, that certain antigens appear to be dominant and are frequently referred to as capsular antigens. There may be, as in the *Pneumococcus*,

a surface covering of type-specific polysaccharide; it is also possible that the surface structure may be such that certain antigenic groupings, owing to their number and steric configuration, may interfere with the reaction of antibodies at other, different specific antigenic sites. If this were so, then, in the absence of specific antibody for some dominant surface grouping, only a limited number of antibody molecules against other groups could attach to the cell surface. Within certain limits the number of antibody molecules associated with the bacterial cell surface will determine the rate at which the particle is removed from the circulation of the animal by the fixed phagocytic cells of the liver and spleen. Hence so far as removal and destruction of bacteria is concerned, the number of antibody molecules that can react with the cell is of the utmost importance. There are a number of examples where it is known that certain antigenic sites inhibit in this manner. For instance factor 5, part of the O somatic antigen of *Salmonella typhimurium* (O, 1.4.5.12) will inhibit the reaction of antibodies with antigenic sites 4 and 12 (Kauffmann, 1954). Further examples of inhibitory antigens are the K and L antigens of *E. coli* strains and the Vi antigen of some *E. coli* and *Salmonella* strains which inhibit the reaction of antibodies with their O somatic antigens (Springer *et al.*, 1961; Felix, 1952; Kauffmann, 1954; van Oye, 1961). Whether these antigens can be regarded as capsular antigens in the sense of an envelope around the O somatic antigen or whether they react in a similar manner as does factor 5 of the *S. typhimurium* O antigen is not known. However, it is obvious that under certain conditions a specific antigen or antigenic grouping can dominate the cell surface. Such a relationship of antigens to one another would be of extreme importance in determining pathogenicity if the dominant antigen of the parasite should resemble a host component sufficiently not to be antigenic in this host. The quantitative relationship of an antigen of this kind to other cell surface antigens could conceivably determine the rate at which the parasite is destroyed. This quantitative relationship is a factor which cannot be too strongly stressed since the mere possession of a potential antigen or antigenic group by the parasite related to a host tissue component would not be important in determining the outcome of the interaction between host and parasite, in view of the ability of the host to form antibodies reacting with the many other nonself antigens.

V. Experimental Data Supporting the Role of the Antigenic Relationship of Host and Parasite in Determining Host Susceptibility to Infection

A. SUSCEPTIBILITY AND RESISTANCE OF CERTAIN MOUSE STRAINS TO *Salmonella typhimurium* INFECTION

Recent studies concerned with the phagocytosis of bacteria by mouse peritoneal macrophages and polymorphonuclear cells have stressed the importance of antibodies in determining not only the rate of phagocytosis, but also the rate of intracellular killing of the ingested bacteria (Rowley, 1958; Jenkin, 1963a,b). Such studies have been conducted in strains of mice either susceptible or resistant to infection with *S. typhimurium*, a natural pathogen of mice. They have suggested that the susceptibility of mice to infection by this parasite lies in an antigenic similarity between the host and parasite resulting in either inhibition or alteration in the rate of production of antibody against the cross-reacting antigen (Rowley and Jenkin, 1962; Jenkin, 1962). It was found quite early in this work that the rates of phagocytosis of avirulent and virulent strains of *S. typhimurium* by peritoneal macrophages harvested from a susceptible strain of mouse differed markedly (Jenkin and Benacerraf, 1960). Virulent strains treated with serum from the susceptible mouse were always phagocytized poorly, and of the phagocytized bacteria a high percentage survived and multiplied intracellularly. In contrast, the avirulent strains were always ingested more rapidly and few survived. In spite of the suggestion that resistance to this infection observed in some strains of animals resided in some intrinsic property of the resistant host's macrophages (Furness and Ferreira, 1959; Oakberg, 1946), no evidence for this could be found. In fact, it has been repeatedly shown that macrophages from a strain of mouse, naturally resistant to *S. typhimurium*, and from rats were no better than macrophages from the susceptible host in dealing with the parasite if they were presented with the bacteria pretreated with serum from the susceptible animal (Rowley and Jenkin, 1962). In contrast, macrophages from the susceptible host proved just as efficient a bactericidal system as did macrophages from the resistant animal if the bacteria had first been treated with serum from the naturally resistant animal. These findings are further supported by the observations that a high percentage of the challenged mice survive if naturally susceptible animals are challenged with a normally lethal dose of bacteria that have been opsonized with serum obtained from animals, such as rats, normally resistant to this infection (Jenkin and Rowley, 1959).

Basically these findings are analogous to the results obtained from experimental infections with *Plasmodium lophurae* in two strains of ducks, one naturally resistant and the other susceptible to the parasite (Ben-Harel, 1951). They also support the findings of Robertson and Sia (1927) that resistance of the dog to pneumococcal infection could be correlated with the presence of serum opsonins, rather than with any unusual property of that animal's phagocytic cells.

Extending the above observations it was found that a proportion of the opsonic activity of serum against the parasite *S. typhimurium* could be removed by absorbing the serum with tissues from a susceptible host (Jenkin, 1962). Further it has been found that if a particular strain of mouse naturally resistant to this infection is immunized with red blood cells from the susceptible host, the opsonic activity of its serum is increased against the virulent strain of *S. typhimurium*. This increased opsonic activity could be removed from the serum by absorbing with erythrocytes from the susceptible mouse (Jenkin, 1963a).

Unlike many other experimental infections of mice, it is generally considered almost impossible to protect mice against virulent *S. typhimurium* infection by prior immunization either with a heat-killed vaccine, or by nonspecific means, such as with lipopolysaccharides from Gram-negative bacteria, or by prior treatment with living *Bacillus Calmette-Guérin* (BCG). The protection afforded by such treatments may in general be measured by increased survival time of the immunized population rather than by any great differences in over-all mortality. However, if susceptible mice are first treated with a living culture of the avirulent strain and some time afterward are challenged with virulent *S. typhimurium*, they may be then 100–10,000 times more resistant to infection than untreated controls. The protection afforded by pre-challenge with avirulent organisms is associated entirely with changes in serum antibodies and may be passively transferred to other mice. Likewise animals that survive a challenge with the virulent strain are resistant to reinfection on subsequent challenge (Jenkin and Rowley, 1963). Such convalescent mice may harbor the organism in the liver, spleen, and lymph nodes for long periods of time. Some preliminary studies on this "carrier" state have already been published by Hobson (1956, 1957a,b). At first sight the ability to protect mice against this infection might appear contradictory to the general hypothesis of susceptibility resulting from failure of an antibody response owing to shared antigens between host and parasite. It is interesting, therefore, to record that Rowley (1963) has found that the red cells of these carrier mice are Coombs positive suggesting the production of autoantibody.

B. SUSCEPTIBILITY OF TURKEYS TO ROUS SARCOMA VIRUS

Some of the most interesting data on the significance of shared antigens between host and parasite in determining host susceptibility to infection have been published by Harris (1956) concerning the establishment of the Rous sarcoma virus, a parasite of chickens, in turkeys which are normally resistant. In experiments concerned with the production of tolerance to cells of foreign species, turkey embryos were injected intravenously with adult chicken blood (Simonsen, 1955). Two out of three of these turkeys, which had been injected with chicken blood on the thirteenth day of incubation and subsequently challenged at 8 weeks of age by intramuscular inoculation with cell-free virus agent, developed sarcomas at the inoculation site (Simonsen and Harris, cited by Simonsen, 1955).

In a more detailed extension of this work, Harris (1956) found that tolerance to the Rous sarcoma virus could be transferred to turkeys by the injection of the cellular elements of chicken blood into turkey embryos. Turkey embryos so treated, when challenged at 5-6 weeks of age, were found to be susceptible to the viral agent. The time period in the life of the turkey poult during which it was possible to influence its tumor virus susceptibility was short, since by 72 hours after hatching the immune mechanism of the turkey against the parasite was established. The antigens responsible for producing susceptibility of turkeys to the Rous sarcoma virus were stable to freeze-drying and relatively stable to heating at 55°C. for 40 minutes, though the potency of the antigenic mixture was considerably reduced by this treatment.

It is interesting to note that blood from older chickens is capable of neutralizing the virus for chicks. If, indeed, as these results strongly suggest, the chicken and Rous sarcoma agent share common antigenic components, then the ability of blood from ageing chickens to neutralize the virus would suggest that the serum factors involved might be of the nature of autoantibodies. Taken together with the observations of Rowley (1963) that mice infected with *S. typhimurium* develop a positive Coombs test, this would suggest that autoantibodies in themselves do not necessarily result in disease. The antigens conferring tolerance in turkeys to the Rous sarcoma virus were found to be nonspecific, in that blood from different strains of fowls, pigeon blood, guinea pig and sheep erythrocytes, human group A cells were all active, whereas rat and human group O cells were not. The apparent nonspecificity of the antigens is probably due to a common antigen or antigen grouping associated with all these various materials which is in reality quite a specific moiety.

Fractionation of soluble cell-free extracts of chicken erythrocytes suggested that the material was a polysaccharide (Harris and Simons, 1958) related to blood group A substance. The latter conclusion was based on the ability of the unknown polysaccharide in relatively low concentrations to inhibit the agglutination of human group A cells by a specific antiserum.

More recently Dourmashkin *et al.* (1962) have suggested, with some supporting evidence based on electron microscopy of the Rous sarcoma agent, that the virus may contain an outer membrane derived from the host cell membrane. With regard to this latter work it is extremely interesting to note the early observations of Holtman (1939) who found that strains of bacteria such as *Salmonella typhi* when grown on media containing the Forssman antigen, or in collodion sacs implanted in the peritoneal cavity of guinea pigs, acquired this heterophile antigen. From the nature of his experiments, which involved repeated subculture in media that contained no Forssman antigen, it is unlikely that the demonstration of the presence of this antigen in the bacterial cell could arise from carry-over of antigen from its original media. Recently Collins and Rowley (1963) have confirmed these results in a somewhat different system. These latter workers have found that *Salmonella enteritidis* grown on a medium containing mouse tissues acquired an antigen (or antigens) which cross reacted with antigen (or antigens) of the mouse red blood cell. Great care was taken throughout these experiments to avoid any possibility of antigen carryover from the medium. These observations regarding the expression of apparently new antigenic specificities by bacteria when grown in a rich medium are obviously of great importance in relationship to pathogenicity, and we should bear in mind that potentially pathogenic strains, which have been maintained by growth on artificial media, may differ significantly in their antigenic structure compared with the same bacteria growing in their hosts. It is a well recognized fact that many pathogens may lose their virulence when maintained under laboratory cultural conditions, though this loss may be corrected by animal passage.

Perhaps the best evidence for the expression of new antigenic specificities by a potential pathogenic organism growing *in vivo* has been produced by Burrows and Bacon (1956). They found that virulent and avirulent strains of *Pasteurella pestis*, when grown on agar slopes at 28°C., were both susceptible to phagocytosis by mouse polymorphonuclear leucocytes. However, after a short period of *in vivo* growth, virulent organisms acquired the ability to resist phagocytosis by these cells (Burrows and Bacon, 1954). The change rendering these bacteria

resistant to phagocytosis could be brought about *in vitro* under certain well-defined conditions, though according to these workers great difficulty was encountered in obtaining reproducible results. The ability to resist phagocytosis occurred in the absence of any visible capsulation and could be shown to arise from the expression of two new surface antigens termed W and V (Burrows and Bacon, 1956). Antisera produced in rabbits and containing antibodies to the V and W antigens rendered normally resistant bacteria sensitive to phagocytosis. Resistant organisms obtained from moribund mice show very poor antigenicity in the mouse. It would be extremely interesting to know the chemical nature of these antigens and to determine whether they have any cross reactivity with mouse tissue components.

C. RECURRENT INFECTIONS IN HUMANS

In certain instances it has been found that individuals suffering from recurrent bacterial infections are lacking or deficient in the plasma proteins usually associated with antibacterial immunity. These immunological deficiency diseases have been amply reviewed by Good *et al.* (1962). However, it has not always been possible to place certain patients whose resistance to infection is low into the various categories that characterize immune globulin deficiency, and it is these individuals that are of particular interest.

Kempe (1960) studying generalized vaccinia gangrenosum has observed that patients with this disease have normal amounts of γ -globulin and respond adequately to immunization with other antigens. However, he was unable to find any circulating antibody against the vaccinia virus and at present passive administration of specific antibody from other individuals actively immunized against this agent has been found to be the only beneficial treatment.

Quie and Wannamaker (1960) have also found that children who have suffered from numerous recurrent staphylococcal infections lack antibodies which are inhibitory of the Muller phenomenon. These patients were found to be immunologically competent in all other respects.

It is tempting to speculate that in these otherwise normal individuals their lack of immune response to specific agents may be due to the sharing of antigenic components between the host and parasite.

As a corollary to the observations that certain bacteria possess blood group antigens one might expect, for instance, that individuals of blood group A, and hence possessing the isoantibody B, would be better protected against infection by an organism having blood group B

activity than would individuals of blood group B. Clinically, of course, this would be an extremely difficult matter to prove and it has been suggested (Springer and Williamson, 1962) that no such correlation exists. However, as stated previously, resistance or susceptibility of an individual to a bacterium containing a blood group active antigen would depend on the quantity of antigen present at the bacterial surface. Recently Pavillard *et al.* (1963) have found that sera from individuals of blood group O possessing the β -isoagglutinin were highly opsonic for *E. coli* (O, 86), an organism having blood group B activity. The opsonin could be absorbed out with blood group B cells together with a corresponding fall in the β -isoagglutinin titer of the serum. These experiments leave little doubt that β -isoagglutinins may act as opsonins for bacteria possessing blood group B activity. The opsonized bacteria are rapidly phagocytized and killed by cells of the reticulo-endothelial system. These experiments suggest that a pathogenic organism, having a dominant surface antigen cross reacting with a blood group antigen, would be correspondingly more virulent for individuals possessing that antigen than for those lacking it and hence having the complementary isoagglutinin. Further examples of this type could be quoted but unfortunately, apart from their suggestive nature, they do not provide critical tests of the fundamental concept that host susceptibility to a parasite may reside in the sharing of antigens between the two. The evidence does, however, show that host susceptibility to infection by a particular organism in many instances is associated with lack or delay in antibody production on the part of the host. The reason for the lack of or delay in antibody production is of fundamental importance. By analogy with the phenomenon of immune tolerance the purpose of this review was to suggest that heterophile antigens might have played and still do play an important role in the dynamics of the host-parasite relationship. However, until a direct chemical approach to this problem can be made in certain specific and well-defined situations, final proof for this hypothesis will be lacking. At the moment it must be regarded as speculative.

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