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PREFACE

The primary purpose of *Advances in Immunology* will be to present timely reviews of various topics in immunology that not only will serve to keep investigators informed but will also try to unify the concepts underlying this highly diversified yet technically specialized subject. Each volume will contain about eight reviews on different aspects of immunology, broadly defined so as to include such subjects as immunochemistry, antibody synthesis, biological actions of antibodies, immunological unresponsiveness, mechanisms in innate and acquired immunity not involving antibodies, and specialized immunological techniques. The articles will stress fundamental concepts but at the same time will attempt to evaluate the experimental approaches.

The reviews in this first volume are concerned mainly with some of the more biological aspects of immunology—partly because of intense interest in these subjects at the present time and partly because reviews on more chemical aspects have appeared elsewhere or will be included in subsequent volumes of *Advances in Immunology*.

We hope that immunologists as a whole will welcome Abraham G. Osler's review on "Functions of the Complement System," including its possible role in pathological processes, and Abram B. Stavitsky's survey of "*In Vitro* Studies of the Antibody Response" in relation to theories of induction and to the biochemistry of antibody synthesis. The subject of "Immunological Tolerance" is very thoroughly covered in two reviews, one by Richard T. Smith in respect to nonliving antigens, and one by M. Hašek, A. Lengerová, and T. Hraba in relation to transplantation immunity. Two fields of study, in which rapid progress in our understanding has been made in recent years, are "Delayed Hypersensitivity to Simple Protein Antigens," and the "Fate and Biological Action of Antigen–Antibody Complexes." These are surveyed by P. G. H. Gell and B. Benacerraf, and by William O. Weigle, respectively. The other two reviews concern aspects of immunology which touch upon the interests of workers on cancer research and virus diseases. One on "The Antigenic Structure of Tumors" is by the late P. A. Gorer and is the last scientific paper he wrote before his untimely death. The other one on

the "Duration of Immunity in Virus Diseases" is by J. H. Hale and is discussed in relation to the possible persistence or reintroduction of the viruses.

The editors take pleasure in expressing to the contributors their appreciation for the great effort involved in writing the reviews and for their tolerance of the various frustrations associated with the starting of a new review series.

October, 1961

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Transplantation Immunity and Tolerance

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I. Introduction

Transplantation immunity and immunological tolerance are, under certain conditions, alternative reactions of the same organism to the same antigenic stimulus—namely, transplantation of tissues or cells from an antigenically different individual. Which of the two alternatives will take place is decided by the circumstances of the first experience by the reacting individual of the given antigens, especially important being the stage of development at which this first experience occurs. In principle, if the first contact takes place early in ontogenesis, when the antigen is tolerated by the organism simply as a consequence of its immunological immaturity, this first experience may cause the capacity for tolerance of this antigen to be retained even after the development of immunological maturity of the organism and, perhaps, throughout its whole life span. By contrast, if an immune reaction is induced by the first introduction of the antigen into the organism, it will as a rule recur at all further contacts. Entry of antigens into an organism which is not yet capable of producing an immune response is normally rather exceptional, and consequently, the first alternative is a much rarer event than the second.

The occurrence of transplantation immunity is a rule and transplantation tolerance is an exception to this rule. In general, the organism

reacts immunologically against the antigens of transplanted homologous tissues, which are thereby destroyed and rejected. The mechanism underlying this regular reaction is the subject of the study of transplantation immunity. The situation in which, under normal conditions, the organism does not react immunologically against tissue antigens of another organism, but tolerates them constitutes an exception to the rule. Such exceptions are the starting points for the study of the mechanism of immunological tolerance.

The fact that the antigen can be tolerated provides an apparent contradiction in terms, since antigens are defined by their capacity to induce an immune response (formation of antibodies) in the recipient. The definition of an antigen is, however, always relative. No substance or complex can be denoted as antigenic in isolation but only in relation to an appropriate recipient, because what is antigenic in one system need not be antigenic in another. For example, the cells of an organism induce the formation of antibodies if administered in a suitable way to another (antigenically different) organism, but they do not elicit a similar reaction in their own maternal organism. It would seem, therefore, that the definition of the antigen must somehow tacitly include a requirement that it be heterogeneic. Even this, however, is insufficient. Under certain conditions the organism tolerates a potential antigen, in the same way as it tolerates the constituents of its own body, and, on the other hand, under different conditions, it can react against a constituent of its own body as it would against a foreign antigen in the usual sense of the word, by forming antibodies. The relationship between experimentally induced tolerance and immunity is, however, not reciprocal. The majority of foreign "antigens" capable of inducing a state of immunological tolerance in a given organism are as a rule able to induce an immune response in it under different conditions, but, in contrast, only a small proportion of antigens producing immunity seems to be able to elicit tolerance as well.

This distinction between antigens to which tolerance can be induced on the one hand and immunity on the other may, perhaps, arise from the different rates at which the two potential responses of the organism come in play under normal conditions. It is possible that under special conditions the development of tolerance to foreign antigens, on the basis of the "first experience," is in principle identical with the mechanism by which, under normal conditions, the organism becomes tolerant of the constituents of its own body. In teleological terms this would mean that the mechanism of tolerance is designed only for a restricted spectrum of the body's own antigens, as a natural defense mechanism against autoimmunity. However, since the antigenic makeup of an individual is the

result of a random event (i.e., of a random recombination of the respective genetic determinants in the formation of a zygote), the spectrum of antigens to which the organism can become tolerant would have to be somewhat wider in order to embrace equally all isoantigens of the given species. The species, which represents all the possibilities of free combination of the antigenic determinants during the process of fertilization, might, therefore, represent at the same time the natural limits for the induction of tolerance under normal conditions. (This begs the question whether experimentally induced tolerance of more remote antigens, if it occurs at all, is the result of a more or less successful application of a mechanism designed normally only for isoantigens, or whether it is a qualitatively different mechanism partially imitating the results of tolerance.) Although such a concept may be far from justified, the fact is that the effectiveness of experimentally induced tolerance of cellular antigens resembles tolerance of the body's own constituents only in some and not all instances when the isoantigens are employed for its induction. Insofar as we are concerned with immunological tolerance and transplantation immunity as two alternative reactions of the organism to the same stimulus, the limited range of tolerance confines this study to the sphere of isoantigens.

While the natural positive role of immunological tolerance in this sphere is readily understandable, the same cannot be said of the role of transplantation immunity. To postulate a natural defense mechanism against surgically transferred foreign tissue seems as inacceptable as the idea that transplantation immunity is an artifact produced by the artifact of transplantation. Nor is it an explanation to regard transplantation immunity as an aberrant form of bacterial allergy directed against the transplanted tissue instead of invading pathogenic microorganisms. The defensive value of bacterial allergy is obscure and to suppose that bacterial allergy is the primary mechanism and that transplantation immunity is derived from it would be quite arbitrary, reflecting evidently the time sequence of their discovery.

It seems far more probable that transplantation immunity and bacterial allergy represent two different manifestations of a more general reaction mechanism based primarily on the ability of the organism to recognize not self from self. What should be, however, the general biological significance of this ability to recognize and destroy "not self"? At first glance it may appear that under normal conditions such an ability would be harmful rather than useful to the organism, e.g., the relation between mother and fetus fulfills the fundamental condition for occurrence of the homotransplantation reaction. Some other mechanism, there-

fore, must be presumed to protect the fetus from the reaction of the mother.

The question of whether the mechanism involved in the homograft reaction plays a positive role in development was discussed recently by Thomas (1959). He suggested two possibilities. First, a mechanism might be involved controlling the uniformity of the cells of the organism and acting against a possible genetic divergence, in other words a mechanism balancing the action of such somatic mutations as might manifest themselves by changes of antigenic specificity. An important part of this biological function could then be the defense against malignant growth based on the immune reaction against a specific tumor antigen produced in the course of malignant transformation.

The second speculation of Thomas is that transplantation immunity could be mediated by a mechanism normally responsible for the "physiological degeneration" of the placenta at the final stage of pregnancy. This concept, therefore, points up a real situation in which under normal physiological conditions the breakdown and rejection of the healthy tissue occur accompanied by pathological changes strikingly recalling the picture of a homograft destruction by transplantation immunity. Both ideas of Thomas are approachable experimentally.

The term "transplantation immunity" was coined to express the discovery that transplantation incompatibility is a phenomenon which is immunological in nature. Although this concept is relatively old (Schöne, 1912), it for long lacked satisfactory experimental evidence owing to failure to detect antibody formation in the organism reacting against foreign tissue. Indirect evidence of basic importance was provided, and older findings rightly interpreted, by Medawar, in the accelerated breakdown of the second graft from the same donor. Such destruction of homografts is commonly thought of in terms of the specific cellular reaction. However, the reactions of the organism against homo- or heterologous tissues can be more varied than this, and the study of their relation to immunological tolerance covers, in fact, a wide field of immunology, namely, not only transplantation of normal and tumor tissues but also transfusion, the relationship between mother and fetus, and autoimmunity. The elucidation of the mechanism of transplantation immunity and immunological tolerance and their relationship is, therefore, not only one of the fundamental problems of any immunological theory, but also a matter of considerable general biological importance.

II. Immunology of Homograft Reactions

Surgical transfer of tissues between two individuals of the same species almost invariably ends in the destruction of a homograft by an immune process. The biological basis of this generalization is best illustrated by a few rare but well-defined exceptions. One of these is the success of transplantation between monozygotic twins (K. H. Bauer, 1927; Padgett, 1932; Hume *et al.*, 1955) which is evidently due to their genetic identity. Genetic diversity is on the contrary a practically absolute condition for tissue incompatibility; this reflects in turn the fact that genetic disparity (for this purpose at least) is also complete, or, in other words, that each individual of the given species has a unique makeup of genetically controlled factors by which individual specificity is determined.

A second exception of the rule of tissue incompatibility has helped to elucidate the nature of the genetic disparity between recipient and donor of transplanted tissue which is necessary for the development of transplantation immunity. The F_1 hybrids between two highly inbred strains are universal recipients of transplanted tissues not only from individuals of both parental strains but also from their progeny in the first and further generations. This was demonstrated by Little and Johnson (1922) and denotes clearly that genetic disparity of the graft is not sufficient in itself for manifestation of incompatibility to occur in the recipient. The F_1 hybrids are universal recipients because they represent a complete combination of tissue compatibility factors characteristic of both parental strains, so that any disparity of tissues derived from parents or from their own progeny is a negative one, due to the absence of some of their genetic determinants. A transplantation reaction to a graft can be stimulated only by a positive difference between the donor and recipient, i.e., by the genetically determined presence of some chemical constituent of the donor tissues which is absent from the recipient.

Such constituents, or isoantigens, may broadly be defined as any genetically controlled positive differences between individuals of the same species which are capable of inducing some type of immune reaction. Their physiological role is largely unknown and their chemical nature is also far from being explained satisfactorily. Proof of their existence is based so far only on their ability to induce immune reactions or to combine with specific antibodies.

Specific isoantigens of the given individual involved in homografting seem to be genotypically fully represented in the cells of a different histogenetic origin, although the possibility is not excluded that their phenotypical representation may vary to some extent. Incomplete repre-

sentation of the individual specific antigens on the surface of the cells of certain tissues (e.g., endocrine glands) could account for their apparently greater resistance to transplantation immunity, the latter being, in fact, weaker and directed against only a limited number of the host's individual antigens. Exceptional behavior of homografts of endocrine glands is, however, far from being unequivocally demonstrated and, on the contrary, more detailed experiments conducted by Krohn (1959) with ovarian homografts in mice show that their sensitivity to transplantation immunity is not weaker than that, for example, of skin.

Organ-specific antigenic differences within the individual, capable under certain conditions of inducing serum (Witebsky and Rose, 1956; Roitt *et al.*, 1956) as well as cell-bound (Lipton and Freund, 1953) autoantibody formation, may induce an immune reaction also in other individuals of the same species (Witebsky and Rose, 1956; Rose and Witebsky, 1956). There is no evidence, however, that they act at the same time as isoantigens in this reaction, i.e., that they lead to antibody formation against individual-specific in addition to organ-specific antigens. There is some evidence for the existence of individual-specific differences in organ-specific antigens (Oudin, 1956a,b; Dray and Young, 1958; Dubiski *et al.*, 1959), but not for their participation in the induction of transplantation immunity or, especially, of immunological tolerance. The fact that transplantation immunity to a certain tissue can be produced by means of another tissue from the same donor (Medawar, 1946) does not conflict with the eventual participation of organ-specific antigens; in order to obtain success in inducing and testing transplantation immunity by different tissues, it is only necessary for those two tissues to share at least one antigen in common. By contrast, when attempting to induce and test tolerance with tissues of different origin, a negative result implies that each specific antigen of the challenging tissue is not represented in the antigenic complex of the tissue used to induce tolerance. Tolerance induced with cells of mesenchymal origin, however, also extends to skin (Billingham *et al.*, 1952) and other tissues (Simonsen, 1955a; Medawar and Russel, 1958), suggesting that, if the organ-specific antigens play a role at all, they cannot display any individual-specific differences.

Some instances of successful homotransplantation are only apparent exceptions to the rule of tissue incompatibility. Thus, the success usually obtained with orthotopic corneal homografts is not conditioned by the special properties of the graft, but by those of the site of implantation. There is unequivocal evidence that corneal homografts transplanted in a heterotopic site (for example, placed on the skin; Billingham and Boswell, 1953) are destroyed, and, on the contrary, skin homografts survive when

placed on the cornea (Bednyakova, 1955) and in the anterior chamber of the eye (Medawar, 1948). Success probably depends on the absence of vascularization, which seems to play an important role in homograft reactions (Mervin and Hill, 1954). An exceptional site for transplantation is also the brain (Murphy, 1926); here even vascularized skin homografts may survive although they would be destroyed by the recipient if grafted orthotopically. The special situation noted in respect of homografts in the brain appears to be due to the absence of lymphatic vessels which constitute the usual channels for the antigenic stimulus to reach the reactive cells. Consequently, when homografts are placed on the brain sensitization of the recipient does not occur.

Similar special properties of certain tissues and transplantation sites, which give rise to exceptions interesting both from practical and theoretical point of view, are not of fundamental importance to a study of basic laws of transplantation immunity, but they can sometimes obscure the interpretation of experimental results. For this reason, skin transplantation became the basic model for the study of transplantation immunity; skin, if transferred orthotopically, does not possess any subtle peculiarities and, moreover, it is endowed with further advantages, such as marked sensitivity to the immune reaction which it induces on being grafted, the ease of the technical procedure, availability of material, etc. The widespread use of skin in tissue transplantation investigations is without doubt also due to the fact that by this technique was worked out the experimental and theoretical foundation represented by the pioneering work of Medawar (1943, 1944, 1945, 1946).

However, if an attempt is made to classify the known categories of transfer of homologous tissue according to the type of actively acquired immunity which is induced in the recipient, then skin grafting represents only one extreme, namely, that in which a specific cellular reaction and cell-bound antibodies are largely or perhaps exclusively involved. At the other extreme we have blood transfusion, where immunity is mediated by serum antibodies. If blood transfusion is at the opposite pole to skin grafting, it is necessary to exclude cases in which natural immunity exists against the transfused blood. Natural immunity has no known analogy in skin homografting, and such unique situations as the human ABO system must be excluded, since they give a false picture of this type of homograft reaction.

As in other attempts to classify biological phenomena, here also there are inherent difficulties. All gradations can be found between the more or less artificially defined extreme categories, and to define strict limits becomes, in fact, meaningless. If the types of immune reactions against

homografts are grouped according to the type of antibodies that they induce, the origin of the most typical tissues, or the nature of antigens responsible, there can always be found intermediate categories which represent a combination or interaction of the extremes. Nevertheless, however imperfect such a classification may be, it serves a useful purpose until we know enough to decide whether there should be more categories or, in fact, only a single category.

A. Types of Reactions against Cellular Isoantigens

The chief evidence for the homograft reaction being an immunological matter appears to be: (a) the second-set phenomenon; (b) the possibility of transferring sensitivity by means of immunologically competent cells, and in some cases by isoimmune serum; and (c) the finding that induced immunological tolerance based on specific inhibition of the immune response makes possible even a permanent take of the homograft.

A piece of fundamental, but indirect, evidence for an immune mechanism is provided by the clear demonstration of the second-set phenomenon (Gibson and Medawar, 1943, Medawar, 1944, 1945), i.e., accelerated destruction of a second skin homograft from a donor of the same genotype. The first graft heals in, forms vascular and lymphatic anastomoses, and has all the appearances of an autograft. Only after some time, as a rule several days, round cell infiltration appears, vascular disorders and necrosis occurs, and finally the graft is rejected. By contrast, the second-set graft has no period of normal take. Vascularization is incomplete, round cell infiltration of the bed and the bottom layers of the graft occurs immediately, and breakdown takes place rather more rapidly than with the first homograft. In any given combination, the survival time of the homograft is exclusively determined by the type and the extent of the isoantigenic differences between the donor and the recipient if other conditions (including the size of the graft) are kept constant. Thus, in the case of transfers between members of two inbred strains, the median survival time of the grafts is nearly constant and exhibits a minimum scatter. In skin homotransplantation between the mice of A and CBA strains, the destruction of the CBA graft on A recipients is accomplished in 10.2 ± 0.3 days, whereas the destruction of the second CBA graft transplanted to the recipient two weeks after the breakdown of the first one is completed in less than 6 days (Billingham *et al.*, 1954a). These quantitative data are strictly reproducible, and, in this host–donor combination, the histological picture of the homograft at a given time after transplantation can be used for the detection of the immune state (Billingham *et al.*, 1956b).

1. Circulating Antibodies

It is not quite easy to appraise the importance of serum antibodies in transplantation immunity; first because their occurrence (as detected by usual methods at least) is not general, and second because the mere presence of serum antibodies, revealed by serological reactions *in vitro,* provides no evidence of their participation in homograft destruction. A relevant role may be attributed only to those serum antibodies whose biological effectiveness has been clearly demonstrated.

a. Antibodies Demonstrable by Serological Reactions in Vitro. In homotransplantation most attention has been paid to the formation of hemagglutinins. Other serological reactions performed *in vitro* with tissue homogenates or fractions gave mostly negative or unreliable results. Our limited knowledge of antibodies giving such reactions may perhaps lie not in their absence but in the inadequacy of detection systems so far used (unsuitable antigenic preparations, insufficiently sensitive reactions, etc.).

The occurrence of hemagglutinins is, however, regular after transplantation of tissues in mice (Gorer, 1937, 1947), in which the tumor transplant seems to provide an even better stimulus than immunization with blood. Moreover, their formation is induced in some, but not all, cases not only by full thickness skin grafts (which could contain some erythrocytes) or mesenchymal tissues of the dermis, but also by grafts of isolated epidermis (Amos *et al.,* 1954). Such hemagglutinins are, as a rule, of the incomplete type.

Hemagglutinins seem to occur less regularly (perhaps as a result of less intensive investigation) after homotransplantation of tissues in other species. However, Zotikov (1956) unlike Medawar (1946) has found the formation of complete hemaglutinins after homotransplantation of skin in rabbits which are known, of course, to be weak producers of isoagglutinins.

b. Antibodies Demonstrated by Their Biological Effect. The second group of antibodies occurring as a reaction against the graft are those demonstrated by their biological effect on the cells of the graft. Such antibodies were detected by Gorer (1942). Leukemic cells incubated *in vitro* with the serum of the animal immunized with a tumor graft lose their ability to induce tumor growth after inoculation into a sensitive recipient. These neutralizing antibodies are unlikely to be identical with hemagglutinins, because the latter can be removed from an immune serum by absorption without decreasing its neutralizing capacity. Billingham and Sparrow (1954) also found a neutralizing effect on isolated

epidermal cells, demonstrated by a decreased capacity to grow in the original donor after temporary contact *in vitro* with the serum of animals which had rejected a skin graft of the same origin.

Other methods have since been employed to demonstrate antibodies in homograft reactions, such as the demonstration of cytotoxic antibodies *in vitro* (Gorer and O'Gorman, 1956) and passive transfer of homograft immunity by serum (Gorer and Amos, 1956; Amos and Day, 1957; Siskind and Thomas, 1959). These two methods have largely been used for study of transplantation immunity to tumor tissues. It was shown that insofar as serum exhibits a cytotoxic effect *in vitro*, it can as a rule transfer passive immunity (Winn, 1960). However, great differences are found in the reaction of different tumors against cytotoxic sera (Gorer and Kaliss, 1959). Whereas leukemia and other lymphoid tumors are inhibited or destroyed by the action of antibodies detected by the aforementioned tests, other tumors remain not only unimpaired, but their growth in incompatible hosts is often enhanced. Such immunological enhancement (Kaliss, 1958) has been most studied with sarcoma 1 as a typical representative of the second group of tumors.

Between these two groups of tumors which are destroyed by immune serum in the first instance and enhanced in the second, there exists an intermediate group in which different doses of antiserum display a different effect. On the whole, the greater the dose of antiserum the better the inhibition of the tumor growth, and the smaller the dose the greater the probability that enhancement will occur. The difference in the reaction of tumors to the action of antiserum seems to be determined by the properties of the normal tissue from which the tumor originated, but differences in sensitivity to passive transfer of isoimmune serum has, however, been studied so far only in a few normal tissues.

2. *Specific Cellular Reaction*

The failure of attempts to provide evidence for a general role of serum antibodies has no bearing on the concept of homograft reaction as an immune process. There is ample evidence for the decisive part played by the second fundamental immune mechanism—hypersensitivity of the delayed type. The homograft reaction resembles the best known representatives of this category (tuberculin hypersensitivity and drug allergy) in the following features: the time of the onset of the reaction; the histological picture; and, especially, in the fact that each is transferable to other individuals by lymphoid cells of the sensitized donor. This was shown by Mitchison for tumors (1953) and also demonstrated for normal tissues (Billingham *et al.*, 1954b). This evidence is, however, not entirely

unequivocal and does not preclude the possibility that humoral antibodies may also be involved in this reaction and even be responsible for it. In transfers of lymphoid cells, cells forming humoral antibodies are transferred at the same time as cells responsible for the reaction of the delayed-type hypersensitivity (insofar as they are really different types of cells).

Further evidence for a decisive role of delayed hypersensitivity in immune reactions against homograft is provided by experiments with semipermeable chambers whose walls are permeable to antibody molecules but not to cells. Epidermal cells and other tissue cells implanted in an immune recipient inside such a chamber are not destroyed. However, the cells perish if they are mixed with spleen cells from an immune recipient before being implanted in a semipermeable chamber in a donor animal (Algire, 1954; Algire *et al.*, 1954). The results of these experiments may be influenced by other factors, for example, by the amount of the complement penetrating into the chambers (Amos, 1960) or the technique of immunization of the recipient (Algire, 1959). It will, therefore, be necessary to submit such findings to further analysis, perhaps by means of an improved method.

Cellular types of immunity manifest themselves in a characteristic way (in man and in guinea pigs at least) by skin hypersensitivity of the delayed type. Brent *et al.* (1958) demonstrated that the homograft reaction against a skin graft in guinea pigs can also be expressed as skin hypersensitivity. In such cases the time of the onset of the reaction—a latent period of 5–8 hours, with maximal reaction after 24–48 hours—serves as a criterion for delayed-type hypersensitivity.

Perhaps the best evidence for the view that the delayed hypersensitivity reaction is sufficient to cause skin homograft destruction is provided by the transfer of homograft sensitivity in man with deoxyribonuclease (DNase)-treated leucocyte extracts (Lawrence *et al.*, 1960). With such extracts it is possible to transfer delayed sensitivity but not serum antibody formation.

3. *Graft Rejection*

Under certain experimental conditions, skin homograft destruction can be accomplished by a reaction of delayed-type hypersensitivity alone without the participation of humoral antibodies (Lawrence *et al.*, 1960). By contrast, grafts of normal or malignant lymphoid tissues can be destroyed by humoral antibodies without the participation of delayed hypersensitivity reaction. The question is, however, what is the relationship between these two types of immunity under normal conditions.

It is known that either kind of tissue can induce either type of reaction. For example, when transplantation immunity is produced by a tumor which requires a delayed hypersensitivity reaction for its destruction, serum antibodies are also produced and are demonstrable not by the inhibition of this tumor but by cytotoxic effects on the lymphoid cells of the same host (Winn, 1960). On the other hand, lymphoid cells can elicit an effective transplantation immunity to skin, and thus hypersensitivity of the delayed type. How far are both types of immune reaction involved in the destruction of all types of grafts?

Where skin transplantation is concerned humoral antibodies seem to be responsible for the so-called "white graft" reaction. This is a reaction resulting in rejection of a graft transferred at the time or soon after immune destruction of a previous graft from the same donor. It appears to be a *hyperimmune* reaction characterized by complete failure of graft vascularization (in contrast to the second-set reaction in which at least partial vascularization is observed). White graft reaction could not be transferred by leucocyte extracts in man (Lawrence *et al.*, 1960). It may, however, be accomplished by means of hyperimmune serum in rabbits and mice (Stetson and Demopoulos, 1958; Chutná, 1959; Chutná and Pokorná, 1961) after immunization with the donor's spleen in Freund's adjuvant. Neither the relation of antibodies responsible for the white graft reaction to antibodies displaying cytotoxic effects on lymphoid tissues, nor their participation in other reactions against skin homografts, is clear so far. Until now, attempts to destroy tolerated skin grafts by serum antibodies failed (Brent *et al.*, 1959; Stetson, 1959; Hašek and Hort, 1960), whereas tolerance was abolished by transfer of lymphoid cells from a normal or immune animal (Billingham *et al.*, 1954b, 1956a). It is possible that serum antibodies are also involved in the inhibition of the epithelium proliferation in first-set skin homografts observed with combinations of mouse strains differing at the H-2 locus (Hašková, 1961).

4. Enhancement

In early attempts to produce enhancement, lyophilized tumor or normal tissue was used (Casey, 1941; Snell *et al.*, 1948; Kaliss and Snell, 1951). With certain antigenic combinations, it is possible in this way to obtain improved growth of a homologous tumor, which in some cases may even kill the pretreated host.

An important contribution, which throws much light on the mechanism of enhancement, is the finding that enhancement can be transferred by means of serum obtained from animals pretreated with lyophilized tissue (Kaliss *et al.*, 1953). It was later found that all isoimmune sera

were effective, irrespective of the manner of immunization, including even immunization by viable tumors (Kaliss, 1956). The effective agent of the serum seems to be antibodies; it has not, however, been found which type is involved.

The discovery of enhancement of tumor growth has raised the question of the extent to which the same principle could be applied to homografting of normal tissues. Unfortunately, the effectiveness of enhancement of normal and tumor tissues was found in practice to be inversely proportional to the practical usefulness which successful results might have. In other words, the possibility of enhancement of transplanted normal tissue is much more limited than that found with tumors. In normal tissues (skin, Billingham *et al.*, 1956c; ovary, Parkes, 1956, 1958) enhancement leads to only a slight prolongation of homograft survival. Enhancement of skin grafts was successfully induced by intravenous injections of dissociated epidermal cells (Billingham and Sparrow, 1955). The transfer of enhancement by serum has not been closely studied in normal tissues, but appears to be a possibility (Medawar, 1959). The difference in the effectiveness of enhancement of normal and tumor tissue is perhaps due to the difference in their growth capacities. The death of the pretreated host as a consequence of a progressive tumor growth may prevent further development of immune reactions which could later result in the tumor regression. The longer survival of enhanced tumor homografts compared with normal tissue could also reflect the higher resistance to transplantation immunity of tumors, which often progress in spite of an immune reaction sufficient to destroy, for example, a skin graft.

The basis for enhancement is probably some interaction between serum antibody formation and the delayed hypersensitivity reaction. A similar phenomenon was observed with hypersensitivity to tuberculin; intravenous immunization with tuberculin (Boyden, 1957) or tuberculous bacteria (Arima *et al.*, 1958) leading to circulating antibody formation retards the development of the tuberculin skin reaction on subsequent intracutaneous immunization with bacteria. Enhancement can, however, be induced by injecting immune serum quite a long time after implantation of the tumor, at a time when transplantation immunity is already developing in the recipient. Consequently, the interaction between different immune reactions is not in the afferent or central part of the reaction chain, but in the efferent one; i.e., in the reaction of the effector components of these different types of immune response to the antigen on the graft cells, perhaps in a similar way to the interaction of reagins with blocking antibodies in allergy.

It cannot be excluded, however, that the enhancing effectiveness of serum antibodies rests (entirely or at least partially) in a modification of the tumor cells themselves affecting transplantability of the tumor (Kaliss, 1958).

B. Cellular Isoantigens

1. Genetic Analysis

The first individual antigenic differences were demonstrated by serological methods on erythrocytes (Ehrlich and Morgenroth, 1900; Landsteiner, 1900) and later they were found to be inherited as Mendelian dominants (Dungern and Hirszfeld, 1910, 1911a,b). Relatively early, the individual specificity of the antigenic makeup of erythrocytes in cattle (Todd and White, 1910) and in chickens (Todd, 1913) was detected and correctly interpreted as a consequence of a unique combination of a certain number of antigens from a larger, but limited, set of individual antigens occurring in a given species. This hypothesis was confirmed in principle by further investigations of blood groups in man and in other species. A similar conclusion was reached by a genetic analysis of individual antigens responsible for transplantation immunity to malignant and normal tissues in mice (Little, 1914; Snell, 1952). An estimate was made of the number of separately segregating genes governing tissue transplantation in mice; this figure was found to be not less than 15, in a combination of rather closely related A and CBA strains, as indicated by the number of successful takes of the skin grafted from the members of one or other of two inbred parental strains to their F_2 progeny (A. D. Barnes and Krohn, 1957). Similar results had been obtained much earlier with other strain combination in tumor grafting experiments of Little and Tyzzer (1916). The corresponding loci were called histocompatibility (H) loci; that they are not equally strong is revealed by the widely differing survival times of homografts of tissues controlled by them. The known histocompatibility loci in mice are H-1, H-2, and H-3 and they belong to the first, ninth, and fifth linkage group. The best analyzed and strongest H-2 locus was found to be complex in structure consisting of at least 10 alleles. In addition, a relatively weak sex-linked transplantation antigen was discovered in mice (Eichwald and Silmser, 1955, 1956) supposed to be controlled by a histocompatibility factor located on the Y-chromosome.

A genetic analysis was also carried out in outbred animals by Medawar (1945) in rabbits and by Hildemann and Owen (1956) in goldfish. A number of homografts was exchanged in groups of animals in such a way that each of them received grafts from all the others. From

the fact that all the homografts failed and from the size of experimental groups, estimates could be made of the minimum number of histocompatibility loci necessary to provide the given number of mutually incompatible combinations. There were at least seven distinct antigens at work in the group of 22 rabbits used in the former experiment and at least three loci must have governed the antigenic diversity of the progeny of the single pair of fish used in the latter. In human beings, at least 20 distinct histocompatibility factors are supposed to occur (Longmire *et al.*, 1947).

The properties of tissues subject to control by histocompatibility genes, as manifested in homotransplantation, are responsible not only for occurrence of the homograft reaction as such, but also for its strength; it seems, therefore, that the potential antigenicity of the tissues is, in fact, inherited as a quantitative character.

The endowment of animals of different species with strong histocompatibility genes need not necessarily be equal; the survival times of skin homografts are surprisingly long in outbred golden hamsters, all derived from a single breeding pair many years ago (Adams *et al.*, 1956; Billingham and Hildemann, 1958; Skowron-Cendrzak and Spissak-Plonka, 1958). This common origin, however, could hardly in itself explain results of skin grafting which are unexpected from the experience with other species, since mutations are known to contribute to the inborn antigenic makeup.

2. *The Relationship between Isoantigens*

Gorer (1937) found that the strongest histocompatibility locus H-2 is also responsible for the formation of blood cell antigens. In addition, he proved serologically that blood cell antigens are present in tissue cells (Gorer, 1942), including epidermal cells (Amos *et al.*, 1954), and often in a much larger amount than in erythrocytes. Consequently, the assumption was easily made that they were the cause of transplantation immunity to skin. Such a conclusion is, however, at variance with Medawar's finding that skin transplantation immunity in rabbits cannot be induced by immunization with erythrocytes, but only with the whole blood, effectively with leucocytes (Medawar, 1946).

For species in which a sufficient number of blood groups is known, it is not yet clear whether a simple relationship exists between the intensity of the homograft reaction and the antigenic disparity of donor and recipient, as expressed by their differences in blood groups. This question cannot be clarified in mice, since, although an ideal conformity exists between the presence of histocompatibility and blood cell antigens

conditioned by the H-2 locus, no agglutinins against other blood cell antigens are known apart from those actually governed by the H-2 locus. Such negative evidence is insufficient to indicate that no other individual red cell antigens exist in mice, nor, if they do exist, that there is no relationship between them and other loci. Any study of the correlation between erythrocyte and transplantation antigens requires a thorough knowledge of red cell individual antigens, such as we have for cattle, chickens, and man. As regards cattle, we are aware only of the negative findings of Kozelka (1933) who looked for individual differences by means of rabbit antisera; since only a small number of antigens were identified in this way, his findings must be treated with reserve. Results obtained so far in man cannot be considered conclusive either (Woodruff and Allen, 1953).

3. *The Chemical Nature of Isoantigens*

The fundamental experiments on isolation of transplantation antigens were carried out by Medawar's group (Billingham *et al.*, 1956b), who succeeded in eliciting transplantation immunity by injecting spleen cells disrupted by means of ultrasonic vibration. Further analysis of cellular fractions has shown transplantation antigens to reside in the nuclei, from which they can be extracted and are then inactivated by DNase but not by trypsin. On the basis of these results, the authors assumed the antigenically active component to be the deoxyribonucleoprotein, particularly the deoxyribonucleic acid (DNA). However Hašková and Hrubešová (1958) found that transplantation immunity to a skin graft cannot be induced by means of purified preparations of tissue DNA. Medawar (1958a) came to the same conclusion, and on the basis of further experiments put forward the hypothesis that the activity of nuclear fractions in producing transplantation immunity was due to admixture of traces of polysaccharides akin to blood group substances. This suggestion is supported by the finding that transplantation antigens can be inactivated by the extracts from *Trichomonas foetus* (Billingham *et al.*, 1958) which are known to inactivate blood group substances. Extracts of *Trichomonas foetus* also contain other enzymes, and it is, therefore, not sure that the same one is responsible for inactivation both of transplantation antigens and of blood group substances. Experiments on inactivation of transplantation antigens by oxidizing agents are also inconclusive. Preparations extracted from tissue cells have mainly been used to study the nature of transplantation antigens, but Hašková and Hilgert (1961) showed the supernatant of tumor ascitic fluid also to be an appropriate source.

Owing to the obstacles met in precisely defining the chemical nature of isoantigens, it is difficult to resolve the question of the relationship between their different types. It is very likely that antigens producing enhancement and transplantation immunity are identical. This assumption is supported by evidence that both phenomena may be produced by means of the same preparations, and it depends only on the dose, the route of administration, and the time interval between inoculation and testing to determine which phenomenon will appear in any given case. By employing appropriate combinations of donor and recipient, Kaliss (1952) was able to induce tumor homograft immunity with small doses of lyophilizate and enhancement with large ones. More recently, Hašková and Svoboda (1961)using C57BL mice pretreated with repeated doses of lyophilized A strain sarcoma 1 found that skin graft immunity could be detected 4 days after the last dose but that this could have entirely disappeared by the tenth day, when enhancement of the tumor is normally tested. Immunity followed by enhancement was likewise observed after repeated homotransplantations of tumors (Kaliss and Bryant, 1958).

As to the relationship between transplantation and red cell antigens, the finding of Hildemann and Medawar (1959) that cellular extracts containing transplantation antigens cannot block antibodies against the hemagglutinins concerned seems to be evidence against their identity. It is still possible, however, that, although the determinant groups may be alike in both types of antigens, they cannot always act in both capacities—e.g., one might be blocked either by secondary attachment to another substance or for reasons of steric hindrance when in complex form.

On the other hand, the fact that at least some red cell antigens have been shown to be present on tissue cells is compatible with, though it does not prove the assumption that the formation of red cell and transplantation antigens is controlled by a single genetic system, i.e., that the occurrence of the H-2 locus in mice is a general phenomenon. The inadequacy of the techniques employed could be responsible, among other factors, for some unsuccessful attempts to show the presence in tissues of traces of red cell antigens.

If the idea of a common genetic control of isoantigens holds true, the question of their different phenotypic manifestations remains to be clarified. At the time when transplantation antigens were assumed to be the deoxyribonucleoproteins, Billingham *et al.* (1956b,c) expressed the hypothesis that the transplantation antigen is itself the material mediating the genetic information (i.e., the gene) whereas the red cell antigen (responsible also for enhancement) is its product. The finding that pure

DNA is not transplantation antigen upsets this hypothesis. The current view is that the antigenically determinant substances are identical both in transplantation and in red cell antigens. The different manifestations of these two types of isoantigens depend, however, on their attachment to other substances, which can act, for example, as an adjuvant (Hilgert and Pokorná, 1961). Thus, the nature of the immune reaction induced by antigens with the same specificity could be modified qualitatively or, in the last resort, perhaps, only quantitatively, as a result of differences in the complex involved. To seek additional evidence in support of this idea is one of the more promising approaches toward elucidation of the nature of isoantigens.

C. Graft-versus-Host Reaction

A basic condition for producing transplantation immunity is *in vivo* interaction of normal immunologically competent cells with cells differing in genetic origin such that the cells inducing the reaction possess at least one transplantation antigen which is absent from the reacting cells. For this reaction to be readily detectable, a second condition should be fulfilled, namely, that the cells which stimulate the reaction should not themselves react immunologically, at least against the antigens of the stimulated cells; this means that if the stimulating cells are also immunologically competent, the genetic constitution of the stimulated cells should not permit them to act as an antigenic stimulus for the former, since this would result in a simultaneous two-way reaction. (The result of such a two-way reaction would not be predictable, because it would depend on the mutual genetic constitution and the quantitative relationship between the two types of cells and, perhaps, also on extraneous factors such as differences in fitness of the environment for the mutually reacting cells.) This general statement intentionally omits any distinction between host and graft. Experimental results in recent years showed convincingly that it is not essential in transplantation reactions that the *host* (as a whole organism) should react against the *graft* (cells, tissues, or a whole organ) and cause graft destruction. If the general conditions are fulfilled, transplantation reactions can also develop in the opposite sense and eventually end in killing the host. If we return to the definition of conditions necessary for transplantation immunity, it is clear that we can find experimental and naturally occurring situations in which a graft-versus-host reaction can occur.

Nevertheless, it must be stressed that the concept of the graft-versus-host reaction was not always obvious. It was first put forward by Simonsen (1953) in his interpretation of interstitial cellular infiltration in kidney

homografts. In spite of the fact, shown only recently, that in that case a graft-versus-host reaction was not actually involved, a principle of biological validity was expressed. This principle has been confirmed later in a series of other experimental models, even though it arose as a consequence of incorrect interpretation of one experimental finding.

At the time of Simonsen's original finding, the existence of immunologically competent elements in peripheral blood was not yet known. Consequently, in evaluating the proliferating cells in histological sections of homotransplanted kidney, no attention was paid to the possibility that they originated in the host. Likewise the finding of Dempster (1953) relating to suppression of this cellular reaction by previous irradiation of the kidney homograft with a dose of 250 r appeared to be consistent with the conception of a graft-versus-host reaction. Only after immunologically active cells had been demonstrated in peripheral blood (Simonsen, 1957; Cole *et al.*, 1959) and shown to be capable of proliferation (Bond and Cronkite, 1958) was it possible to explain the original finding as a host-versus-graft reaction. The same applies to the enhancement of the infiltrating cellular response which follows transplantation of a renal homograft back into the original donor, which is due to his reaction against cells of the first recipient which settled and multiplied in the renal homograft and were transferred with the graft when it was transplanted back. The host origin of the infiltrating cells in kidney homografts was demonstrated by a direct method (Porter and Calne, 1960). The inhibitory effect of irradiation on the cellular infiltration in a renal homograft was not confirmed in a more extensive investigation by Fowler and West (1960); in spite of using a dose of 3000 r, the histological picture in the irradiated and nonirradiated kidney was not distinguishable. However, the cellular infiltration was prevented by the irradiation of the host (Mannick *et al.*, 1959; Murray *et al.*, 1960; Zukoski *et al.*, 1960).

Pathological manifestations of the graft-versus-host reaction are denoted as "homologous" or "heterologous" disease. This appears in two main forms.

1. Splenomegaly and Runt Disease

When fragments of adult chicken spleen are transplanted onto the chorioallantois of chicken embryos, a significant enlargement of embryonic spleen takes place within a few days. This phenomenon had been recognized for some time (Danchakoff, 1916; Murphy, 1916; Willier, 1924; Weiss, 1950) and used to be interpreted as an organ-specific growth stimulation of the embryonic spleen induced by subcellular constituents of adult cells (Ebert, 1951, 1954). Simonsen (1957) showed,

however, that the difference in age of recipient and donor is not the main factor. What matters is their genetic diversity, without which adult cells do not induce enlargement of the embryonic spleen. At the same time, positive results can be produced even when donor and recipient are of the same age, provided that their genetic diversity is adequate. Thus genetic disparity is a necessary requirement, but disparity in the age of donor and recipient is of no fundamental importance for the development of the immune reaction between graft and host. It may, nevertheless, be a main factor in revealing the reaction provided that the other conditions are fulfilled. Owing to the immunological immaturity of the recipient only one of the potential reactions develops, namely, that of the adult graft against the nonreactive host, and it can easily be detected.

Spleen enlargement after injection into embryos or very young animals of adult homologous spleen cells is currently interpreted as due to multiplication of transplanted donor cells stimulated by the contact with the recipient's antigens. However, it may perhaps be necessary to revise this idea in the light of the recent finding that the dividing cells in the spleen of young chick embryos or of mice (3–10 day old), injected at birth with adult homologous spleen cells, are practically all of the host's origin (direct cytological evidence; see Biggs and Payne, 1959; Davies and Doak, 1960). This result, if confirmed, will not affect the basic conception but only the detailed interpretation of the splenomegaly. It might imply that immune reaction on the part of the implanted cells need not cause them to divide at a given stage and yet they might stimulate reparatory multiplication of the recipient's spleen cells.

In chickens injected intravenously several days before hatching with adult homologous spleen cells anemia appears, resulting as a rule in death of birds within 2 weeks of hatching. The blood of anemic animals gives a positive direct Coombs test, which again points to the graft-versus-host reaction as the immunological mechanism underlying this hemolytic disease (Simonsen, 1957).

Billingham and Brent (1957) described an analogous situation in young mice injected with homologous spleen cells immediately after birth. A conspicuous retardation in the growth, accompanied by the involution of the lymphoid tissues, occurs in animals so treated. The authors called this "runt disease" and ruled out the possibility that it might be caused by some latent pathogenic factor transferred from the donor.

2. *Secondary Disease in Radiation Chimeras*

The second situation which is at present almost generally interpreted as a manifestation of the graft-versus-host reaction is the secondary dis-

ease and death which occurs in lethally irradiated animals given a therapeutic dose of adult homo- or heterologous blood-forming cells in order to prevent radiation death from hemapoietic failure. Assuming that the radiation dose was really sufficient for a permanent or at least long-term inhibition of the immunological reaction on the part of the recipient (which is the critical condition for the success of this therapy), the implication is that all immunological symptoms of this syndrome must be attributed to a reaction on the part of the graft. This concept received support from a series of experimental studies indicating that immunologically competent cells of donor specificity are transplanted together with spleen or bone marrow cells (Ford *et al.*, 1956; Mitchison, 1956; Feldman and Yaffe, 1958) which, if the necessary genetic conditions are fulfilled, react immunologically against the irradiated recipient. Moreover, if the donor of hemapoietic cells was presensitized against the recipient (E. E. Schwartz *et al.*, 1957; Cosgrove *et al.*, 1959), the injection of these cells corresponds at the same time to the adoptive transfer of immunity (Mitchison, 1956) except that the immunity is directed against all tissues of the recipient; the chief target of this reaction, however, is lymphoid tissue, in which the reacting cells settle owing to their "homing instinct." This is revealed by post mortem examination of radiation chimeras (Congdon *et al.*, 1958).

3. *Experimental Analysis of the Mechanism of Homologous Disease*

When a nonreactive recipient receives homologous immunologically competent cells, the conditions for a two-way reaction are at least potentially fulfilled (assuming that the immunological reactivity of the host can appear or recover early). Semi-isologous combinations, permitting reaction in only one direction are, therefore, more suitable for analysis of "homologous disease." Cock and Simonsen (1958), employing two inbred lines of chickens and their F_1 hybrids, demonstrated that their findings, earlier interpreted as manifestations of the graft-versus-host reaction, can in practice only be induced when the genetic combination of the donor and recipient is suitable. Adult spleen or blood cells from one strain produce enlargement of the spleen and hemolytic anemia in F_1 hybrids between this and another strain, whereas adult isologous cells or F_1 hybrid cells do not.

Simonsen and Jensen (1959) also elaborated in detail the qualitative aspect of the graft-versus-host reaction and pointed out the importance of the age of the recipient at the time of implantation of adult spleen cells and of the genetic disparity of the donor and recipient in relation to the degree of spleen and liver enlargement produced. They also

showed that the absence of reaction in some combinations of F_1 newborn recipients and adult parental donors cannot be considered as evidence for recessively determined transplantation antigens (the original interpretation; Simonsen, 1958), but rather as evidence for a gene–dose effect in dominant but weak histocompatibility systems. After presensitization of the donor against the recipient, the reaction occurs even in such otherwise negative combinations.

Other authors (Koller, 1957; Trentin, 1957; Uphoff, 1957; Uphoff and Law, 1958) likewise showed that delayed mortality in radiation chimeras occurs mainly when F_1 hybrids of two different strains are used as recipients after irradiation and the bone marrow is derived from donors belonging to one or other parental strain, i.e., when the combination allows only the graft-versus-host reaction. In a control experiment Uphoff (1957) has, however, shown that parental bone marrow can have in F_1 hybrids a therapeutic effect comparable to that of isologous marrow provided that a combination of parental strains identical at the H-2 locus is involved.

In some combinations of irradiated F_1 hybrids, delayed mortality appears after application of normal parental spleen cells, but not of normal bone marrow. Previous sensitization of the donors against the recipient, however, enables even parental bone marrow to produce appreciable mortality in irradiated F_1 hybrids: this may be due to a difference in the content of immunologically active elements of bone marrow in normal and presensitized animals of some strains (E. E. Schwartz *et al.*, 1957; Cosgrove *et al.*, 1959).

When F_1 hybrids are used as recipients and one of the parental strains as donors of immunologically active graft, the conditions are fulfilled for a graft-versus-host reaction, even if the host's immunological reactivity were not inhibited by irradiation. This is because the F_1 hybrid, as a consequence of its genetic constitution, is naturally tolerant of antigens of both parents (insofar as they are all dominant). Attempts at implantation of lymphoid parental cells into unirradiated adult F_1 hybrids were made by Trentin (1958) as well as by Gorer and Boyse (1959); they found that even in this situation pathological changes and death occur in F_1 hybrids comparable to the picture of the graft-versus-host reaction in irradiated animals.

Further indirect evidence in favor of the hypothesis that a graft-versus-host reaction underlies "homologous disease" is provided by the fact that both these phenomena can be prevented by inoculating immunologically immature (e.g., embryonic) cells (Lengerová, 1958; Simonsen, 1957; Barnes *et al.*, 1958a; Uphoff, 1958) instead of cells from adult donors. The immunological immaturity of foreign cells at the time

of implantation, however, would not in itself be sufficient to prevent the later reaction against the host, since it must be assumed that these cells, even in a new environment, sooner or later reach immunological maturity. Insofar as no reaction against the recipient occurs, it must be because the antigenic stimulus, which would lead to acquired immunity in adult cells, results in acquired tolerance in embryonic cells.

4. The Relative Nature of the Terms "Host" and "Graft"

It is usual to assign cases of the graft-versus-host reaction to a special group of transplantation reactions. This is based on the distinction of host (the whole organism reacting as a rule against the graft) and graft (the part of the organism which as a rule induces the reaction). From the point of view of the actual mechanism underlying transplantation reaction, however, the relative nature of such a distinction is apparent in certain experimental situations, such as a transplantation reaction between two whole organisms or between two grafts. The first example is represented by the parabiotic union of two adult organisms with a different genetic constitution (Finerty, 1952) in which an immunological reaction occurs in one part or the other (Koldovský and Skowron-Czendrzak, 1959; Trentin, 1959; Stark *et al.*, 1960a), or, eventually, in both parts at the same time, according to the antigenic constitution of the parabionts. In the extreme case, either of them act simultaneously as the source of the antigenic stimulus (i.e., actually the "graft") and as the part reacting against the antigenic stimulus (i.e., actually the "host"). The second instance is a mutual reaction between immunologically active grafts of different genetic origin ("graft-versus-graft" reaction), occurring in an environment provided by a nonreactive host. Here, in the extreme case, each part can both react and induce the reaction, representing graft as well as host at the same time.

In conclusion, an attempt could be made to review situations in which the graft-versus-host reaction occurs or could occur under natural conditions or in cases which may appear in the clinical practice.

5. The Risk of the Graft-versus-Host Reaction in Natural or Clinical Situations

The immunological relationship between mother and fetus still continues to be a subject of speculation because there is still no explanation why a homograft reaction against the fetus does not regularly appear. Explanation in terms of a "placental" barrier is not satisfactory, because the placenta itself is also of fetal origin; it should act, therefore, as an antigenic stimulus in the same way as the fetus, unless some so far un-

discovered mechanism prevents it. There are instances in which the barrier function of the placenta fails and the fetus becomes the source of an antigenic stimulus for the mother, which can result in fatal consequences such as are familiar in the case of Rh incompatibility. If in such cases passage of cellular elements from the fetus into mother (through a placental leakage) occurs, then the possibility that maternal cells can pass in the opposite direction may also be assumed. In such a situation, it would be worthwhile paying attention not only to the part played by serum antibodies in hemolytic disease of the newborn but also to any contribution by a specific cellular reaction of the graft (i.e., of the immunologically competent maternal cells implanted to the fetus) against the host.

If in hemolytic disease of the newborn, the graft-versus-host reaction does not occur naturally, the conditions for it can be created artificially, namely, by exchange transfusion of fresh blood from adult donors, a procedure used therapeutically in this disease. This risk is quite real, because the children undergoing this treatment are at a critical stage of development, at which it is not possible to foretell in any given case whether the recipient will react against foreign cells by immunity or tolerance. The possibility cannot be, therefore, precluded that immunologically active elements from the donor blood can be permanently implanted in the recipient, proliferate there, and react against his antigens.

In all cases mentioned so far, the graft-versus-host reaction is harmful and results in some form of homologous or heterologous disease manifested by hemolytic anemia or runt disease in newborns, or secondary disease in radiation chimeras. Attempts have been made to extend the principle of the graft-versus-host reaction to the interpretation of some autoimmune and neoplastic diseases (H. S. Kaplan and Smithers, 1959; I. Green *et al.*, 1960; Tyler, 1961). For example, one such speculation is that a tumor cell originates by a somatic mutation accompanied by an antigenic deletion (Weiler, 1959); if such a cell were immunologically competent, this change would render it capable of reacting against its own lost antigen occurring in other tissues. Experimental evidence for this hypothesis is so far, however, not strong.

Finally it should be mentioned that attempts have been made to use the graft-versus-host reaction for the benefit of the host, namely, in the therapy of some malignant diseases. Insofar as such a reaction is directed also against normal tissues of the recipient, the success of such attempts is rather problematical (Barnes *et al.*, 1956; Barnes and Loutit, 1957; Mathé and Bernard, 1958, 1959). Nevertheless a promising approach seems to be the use of specific antitumor therapy based on the adoptive

(and/or passive) transfer of immunity against a specific tumor antigen, i.e., one peculiar to tumor cells (in addition to those shared with normal cells). This procedure appears, in fact, to be effective (Koldovský and Lengerová, 1960), but its practical success depends on the sensitivity of the tumor in question to such immunotherapy alone or in combination with irradiation.

III. Immunological Tolerance

The concept of immunological tolerance which was being shaped for several years in independent trends of immunogenetic and immunological investigations actually emerged at the moment when these different approaches met. Owen (1945) discovered the occurrence of erythrocyte mosaicism in dizygotic twin cattle and explained it by an interchange of primordial hematopoietic cells through vascular anastomoses between the co-twins. The continued existence of this cellular chimera was interpreted by Burnet and Fenner (1949) in an original way. They advanced the hypothesis that the immunological system of the organism becomes nonreactive to antigens with which it comes into contact in embryonic life; the normal function of this mechanism was to ensure the nonantigenicity of selfcomponents, and it was thought that this could be extended to a foreign antigen artificially introduced into the embryo. Further evidence for an anomalous situation in dizygotic twin cattle, provided by the taking of skin homografts exchanged between the partners, was discovered by Medawar and his associates (Anderson *et al.*, 1951; Billingham *et al.*, 1952) who called it tolerance.

The first attempt to induce immunological tolerance experimentally, which was made by Burnet to prove his hypothesis, was not successful (Burnet *et al.*, 1950). Although this failure could perhaps have been in some degree due to technical limitations, its cause seemed to be of a more fundamental nature and to reside in the very distantly related antigens (influenza virus, bacteriophage, and human blood cells) used for the induction of tolerance in chicken embryos. It was argued that the hypothesis as expressed by these authors need not have general validity, being restricted perhaps only to antigens relatively closely related to the recipient. [The earlier finding by Traub (1936, 1938, 1939) concerning congenital atypical choriomeningitis, the mechanism of which Burnet considered identical to that of tolerance in dizygotic twins, would then represent an exception; of course, the criterion of the "distance" between antigen and recipient in a given combination is always more or less arbitrary.] Consequently, later attempts based on the artificial introduction of homologous cells into the circulation of embryos had a much

greater chance of being successful since this was an experimental imitation of the situation in dizygotic cattle twins. Exposure of embryos to isoantigens led to the inhibition of serum antibody formation and to tolerance of a skin homograft in adult life (Billingham *et al.*, 1953; Hašek, 1953a).

Further attempts to induce immunological tolerance to more remote complex antigens were more or less unsuccessful (Buxton, 1954; Hašek, 1954; Kerr and Robertson, 1954). The phenomenon seemed to remain restricted mainly to the sphere of isoantigens until it was found that by means of purified antigens of heterologous origin (e.g., by serum proteins) specific immunological tolerance can be induced, which is in many respects comparable with that to cellular isoantigens (Hanan and Oyama, 1954; Cinader and Dubert, 1955; Dixon and Maurer, 1955; Smith and Bridges, 1956, 1958).

The question is whether the mechanism underlying the two phenomena is identical and, if so, why is it easier to induce tolerance to purified heterologous antigens than to complex cellular antigens from the same source. The most acceptable explanation seems to be that the induction of tolerance to an antigenic complex is the result of a series of separate events, the probability of each of which depends on the quantitative representation of the individual antigen in the complex. The probability of tolerance of a whole complex, in contrast to that of a single purified antigen, would then be far lower, since it would require simultaneous success in all the individual events. It cannot be excluded, however, that the inducibility of tolerance as compared with immunity is more strictly limited by genetic factors, and that tolerance takes place at the level of more fundamental cellular processes than those concerned with the actual elaboration of antibody.

The term of "induced immunological tolerance" is not quite unequivocal at present. The term has been used in a broad sense to include all known types of induced immunological nonreactivity (Chase, 1959a); it is also used in the narrower sense defined originally by Medawar and his associates (Billingham *et al.*, 1953, 1956a). It is, therefore, necessary to define the term "immunological tolerance" for our purposes. We run into the usual difficulty met by attempts to include under one common term phenomena which are apparently similar but whose mechanisms are not yet clear. Under these circumstances, the definition can be no more than a description of the conditions under which the phenomena occur. By immunological tolerance will be understood only specific immunological nonreactivity induced by the exposure of the embryo or the very young organism to a potential antigen. By defining tolerance in

terms of the conditions underlying its induction (i.e., the developmental period of the organism as a whole), we may be excluding some phenomena with an identical mechanism, at least at the cellular level. The definition, therefore, might require some modification when relating facts about tolerance to kindred phenomena.

A. Methods of Inducing and Testing Immunological Tolerance

1. Induction of Tolerance in the Prenatal Period

a. Natural Embryonic Parabiosis. The spontaneous induction of immunological tolerance in dizygotic twins is a frequent finding in cattle (Owen, 1945, 1946; Owen *et al.*, 1946; Anderson *et al.*, 1951; Billingham *et al.*, 1952; Billingham and Lampkin, 1957). In sheep (Lampkin, 1953; Stormont *et al.*, 1953; Hraba *et al.*, 1956), man (Dunsford *et al.*, 1953; Booth *et al.*, 1957; Nicholas *et al.*, 1957), and chickens (Billingham *et al.*, 1956a) it is known to occur far more rarely. In all these cases, the tolerance is the result of natural embryonic parabiosis in which the embryonic blood is exchanged between the partners, due to vascular anastomoses. The differences in the incidence of tolerance in the species just mentioned correspond to the differences in occurrence of hemochorial anastomoses which are known from the findings of embryologists (Keller and Tandler, 1916; Petskoy, 1955; Smidt, 1955).

The methods used for the experimental induction of tolerance are, in fact, always more or less perfect imitations of natural embryonic parabiosis or the artificial reproduction of the conditions resulting from it, i.e., exposure of the immunologically immature individual to a potential antigen.

b. Experimental Embryonic Parabiosis. Experimental embryonic parabiosis is the closest imitation of natural parabiosis (Hašek, 1953b); it is easily accomplished in bird embryos by the fusion of chorioallantoic membranes of two embryos, stimulated by depositing a fragment of embryonic tissue on the chorioallantois. This technique has been employed for the induction of immunological tolerance between two or more individuals in intraspecies relationships (Lazzarini, 1960) as well as in interspecies and intergeneric relationships (Hašek, 1954).

c. The Relation of Mother and Fetus. The relationship between mother and fetus in the case of leakage of the placental barrier seems to be an example of artificially induced one-way embryonic parabiosis. Tolerance toward the antigenic spectrum of the mother is induced quite exceptionally under normal conditions (Billingham *et al.*, 1956a). Indirect evidence has been obtained for the possibility of the spontaneous transplacental induction of tolerance only to the maternal Rh antigen (Owen

et al., 1954; Ward *et al.*, 1957). Specific tolerance to homografts of maternal origin was induced in the progeny of pregnant females treated by various agents which are supposed to increase the permeability of the placenta (Lengerová, 1957; Nathan *et al.*, 1960). Transplacental induction of tolerance need not, however, apply only to antigens of the mother. Tolerance occurring spontaneously in this way can also explain atypical congenital infections caused by the lymphocytic choriomeningitis virus, as described by Traub (1939) in mice, and the presence of maternal melanomatous tumor cells in man (Holland, 1933; Wells, 1940). The transplacental induction of tolerance to proteins was used experimentally in rabbits and guinea pigs (Humphrey, 1961).

d. Intraembryonic Injections of Cells. A looser imitation of the conditions of natural embryonic parabiosis is the intraembryonic injection of blood or of homogenates of different tissues (which seem to be more effective, presumably because they contain more cells capable of multiplying in the recipient). This technique for the induction of tolerance was first successfully used by Billingham *et al.* (1953). They inoculated mice and chicken embryos with viable cells (homogenates of mouse testis, kidney, and spleen and embryonic chicken blood). In the same way, tolerance was induced in various species of experimental animals, mammals and birds, both in interspecies and in more distant relationships between the donor and recipient.

e. Transplantation of Solid Tissue in Embryos. The direct transplantation of solid tissue into embryos is also a possible way of conferring immunological tolerance. The possibility of successful transfer of foreign tissue grafts to embryos, already known for many years, used to be explained simply by the immunological immaturity of the recipient (Murphy, 1914; Danchakoff, 1916). Moreover, evidence was not lacking that homologous cells transferred to the avian embryo (melanoblast, neural crest tissue, skin, and limb buds) could further differentiate and survive, even after immunological maturity was reached (Eastlick, 1941; Rawles, 1944, 1945, 1952; Weiss and Andres, 1952). This was already the consequence of acquired tolerance, but such experiments, made for other purposes, did not receive careful attention from immunologists. In cold-blooded animals, a series of successful homo- and heterotransplants were likewise made in early ontogenesis which persisted up to the time of immunological maturity (for references see Hildemann and Haas, 1959). A great technical success was the recent transplantation of the forebrain region in bird embryos before the establishment of the circulation and the obtaining of viable chicks bearing a homograft of a part of the head (Martinović and Pavlović, 1958).

2. *Induction of Tolerance in the Postnatal Period*

Some early successful homotransplantations (Danforth, 1929) and/or parabiosis carried out in the early postnatal period (Schwind, 1938; Lapchinsky, 1941) may be interpreted on the assumption that the capacity of achieving a state of immunological tolerance need not end with the fetal period, but may persist for a shorter or longer period of time after birth or hatching. The successful transfers of tumors in newborn animals can be explained in a similar way (Ehrlich, 1906). More recent experiments have confirmed the possibility of inducing tolerance after the embryonic stage in some animal species (Hraba and Hašek, 1956; Medawar and Woodruff, 1958) by means of skin graft transfers (Cannon and Longmire, 1952) or by single injections of blood or tissue homogenates (Hašek, 1955; Woodruff and Simpson, 1955; Billingham and Brent, 1957). A more drastic technique for induction of tolerance in the postnatal period seems to be virtual exsanguination and transfusion of fresh blood, successfully employed in puppies (Puza and Gomboš, 1958). In human newborns treated in this way for hemolytic disease tolerance was also occasionally induced (Albert *et al.*, 1959; Fowler *et al.*, 1960; Mariš, 1961).

Repeated injections of antigens (soluble proteins; Hanan and Oyama, 1954) or blood cells (Hašková and Pokorná, 1956) in the postnatal period, begun immediately after birth, can likewise induce tolerance.

3. *The Importance of Experimental Conditions in the Induction of Tolerance*

In principle, the successful induction of immunological tolerance depends to a great extent, but not primarily, on the conditions involved. Of great importance is the time of administration of an appropriate antigen; its amount (Smith and Bridges, 1958); the duration of its action (a fractionated dose being more effective than a single one; Hašková, 1957); the presence of antigen at the time critical for the induction of tolerance (Simonsen, 1956); the form of its presentation (protein antigen bound to antibody did not elicit the tolerance; Smith, 1960); and sometimes the route of administration (Billingham and Brent, 1959).

When inducing tolerance with viable cells, their origin may play a critical role and this partly accounts for the different effectiveness of various methods of induction. Following experimental embryonic parabiosis, all degrees were found from permanent tolerance of skin grafts (lasting more than 4 years in chickens) through long-term but impermanent, to only short-term tolerance (Hašek, 1954; Hort *et al.*, 1961). There are no entirely negative results after successful embryonic para-

biosis between individuals of the same species. One of the causes underlying the great effectiveness of embryonic parabiosis is certainly the embryonic origin of inducing cells, so that the conditions are fulfilled for tolerance between the partners in both directions.

Intraembryonic or postembryonic injections, using as a rule cells from adult spleen or other tissue containing immunologically competent elements, lead to the conditions for tolerance being fulfilled only on the part of the recipient, whereas a reaction by the graft against the host may occur, often resulting in its death (Billingham and Brent, 1957; Simonsen, 1957). The relatively lesser effectiveness of these methods can thus be explained by the fact that the ratio between tolerant and nontolerant individuals is changed in favor of the nontolerant, owing to the mortality of the tolerant animals (Billingham and Brent, 1959). There is no objection, however, to the use of immunologically immature cells for the induction of immunological tolerance; antigenic maturity considerably precedes immunological maturity (Chutná and Hašková, 1959; Medawar, 1959; Hašek, 1960a), so that appropriate embryonic cells are, in principle, capable of eliciting and acquiring tolerance at the same time. Tolerance can, in fact, be successfully induced without the accompanying undesirable effects of runt disease and neonatal mortality, by means of immunologically immature cells (Hašek and Hašková, 1958; Simonsen, 1957) or adult cells whose immunological competence is either lacking (Terasaki, 1959) or does not apply to the recipient (Billingham and Brent, 1959).

Although there is no cause and effect relationship between runt disease and tolerance, there is a striking parallel between the two in regard to the cells which induce them most effectively; these are spleen cells, lymphoid tissue, bone marrow cells, and leucocytes. The decisive factor does not seem to be the immunological competence of these cells, but their ability to settle in the lymphoid tissues of the recipient, so that the tolerance-inducing stimulus successfully reaches the relevant sites in the recipient. This does not, however, always hold true. Tolerance in ducks can also be induced by skin homografts irradiated with a dose of 1500 r (Hašek, 1961). The activity of the lymphoid elements of skin is greatly reduced by this dose, as is evident from the inhibition of splenomegaly resulting from a similar irradiation of spleen or skin grafts transferred to the chorioallantois of chicken embryos (Kryukova, 1959; Mun *et al.*, 1959; Puza *et al.*, 1959). There is an inconsistency in the findings of Medawar and Woodruff (1958) and of Billingham and Silvers (1960) which has also not yet been resolved; the former succeeded in eliciting tolerance by skin grafts in rats, whereas the latter failed to induce tolerance to sex

antigens by skin grafts in mice, and they attribute their failure to the absence of lymphoid elements in the skin of mammals. It is not impossible, however, that the incapacity of some types of cells or tissues to induce immunological tolerance is connected with the phenotypic variability of diversely differentiated tissue elements and their failure to provide a complete antigenic stimulus in appropriate form and amount.

4. *Tests for Immunological Tolerance*

a. Tissue Transplants. Immunological tolerance has been tested by various means. The homograft reaction is a very sensitive test (even if the capacity for serum isoantibody formation is weak); as a rule, transplants of skin (Billingham *et al.*, 1953) or tumors (Koprowski, 1955) are used; in special cases, the persistence of erythrocyte chimerism proves the successful induction of tolerance (Owen, 1945, 1954).

b. Formation of Serum Antibodies. Another method for the demonstration of immunological tolerance consists in the follow-up of serum antibody formation and/or the rate of elimination of antigens from the circulation. The capacity for antibody production has been investigated after the induction of tolerance toward homologous (Hašek, 1953a) and heterologous cells (Hašek and Hraba, 1955a,b, Simonsen, 1955b), as well as to bacterial antigens (Buxton, 1954), serum proteins (Hanan and Oyama, 1954), and enzymes (Bussard, 1957; Cinader *et al.*, 1958). The persistence of antigen in the circulation has been studied in proteins (Dixon and Maurer, 1955) and blood cells (Mitchison, 1959).

c. Skin Tests. Skin tests for a hypersensitive state have only occasionally been used in the study of immunological tolerance (D.W. Weiss and Wells, 1957; Turk and Humphrey, 1961).

d. Susceptibility to Infective Agents. The success of the induction of tolerance to an infective agent has been tested, as a rule, more on the basis of antibody formation (Buxton, 1954) or skin sensitivity (Weiss and Wells, 1957) than according to the course of the infection. After BCG injections into guinea pig embryos (Milgrom *et al.*, 1958), the tolerance to the infective agent manifested itself rather paradoxically—the infection with *Mycobacterium tuberculosis* assumed a milder form and the experimental animals survived longer. This seems to be analogous to older findings by Traub (1939) in mice where, after viral infection *in utero*, lymphocytic choriomeningitis followed without antibody formation and the clinical signs of the disease. A favorable effect of tolerance could be explained by assuming that a strong immune reaction is detrimental to the infected organism. This could account for the finding that the pathogenic action of choriomeningitis virus in mice can also be

reduced by some nonspecific inhibitors of immunological reactivity (Rowe, 1956).

e. Autoimmunity to Tissue Antigens. This method is based on the idea that immunological tolerance is the means by which immunological unresponsiveness is secured against the body's own constituents which are in contact with the mesenchymal cells during their maturation. This assumption seems to be consonant with the findings that autoimmunity can be induced against antigens which are not normally in contact with mesenchymal cells during embryogenesis—such antigens either develop too late (e.g., antigens of sperm) or are kept apart from the mesenchymal cells under normal conditions of development (e.g., thyroglobulin and antigens of the eye lens).

Paterson (1958) made attempts to prove this hypothesis by inoculating brain homogenates into newborn rats. In animals assumed to be tolerant owing to this pretreatment, it was impossible to induce allergic encephalomyelitis by inoculations of the same material in a complete Freund's adjuvant. It is not quite clear, however, whether the inability to induce an autoimmune disease is really the result of tolerance to organ antigens of the central nervous system or of some other mechanism (Kies and Alvord, 1958; Svet-Moldavskaya and Svet-Moldavsky, 1958; Svet-Moldavsky *et al.*, 1959).

B. The Occurrence of Immunological Tolerance and the Duration of the Adaptive Period in Different Species

Immunological tolerance is evidently a general phenomenon since it has been found or induced in all species investigated. In addition to mammals and birds, tolerance was successfully induced in frogs (Hildemann and Haas, 1959; Vyazov and Sorokina, 1961). The stress upon the early developmental stage as a condition for the induction of tolerance is not accidental; the period of time during which this type of unresponsiveness can be induced, the so-called adaptive period, seems to end at the same time as fetal development, when a rather sudden change in the reactivity of the developing individual takes place. The same doses of antigen can induce either tolerance or immunity depending on whether given before or shortly after the end of the adaptive period (Hašková and Pokorná, 1956; Smith and Bridges, 1956). The main differences in the duration of the adaptive period are a property of the species, conditioned evidently by a varying degree of maturity of immune systems at the time of hatching or birth. Judging by the stage at which homograft tolerance can no longer be produced, the animal species investigated so

far can be classified into the following groups: (a) sheep and rabbit (the adaptive period ends before birth), (b) rat and duck (the adaptive period persists for several days after birth), and (c) mouse, dog, chicken, and turkey representing a transitional group. In addition to species differences, there is an appreciable individual variability in the duration of the tolerance responsive phase. The adaptive period for the fairly weak sex antigen in contrast to other isoantigens is relatively long (Billingham and Silvers, 1960).

The nature of the antigen also plays an important part. The question is, however, to what extent the nature of the antigen is itself responsible for the varying duration of the adaptive period and/or a different maturation rate of the normal immune response mechanisms. An extreme example of such a situation is provided by the adaptive period in the rabbit, in which tolerance to homologous cells could no longer be induced after the twenty-second day of embryogenesis (Porter, 1960), whereas tolerance to soluble proteins was conferred as late as the fifteenth day after birth (Smith and Bridges, 1956). Such a comparison of the duration of the adaptive period to different antigens has the disadvantage that two variables are included: antigen and the system of testing tolerance. It is not impossible that the delayed-type reaction and serum antibody formation are two reaction mechanisms, more or less independent of each other, maturing at a different rate in the process of ontogenesis; this might be inferred from the finding that the specific cellular reaction can precede the onset of serum antibody formation (Schinkel and Ferguson, 1953; Šterzl *et al.*, 1960).

A much more appropriate model for the solution of this question would be a system making it possible to test tolerance of the same antigen by comparing its inhibition effect on both fundamental specific immune mechanisms. In the original experiments, tolerance induced with viable cells involved both the cellular type of immunity and serum antibody formation.

When inducing tolerance with erythrocytes, only the formation of aglutinins was suppressed, whereas the skin graft was not tolerated (Hašek *et al.*, 1955, 1956; Billingham *et al.*, 1956d). On the other hand, tolerance of skin homo- (Štark *et al.*, 1960b) and heterografts (Hašek and Hort, 1960) was found in animals producing hemagglutinins against the donor's cells.

Neither of these findings can be taken as decisive evidence for the dissociation of the two specific types of immune response by means of tolerance. A difference in the sensitivity of the two detection systems could imitate such a dissociation and moreover, the identity of antigens

inducing the antibody formation and specific cellular response is open to doubt in these cases.

The antigens were, however, identical in the experiments of Turk and Humphrey (1961), who induced tolerance to bovine serum albumin (BSA) and human γ-globulin in guinea pigs in respect both of delayed-type hypersensitivity and of serum antibody formation. This result seems to indicate that, if under appropriate conditions a given antigen can induce different types of immune reactions (the relationship between which has not as yet been clarified), then tolerance to this antigen might cover all potential types of specific immune response.

C. Basic Facts of Immunological Tolerance

A logical consequence of immunological tolerance induced with cells capable of reproduction is that these cells or their progeny persist in the tolerant individual. Animals rendered tolerant appeared, in fact, to be cell chimeras after embryonic parabiosis (Billingham *et al.*, 1956a) as well as after inoculation with viable cells (Martinez *et al.*, 1958; Medawar, 1958b; Billingham and Brent, 1959). Tests for immunological tolerance made in such individuals (skin homograft or antibody formation) only confirm that tolerance toward the donor whose cells are already present in the tolerant recipient can extend to cells of different histogenetic origin.

Tolerance induced with viable cells capable of reproduction can persist permanently under optimal conditions. On the other hand, tolerance of nonreproducing antigens is a transient state even though it persists sometimes for a very long time (Humphrey, 1961), and its duration can be prolonged by the further administration of antigen (Smith and Bridges, 1958; Smith, 1960).

If the mechanism underlying both phenomena were the same, then the reproduction of viable cells could be not only a consequence of the state of tolerance induced by them, but also a necessary condition for its maintenance. This would be in agreement with the fact that tolerance induced with cells unable to reproduce can be maintained only by the repeated introduction of these cells (Mitchison, 1959). The fate of antigen in a tolerant organism could be, therefore, a convenient criterion for the classification of the facts of immunological tolerance.

1. Manifestations of Tolerance Induced with Viable Cells

a. Compatibility of Homografts. Immunological tolerance induced with blood or spleen homogenates, and usually tested with skin grafts, can also be employed for homografting other tissues. Such attempts were

successfully made with adrenal cortical tissue (Medawar and Russel, 1958), thyroid gland (Woodruff and Sparrow, 1958), ovary (Martinez *et al.*, 1956; Krohn, 1958), bone (Nisbet *et al.*, 1960), and kidney (Gomboš *et al.*, 1960; Puza *et al.*, 1961).

Medawar's group provided convincing evidence that acquired tolerance to a homograft is primarily due to a modification of the immunological system of the recipient. By adoptive transfer of isologous lymph node cells from normal or preimmunized mice into a tolerant animal, tolerance can be abolished (Billingham *et al.*, 1953, 1956a). Thus, tolerance may be a central failure of antibody formation due to a systemic modification at the cellular level (at the level of lymphoid cells). The tolerated graft may also retain some antigenic properties of the donor at least at the time of the experiment.

Although there is indirect evidence that adaptation of the graft facilitates its transplantability, it cannot be considered as unequivocal proof that such adaptation is involved in the actual induction of tolerance. In induction of tolerance in newly hatched chicks by means of skin grafts from adult and newborn donors, the greater effectiveness of the latter was interpreted in favor of the adaptability of the young graft (Cannon *et al.*, 1954). Weber *et al.* (1954) described rejection of a second homograft in adult chickens which still tolerated the first graft from the same donor, transplanted when both the recipient and donor were newly hatched.

Evidence against the idea that adaptation of the graft from a young donor participates in its becoming tolerated is provided by the finding in ducks that there was no difference in the effectiveness of skin graft from newly hatched and adult donors. The difference between the two results might be partly the result of the longer adaptive period in ducks. Second grafts from the same donors transferred to ducks 40 days later suffered the same fate at those transplanted at birth. If the second transplantation took place 200 days later, however, only half the grafts were successful (Hašek, 1961). The fate of grafts transplanted back to the donor after a prolonged period of being tolerated by the homologous host has received little attention. Cannon (1957) found that as few as half the homografts originally exchanged between newly hatched chickens could be successfully transferred back onto the original donor in adult life.

These findings cannot, therefore, be interpreted as evidence for a primary role played by the adaptation of the graft in the induction of tolerance, but rather as support for the view that tolerance provides the conditions for the graft to undergo some changes which eventually make

possible its survival even on an incompletely tolerant animal whose tolerance disappears with time. This idea would be in agreement with the finding of Woodruff (1957) that in rats the later of the two grafts from an adult donor need not take, although the first one continues to be tolerated. The survival of a chicken skin heterograft in a turkey (an embryonic parabiont) at the time of disappearance of chimerism and of the onset of antibody formation (Hašek and Hort, 1960) could also have a similar explanation. As a rule, the take of a skin graft is a highly sensitive indicator of complete tolerance, but its long-term survival is not incompatible with a state of incomplete or even lost tolerance.

The nature of changes which might occur in the long-term tolerated graft was discussed by Woodruff (1959). He pointed out especially the possibility of stromal replacement and of adaptation at the cellular level; these would imply changes of a phenotypic nature (e.g., diminished accessibility of nontolerated antigens) and eventually also changes of a genetic nature (due, e.g., to the exposure of the graft cells to the host's DNA). Somatic antigenic recombination in interspecies chimeras (turkey-chicken) was, however, not found (Hašek *et al.*, 1960).

b. Compatibility of Heterografts. A heterograft of normal tissue is tolerated only rarely. After interspecies embryonic parabiosis between members of closely related species, long-term or permanent tolerance of a skin heterograft (Hašek *et al.*, 1960) and long-term erythrocyte chimerism (Hraba, 1956; Hort, 1957; Hašek *et al.*, 1960) occurred only exceptionally. In general, the heterologous tolerance was only of short duration (Hašek, 1954; Billingham *et al.*, 1956a), and techniques other than embryonic parabiosis were even less effective in inducing tolerance. Injections of heterologous blood (Billingham and Brent, 1956), bone marrow (Hašková and Hašek, 1957), and tumor cells (Green and Lorincz, 1956) in birds as well as rabbit (Egdahl and Varco, 1957), and human tumor cells (Wallace, 1956) into young rats elicited only short-term tolerance, if any.

c. Susceptibility to Tumor Grafts. One of the tests for induced immunological tolerance is the susceptibility of the pretreated recipient to homo- or heterologous tumors. This criterion does not, however, provide results entirely consistent with those from the transplantation of normal tissues. Tolerance tested by tumors seems usually to be more effective, including, in addition to successful homotransplantation (Koprowski, 1955) more or less successful heterotransplantation (Bollag, 1955; Grozdanovič, 1956; Koprowski *et al.*, 1956; Ageenko, 1959; Radzikhovskaya, 1959; Iversen and Sørensen, 1960). Tumor growth which as a rule kills the host is, of course, only a short-term test for tolerance, and, in addi-

tion, certain specific properties inherent to tumor tissues might make possible both their resistance to weak immunity and perhaps some forms of graft adaptation.

Koprowski (1955; Koprowski *et al.*, 1956) demonstrated a marked adaptation manifested in the capacity of the passaged tumor to grow later even on an untreated recipient. The mechanism of this adaptation in homologous relationships was studied in detail by Feldman and Sachs (1958). They assume that the resistance of adapted tumors is based on neutralization (either extracellular or intracellular) of the homograft reaction on the part of the recipient by means of a high production of isoantigens. Another type of adaptation was described earlier by Barrett and Deringer (1950, 1952) following the passage of the tumor from the parental strain on F_1 hybrids, and this mechanism was explained as resulting from the contact of tumor cells with foreign tissue antigens in the absence of any immunological reaction (Klein and Klein, 1956). In a "tolerant" environment the effectiveness of the adaptive mechanism could be further increased by an immune selection of the modified cells as a consequence of an incomplete tolerance.

Tumors transferable by means of a filtrable agent occupy an exceptional position. Particular attention was devoted to the transfer of chicken Rous sarcoma virus to turkeys (Simonsen, 1955b; Harris, 1956; Svoboda and Hašek, 1956) and to ducks (Svoboda, 1958), in which tolerance was induced to normal tissue chicken antigens. Tolerance to a leukemogenic agent in mice was described by Duplan and Monnot (1958). The analysis of the mechanism of the tolerance to Rous sarcoma showed that, in this case, tolerance was not related to virus antigens (Svoboda, 1961) but probably to cells in which (as a consequence of their malignant transformation by virus) some nonviral chicken antigens occurred (Hort *et al.*, 1960). The successful growth of a virus-induced chicken tumor in a heterologous recipient does not require tolerance to a complete spectrum of chicken antigens (Svoboda and Hašek, 1956; Hort *et al.*, 1960), and susceptibility can be induced even by lyophilized chicken blood and other preparations (Harris and Simons, 1958; Svoboda, 1958, 1960a,b).

The question is, however, whether the successful induction of tumors in newborn animals by means of tumor virus is not accompanied by tolerance of the virus-modified cell, even in cases in which the effect of tolerance to the virus itself can be excluded (Sachs *et al.*, 1959; Ham *et al.*, 1960).

d. Suppression of Antibody Formation. The formation of isoantibodies (agglutinins) affords a possible test of tolerance only in species

whose members are good producers of well detectable isoagglutinins. These conditions are very well fulfilled in chickens in which the tolerance induced by means of embryonic parabiosis manifests itself by a complete suppression of antibody formation against the partner's erythrocytes. This state persists even after repeated immunizations (Hašek, 1953a). The nonreactivity sometimes lasted for a number of years and remained even in animals from which erythrocyte chimerism had disappeared (Hašek and Hraba, 1955a). The elimination of the partner's erythrocytes (detected by Cr^{51} labeling) proceeded in parabionts at the same rate as the elimination of autologous erythrocytes (Hort *et al.*, 1961).

In chickens, injections of blood also lead to a similar suppression of isoagglutinin production (Billingham *et al.*, 1956d; Hašek *et al.*, 1956).

When interspecies differences are concerned, the changes in antibody formation are far less pronounced. Following single injections of heterologous blood, antibody formation, if affected at all, was only decreased (Simonsen, 1955b, 1956; J. A. Bauer *et al.*, 1956; Hašek, 1956a; Owen, 1956; Puza and Molnár, 1956; Grozdanovič, 1957). Complete inhibition was found only in closely related species (Hašek, 1956b) or when testing tolerance in very young animals by a single immunizing dose (Simonsen, 1955b; Hraba 1956).

Even after embryonic parabiosis, heteroagglutinin formation is only decreased (Frenzl *et al.*, 1955; Hašek and Hraba, 1955a; Hort, 1957), except in cases of parabiosis between members of some closely related species, in which the chimerism may persist throughout life. Tolerance may, therefore, include even the inability to form "natural" antibodies (Hraba, 1956; Hašek *et al.*, 1960).

The phenomena of tolerance induced by heterologous cells already represent a category transitional between tolerance to self-reproducing antigens, on the one hand, and to nonreproducing antigens, on the other. In spite of the potentiality of heterologous cells to reproduce in a tolerant recipient, chimerism is mostly not established at all or disappears early, and the source of further antigen is abolished. Nevertheless, a form of partial tolerance may persist, sometimes for many months (Hašek and Hraba, 1955a; Hašek *et al.*, 1960). Persistence of chimerism, however, requires complete tolerance on the part of the heterologous recipient. This condition is not necessarily sufficient, however, as factors of a nonimmune nature might hinder the proliferation of cells in a heterologous environment (Barnes *et al.*, 1959; Goodman and Smith, 1959). Consequently, it may be asked whether the disappearance of chimerism should be regarded as a consequence of partial tolerance or whether

partial tolerance is a result of the disappearance of chimerism for some other reason. A partial answer is provided by experiments in which the state of chimerism is artificially maintained by the repeated administration of heterologous cells (Hašek, 1956a; Hašková, 1957; Nossal, 1957, 1958). Such a procedure is quite effective, when carried out between fairly closely related species, but no results comparable to tolerance to isoantigens are obtained in more distant relationships (Hašek, 1960b). It seems, therefore, that in heterologous relationships the disappearance of chimerism is a consequence of incomplete tolerance, and not vice versa, but participation of some other factors cannot be excluded.

2. *Tolerance to Nonreproducing Antigens*

The use of antigens incapable of reproduction offers some advantages for the study of tolerance. Tolerance to purified, chemically defined antigens may represent a model system which could clarify the mechanism underlying the tolerance of complex cellular antigens. Moreover, it provides an approach to the question whether persistence of a self-reproducing antigen is the cause or the consequence of the maintenance of the state of tolerance.

a. Tolerance to Nonreproducing Cells. Tolerance induced by means of cells which cannot reproduce can serve as a model for studying the import of the continual supply of antigen provided by viable cells. In chickens, Mitchison (1959) used homologous blood irradiated with a dose of 4000 r and so obtained cells incapable of dividing; by repeated administration of such blood cells labeled with Cr^{51} he could maintain the presence of foreign cells in the circulation and a nonimmune elimination. The state of tolerance disappeared quite rapidly (between the second and the twenty-fifth day) after the disappearance of foreign erythrocytes from the circulation. This is in contrast to previous findings (Hašek and Hraba, 1955a; Billingham *et al.*, 1956d; Hašek *et al.*, 1956), in which the evidence for the disappearance of chimerism is, however, not so convincing. The extremely rapid disappearance of tolerance in Mitchison's experiments may be the reflection either of a higher sensitivity of the detection technique or of a special situation in birds as compared with mammals (Tempelis *et al.*, 1958a,b). It is also possible that incomplete tolerance might have been involved.

b. Tolerance Induced by Isoantigenic Preparations. Attempts have been made to induce tolerance to homografts by means of isoantigenic preparations obtained from cells. Crude aqueous extracts which were effective in inducing immunity (Billingham *et al.*, 1956b) were ineffective in inducing tolerance (Hašková and Majer, 1959; Medawar, 1959).

Part of the antigens may possibly be destroyed during preparation, and the remaining antigens, although sufficient for the induction of immunity, may be incapable of inducing complete tolerance which is necessary for the acceptance of a skin homograft. Tolerance of a male graft in females, i.e., of the sex antigen could, however, be induced by means of cell-free material (Billingham and Silvers, 1960). In principle, there is no reason why tolerance should not be induced by cell-free material, but the question is how long such tolerance could be maintained.

c. Tolerance Induced by Serum Proteins. The administration of foreign soluble proteins into very young animals can induce a specific inhibition of antibody formation against the antigen employed. This type of induced immunological tolerance has received particular attention in relation to mammalian serum proteins, especially in rabbits (Hanan and Oyama, 1954), later in chickens (Wolfe *et al.*, 1957), mice (Terres and Hughes, 1959), rats (Kryukova, 1957), goats (Carter and Cinader, 1960), and guinea pigs (Humphrey and Turk, 1961). Although a high degree of tolerance to purified serum proteins was obtained in rabbits (Dixon and Maurer, 1955; Cinader and Dubert, 1955; Smith and Bridges, 1956; Curtain, 1959; Humphrey, 1961), only short-term and partial (Tempelis *et al.*, 1958a, b; Stevens *et al.*, 1958) or quite insignificant tolerance (Majer and Pokorná, 1959) was obtained, as a rule, in chickens. This difference might be due to a quantitatively larger antibody-formation mechanism in the chicken as compared with the rabbit (Hirata and Schechtman, 1960) or to a more rapid catabolism of antigens in chickens leading to the power of inducing tolerance to be decreased. The comparison of tolerance between these two animal species is, of course, performed in relation to antigens whose source (i.e., cattle, man) is of different phylogenetic distance; therefore, the difference in inducibility of tolerance might also be a reflection of this factor. Although experimental evidence is as yet lacking, the phylogenetic relationship between the source of antigen and the recipient might affect the degree or even the possibility of inducing tolerance toward purified protein antigens, as in the case of cellular antigens. To settle this, it would be necessary to test individuals of the same species for tolerance induced by analogous substances (e.g., serum albumins) obtained from individuals of species of different phylogenetic distance. Such experiments have not yet been carried out.

In induction of tolerance by means of whole serum, it is usually possible to obtain complete tolerance of the main components only (Dixon and Maurer, 1955; Hraba, 1959). Apparently, the relative proportions of individual components are important here.

Tolerance induced by a single injection of protein antigen disappears with time, its duration corresponds to the inducing dose, and it can be repeatedly prolonged by the administration of small amounts of the respective antigen (Smith and Bridges, 1958). The question of whether or not the presence of antigen in the organism is necessary for the maintenance of the state of tolerance already induced and what is its function has not yet been resolved. Denhardt and Owen (1960) did not abolish tolerance to BSA by X-irradiation in rabbits although the antigen is thought to be for the most part removed from the body as a result of radiation death of cells critically concerned with the storage of antigens. However Dresser (1960), using a more telling experimental design, found that, in mice, tolerance of bovine γ-globulin disappeared rapidly if antigen was diluted in the course of provoked mitosis in lymphoid cells. If so, it would imply that antigen given to a young, immunologically immature organism, would actually only postpone the onset of the specific immune reaction for so long as its concentration sufficed—perhaps to maintain some adaptation of cells already tolerant or to induce tolerance in newly differentiating cells.

The localization of antigen in a tolerant organism is also obscure. It has been demonstrated that tolerance can persist even when antigen is being removed from the circulation (Smith and Bridges, 1958; Dresser, 1960; Humphrey, 1961). On the basis of their results, Smith and Bridges (1958) calculated that tolerance disappeared at a time when the amount of antigen persisting in the circulation from the inducing dose dropped below 10^{12} to 10^{13} molecules. These figures, however, do not hold true in general. Humphrey (1961) showed that tolerance might persist even after it was calculated that all molecules of antigen had left the circulation; after a single dose of protein antigen to rabbits at birth, long-term tolerance (up to 123 weeks) was observed in more than half of the animals. Such duration is comparable to that occurring in tolerance to living cells; thus the question arises whether or not maintenance of the change, when once induced by a nonreproducing antigen, requires a further supply of antigen. It seems highly probable that, if the antigen must be present in the organism to maintain the tolerance, some intracellular localization is involved. It does not seem, however, that the antigen impresses its specificity upon some reproducing cellular structure in the induction of tolerance.

d. Tolerance to Other Antigens. Among other antigens, mainly microorganisms and viruses were used for the induction of tolerance. With these only depression of antibody formation was observed (Buxton, 1954; Kerr and Robertson, 1954; Šterzl and Trnka, 1957; Friedman and Gaby,

1960a,b), and in several experiments no alteration of the immunological reactivity was demonstrated at all (Burnet *et al.*, 1950; Cohn, 1957; Smith and Bridges, 1958). The small quantity and the complexity of antigens used for the induction might be the cause of these failures.

Quite isolated, however, is the finding by Traub (1936, 1938, 1939) concerning the suppression of the immune reaction and the latent course of lymphocytic choriomeningitis after transplacental virus infection. The mild course of this infection in newborn mice was confirmed experimentally (Whitney, 1951; Hotchin, 1957), but the alterations of immunological reactivity were not followed simultaneously. No other appropriate model has yet been found which takes advantage of the multiplication of a noncytopathogenic virus in the embryo for obtaining a large amount of tolerance-inducing antigen. In an experiment in which this property of the virus was utilized, tolerance was not demonstrated (Burnet *et al.*, 1950) and the cause of this was not analyzed. Using more or less purified and markedly foreign antigens (Cohn, 1957; Smith and Bridges, 1958), the only success was obtained in rabbits with yeast glucose-6 phosphate dehydrogenase (Bussard, 1957, 1960). These experiments seem to be a promising approach to the solution of the question of whether it is, in principle, possible to induce tolerance to any foreign antigen, or whether there are some limits of a physiological or genetic nature (Denhardt, 1960).

3. *Quantitative Manifestations of Immunological Tolerance*

Medawar and his associates (Billingham *et al.*, 1953) expressed the view that immunological tolerance is not an "all-or-nothing" phenomenon, but that it occurs in all quantitative stages from complete nonreactivity to normal reactivity. Quantitative manifestations of tolerance can primarily be evaluated with regard to duration, and afterward by the degree of specific immune reaction which a treated organism is capable of developing against the respective antigen (the degree of this reaction may also reflect the proportion of the antigens in an antigenic spectrum to which tolerance was actually achieved). Lastly, from a different angle, variability in the specificity of tolerance could be a quantitative phenomenon. It remains an open question, however, whether the appearances of partial or imperfect tolerance are all based on the same mechanism as complete tolerance, or whether sometimes the immunological reactivity of the treated organism may not be depressed in other ways.

In complete tolerance, we are dealing with a complete inhibition of antibody formation against the whole spectrum of antigens in the inducing complex, to a degree which exceeds any variations in immunological

responsiveness in normal animals. (This statement is not wholly accurate as far as tolerance to "weak" antigens is involved, e.g., to BSA, since 5–20% of normal adult rabbits fail to form antibodies against it (Dixon and Maurer, 1955; Smith and Bridges, 1958).

Partial tolerance of a skin graft, for example, is manifested only by a survival time which is significantly prolonged in relation to untreated controls. Moreover, a trace of the tolerance is also revealed by the survival time of a second graft from the same donor transplanted after the first was rejected (Billingham *et al.*, 1956a). Partial tolerance is also manifested by decreased titers of serum antibodies compared with control animals (Buxton, 1954; Hašek and Hraba, 1955b; Simonsen, 1955b). No matter which of the available tests is used to detect partial tolerance, it seems that for some reason or other tolerance has not been attained to all the components of the inducing antigenic complex. As a rule, detection systems are lacking to demonstrate whether partial tolerance is a manifestation of split tolerance, i.e., complete tolerance to some antigenic constituent of the inducing complex which is, however, partly masked by an immune response to other components. A simple example is partial tolerance to serum, which seems to be a result of split tolerance to the most abundantly represented components (Dixon and Maurer, 1955; Hraba, 1959).

There are also a few detection models of split tolerance to cellular antigens. Thus tolerance to sex antigen in mice (Billingham and Silvers, 1958; Mariani *et al.*, 1959) can even be induced by homologous male cells which are not tolerated themselves (Billingham and Silvers, 1960; Lustgraaf *et al.*, 1960). A similar situation is encountered in the majority of heterologous embryonic parabionts in which the erythrocyte chimerism disappears, as a rule, within 3 weeks after hatching, but a partial depression of heteroagglutinin formation lasts for 6 months at least (Hašek and Hraba, 1955a; Hašek *et al.* 1960). That split tolerance is the mechanism of partial tolerance in a heterologous combination is suggested by the finding of a long-lasting susceptibility to virus-induced Rous sarcoma in turkeys, partially tolerant of chicken cells. The turkeys, from which chicken erythrocytes disappeared within 3 weeks of ending embryonic parabiosis and which formed agglutinins and repeatedly rejected skin heterografts, died from virus transferred Rous sarcoma (Hort *et al.*, 1960). Thus split tolerance to cellular antigens can persist, often for a long time, even after the source of further doses of antigens has disappeared.

Although in many instances partial tolerance might be a quantitative outcome of two or more qualitatively different concurrent processes

(tolerance and immunity), this need not hold in general. Despite the fact that partial tolerance even to a single protein antigen (Bussard, 1957; Wolfe *et al.*, 1957; Stevens *et al.*, 1958; Hirata and Schechtman, 1960) could be traced back to split tolerance at the level of antigenic determinants, it may still be true that many types of partial tolerance are due to quantitative phenomena. Thus a partially tolerant animal might possess fewer cells capable of forming normal amounts of antibodies or a normal complement of cells might produce less than the usual amount of antibodies.

There is marked variation in the extent to which different antigens elicit tolerance. The most important factor appears to be the number of antigenic determinants to which the individual recipient can make an immune response, and this is likely to be greater for antigens derived from phylogenetically distant rather than from closely related sources. The more separate immune responses to an antigen are possible, the less will be the likelihood of simultaneous modification of them all, i.e., of obtaining complete tolerance.

Other factors have also to be considered. For example, differences in rates of catabolism between antigens depending on their chemical natures (e.g., protein and polysaccharide) or on the form in which they are administered may be of great importance in deciding whether complete or partial tolerance will occur.

Experiments on antigens specific for organs (Feldman and Yaffe, 1957), sex (Billingham and Silvers, 1960), or tumors (Levi *et al.*, 1959; Zilber, 1959, 1960) have been used as evidence for the specificity of tolerance, and at the same time the specificity of tolerance has been taken for granted and the results have been regarded as demonstrating the existence of the specific antigens in question. Two experimental designs have been used. In one, tolerance is induced by means of an antigenic complex which lacks the specific antigen in question, and the presence of the latter can be revealed by development of immune reactions against it. In the other, the specific antigen is present in the antigenic complex used to induce tolerance, but is the only component to which tolerance results; this can later be detected in an appropriate system.

Animals rendered tolerant to tissue cells of a given donor may show partial tolerance toward tissues of a homologous donor, and this could be interpreted as "nonspecificity." It may, in fact, be due to antigenic overlap, and the extent of such overlap within a noninbred population and between inbred strains can be quantitatively expressed, either in terms of immunity (Berrian and Jacobs, 1959; Lengerová and Chutná, 1960) or of tolerance (Billingham and Brent, 1959). Such antigenic over-

lap is essential for the induction of polyvalent immunological tolerance between host and graft (Hašek and Hašková, 1958; Billingham and Silvers, 1959) as well as between the individual components of a pooled graft (Lengerová, 1960).

The idea behind the attempts to induce polyvalent immunological tolerance was to expose an immature immunological system to a wide spectrum of antigens of different origin and thus increase the probability of tolerance being also related to tissues from a randomly selected donor whose cells were not employed for the induction. Positive results confirm this assumption as being correct.

Experimental results interpreted as evidence for (Cannon *et al.*, 1958 a,b) or as admitting the nonspecificity of tolerance (Hildermann and Haas, 1959) can also be interpreted in terms of specificity. The argument that tolerance can include tissues of two donors which would destroy each other's graft can be answered on genetic grounds by assuming that the antigenic assortment of each of the two donors is included within the whole spectrum of antigens to which the recipient has natural *plus* acquired tolerance.

This, however, does not exclude the possibility that tolerance is not strictly specific at the molecular level, especially when antigens with a closely related structure are concerned. Some findings in heterologous systems can hardly be interpreted otherwise than by the nonspecificity of tolerance. Thus, in heterologous tolerance, the capacity for the reaction against other species may be maintained, but the capacity to recognize antigenic differences within the species can be more or less suppressed. This difference is related to species differences in the donor and in the host (Hašek, 1954; Hašek and Hraba, 1955a, Hašek *et al.*, 1959, 1960; Hašek and Hort, 1960). With regard to specificity, therefore, heterologous tolerance is scarcely comparable with homologous tolerance. Hence, there may be different mechanisms. It is not impossible that the exposure of the immature immunological machinery to a completely foreign antigenic stimulus is so unphysiological an interference that it leads to some more or less nonspecific damage instead of to a specific modification. For example, some key genetic information might be suppressed, or the recognition mechanism might have deteriorated to the extent that it had become very insensitive to weak stimuli. The result might perhaps bear some analogy to the influence of nonspecific inhibitors of immunological reactivity such as irradiation (Clemmesen, 1940; Makinodan and Gengozian, 1960).

Even if analyzed by means of purified antigens, the question of specificity of tolerance is not solved unequivocally so far. Contradictory re-

sults have apparently been due to the combinations of inducing and testing antigens used. For example, Cinader and Dubert (1955) demonstrated the specificity of tolerance to BSA in rabbits by using tobacco mosaic virus as a control antigen in order to avoid the possibility of a cross-reaction, whereas Hanan and Oyama (1954) found a certain nonspecificity of tolerance to BSA with egg albumin as a control—although these two antigens give a cross-reaction (Maurer, 1954). In the case of Cinader and Dubert's findings, it may be objected, however, that the antigens employed were too different to ensure adequate sensitivity for the detection of small deviations from strict specificity. The solution of the question of the specificity of tolerance will, therefore, require careful experimental design and antigens of a known chemical structure.

D. The Relationship of Immunological Tolerance to Some Tolerancelike Phenomena

The term "immunological tolerance" implies a certain vagueness as it is defined rather by its conditions than by its causes. If any condition not critical for the actual mechanism of tolerance is stressed in its definition, some basically similar phenomena could be excluded because it did not fulfill this subsidiary condition. Several phenomena giving similar results are known, and it is appropriate, therefore, to examine in which fundamental features they conform with immunological tolerance and in which they differ.

Inhibition of immune reactions induced by irradiation, cortisone, and pathological anergy, such as agammaglobulinemia (Good *et al.*, 1960) or Hodgkin's disease (Kelly *et al.*, 1960), cannot be classed among phenomena of tolerance. In all these states the inhibition is nonspecific, of a generalized nature, and the antigenic stimulus does not take part in its induction. The specific inhibition of immune reactions induced by 6-mercaptopurine (R. Schwartz and Dameshek, 1959) will be left aside, because the experimental evidence is rather limited and partly contradictory (Schwartz and Dameshek, 1960; Meeker *et al.*, 1960).

1. Immunological Paralysis and Protein Overloading

Immunological paralysis may be cited as the first phenomenon. It is long-lasting (15 to 18 months at the minimum) and specific. The earliest example is the nonreactivity induced by the administration of large amounts of pneumococcal polysaccharides to adult animals (Perlzweig and Steffen, 1923; Schiemann and Casper, 1927; Felton and Ottinger, 1942; Felton, 1949). The antigen may be demonstrated in the tissues of the "paralyzed" animals as long as 1 year after administration (M. H. Kaplan

et al., 1950; Felton *et al.*, 1955a; Stark, 1955). Passively transferred antibodies are rapidly eliminated and the protection against the infection in "paralyzed" animals requires a many times larger amount of serum than in controls (Dixon *et al.*, 1955; Felton *et al.*, 1955b). The paralysis can be induced even in an already immune animal if sufficient antigen is given (Felton *et al.*, 1955b).

It is not yet known what immunological paralysis and immunological unresponsiveness produced in adults by protein overloading (Dixon and Maurer, 1955) have in common. This latter is relatively short-term, lasting only as long as the antigen persists in the circulation.

It has often been maintained that immunological paralysis does not involve the suppression of antibody formation, that the paralyzed animal produces antibodies which are, however, neutralized by the antigen present in the organism as fast as they are formed so that this state imitates immunological nonreactivity (Kaplan *et al.*, 1950; Dixon *et al.*, 1955; Stark, 1955). This hypothesis is not, however, based on direct evidence of antibody formation against the paralyzing antigen, but on the rapid disappearance of passively introduced specific antibodies; it is further based on the observation that the specific radioactivity of labeled pneumococcal polysaccharide remains unaltered in the tissues of the paralyzed animal for at least a year, while the amount of antigen as demonstrated by immunological methods decreases substantially with time (Stark, 1955). Against the evidence of the disappearance of passively introduced antibodies, it may be objected that this was followed only relatively early after the induction of paralysis and that this effect was caused by the excess of antigen employed, and does not participate in the actual processes of the establishing and maintenance of paralysis. Furthermore the neutralization hypothesis conflicts with the results of Sercarz and Coons (1959), who were unable to demonstrate any specific antibody formation in lymph node and spleen cells of the animals paralyzed with pneumococcal polysaccharide or overloaded by protein; this finding seems to indicate that in paralysis some more fundamental step of the process of antibody formation is involved. Finally in the case of unresponsiveness resulting from protein overloading in adults as well as of tolerance, even the final phase of elimination of antigen from the circulation is of nonimmune type (Dixon and Maurer, 1955). It is therefore unlikely that the excess of antigen masks the formation of antibodies, since even minimal amounts of these should be sufficient to cause accelerated elimination of the remaining traces of antigen in the final phase.

The experiments with protein antigens may be of special importance for the comparison of the two phenomena described in the foregoing

with immunological tolerance. The data concerning the dose by which tolerance and overloading may be induced and maintained indicate their difference. Smith and Bridges (1958) found that the same dose of BSA per kilogram weight which was sufficient to induce tolerance in rabbits within the first 2 weeks after birth induced antibody formation from the third week. In the experiment of Dixon and Maurer (1955), the dose of antigen per kilogram weight which in newborn rabbits caused unresponsiveness lasting for at least from 10 to 11 months, had a much shorter effect in adults; reactivity reappeared after the disappearance of antigens from the circulation, i.e., after 3–4 months. Even if the tolerance induced in newborns was prolonged by repeated challenges with antigen, the same challenges in adults did not affect the duration of their unresponsiveness in the same way.

It is more difficult to compare the immunological paralysis and overloading with tolerance to cellular antigens. Owing to lack of appropriate models, situations were always compared which differed from each other by two variables, namely, the type of antigen and the type of response. As far as homologous cells are concerned, which are the basic model for the induction of tolerance, no success was achieved in inducing a state comparable with paralysis even when repeated large doses of cellular antigen were administered in adult life (Castermans, 1958). The prolonged survival of very large skin homografts in rats observed in the experiments by Zotikov *et al.* (1960) cannot be considered as direct evidence for the inhibition of the immune reaction with a large amount of antigen, because of the influence of postoperative trauma. A strong reason for adopting a skeptical attitude to the possibility of inducing paralysis with homologous cells is the existence of runt disease itself and of other manifestations of the graft-versus-host reaction. If quantitative relations were primarily involved, no other situation could offer more ideal conditions for overloading the immunologically active cells with an excess of antigen.

The history of immunological paralysis begins, in fact, with the use of purified polysaccharides. We know of no evidence that paralysis was induced by penumococci as such and neither was it possible to induce tolerance to polysaccharides in newborn animals with them (Cohn, 1957). However incomplete these data are, they are suggestive that the form of presentation of antigen could be decisive for the success or failure of these experiments; it may be imagined that antigens soluble or insoluble, or free or bound to various structures or to cells could have different chances. An answer to this seemingly minor question could contribute significantly to the understanding of the individual phenomena of induced immunological nonreactivity.

2. *The Sulzberger-Chase Phenomenon*

The Sulzberger-Chase phenomenon is a state of immunological unresponsiveness to chemical allergens given by a "wrong" route. It was observed for the first time by Sulzberger (1929, 1930). Guinea pigs given Neosalvarsan intravenously lose their capacity to be sensitized to it. Chase (1946, 1949) found, for a series of chemical allergens, that their prior introduction *per os* into adult guinea pigs prevented the induction of contact sensitivity to these haptens. This inhibition is specific and cannot be induced in animals already sensitized. In animals sensitized by feeding with picryl chloride, antibody formation against "picrylated" guinea pig serum proteins was inhibited, but was even accelerated to picryl groups bound to bovine globulin (Battisto and Chase, 1955a).

The Sulzberger-Chase inhibition of contact sensitivity and immunological tolerance to foreign proteins conform in the following manifestations:

1. Passively introduced antibodies display a normal disappearance rate, not an accelerated one as in immunological paralysis (Battisto and Chase, 1955b).

2. Unresponsiveness can be abolished by the adoptive transfer of immunity by cells of the sensitized individuals (Chase, 1959b).

3. The lymphoid cells of a nonresponsive animal, transferred to a normal individual, do not react even in a new environment (Battisto and Chase, 1955b).

These similarities, however, need not involve a parallel between the mechanisms governing these states. In view of the insufficient evidence for antibody formation against the respective allergens, the alternative must be considered that the Sulzberger-Chase phenomenon is akin to enhancement. It would be necessary to postulate an undefined interference between delayed-type hypersensitivity reaction and some other immune reaction induced by allergen pretreatment.

3. *Immunological Neutrality Induced in Adult Life*

The final question is the relationship between tolerance and some phenomena whose common feature is that they always represent relatively rare exceptions to the usual experimental course, e.g., where transplantation immunity with its normal consequences is expected, some state of immunological neutrality appears instead. Since these phenomena occur in adult organisms, or in cells derived from them, they cannot immediately be explained by the mechanism of immunological tolerance.

The first such phenomenon occurs sometimes in lethally irradiated

organisms which have recovered from hematopoietic failure by means of successful homotransplantation or even heterotransplantation of hematopoietic cells. The short-term success of transplantation of foreign hematopoietic cells and the development of a radiation chimera does not depend, however, on a specific modification of the immune mechanism by the respective antigens, but on its nonspecific inhibition by damage to immunologically competent cells and tissues of the irradiated recipient. Only the prolonged persistence of radiation chimeras, however, poses the question of why the recovering immunological activity (regardless of whether of donor or of host origin) is not primarily directed against the second antigenically different component of the chimeric organism and does not lead to its elimination. The answer is that this does, in fact, occur very often. Immunologically competent cells of the graft do react against the host or, on the contrary, the host's lymphoid tissues do regenerate (sometimes from a few cells surviving irradiation) and display an immune reaction to the graft.

However, from the point of view of tolerance, the exceptions to such a usual fate of homologous or heterologous radiation chimeras are of particular interest. A state of "peaceful coexistence" between antigenically different tissues of the donor and recipient is sometimes reached while ability to react against other antigens is retained. The inhibition of the reactivity to the second component of the chimera is, therefore, a specific modification evidently due to exposure to the respective antigens (Barnes *et al.*, 1957).

A similar mechanism seems to account for the immunological neutrality of the graft to its host which is sometimes found in individuals rendered tolerant as a result of neonatal inoculation with homologous adult spleen cells. In some instances, the graft-versus-host reaction does not take place, or at least does not lead to the usual consequences. Insofar as the state of chimerism persists, it can be inferred that besides the tolerance of the host to the graft some tolerancelike state of the graft toward the host must have been induced.

The third case to be mentioned here is an abnormal course of parabiosis between adult animals in which stable unions can exceptionally occur; that their cause is a specific immunological neutrality is indicated not only by the absence of "parabiotic intoxication" in the partner, against which the reaction is normally directed, but also by tolerance to a skin graft of the partner's genotype (Rubin, 1959; Skowron-Cendrzak and Konieczna-Marczynska, 1959; Martinez *et al.*, 1960).

Hesitation in explaining these states by immunological tolerance is only due to their being induced in adult organisms or in cells derived

from them. It is possible that even in the adult organism there persists throughout life a stem line of cells capable of acquiring immunological tolerance (Lederberg, 1958). It remains, however, to explain the fate of cells no longer possessing such an ability. If these cells were not affected in some way, they would normally react to an antigenic stimulus by immune reactions which would mask the behavior of the tolerant component.

For the type of radiation chimeras in which the host's lymphoid tissues regenerate without the elimination of the donor's cells, the explanation was suggested that immunologically mature cells were destroyed by irradiation (Barnes *et al.*, 1958b); if regeneration proceeded from the functionally immature stem cells, the immunological neutrality of the host toward the graft would be due to immunological tolerance and no other mechanism would be necessary. This explanation is acceptable, but it does not solve the mechanisms underlying the immunological neutrality of the graft toward the host; immunologically competent cells of donor origin were not irradiated and, therefore, not exposed to any selection pressure other perhaps than that of excess of host antigens. This factor is a common feature of all the aforementioned experimental situations in which exceptionally the usual manifestations of the immunological reaction do not take place. The question is whether excess of antigen is the critical factor involved.

In normal homografting, in which failure of the reaction is practically unknown, the quantitative relationship between the reacting and inducing components is far more in favor of the former than in the systems discussed where exceptions sometimes occur. Simonsen (1960) expressed the view that these quantitative relationships could decide whether a "productive" or "exhaustive" sensitization would take place. It is also assumed that the stronger the antigenic stimulus the stronger the reaction induced and that, for example, the cells of the graft which are strongly stimulated (owing to the overload with host's antigens) can suffer a greater degree of damage as a result of their own reactivity; this can manifest itself in their decreased viability, leading to a secondary weakening of their functional capacity or even to complete immunological inertness or death. Such an assumption would be in agreement with the rapid disappearance of parental spleen cells injected into F_1 hybrids (Gorer and Boyse, 1959) which is interpreted as allergic death of the graft.

Simonsen (1960) provided experimental evidence that immunologically competent cells from an adult organism can pass over from a state of "productive" sensitization into a tolerancelike state specific for the

given antigen. On the basis of his further experiments, Simonsen (1961) favors the view that this state is essentially identical with immunological tolerance. Insofar as its induction in adult life seems to depend only on a larger dose of antigen, he is then inclined to equate tolerance and paralysis. The experiments supporting this conclusion still require some elucidation. The main evidence is that adult parental spleen cells injected into newborn F_1 hybrids rapidly lose their capacity to induce splenomegaly when passaged into new F_1 hybrids identical with the original ones, although they retain this capacity in respect to other newborn hosts. In spite of this control, the question arises as to what amount of the first donor's cells are passaged with the first chimeric spleen into the second F_1 hybrid host. In view of the findings that the dividing spleen cells, under similar conditions, are mostly derived from the host (Biggs and Payne, 1959; Davies and Doak, 1960), the suppression of immunological activity of passaged cells might, at least partly, be a question of their quantity. Moreover, it is necessary to explain why it is impossible to elicit a tolerancelike state in the first host with the same success as in the second, although the qualitative relations are identical (the relationship between the antigenic constitution of the donor and host) and the quantitative relations are similar. The inhibition of foreign spleen reaction in radiation chimeras is analogous except that it is induced by the previous incubation *in vitro* of donor spleen cells with a homogenate of the host's liver (Upton *et al.*, 1959). In this case one would have thought that *in vitro* contact of immunologically active cells with the donor's antigens (represented by a homogenate in which there may be many disrupted cells) could afford different conditions for "neutralization" than contact *in vivo*.

On the basis of the material available, it is impossible to decide unequivocally the relationship between immunological tolerance and immunological neutrality reached in adult life. It cannot be excluded, of course, that a condition of immunological immaturity is not critical for the induction of tolerance and that an identical phenomenon can be elicited even in adult life. Nevertheless, such immaturity seems to affect the ratio of successful to unsuccessful cases so much that it should not be omitted from the definition of tolerance.

Moreover, the question is whether immunological neutralization in adult life is due to a selection of certain cells capable of undergoing some profound alteration while the remainder are eliminated or to a modification of all adult cells under appropriate conditions. Nor can it be excluded that the final state only represents a dynamic balance between continual damaging and regeneration of target cells; the immune

reaction suppressed by the excess of antigen would thus escape detection by the usual tests (Trentin, 1957; Barnes and Loutit, 1959).

Such a state of "practical tolerance" could be very important from the clinical point of view because an imperfect state of tolerance would be the price of the ease with which it was induced. In this connection, it is appropriate to recall the recent cases of clinical homotransplantation of kidney (Küss *et al.*, 1960; Merrill *et al.*, 1960) in which the initial immunological crisis was overcome spontaneously. The fact that only a temporary balance was involved would suggest that the state was a relatively labile one. Further investigations should throw light on the conditions under which productive sensitization is spontaneously overcome. The extent of genetic diversity between the donor and recipient is probably an important factor.

E. Possible Mechanisms of Immunological Tolerance

The interpretation of immunological tolerance can be dealt with either separately or as a part of the mechanism of antibody formation. Irrespective of this, however, it is possible to divide the different views into two categories according to the difference they assume between a tolerant and a normal organism. The first group (can be called "negative") assumes that the induction of tolerance implies the loss of a certain capacity, whereas the second group (which can be called "positive") is characterized by the idea that tolerance is a consequence of the acquisition of some capacity. Both groups can be further divided into "active" and "passive" subgroups. It can be imagined that the loss of capacity to perform a certain function can assume two different forms: (a) the elimination of the relevant mechanism and (b) the blocking of a mechanism which is potentially retained. The acquisition of the capacity to perform a certain function could be similarly accomplished by one of the two alternatives: (a) by the development of a new mechanism or (b) by the retention of a mechanism which would spontaneously disappear under normal conditions of development.

The concepts of the mechanism of immunological tolerance could be symbolically characterized and classified in the following scheme:

	Negative	Positive
Active	Elimination	Adaptation
Passive	Blocking	Retention

The elimination mechanism of tolerance can be conceived at different levels. The clonal selection theory of antibody formation (Burnet, 1959) explains immunological tolerance as a consequence of the elimination of

the clone of cells predetermined to form a specific antibody against the given antigen. In the early development of the clone, before the immunological mechanism has become functionally mature, a tolerance-responsive phase is assumed in which the contact of cells with the antigen leads to their being killed and eliminated. By means of some subsidiary hypotheses (e.g., that of reoccurrence of the eliminated clones by somatic mutations) this concept can be developed to be consistent with all known experimental facts (which by no means implies, of course, that it must hold true). By insisting on a one clone—one antibody relation, it would be difficult for the clonal selection theory to explain the disproportion in the range of action of immunity and tolerance; but on passing from the molecular level to the level of antigenic determinants and especially on admitting a one clone–several antibodies relation (and also the concept perhaps that the image of the antigenic determinant in an immature mesenchymal cell can be somewhat shadowy), it becomes easy to explain both the tolerance–immunity relationship and various quantitative manifestations of tolerance, including the significance of the phylogenetic distance between the recipient and the source of antigen and some manifestations of nonspecificity of tolerance.

The elimination mechanism can be imagined even at the level of organellae or of molecules but it would still require pre-existing genetic information and specialization of mesenchymal cells. Tolerance would not then be realized by the elimination of cells, but of subcellular structures; the main difference would only be that in the former case tolerance results from the absence of nontolerant cells from the organism, whereas in the latter actual tolerant cells would be present.

The ideas concerning blocking of antibody-forming function need not be confined to blocking of formed antibodies, as in the original concept of immunological paralysis. As with the elimination of a structure, blocking of function can also be imagined at a different level; the deeper it is to the very basis of function, the more easily can the concept of blocking be brought into conformity with the evidence for the importance of the immunological immaturity of the organism. If the blocked function is to be potentially retained, the continual presence of the blocking agent seems to be necessary: such a role could be played by the antigen incorporated in the cells. Should persistence of antigen (as some findings seem to suggest) turn out to be a critical condition for the state of tolerance to be maintained this would support the concept of a blocking rather than an elimination mechanism. The developmental stage of mesenchymal cells could decide which of the two important sites in the cell will be accessible and, therefore, occupied by the antigen (Mitchison,

1959). At the time of immunological maturity the antigen could penetrate into one of these sites and act as a releaser of antibody production, whereas in an immature cell the other site could be open to the antigen which would function as a repressor of antibody formation (Szilard, 1960). On these assumptions, the present findings on tolerance could be likewise interpreted by means of the blockade of antibody formation in the wider sense.

The adaptation mechanisms could be different depending on the nature of the tolerance-inducing antigen. A nonliving antigen could hardly be imagined in other than an elective function, diverting the process which would normally lead to antibody formation into some other of the possible pathways (Denhardt, 1960). Such adaptation could be a switch, shunting the potential antigen so that it is broken down fast enough to prevent it acting as a real antigen (Humphrey, 1956). Medawar (1961) suggested that an analogy could be drawn between adaptive enzyme formation in bacteria and immunological tolerance rather than between adaptive enzyme and antibody formation as suggested by Burnet and Fenner (1949). The nonliving antigen incorporated into the cytoplasm could also induce a change in the epigenetic machinery of the cell and thus affect the differentiation potentiality of the nucleus (Markert, 1960). In this way a "foreign" environment for the nucleus could arise in its own cell with the consequences known in nucleus transplantation. The adaptation changes induced by a nonliving antigen could at most represent an imitation of hereditary changes at the cellular level (*Dauermodifikation*) and their maintenance would, therefore, require the presence of the inducing principle, i.e., the antigen.

Adaptation changes similar to those induced by nonliving antigen could probably also be induced by the antigen represented by living cells. In an extreme case, this antigen could also have an instructive function, i.e., it could pass on new, specific information to the genetic structure of mesenchymal cells (transformation, transduction, vegetative hybridization). The present evidence is rather against effects of this kind inducing tolerance with living cells (Owen, 1945; Hašek *et al.*, 1959; Stone *et al.*, 1960). Nevertheless, if the living cells were to function in some similar way, it would allow at least two different mechanisms for tolerance—toward a nonliving and living antigen—which would greatly facilitate the interpretation of some experimental data.

The borderline between adaptation and retention of a function (as possible mechanisms of immunological tolerance) is not sharp, at least as far as the adaptation of the elective type is concerned. For example, the normal development of a certain function can end in its spontaneous

elimination. If this were prevented, the function would be retained and in comparison with the normal state after elimination, would, in fact, represent something new. Haldane, for example, put forward the hypothesis that the embryo might possess the capacity of metabolizing and disposing of some substances which an adult organism cannot break down, but produces antibodies against them instead. It could be assumed that the developing organism would normally retain this capacity (probably due to certain enzymatic systems) only for potential antigens of its own body which are present in the organism at the critical time when some embryonic enzyme systems are disappearing. Another potential antigen artificially present at this period, however, could cause any embryonic capacity to break down this antigen (if it ever existed) to be retained as well. The limits determining inducibility of tolerance would evidently be genetic in nature.

On comparing the various concepts of the mechanisms of immunological tolerance with the main experimental data given in the previous sections, it is seen that each of them may fit better or worse according to the circumstances. Some manifestations of tolerance strongly suggest that something more than the suppression of antibody formation below a threshold is involved, i.e., that a deeper change is involved already representing a new state. However, even the term "new" may be quite relative as is apparent, for example, when comparing the concepts of adaptation and retention of function. In contrast, the results of some less successful attempts to induce tolerance seem to imply a quantitative modification of the process of antibody formation and could, therefore, be explained by a far more superficial change.

It seems, therefore, wiser, at least at the present stage of knowledge, to admit all possibilities rather than to exclude any one of them. The idea that the same effect must always have the same cause is probably the reflection of the tendency to simplify and to adjust the problems so that they are more digestible for human comprehension rather than to give a true picture of the situation occurring in nature, which might prefer greater diversity.

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Immunological Tolerance of Nonliving Antigens[1]

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I. Introduction

Specific repression of the usual manifestations of the vertebrate immune response through exposure to nonliving antigens is the common feature which identifies the group of phenomena under consideration in this review.[2] Nonliving, for these purposes, has been interpreted as nonreplicating—giving sufficient breadth to include the range of antigens from defined chemicals, proteins, and carbohydrates to red blood cells, bacterial antigens, and similar complex mixtures. By this definition the antigen evoking tolerance need not necessarily originate extrinsically, so that the immunological role of the host's own tissue components and cell products may be considered.

[1] Support for these studies came in part from the National Institutes of Health, (A-2964), the American Heart Association, the Arthritis and Rheumatism Foundation, and the American Medical Association.

[2] Use of the term "immunological unresponsiveness" in this connection was originally suggested by Chase and co-workers and used by Dixon and by the author in his own publications. At that time, mechanisms were not apparent which linked specific failure of antibody production to heterologous proteins to the tolerance of homografts experimentally contrived earlier by Billingham, Brent, and Medawar (1956), and termed "acquired tolerance." By general usage, by discussion in symposia and reviews, and by developments indicating related mechanisms since these earlier terms were devised, the hybrid term "immunological tolerance" appears to have gained acceptance and will be used here for all of these phenomena.

With the growth of experimental data, it has become evident that the various experimental models interpreted as tolerance of nonliving antigens are not unrelated to those in which tolerance of living material is demonstrated. However, the exploration of homograft and tumor tolerance and of parabiotic union employ detection and measurement techniques which are new tools to the immunologist and involve special genetic considerations. These are reviewed elsewhere in this issue (see article by Hašek *et al.*) and will not be dealt with here, except as they are interpretable in terms of concepts revealed through studies of nonliving antigens. Nor will attempts be made to include nonspecific suppression of immune mechanisms or absence or defects of the immune response which occur in certain congenital and acquired diseases. Concerning these phenomena, as well as tolerance, the reader is referred to comprehensive reviews by Chase (1959b), Lawrence (1959), and others.

The thesis of this presentation will be the central role of antigen itself in the induction and maintenance of the tolerant state. As a first approximation of a mechanism of tolerance such emphasis appears justified by available data; but, as will be shown, requires much further experimental support before it can be considered established.

Data derived from the individual models will first be considered separately and with some thoroughness.[3] With all the data at hand, evidence, where it exists, for grouping these models on common fundamental mechanisms will be marshalled and discrepancies in fitting formulations to fact brought out. With this, some preliminary concepts of the nature of the tolerant state can be adduced, and various hypotheses measured to the data. The many questions which will be raised but remain unanswered, it is hoped, will stimulate interest in this important field and lead to the development of further experimental approaches.

II. The Experimental Models

Whereas at least six different examples (Wells and Osborne, 1911; Traub, 1936, 1938, 1939; Sulzberger, 1929; Felton and Ottinger, 1942; Owen, 1945; Chase, 1946) of specific suppression of immunity were investigated prior to Burnet and Fenner's book in 1949, its publication (and prepublication discussions) can properly be said to have stimulated much current work in the field of immunological tolerance. As Chase (1959b) has pointed out, the biological milieu was fully prepared for the experimental work triggered by Burnet's formulations. Owen's (1945)

[3] The reader who is familiar with the details of available experimental data may wish to refer to the summary in Table IX and bypass Section II.

incisive observations on chimerism in cattle provided the basis for Burnet's prediction that embryonic exposure to antigenic stimuli would lead to unresponsiveness to this antigen in later life. Medawar and colleagues (Billingham *et al.*, 1953) then established homograft tolerance in mice, through prenatal injection, as an experimental model. The field had so grown in scope that by 1956 acquired tolerance was already the subject of a symposium at the Royal Society.

Most of the early work with tolerance involved tissues and other living systems. More recently, use of partially defined nonreplicating antigens in studies of tolerance has attracted many investigators. These tend to minimize the variables, allow the use of classic immunological tools—including quantitation—and permit studies on the fate of the antigen itself. The antigens used have been for the most part of the widely available easily purified serum components of bovine and human origin. However, the erythrocyte has proved advantageous in many respects as an antigen, and its use has yielded valuable quantitative and qualitative data concerning certain mechanisms governing the tolerant state. Bacterial and more complex antigens, however, used in similar experiments have thus far produced less data of fundamental interest except in the case of polysaccharides in adult animals.

Upon this brief historical background, individual experimental models will now be considered in an arbitrary order depending on the mode of induction.

A. Tolerance Induced by Exposure in Perinatal Period of Life, by Species

Inasmuch as susceptibility to induction of tolerance in the neonatal period around the time of birth varies in different species, each model will be described with similar models within the same species. Comparison of data within species appears less hazardous than between species.

1. Studies in Rabbits

The first demonstration of inhibition of the response to protein antigen was in the study of Hanan and Oyama (1954). These experiments were originally designed to determine the effects of an immune response to BSA (bovine serum albumin) on γ-globulin levels in young rabbits. Repeated injections were given starting by injecting three times a week increments of 0.1 mg. up to 1.0 mg. intraperitoneally for 1 month and intravenously thereafter for 114 or 181 days. Compared to littermate controls, these injected animals did not form anti-BSA precipitins, sensitize guinea pigs for passive anaphylaxis, or give an Arthus reaction. While

most of them had some antibody response to injection of Ea (egg albumin), the response was diminished in comparison to controls. They interpreted their experiment as an example of immune tolerance as predicted by Burnet.

Dixon and Maurer (1955a, b) used a similar experimental design to investigate the fate of large amounts of heterologous protein in rabbits. Rabbits were injected either with whole human plasma or with BSA for the first 98–112 days of life subcutaneously, six times weekly, 500 mg. per kilogram daily. They found that, in contrast to normal adult rabbits, animals injected from shortly after birth with noniodinated proteins, regularly showed a nonimmune type of elimination of lightly iodinated proteins from their serum and produced no antibody to BSA. The animals were unresponsive up to 11 months after cessation of infusion as shown by repeated challenge. The specificity of the unresponsive state was demonstrated by antibody response to other administered antigens. Too, small amounts of antibody to trace components of whole serum were found in the groups infused with whole plasma (antibody against fractions I, II, III, IV, in the human plasma-infused group and anti-BGG in the BSA group). They showed, incidentally, that offspring of unresponsive animals were fully competent immunologically, eliminating the possibility of a heritable change as explaining the unresponsive state. These workers first performed the important experiment wherein remaining circulating antigen in unresponsive rabbits was bound and cleared by passively transferred antibody. They found that this antibody had a half-life identical to fully responsive animals and that (by inspection of their data) unresponsiveness was still present after this antibody had been eliminated.

Shortly after the aforementioned paper appeared, Cinader and Dubert (1955, 1956) reported the first of a series of studies of the mechanism of immunological tolerance (Cinader, 1957a, b; Cinader and Pearce, 1956) of defined proteins, with particular emphasis on the use of S^{35}-labeled azoproteins. The antibody measurements in contrast to the previously mentioned studies were made by Boyden's tannic acid-treated cell technique, which is much more sensitive than the precipitin method. They showed that rabbits injected from birth with HSA (human serum albumin) were unresponsive to this antigen at maturity and that this tolerant state did not preclude responses to tobacco mosaic virus. Only one of six animals tolerant of HSA produced antibody to benzene-*p*-sulfonic acid azo HSA directed to the prosthetic group, but *not* at the same time to HSA. In a later investigation of this same model, they attempted to induce tolerance of the azoprotein by neonatal injection. A high pro-

portion of animals given 20–80 mg. in the first 10 days of life failed to form detectable antibody when challenged with the same azoprotein at 71–80 days of age; those responding had much lower antibody titers than controls. Curiously, these partial responders, upon later rechallenge, had lower titers than after the original challenge. Elimination of HSA from the serum was exponential with a half-life of 5.6 days in azo HSA-tolerant rabbits—the *same* as HSA in HSA-tolerant animals. In contrast, animals injected at birth with comparable amounts of azo bovine ribonuclease or azo rabbit serum formed antibodies, on challenge with azo HSA, which did *not* differ from littermate controls except that the azo bovine ribonuclease groups made antibody possibly of lower avidity than controls. Thus it appeared that tolerance of the azo group, per se, is not induced by neonatal injection; tolerance was of the determinants of HSA, or modifications of the azoprotein conjugate incidental to the actual coupling process.

Since 1954, quantitative investigations of protein tolerance models have been carried on simultaneously in several laboratories throughout the world. Dubert and Paraf (1957) have defined the minimal amounts of BSA and the age limits within which unresponsiveness can be induced in rabbits lasting into early maturity (2 kg.). Table I gives a summary analysis of their data.

TABLE I[a]

DOSE AND AGE EFFECTS IN PRODUCING BSA TOLERANCE IN RABBITS

Amount injected I. P. in each dose (μg.)	Number tolerance induced / Number in group / Number of daily injections: 1	2	5	20	Total
10	1/2	—	2/2	3/7	6/11
200	2/7	5/7	6/7	—	13/21
1000	2/2	2/2	1/1	8/8	13/13
Controls					1/16

Amount in daily dose given for first 20 days (μg.)	Age at injection, days: 2	5	7	10
10	2/4	1/2	—	—
200	1/1	4/4	—	0/3
1000	1/1	—	3/4	0/2

NOTE: The indicated dose of BSA was injected intraperitoneally daily for the indicated number of injections. The results are expressed as the proportion of rabbits showing no anti-BSA after challenge when they weighed 2 kg. and thus are assumed to be tolerant.

[a] After Dubert and Paraf, 1957.

Stimulated by Burnet's book, the first experiments of Billingham, Brent, and Medawar, and the report of Hanan and Oyama, the author and his colleagues (Smith and Bridges, 1956, 1958; Smith *et al.*, 1957; Eitzman and Smith, 1959a, b; Garvey *et al.*, 1960; Smith, 1960a, b) undertook to define more completely the model of protein tolerance in rabbits and to investigate some of the underlying immunological mechanisms.

The results of this series of investigations may be summarized briefly as follows:

(a) The relation between dose and duration of tolerance was determined by giving a single intraperitoneal injection of BSA in the first few days of the neonatal period, and testing the immunologic response at intervals. The duration of complete unresponsiveness appeared to vary directly with the amount given at birth. For example, injection of 100 mg. yielded tolerance lasting 135–189 days; 10 mg., 90–135 days; 1 mg., 70–90 days; 0.1 mg., 47–65 days. One hundred milligrams as a single injection induced tolerance of 90–120 days duration given up to the tenth day; less consistently at 10–15 days, and not after this. Partial tolerance, that is, decreased antibody levels as compared with controls, was not evaluated in detail in any of these studies. The unresponsive state appeared conceivably to represent simply a protein loading phenomenon analogous to experiments in adult rabbits reported by Dixon and Maurer (1955a, b). In similar experiments, however, in which a single weight-graded amount of BSA (200 mg. per kilogram) was injected at varying ages, tolerance was induced in animals injected before 15 days of age; antibody production was elicited when it was given after this.

(b) Prolongation of the tolerant state was found to depend on the provision of additional increments of antigen while the animal was still completely tolerant. For example, data from one experiment (Table II) showed that on the order of 10 μg. BSA was required to extend tolerance from 45 to 70 days. This observation provided an explanation of the apparent permanence of the tolerant state in earlier reports and in the early experiments of the author, i.e., the increments of antigen required to *challenge* the tolerant state insured at the same time its longevity.

(c) The high degree of specificity of tolerance was again shown; however, no interference with Ea antibody production in BSA-tolerant animals was found as reported by Hanan and Oyama (1954). Some evidence suggested that BSA-tolerant rabbits could discriminate the alterations incident to lightly radio-iodinating BSA. For example, the half-life of I^{131}-BSA (1–3 I^{131} atoms per molecule) in BSA-tolerant animals measured by its radioactivity was 4.2 days, but measured immunochemically

(presumably the carrier BSA for the most part) was 7.8 days. However, rabbits given I^{131}-BSA *at birth,* had similar serum fall-off rates measured either way.

TABLE II

INCREMENTS OF BSA NECESSARY TO PROLONG TOLERANCE IN RABBITS

Group	Results of challenge with 15 mg./kg. at 75 days of age[a]
100 μg. at birth; 20 mg./kg. I.V., at 45 days of age	1/5[b]
100 μg. at birth; none at 45 days	4/5
100 μg. at birth; 10 μg. at 45 days	0/6
100 μg. at birth; 1 μg. at 45 days	2/5
100 μg. at birth; 0.1 μg. at 45 days	3/4

NOTE: 100 μg. BSA was given I.P. to litters of rabbits at birth. At 45 days, one litter was given 20 mg./kg. I.V. (intravenously) and bled serially to determine the production of BSA. The other litters were given the indicated amount of BSA and challenged I.V. at 75 days with BSA (15 mg./kg.). The results indicated that 10 μg. BSA prolonged tolerance at least 30 days (Eitzman and Smith, unpublished data).

[a] Numerator, number of rabbits producing antibody after receiving 20 mg./kg. at 75 days; denominator, number in group.

[b] Results of challenge at 45 days given here.

(d) Cell transfer studies showed that vicarious antibody production by lymph node and spleen cells from BSA-immune rabbits, implanted in the BSA-tolerant animal, did not differ from that in the normal recipient. Neither lymph node and spleen cells from BSA-tolerant animals transferred into normal recipients, nor normal cells implanted into BSA-tolerant recipients resulted in antibody formation. As Weigle and Dixon (1959) have pointed out, a *primary* response to BSA would go undetected because homograft immunity would intercede before the inductive phase was over, precluding detectable antibody production. The use of BGG (bovine γ-globulin) or a similar antigen having a shorter induction time should permit more adequate examination of this point.

(e) Attempts to "break through" BSA tolerance by intensive stimulation with BSA–adjuvant mixtures met with but limited success. When given toward the end of the expected tolerant period, BSA in adjuvants produced some shortening of the duration of the tolerant state as compared with the effect of similar injections of soluble BSA. In a few

experiments, no "break through" by X-irradiation was found—confirming the recent report of Denhardt and Owen (1960), but in contrast to the observation of Nossal (1959) on RBC (red blood cell) tolerance in rats.

(f) Production of tolerance of various antigens was attempted. Only the heterologous proteins have induced a significant unresponsive state: I^{131}-BSA, BSA, S^{35}-sulfanilic acid azo BSA, BGG, HGG (human γ-globulin), human purified macroglobulins, HSA, I^{131}-HSA, horse γ-globulin, and Ea have all been successfully used. The BGG and HGG tolerance was of greater duration (not established with the precision of BSA tolerance) than BSA and HSA tolerance, as has been found by Dixon (1960), and Ea tolerance was of shorter duration than BSA tolerance. In contrast, no significant tolerance to bacterial antigens (streptococci, typhoid bacilli, endotoxins, toxoids, tuberculin, BCG, etc.), to a semipurified bacterial protein (streptococcal M), or to skin homografts (by neonatal injection of adult spleen and lymph node cells) was ever obtained.

(g) A number of experiments, largely unreported as yet, were directed to elucidate the special conditions of the neonatal period which may result in the induction of tolerance (Table III).

1. Nearly quantitative absorption of heterologous protein occurred through the gut of the neonatal rabbit. Tolerance could be regularly induced in this way. The period of increased permeability was of similar duration to that of susceptibility to induction of tolerance.

2. The BSA injected intraperitoneally into the neonatal rabbit appeared to occupy a theoretical space of distribution which was from four to six times that of an adult animal—a space not accountable as increased extracellular fluid volume or renal excretion.

3. After a variable period of equilibration, injected heterologous protein disappeared from the serum exponentially at a rate, corrected for growth, only slightly faster than that in adult animals (Fig. 1). However, in the case of HGG, the serum level during the first 4–10 days remained essentially unchanged. Similar results have been reported by Diechmiller and Dixon (1957) and by Humphrey (1960). The latter author, however, found no period of initial slow decay from serum of γ-globulin as seen in Fig. 1.

Administration of large amounts of bacterial endotoxin simultaneously with BSA in an attempt to effect premature activation of the immune mechanism, in no detectable way affected the age, dose, or fact of induction of tolerance in the neonatal period.[4]

[4] Drs. G. J. Thorbecke and B. Benacerraf, New York University, in preliminary experiments with similar objectives, found no indication of a detectable effect of BCG infection on the induction of BSA tolerance (personal communication, 1960).

TABLE III

CHANGES IN RESPONSE TO BSA DURING EARLY LIFE IN THE RABBIT

Age (days)	A Anti-BSA formed after injecting 20 mg. BSA/kg. Varying intervals after injection (days) 7	14	21	B Tolerance induced by injection of 100 mg. BSA at varying ages	C Absorption of BSA after oral dose of 1 gm./kg. at varying ages	D Calculated BSA "space" following 100 mg. BSA I.P. at varying ages (% body weight)
Birth	0/12[a]	0/12	0/10	21/22	2/4	—
3	—	—	—	11/12	—	27–34.6
7	0/9	1/9	0/8	—	3/3	—
10	—	—	—	3/4	4/4	—
14	0/9	0/8	0/7	5/10	3/4	5.9
21	0/6	4/6	3/6	1/16	1/4	—
28	0/4	2/3	—	—	—	—
30	0/8	3/8 (0.92)[b]	5/8 (3.2)	—	1/4	5.5
60	0/9	8/9 (23.0)	9/9 (20.5)	—	—	—
90	0/8	8/8 (47.5)	8/8 (27.5)	—	—	5.0

NOTE: Summary of some types of experiments characterizing the response of the rabbit to BSA at varying ages. In A, the animals were bled 7, 14, and 21 days after receiving I.P. or I.V. 20 mg./kg. BSA, and the antibody content of serum was determined by the precipitin technique and where measurable, quantitatively. In B, rabbits which received I.P. 100 mg. BSA at the times indicated were challenged at 90 days of age by injection I.V. of 25 mg. BSA/kg. with measurement of antigen disappearance and antibody production. Tolerance was assumed where antigen disappearance was nonimmune in type, and no antibody was detected. In C, rabbits were fed 1 gm./kg. BSA by tube, bled 4 hours later, and the serum examined for precipitins. In D—from serial measurements of serum levels of animals injected I.P. with 100 mg. BSA at birth—calculations were made of space by extrapolating to zero time and dividing this value by amount injected (Smith and Eitzman, 1959, unpublished data).

[a] Numerator, number positive; denominator, number in group.

[b] Mean antibody titer of responders (μg.N/ml.).

5. In an attempt to determine the significance of the form of antigen in the induction of tolerance, either specific precipitates (BSA–rabbit anti-BSA) or identical amounts of soluble BSA were given to newborn rabbits. Those receiving specific precipitates showed immune elimination of BSA and anti-BSA production at 44 days of age, while the littermate controls were completely tolerant (Table IV).

(h) S^{35}-sulfanilic acid-azo BSA was employed in order to determine the fate of injected heterologous proteins and, perhaps, reveal dif-

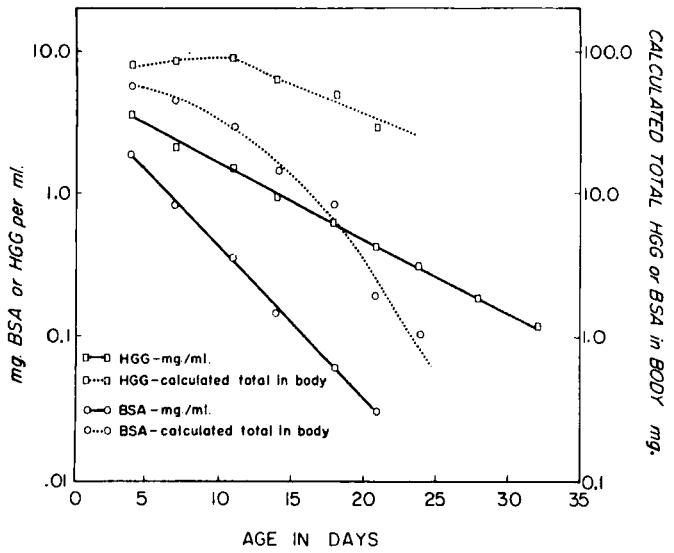

FIG. 1. Results of immunochemical measurements of BSA and HGG in the serum of groups of rabbits (each point represents 6–8 animals) at intervals following intraperitoneal injection of 100 mg. of each on the day of birth. The symbols joined by dotted lines represent calculated total body content. (Total body content equals milligrams per milliliter in serum times 0.2 body weight; 0.2 represents assumed protein "space" value of 20% of total body weight, an assumption consonant with data given elsewhere.) (Smith and Eitzman, unpublished.)

ferences which might exist in the organ or subcellular distribution of the antigen in neonatal rabbits of varying age and in tolerant, as compared with normally responsive, animals (Garvey *et al.*, 1960). This antigen injected into the newborn rabbit had the expected exponential disappearance from serum (half-life = 3 days), but it distributed initially and changed with time differently in each organ studied (Fig. 2); the significance of this pattern of distribution is not yet clear. Comparison of the organ distribution 3 weeks after injection in other groups injected at birth, at 21 days of age, and after maturity revealed differences in liver,

spleen, and thymus uptake in the three groups. For example, relatively increased thymic and liver uptake was found in the mature group and increased spleen uptake occurred in the 21-day group.

In another group of experiments S^{35} distribution in 8-week-old S^{35}-BSA-tolerant animals was determined 3 weeks after rechallenge with S^{35}-BSA and compared with similarly injected normal littermates. These groups were in no way different despite vigorous antibody production in the normal group and nonimmune elimination of antigen in the tolerant group.

TABLE IV

FAILURE OF BSA–ANTI-BSA PRECIPITATE TO INDUCE TOLERANCE IN RABBITS

Form of BSA injected	Results of challenge at 44 days
0.1 mg. (soluble)	1/6[a]
0.1 mg.[b] (ppt. with anti-BSA)	5/7
none	6/6

NOTE: Rabbits were injected at birth intraperitoneally with (a) 0.1 mg. BSA alone; (b) 0.1 mg. BSA as a specific precipitate formed in slight antibody excess with a high titer rabbit anti-BSA; or (c) nothing. At 94 days they were challenged by injecting 20 mg./kg. BSA I.V., and the rate of disappearance of antigen and the presence of precipitating antibody were measured. (Eitzman and Smith, 1959, unpublished data).

[a] Numerator, number of rabbits producing antibody as a result of challenge with 20 mg. BSA/kg. (I.V.) at 44 days of age; denominator, number in group.

[b] Specific precipitate in slight antibody excess with 0.1 mg. BSA., washed 3 times before injection.

Comparison of the distribution of antigen in the subcellular fractions of the two groups revealed only that the tolerent animals had over twice as much radioactivity in the nuclear fraction as the normals. Most of the antigen was in the soluble supernatant. Since the preimmune phase of antigen elimination in normal animals (which eliminate antigen at a similar rate) has not been compared to the tolerant animal the significance of this observation is not established; however, it deserves confirmation and further elaboration.

Humphrey (1956, 1960, and personal communication) has been concerned with similar problems of protein tolerance in rabbits. With regard to tolerance of BSA, HGG, or HSA, he found a duration–dose relationship similar to that described by Smith and co-workers. However, when he considered the point at which only half of a group of animals were tol-

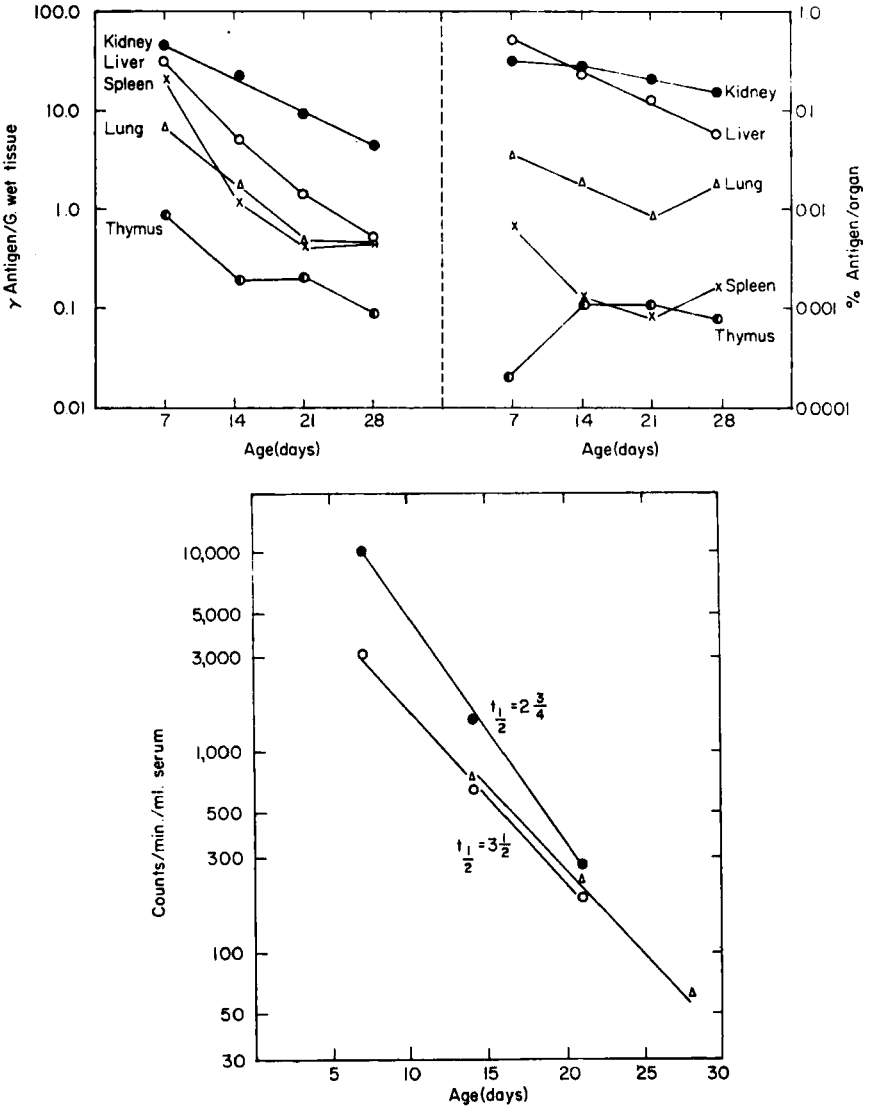

FIG. 2. Elimination of S^{35}-labeled sulfanilic-azo BSA from the tissues and serum of rabbits given 25 mg. of the antigen on the day of birth. Each point represents the average values of 2 or 3 animals in 3 litters. The serum measurements are from these three litters at the time of sacrifice. Values for per cent of antigen in tissues includes an automatic correction for growth of the organ. (From Garvey *et al.*, 1960.)

erant, longer durations were evident. For example, when rabbits received HSA or HGG at birth and were left 12–27 months before being tested, more than half were found to be unresponsive. At this time Humphrey calculates that 10^3 or less molecules of the injected antigen remained in the circulation. In this respect the results appear to differ somewhat from those of Smith and Bridges (1958). Species differences could perhaps account partially for these dissimilar results. It also seems probable that individual rabbits vary greatly in their capacity to respond to BSA (see Johnson *et al.*, 1955); thus, minor technical differences in experiments could be important.

Crampton and his group (Crampton *et al.*, 1959a, b) have followed the fate of heavily iodinated proteins (8–10% I^{131}) in rabbits injected intraperitoneally from birth (30–50 mg. per kilogram every 3 days). When 4–6 months old, these animals were fully tolerant of this protein. The iodoprotein was rapidly cleared from the tolerant animal's serum—usually within 24 hours—and distributed in the various organs exactly the same as in antibody-forming normal adult animals. Most of the antigen localized in the cytoplasmic particles—microsomes presumably, rather than the supernate as found by Garvey *et al.* (1960). These differences in results are probably related to the type of antigen and to the techniques employed and are not necessarily contradictory.

At least three investigators have been concerned with tolerance of complex mixtures of heterologous protein antigens in rabbits. Downe (1955) injected chicken serum into a few newborn rabbits and found no antibody to most components of this antigen upon later challenge. Upon challenge with turkey serum, however, antibody was produced which did not contain the moieties which characteristically cross-react with chicken serum. A possible interpretation of this experiment is that tolerance of cross-reacting determinants could be expressed even when an immune response was being directed toward most determinants of the antigen. Quantitative investigation of this or similar models should prove fruitful. For example, tolerance of the internal determinants of BSA (Ishizaka and Campbell, 1960; Lapresle and Webb, 1960) might be separable from tolerance of other portions of the molecule; or, tolerance of thiol reagent-split macroglobulin fragments might not yield tolerance of intact macroglobulin.

An interesting and provocative use of protein tolerance as an experimental tool is exemplified by the studies of Curtain (1958). Here attempts were made to determine if myeloma proteins arise *de novo* or are possibly represented by trace quantities in normal sera. In one type of experiment, rabbits were injected with approximately 80 mg. of a human

plasma concentrate at birth and then challenged at intervals, starting at 35 days, with this same antigen incorporated into Freunds' adjuvants. At 55 days, no antibodies being detected, groups of the animals were given 3 injections of serum from 5 different patients with multiple myeloma, with subsequent bleedings. No antibody was detected to the specific myeloma proteins. This experiment was interpreted as suggesting that the animals were rendered tolerant of varied myeloma proteins by virtue of trace amounts of each present in the pooled human plasma concentrate. An alternative interpretation would be that tolerance was induced for every determinant group represented on the abnormal protein, but because each was present on at least one type of normally occurring globulin, not necessarily all on the same molecule. Similar experiments revealed no antibodies to two pathological macroglobulins and a cryoglobulin in whole plasma-tolerant animals.

In another series of experiments, tolerance of different Bence-Jones proteins was induced in 13 of 15 animals injected at birth, and challenged at 85 days of age. The tolerant animals were then given 3 injections of the homologous myeloma protein with the result that 3 of 13 remained tolerant of the Bence-Jones protein, 8 made antibody to determinants on the intact protein but not to the Bence-Jones moiety (Ouchterlony technique), and 4 yielded antibody to all components, i.e., tolerance had been reversed. The facility with which tolerance could be broken through was greater in groups challenged at 116 days than at 56 days. These data appear highly pertinent to recent demonstrations that γ-globulins are composed of fragments of lower molecular weight.

Similar experiments revealed that the duration of tolerance of the Bence-Jones protein itself was finite, lasting somewhat longer than 116 days, but could be continued up to 300 days by frequent injections of antigen. The effect of whole myeloma protein on Bence-Jones protein tolerance appears, in principle, to be very similar to the result obtained by Cinader and Pearce (1956) and provides further evidence for the view that tolerance is specific to individual determinant groups and that complete tolerance of an antigen depends on tolerance of all determinants on the antigenic molecule.

A more complex system has been employed by Wyttenbach (1960) to study the problem of tolerance of heterologous tissue proteins. This investigator injected varying amounts (10–100 mg. total dose) of chicken spleen or liver homogenates into rabbits during the first 10 days of life and challenged them at 11 and 20 weeks with the same material (Table V). Compared with uninjected controls at 11 weeks, there was a significant over-all reduction in precipitin titers found in the groups given 25

mg. or more at birth, but only a few sera gave *no* precipitin titer (1 of 49 spleen-injected, 2 of 34 liver-injected). Most interesting, however, was the positive correlation of the number of precipitin bands produced and the serum titer of the injected animals. As many as 7 bands to splenic tissue components and 5 bands to liver components were observed. Furthermore, those injected in the neonatal period had, on the whole, more bands—and curiously, sharper ones—than the controls, though the titers

TABLE V[a]

EFFECT OF NEONATAL INJECTION OF CHICKEN SPLEEN HOMOGENATE IN RABBITS ON THE PRECIPITIN TITER AND NUMBER OF PRECIPITIN BANDS AFTER REINJECTION AT 11 WEEKS OF AGE

Amount injected at birth (mg.)	Number in group	Number with given precipitin titer				
		<100	100–200	400–800	1600–3200	6400
100	4	—	3	1	—	—
50	10	—	3	6	1	—
25	20	1	7	8	3	1
10	15	—	—	4	9	2
Control	16	—	—	—	4	11

Group (injected animals)	Number in group	Number of sera with given number precipitin bands			
		0–1	2–3	4–5	6–7
Sera with 100–200 titers	13	1	8	4	0
Sera with > 400 titers	35	0	15	16	4
Control sera	16	7	9	0	0

NOTE: 10–100 mg. of chicken spleen homogenates were injected into rabbits over the first 10 days of life and again at 11 weeks of age as a challenge. The results of this challenge in terms of the precipitin titer to the whole antigen are given in the upper section for various doses at birth. Precipitin bands visualized in Ouchterlony plates varied in number from 0 to 7, more bands occurring in animals with higher titers, but in both groups greater than in the controls.

[a] After Wyttenbach, 1960.

were lower. Similar, but lesser differences in titers and number of bands in the group challenged at 20 weeks were found. It appeared that neonatal exposure had elicited a primary response to some antigens and tolerance to others. The relevance of these studies to the problems of heterotransplantation tolerance and immunity is clear.

Also concerned with tolerance of tissue proteins were Feldman and Yaffe (1957), who in a brief report described the results of injecting mouse heart or brain saline extracts over a 60-day period after birth. At 70 days, challenge with the same antigens resulted in antibodies which

did not cross-react with the other organ, as did the antibodies produced by control animals. This result suggests that tolerance of some antigen common to the organs was induced, but those unique to the organ gave rise to an antibody response. Unfortunately, Wyttenbach did not examine this point.

Attempts to produce in rabbits tolerance of a yeast enzyme, glucose 6-phosphate dehydrogenase, have been made by Bussard (1957, 1960). Precipitin formation, after reinjection of the yeast-derived antigen at 7 months of age, was inhibited in the animals which received 2 mg. of a preparation with high enzyme activity during the first 10 days of life. Inhibition was said to be total on the first postchallenge bleeding, but only partial after this. Three of 9 of the treated animals had no enzyme-neutralizing capacity at all, others showed partial degrees of neutralization. Rechallenge reduced the number of presumably tolerant animals, but some were said to remain tolerant. Thus, it appears that partial suppression of neutralizing antibody production against a yeast enzyme was induced by neonatal injection. The amount used for neonatal injection is small compared to the amounts of other antigens required for inducing tolerance of this duration. This result suggests that the dose required for tolerance is not similar for all proteins. This point is pertinent to interpretation of such data in terms of homotransplant tolerance. For example, the amounts of individual "transplantation antigens" available would probably be more of the order of 1 mg. than 100 mg. when spleen cells are given at birth in attempts to induce transplantation tolerance.

An experimental basis for the differences in the ABO and Rh isoimmunization potential of humans having blood groups differing from their mothers (see Section II, A,5) has possibly been provided through the studies of Cohen (1958, 1959). He selected offspring of matings lacking the rabbit A-blood group and injected the litters—keeping appropriate controls—with incompatible RBC bearing the A antigen, daily for 15 days, then at least once weekly until 3 months of age. Their data, summarized in Fig. 3, show that suppression of the response to isoimmunization at 4–5 months of age regularly occurred in groups receiving injections of the foreign RBC until 3 months of age. Some suppression of isoimmunization occurred with only 15 injections as compared with controls. As with protein tolerance, a requirement of continuous increments of antigen to sustain the tolerant state is suggested.

The response to staphylococcal toxoid in rabbits injected at birth has been studied by Lindorfer and Subramanyam (1959). Sixty-two days following neonatal injection of a toxoid preparation (1.5 ml. over the first 5 days) the first of 5 intraperitoneal injections of the same antigen was

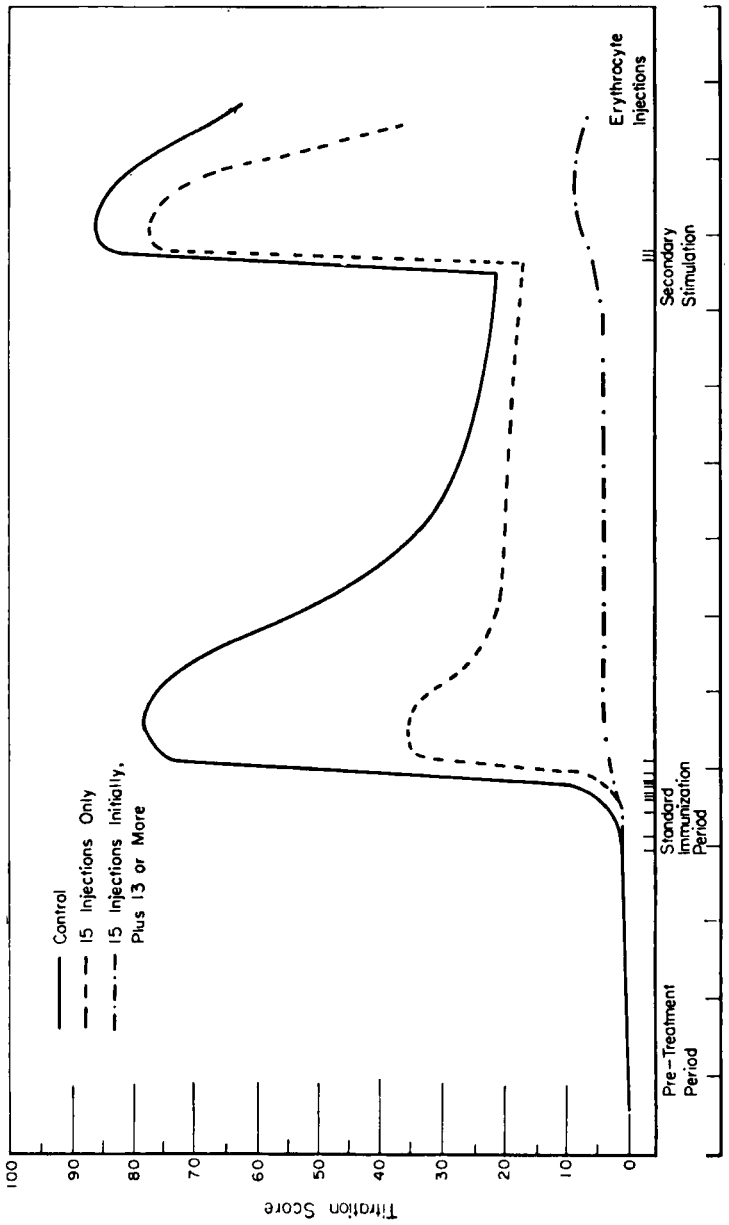

FIG. 3. Effect of injections of rabbit A-erythrocytes into nongroup-A animals 15 or 28 times during the first 3 months of age on the response to immunizing injections at 4 and 11 months of age. Note almost total repression of isoimmunoagglutinin response in the group injected 28 times. (Cohen, 1959.)

given, and the response examined at 92 days of age. In 3 experiments the groups injected at birth had lower anti-hemolysin titers (5- to 10-fold differences) and larger areas of dermatonecrosis than normal animals. This finding is interesting in view of the observations of Quie and Wannamaker (1960) who reported that, in certain young children who have sustained early and repeated staphylococcal infections, a normally present antibody to the staphylococcal Muller factor is absent.

2. *Studies in Chickens*

In view of the background of basic data, ease of administration, and availability of the experimental animals in a fetal state, it was natural that early attempts to induce tolerance were made in chickens by exposure of the chick embryo. Burnet's own attempts to provide an experimental basis for immunological tolerance (Burnet *et al.*, 1950) failed to show an effect with influenza virus, confirming a similar observation of Fox and Laemmert (1947) with yellow fever virus infected embryos. Burnet's attempts using mammalian RBC and bacteriophage were likewise unsuccessful.

Buxton (1954), on the other hand, inoculated chick embryos intravenously with heat-killed *Salmonella pullorum* at 15 days only, at 15 and 20 days, and at 20 days only and measured the agglutinating and nonagglutinating antibody response to oral vaccination 54 days after hatching. From 10- to 100-fold differences in titers were observed in the response of the 15-day and of the 15 and 20-day groups as compared with that of the 20-day injected group. No data on simultaneously injected controls were given.

This result appears to be an example of tolerance and has been so interpreted (Medawar, 1956). However, it seems possible that the 20-day group was, upon challenge, demonstrating a *secondary* type response. This reasoning infers that the embryos had a primary response but were insufficiently mature to produce antibody at 20 days and that the 15- and 15 plus 20-day injected group were immunologically inactive at the time of original exposure and thus demonstrated a primary response to *S. pullorum* with, as would be expected, lower antibody titers. This view is compatible with evidence that certain recognition mechanisms are developed in the chick prior to hatching (Ebert and DeLanney, 1960).

Cohn (1957) has recorded elegant though unsuccessful attempts to induce tolerance of a variety of defined antigens (diphtheria toxoid, arsanilic acid BSA, β-galactosidase, T_2 bacteriophage, and pneumococcus type 2) in the 14-day chick embryo. Maximal practical quantities were employed for a single intravenous injection, which by the nature of the

subject and antigen limited the injected quantity to a total of 50–80 μg. antigen N except in the case of bacteriophage (2.5 μg. N). The antigenic challenge was at 10–12 weeks after hatching. No differences from uninjected controls was found with any of the antigens. From subsequent work (see following), it is apparent that the design of the experiment precluded demonstration of tolerance because too little antigen was injected before hatching and the time interval from hatching to challenge was too long.

Tolerance of heterologous proteins induced in chickens has been the subject of a series of studies by Wolfe and colleagues (Wolfe *et al.*, 1957; Tempelis *et al.*, 1958). Using an experimental design similar to the studies in rabbits, BSA was injected into chicks within a few hours of hatching and the response to reinjection of the antigen determined at 6, 12, and 22 weeks of age. They found unresponsiveness in groups receiving BSA. A typical experiment is given in Table VI.

TABLE VI[a]

RESPONSE OF CHICKENS TO I.V. BSA AT 6 OR 12 WEEKS OF AGE AFTER RECEIVING BSA I.P. ON DAY OF HATCHING

Amount BSA on day of hatching (mg.)	Number of animals	Antibody response at challenge			
		6 weeks of age AbN, μg./ml.		12 weeks of age AbN, μg./ml.	
		Range	Mean	Range	Mean
3.76	15	0–292	132	28–496	270
22.5	14	0–168	61	15–642	204
135	12	0–164	33	72–432	248
None	14	48–304	164	120–582	272

NOTE: Groups of chick embryos were injected I.P. with the indicated amounts of BSA and then reinjected at 6 or 12 weeks of age. The results in terms of precipitin titers are given.

[a] After Tempelis *et al.*, 1958.

They also showed that a lower precipitin titer could be demonstrated in chicks injected with a weight-graded dose up through the twelfth day after hatching. These data indicate that a relationship exists between inducing dose and the degree of tolerance and, less convincingly, the duration of tolerance. Note that partial tolerance was the rule (the proportion with no response has not been given in these papers). The immunological null period for chicks appears different for BSA (Wolfe *et al.*, 1957) than for homografts and bacterial antigens. The characteristic pattern of elimination of BSA from the serum, after challenge in these presumably tolerant chickens, was the *immune* type, not different from normal chickens. As the animals were only partially tolerant, this was

not unexpected; immune type elimination is also characteristic of partially tolerant rabbits. In other experiments, the specificity of this tolerant state was shown by the occurrence of a normal antibody response to HGG. In some respects, therefore, the results in most chickens have features in common with the protein loading model of Dixon and Maurer (1955a, b) as well as with tolerance induced in rabbits by neonatal injection.

Stevens and co-workers (1958) injected 115 mg. HSA into the chick embryo yolk sac at varying ages (days 8–18) and challenged them 6 weeks after hatching. Clear-cut differences were found in injected and control animals, the maximum degree of tolerance being elicited in the group injected at 14 days. [Eight of sixteen failed to show *any* antibody response, and the mean titer was 7 μg. AbN (antibody nitrogen) per milliliter as compared with the mean control titer of 101 μg. AbN per milliliter.] This model appears to give data more comparable to results of studies in rabbits and should be examined more completely.

The fate of S^{35}-labeled BSA in "immunologically suppressed" chickens has been studied by Hirata and collaborators (1960), essentially confirming with this antigen the dose–degree of suppression relationship described by others (see the foregoing). They found by carcass and organ analysis that a total of 68, 205, and 613 μg. of antigen (measured as radioactivity) were present in the animals 63 days after receiving 20, 60, and 180 mg., respectively, of the original antigen. No data on the form or intracellular status of this antigen was given.

Partial tolerance of chicken RBC in chickens (isoagglutinins) (Billingham *et al.*, 1955), chick or goose RBC in ducks (Haskova and Pokorna, 1956; Haskova, 1957), turkey and possibly goose RBC in chickens, and chicken RBC in turkeys (Simonsen, 1955) have all been reported as being induced by injection starting before (Simonsen) and after (Haskova) hatching. The state of partial tolerance induced was not permanent, regardless of when injections were initiated, unless repeated RBC injections were given starting soon after hatching.

An interesting sidelight to Simonsen's work was the enhancement of susceptibility of normally resistant mature turkeys to Rous sarcoma virus by inducing partial tolerance of chicken RBC. Subsequent studies, however (Harris and Simons, 1958), have revealed that human group A erythrocytes, sheep erythrocytes, and chicken tissue extracts all had the property of altering turkey susceptibility—all having in common a Forssman-like heterophile antigen. Whether or not this is an example of tolerance of an antigen widely found in vertebrates remains to be shown.

Some of the critical questions about tolerance were approached by

Mitchison and colleague (Mitchison and Dresser, 1960; Mitchison, 1959). Their experiments were designed to assess the role of RBC antigens in sustaining the tolerant state in different species of chickens. The life span (normally 30 days) was determined by measuring at intervals, the residual Cr^{51}-tagged donor RBC in the circulation of the recipient species. In this way transfusions of foreign RBC could be gauged to the level of residual RBC from the previous injection. Differences in the rate and time of disappearance of tolerated and nontolerated RBC was used for assessing the immunological status of the host with respect to the foreign RBC. Radiation (10,000 r) of the RBC suspension prior to transfusion insured that no viable leucocytes were transfused with the RBC. Complete tolerance (donor RBC disappear exponentially like host RBC, with no agglutinin formation) was induced in the majority of birds injected from hatching. A small proportion developed partial tolerance. It was possible to keep the fully tolerant animals in that condition up to 7 months by providing a continuous supply of foreign RBC. However, if the cells were allowed to be eliminated completely from the circulation, immune clearance always followed (39 animals) the next challenge dose of donor RBC, 25 days or longer after the last cells had disappeared. The data demonstrate concisely the requirement for antigen to sustain the tolerant state. Presumably because this particular antigen is rapidly broken down, eliminated from the circulation, and metabolized in the reticulo-endothelial system, the model provides a very crisp end point.

Attempts by several investigators to induce tolerance of mammalian RBC in chick embryos have failed (Burnet *et al.*, 1950; Brauer *et al.*, 1956; Owen, 1957; Ambrus *et al.*, 1955; Hasek, 1956). However, the explanation of this failure may lie in the timing of the original injection and failure to provide for the need of a continuous source of antigen. Simonsen (1956) succeeded in inducing partial tolerance of human RBC in chickens by starting injections neither too early in embryonic life (which failed to induce tolerance) nor too late and by continuing them after hatching. His study raises the very interesting possibility that tolerance cannot be induced until certain stages of differentiation of mesenchymal cells with an immunological destiny have passed—an interpretation susceptible to further experimental analyses. A similar series of changes in the chick embryo are documented with respect to age and resistance to bacterial endotoxin (Smith and Thomas, 1956).

Recently Friedman and Gaby (1960) have investigated the response to a *Shigella paradysenteriae* soluble antigen in chickens which were given whole cells or the soluble antigen at varying times before and after hatching. As would be expected (Smith and Thomas, 1956), a very

high mortality resulted from the primary toxicity of the antigen in the embryos, particularly when given by the intravenous route. In the survivors, regardless of the route by which the antigen was administered (intravenous, yolk sac, or intramuscular), partial depression of agglutinin titers was found after challenge starting at 50 days. Figure 4 illustrates their results, which might be interpreted as showing that the duration of tolerance was finite and related to the age at injection. Note that injection in the few days before hatching gave the greatest inhibition of the immune response possibly because the embryos survived the largest amounts of antigen at that time. This finding suggests that the degree of

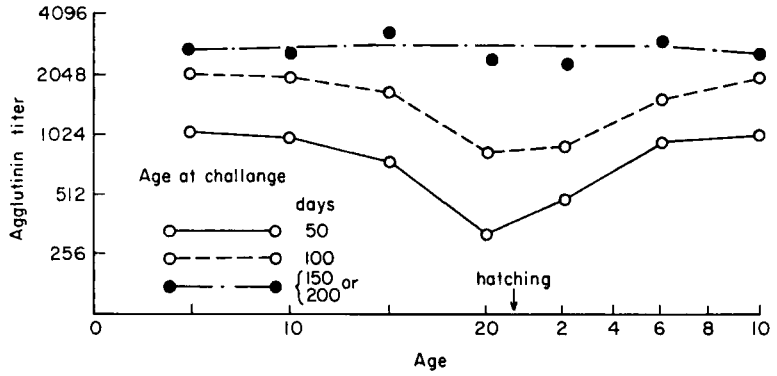

FIG. 4. In this experiment groups of chick embryos were injected with *Shigella* antigen various days before or after hatching. At intervals of 50, 100, or 150–200 days, some of each group were challenged with this antigen. The titer is the geometrical mean maximum titer of the group. Greatest depression is seen in the group receiving antigen on the twentieth day of incubation. (After Friedman and Gaby, 1960.)

depression was dose-dependent. However, arguments similar to those brought out in relation to Buxton's data (page 84) would possibly apply here. It seems possible also that an immunologically significant selection may have been operative in using only the *survivors* of the first dose. C^{14}-labeled *Shigella* antigen given to 10-day embryos was not detected as radioactivity or by immunological means in any tissues 1 week after hatching, although the suppressive effect lasted over 100 days. Further work with this model should prove interesting.

3. *Studies in Mice*

The mouse has received much attention in tissue and tumor tolerance work; however, built-in limitations of this species in producing precipitating antibody have made it, perhaps, a less convenient animal for

study of nonliving antigens. An extensive investigation of tolerance of BSA in mice has been made by Terres and Hughes (1959). Mice, which had been injected shortly after birth, or at 1 week of age with 12.5 mg. BSA as a single injection, or 50 mg. in 4 increments, were compared with uninjected controls at 6 and at 11 or 12 weeks of age. Antibody response to challenge in these two age groups was detected by Farr's technique or by the occurrence of systemic anaphylaxis. The 12.5 mg. failed to induce a significant degree of tolerance at 6 weeks. However, animals receiving 50 mg. as late as the third day of life showed almost complete absence of anaphylaxis and no antibody when challenged at 6 weeks of

TABLE VII

FAILURE OF ANAPHYLACTIC SENSITIZATION TO BSA IN MICE WHICH WERE INJECTED WITH BSA AT BIRTH

Group	Number showing anaphylaxis / Number in group
18 or 36 mg. BSA I.P. at birth, challenged at 7 weeks of age	0/12
Not injected at birth, challenged at 7 weeks of age	15/31
18 or 36 mg. BSA I.P. at birth, challenged at 11 weeks of age	10/13
Not injected at birth, challenged at 11 weeks of age	11/13

NOTE: In these experiments pen-bred Swiss mice were injected with either 18 or 36 mg. BSA I.P. soon after birth. At 7 or 11 weeks of age, sensitization to anaphylaxis was attempted by injecting 10 mg. BSA in Freund's adjuvants. Three weeks later a single I.V. injection of 1 mg. of the antigen was given and anaphylaxis assumed if the animal died (Eitzman and Smith, 1959, unpublished data).

age. Similar groups challenged only at 12 weeks were not different from controls. Thus tolerance of BSA in mice appeared finite in duration, dependent on the dose, and was induced only shortly after birth. Eitzman and Smith (Table VII) obtained essentially the same results using anaphylaxis as a criterion of an immune response. In this instance, injection of 18 or 36 mg. BSA at birth yielded a high percentage of mice that resisted anaphylaxis at 7 weeks of age, but the effect had disappeared by 10 weeks of age.

Apparent effects upon ultimate susceptibility of mice to lymphocytic choriomeningitis virus, following prenatal experience with this virus, were reported by Traub (1936, 1938, 1939). Burnet and Fenner (1949) interpret this as a possible example of tolerance. Nossal (1957b), however, attempting to influence the outcome of influenza infection in mice

by prenatal infection, could find no evidence of tolerance as enhanced susceptibility or decreased antibody formation in comparison to animals infected for the first time. A recent report of Hotchin *et al.* (1960) appears to confirm Traub's earlier observation that early infection with lymphocytic choriomeningitis virus does affect the later expression of immunity in mice. Here the antigen is easily demonstrable in the tissues after maturity and no antibody is detectable. Further investigation of possible tolerance of viruses should prove extremely valuable.

4. Studies in Rats

The response to foreign RBC in the rat has proven also to be a valuable model in elucidating the mechanisms of tolerance. Whereas Brauer and co-workers (1956) failed to show any tolerance of sheep RBC at 11 weeks by giving a single neonatal injection, Ripley (1953), Simonsen (1956), and Nossal (1957a, 1958) have all succeeded in this to some degree. Nossal has examined RBC tolerance in rats most completely, employing human type O or A RBC, guinea pig RBC, C3H mouse RBC, or sheep RBC injections starting within a few hours after birth. He later assayed the agglutinin titer following reinjection of the same donors cells. Providing that injections of cells were made twice weekly for 8 weeks, challenge at 10 weeks of age revealed complete suppression of the agglutinin response. An exception was the group given guinea pig RBC, which showed only partial suppression. If injections were continued, tolerance lasted indefinitely (more than 9 months). Administered hyperimmune serum disappeared from the serum of unresponsive animals at a rate identical to controls, and the animals were still tolerant upon rechallenge 6 weeks later. The specificity of RBC tolerance in rats was also demonstrated convincingly. By stopping twice weekly injections at varying times, and challenging the group at 9 weeks of age, it was shown that complete tolerance required injection for at least 7 weeks of the 8-week period, though degrees of partial tolerance occurred in animals discontinued earlier. A dose-duration effect was shown in that no concentration of a single dose under 10% RBC induced tolerance by the foregoing schedule. Lastly, it was found that partial tolerance of RBC antigens could be induced when injections were started at 3 and 7 days but not later; complete tolerance resulted only in animals in which injections were inaugurated on the first day of life.

Calabresi and Edwards (1958) have also found some degree of depression of agglutinins to human RBC in rats by multiple injections, but their data do not permit analysis of the role of antigen in the same sense as was possible in the foregoing experiments.

Zilber and Kruikova (1957) have recently reported that rats which are given Rous sarcoma virus prenatally often develop a "generalized hemorraghic disorder" with virus proliferation and fail to develop antibodies to this virus upon challenge. This phenomenon appears very closely related to that described in mice by Traub (1936, 1938, 1939).

An important example of tolerance of complex tissues of *homologous* origin is the work of Paterson (1958, 1959a, b) who produced refractoriness to allergic encephalomyelitis in rats by a single neonatal injection of brain tissue. These animals failed to develop the paralytic consequences when administered nervous tissue in adjuvants at 8–10 weeks of age, compared to control animals which regularly became paralyzed. Myelination of the developing brain is known to be quite delayed in most species, and the factors in the brain which produce this CNS (central nervous system) disease develop only postnatally. Perhaps, as has been suggested by Paterson, this absence of a brain antigen denies the fetus the opportunity to acquire tolerance of this component of itself prenatally and thus creates a permanent dependence on the immunological isolation of the brain from tissues of the reticulo-endothelial system.

5. *Studies in Humans*

Obvious limitations to experimental manipulations have restricted observations of tolerance in humans to a few "experiments of nature." These efforts, though leaving many questions unanswered, are suggestive that *Homo sapiens* is not exempt from the phenomena of tolerance. The earliest observations (Dunford *et al.*, 1953) of RBC chimerism in humans led to further search for evidence of tolerance of the blood group antigens. The investigations of Owen *et al.* (1954) revealed suggestive evidence of a lower incidence of anti-Rh antibody development in pregnant Rh-negative women, whose *own* mothers were Rh-positive, than in those with Rh-negative mothers.

A more direct approach to this problem was made by Jakobowicz and colleagues (1959), who determined the hemolytic, saline, and incomplete isoagglutinin response to tetanus toxoid immunization (which carries very strong blood group A activity) in enlisted men whose mother's blood group was known. Table VIII summarizes their findings indicating that a statistically significant reduction in the response to this strong group A stimulation occurred in the group with A mothers—a difference most evident in the incomplete antibody titers. It may be noted that some increase of average anti-B titers occurred despite the absence of this antigen in the toxoid. An unexplained finding was that the titer of tetanus antitoxin developed was inversely related to the anti-hemolysin titer rise.

In studies still in progress (see also Smith, 1960b), Eitzman investigated the fate of horse γ-globulin antitoxin used in treatment of human infants having tetanus neonatorum usually within 1 week of birth. In those surviving the disease, this antigen was eliminated from their serum with exponential decay characteristics (half-life = 14 days), no immune phase of fall off, and no antibody production. The few mature individuals studied who received this material, showed immune-type antigen elimination, but, as has been observed by others, some older, normal children

TABLE VIII[a]
MEAN ANTI-A AND ANTI-B TITERS IN GROUP O TROOPS RECEIVING TETANUS TOXOID

Antibody	Mother's blood group	Mean anti-A titer		Mean anti-B titer	
		Before injection	After injection	Before injection	After injection
Saline agglutinin	O	19.8	326.4	6.8	23.1
	A	14.8	226.3	5.5	18.1
	B	16.0	212.0	7.5	21.9
Hemolysis	O	1.1	24.4	0.6	1.8
	A	0.8	14.6	0.5	1.3
	B	1.2	24.8	0.6	1.1
Incomplete antibodies	O	1.2	68.0	0.6	1.8
	A	0.7	21.6	0.6	1.4
	B	1.9	43.8	0.6	0.8

NOTE: Troops of blood group O received routine immunization with tetanus toxoid, which has demonstrated strong blood group A activity. The results of preimmunization and subsequent determination of isohemagglutinins in saline albumin-supported media or with complement are shown assorted by the blood group of the soldier's mother. The results appear to show reduced capacity to produce isohemagglutinins in soldiers whose mothers blood group was A but also in B to a lesser extent.

[a] After Jakobowicz *et al.*, 1959.

have prolonged elimination times. No opportunity to challenge these infants' putative tolerant state, has yet occurred. Work in progress with chimpanzees should provide more complete data on tolerance in primates.

Other opportunities to examine possible tolerant states, particularly as they relate to the important question of immunological status of autologous components, may exist in certain genetic deficiencies in enzymes or serum proteins in humans. For example, patients having anti-hemophilic globulin (AHG) deficiency, a sex-linked form of hemophilia, occasionally develop marked resistance to the repeated human AHG infusions used in their treatment. Some evidence exists that the "resistance factor" is a γ-globulin with some, but not all, of the characteristics of an antibody (Biggs and Bidwell, 1959; Munroe and Munroe, 1946). Whether genetically determined absence of the antigen has permitted immunological

responsiveness to AHG of homologous origin is an interesting and challenging, but unanswered, question. Other examples possibly exist, such as the response to fibrinogen administration in afibrinogenemic patients, to pepsin or gastrin in patients who have had total gastrectomy, to thyroglobulin in cretins or thyroidectomized individuals. The response of hypogammaglobulinemic children to certain γ-globulin components would also be of interest.

Although perhaps out of context of this review, note should be taken in this section of the phenomenon of continuous release of trophoblasts into the circulation of pregnant women (Boyd, 1956; Douglas *et al.*, 1959). Following Thomas' (1959) provocative analysis of the intrinsic significance of transplantation immunity, suggesting an analogy between normal pregnancy and immunological tolerance, these cells could provide the continuous source of antigen which sustains the "placental homograft." This appears to be a fertile field for further investigation.

6. Studies in Guinea Pigs

The maturity of this species at birth has until recently exempted it from the machinations of the investigator of tolerance, except for the study of Weiss (1958) who found that injection of tuberculin into fetal guinea pigs delayed the development of BCG hypersensitivity when infected at 8 weeks of age, though the effect was temporary. Humphrey and Turk (1961) report that tolerance to BSA and HGG resulted from administration of these antigens at birth, with or without prior administration of large doses of antigen to the mothers. These authors were unable to produce a state of delayed hypersensitivity to these proteins in the guinea pigs as long as the animals remained tolerant in respect of circulating antibody production.

7. Studies in Cattle

The only example of tolerance of nonliving antigen in this species is that reported by Kerr and Robertson (1954) who found depression of immunological activity in calves resulting from the early injection of large amounts of *Trichomonas foetus* antigen. The data are too limited, however, for further evaluation.

B. Tolerance and Tolerancelike Models Involving Induction in Mature Animals

The pertinence of the following examples of specific depression of immunity to the foregoing considerations of tolerance depends on whether they may be related in mechanism or offer some unique oppor-

tunity to understand mechanisms of the classic tolerance models. Those involving nonreplicating antigens—as most do—will be considered here briefly but only as the data are germane to the theme of this review.

1. *Induction by Amounts of Antigen Very Large Compared to an Immunizing Dose*

Three general examples of this type of experimental model have been investigated—those induced by proteins, polysaccharides, or simple chemicals.

a. Proteins. The studies of Dixon and collaborators (Dixon and Maurer, 1955a, b; Weigle and Dixon, 1959) have provided the bulk of the data concerning this model. Essentially, repeated injections (500 mg. per kilogram daily for 37 days) of large amounts of whole plasma or a protein such as BSA were given to mature rabbits. The rates of disappearance from the plasma after cessation of infusions were very much like those of tolerant animals but tended to become more rapid as the levels decreased (Fig. 5). Different components of the complex mixtures disappeared at their characteristic non-immune rate, and antibodies against trace components often appeared during the infusion period. However, within 6 months most animals so injected became capable of immune response to the major components of these proteins again. Johnson *et al.* (1955) have performed similar experiments selecting animals, however, which for genetic or other reasons, tended to remain unresponsive after the infusion period ceased.

Perhaps analogous to these results are experiments in which delay in or prevention of the paralytic consequences of injecting brain–adjuvant mixtures into rabbits, follows preinjection with a relatively large amount of homogenized brain tissue (Condie *et al.*, 1957). It is presently a moot point whether this type of experiment is related to the phenomena of loading or to the enhancement effect (Snell *et al.*, 1960) which appears due to suppression of delayed allergy by increased antibody formation.

In each of the loading models, it appears that no suppression of immune responsiveness has occurred in the sense of tolerance as induced in newborn animals, but that overloading of the capacity to handle the antigen has merely *delayed* immune clearance and antibody formation. The occasional animal which *remains* unresponsive deserves attention (Johnson *et al.*, 1955), however, as a possibly significant exception. The lack of many critical similarities between this and more straightforward examples of tolerance leads to the view that they are probably not entirely comparable in mechanism. However, many of the immunological phenomena brought out in the preceding may be intermediate between

this type of suppression and that induced by neonatal injection. Taliaferro (1957b) has shown another possible intermediate state in his studies of hemolysin systems. Thus, the question cannot be considered as answered yet.

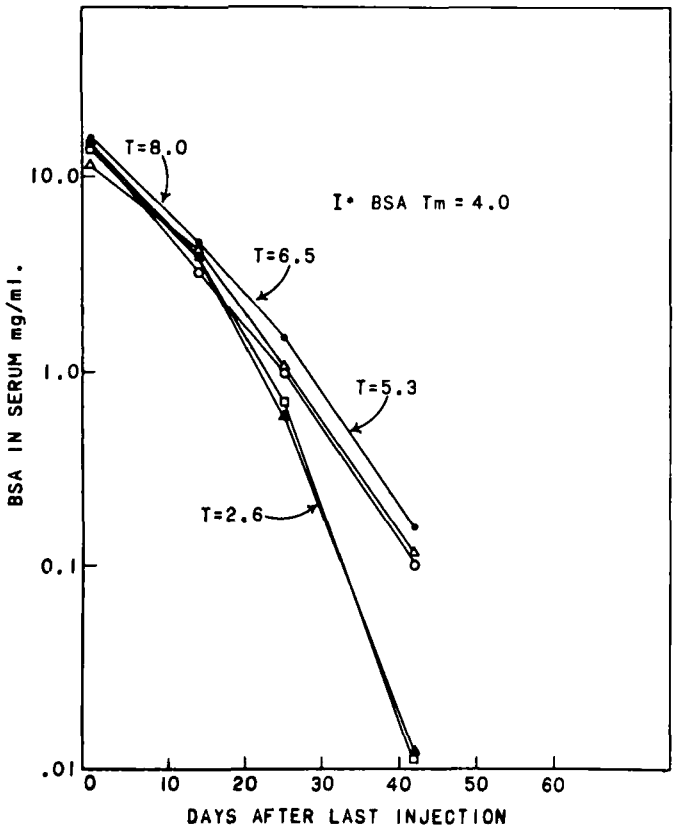

FIG. 5. This figure was constructed from immunochemical determinations of BSA in the serum of individual normal mature rabbits following the last of 43 daily injections of 500 mg. BSA per kilogram. The half-life value (T) of each segment is indicated. Average half-life given by the authors for I^{131}-labeled BSA in these animals was 4 days. (Taken from data of Dixon and Maurer, 1955a.)

b. Polysaccharides. One of the most intriguing phenomenon in immunology is that described first by Felton (Felton and Ottinger, 1942; Felton, 1949; Felton *et al.*, 1955a, b, 1956) and studied later by others (Morgan *et al.*, 1952, 1953, 1957; Stark, 1955, 1959; Sandage and Stark, 1947; Ørskov, 1956; Kaplan *et al.*, 1950; Gitlin *et al.*, 1958; Dixon *et al.*, 1955) as "immune paralysis." Doses of pneumo-

coccal polysaccharide 500 times greater than an immunizing dose produced a long-lasting and total inhibition of the capacity of adult mice to produce neutralizing antibody to this agent. Similar "paralysis" has been found with respect to pneumococcal polysaccharide in humans following severe pneumococcal pneumonia. Some evidence (Morgan *et al.*, 1952, 1957) suggests that a similar phenomenon occurs in rabbits. It should be recalled that each of these polysaccharides has determinants closely related to or identical with host polysaccharide determinants and is very slowly metabolized. Although Dubos has shown that a specific hydrolytic enzyme for the type III pneumococcus can be induced in *Escherichia coli*, evidence for this in mammalian cells does not yet exist. Although a definite dose–induction relationship has been described, the duration of "paralysis" once induced is not known (with various doses of antigen). Paralysis produced by a threshold dose does not appear to have been tested only once in order to ascertain whether it is of finite duration. Here the additional increments of antigen required for testing might be sufficient to perpetuate the "paralyzed" state. The critical studies involving cell transfer—normal and immune cells to "paralyzed" mice, "paralyzed" cells to normal hosts, etc.,—have not been reported to date.

The most disputatious point surrounding this model has been whether or not the "paralyzed" mouse is making antibody. Large amounts of antigen persist in tissue (Kaplan *et al.*, 1950) (Stark, 1959, and prior work) and bind passively transferred antibody to the extent that considerably larger doses are required for passive protection of paralyzed mice from pneumococcal infection. This observation has led to the hypothesis that antibody is being bound and disposed of as rapidly as it is formed—the treadmill hypothesis. The recent studies of Gitlin *et al.* (1958) and Sercarz and Coons (1959) using immunohistochemical techniques demonstrate, however, that detectable anti-pneumococcal polysaccharide antibody is not being formed in the paralyzed animal's tissues. The large amounts of demonstrable residual antigen may, in fact, be irrelevant to maintenance of the tolerant state once induced. No data yet available clearly separates out this type of reversal of immune capacity once present from tolerance of protein antigens induced by neonatal injection; and it would not be surprising if further studies link the models more closely.

c. Simple Chemicals. Perhaps the first experimental model in which an expected immune response was suppressed by prior exposure to antigen is that described by Wells and Osborne (1911). These workers, investigating anaphylactic sensitization of mature guinea pigs to zein, a corn protein, discovered that animals accidently fed green corn diets

became refractory to sensitization. Comparable suppression of dermal delayed-type hypersensitivity resulted in guinea pigs when 6 mg. neoarsphenamine was injected intravenously prior to intradermal sensitization Sulzberger, 1929); Chase (1959b) extended this finding to arsphenamine with apparently similar results.

Chase (1946, 1959a; Chase and Battisto, 1959) first contrived an experimental model in which the suppression, per se, was investigated. Originally, 2,4-dinitrochlorobenzene in olive oil was fed to mature guinea pigs for 6 days, which, after an interval, were given an intradermal sensitizing dose of this chemical. Little or no anaphylactic sensitization occurred in the fed group as contrasted to unfed controls. The specificity of suppression was shown in the 2,4-dinitrochlorobenzene group by expected development of sensitivity to intradermal *o*-chlorobenzoyl chloride. Suppression in the fed group, in these and later experiments, lasted several months (up to 13), but testing of the suppression appeared always to require giving an additional increment of antigen to the animal. Animals already showing anaphylactic sensitization failed to be suppressed by comparable amounts of the antigen.

In later experiments, Chase (1953, 1959a) found that dermal or delayed-type hypersensitivity as well as a circulating antibody response to 1,2,4-trinitrochlorobenzene (picryl chloride) was suppressed simultaneously by feeding 50 mg. in 3-mg. daily increments. With picryl chloride, application to the skin, as well as feeding, produced unresponsiveness, but with less regularity. Passively transferred antibody-containing serum had no effect on delayed hypersensitivity nor was it removed rapidly (Battisto and Chase, 1955) in unresponsive guinea pigs. However, transfer of living reticulo-endothelial cells from sensitive donors endowed contact sensitivity to unresponsive animals. Transfer of cells from unresponsive animals to normals did not result in sensitization.

When fed C^{14}-labeled picryl chloride, guinea pigs excreted most of the antigen in the bowel, leaving too little to detect in the bowel wall by radioautography. This finding indicated that an exceedingly small amount of antigen was needed to induce the tolerant state. In other experiments (Chase, 1949; Battisto and Chase, 1955), it was shown that while sensitization of picryl-unresponsive guinea pigs did not occur when homologous picrylated proteins were injected intracutaneously, picrylated BGG did break through the unresponsive state with the production of antibody, but dermal hypersensitivity remained repressed. An exceedingly strong stimulus—picrylated red cell stroma in Freund's complete adjuvants—broke through the unresponsive state to some extent with respect to hypersensitivity.

The available data on this model show it to differ from protein tolerance in that (a) the role of antigen is not yet clarified and (b) that it is induced in mature animals. Further dose–duration studies, in which the challenge dose is given but on a single occasion, should answer the former question. With respect to the latter, the role of host protein–antigen complexes (Eisen and John, 1957; Eisen and Belman, 1953; Eisen, 1959) appears to be an important ingredient in this unresponsive state. If it were possible to produce similar tolerance with picrylated heterologous protein in mature animals by, say, intravenous injection, it would have to be assumed that this model is indeed unique in the circumstances of its induction. However, we would at present agree with Chase's working interpretation that this is an example of immunological tolerance not unrelated to tolerance induced in the neonatal period. This model still appears most promising in terms of further understanding the mechanisms underlying tolerance.

2. *Induction in X-Irradiated Animals*

Near lethal irradiation of various species results in depression of the primary immune response which, depending on the species, nature of the antigen, the timing of radiation relative to antigen administration, may be complete or partial (Taliaferro, 1957a). Since Makinodan and Gengozian (1960) have recently reviewed this subject, a detailed account will not be given here. The most complete unresponsiveness to nonliving antigens is to heterologous proteins in the rabbit (Dixon *et al.*, 1952; Dixon and Maurer, 1955b; Dixon and Weigle, 1957a, b; Taliaferro, *et al.*, 1952; Taliaferro and Taliaferro, 1954a, b; Hobson *et al.*, 1958). Following injection of BSA, for example, 1–48 hours after 400–600 r total body irradiation, the animals show exponential, nonimmune elimination of the foreign protein from the serum, and no antibody appeared. This effect may be transient when the usual immunizing doses are used, responsiveness being restored after a variable waiting period. In experiments where repeated infusions of foreign protein were started shortly after irradiation (Dixon and Maurer, 1955b; Weigle and Dixon, 1959), the unresponsive state was as permanent by their techniques as that induced in newborn animals and persisted after antigen was no longer detectable in the serum. The rate of elimination from the serum was the same as in tolerant animals. Unpublished studies of this model in the author's laboratory confirm Dixon and Maurer's results and show that Ea unresponsiveness is of much shorter duration than BSA unresponsiveness and that it is very similiar to observations of tolerance induced in the newborn rabbit. In this model,

more dose–duration studies, cell transfer studies, etc., are all needed before it can be concluded that this type of unresponsiveness is the same as that induced in the neonatal period. The evidence available favors the interpretation that X-irradiation before infusion of the antigen has, indeed, altered the responsiveness of the mature animal in a fashion superficially resembling that in the neonatal period. The resemblance is corroborated by the extensive recent investigations of tissue and cell tolerance brought about in supralethally irradiated animals. With other nonliving antigens, irradiation has not resulted in complete unresponsiveness. For example, unsuccessful attempts to induce unresponsiveness of rat RBC in irradiated mice have been reported by Nossal and Larkin (1958).

3. *Induction of Unresponsiveness in Chemically Altered Animals*

A significant advance in the knowledge of the mechanism of immunological tolerance has come through the finding that certain purine antagonists or competitive inhibitors can drastically interfere with the immune response (Malmgren *et al.*, 1952a, b; Dutton *et al.*, 1958; Sterzl and Holub, 1957). With certain antigens, and provided timing and dosage are properly adjusted, the administration of an adequate dose of 6-MP (6-mercaptopurine) (6 mg. per kilogram daily in rabbits) (Schwartz *et al.*, 1958, 1959) results in a nonimmune type of elimination of protein antigens and the absence of all types of antibody formation (Fig. 6), a situation closely resembling immune tolerance induced in the neonatal period. These authors have further shown that such treatment interferes with but does not inhibit the secondary response. Investigations of this model in rabbits in our laboratory (Fish, L., and Smith, R. T., to be published) have revealed other resemblances to neonatal tolerance. For example, the antigen elimination rates are exactly those found in neonatal tolerance by similar methods. Cell transfer and dose–duration studies should be done to test the analogy further.

Humphrey and Turk (1961) failed to inhibit antibody production to BSA or HGG in mature guinea pigs, even when lethal or nonlethal doses (25 mg. per kilogram daily) of 6-MP were used. Nor does it appear possible to interfere with the development of delayed contact allergy to 2,4-dinitrofluorobenzene or to tuberculin allergy in BCG-injected guinea pigs with near lethal doses of this drug (Fish, L., and Smith, R. T.).

Interference with another immunological phenomenon—allergic encephalomyelitis in rabbits—has been recently reported by Hoyer *et al.* (1960). These workers found marked delay in the development of the paralytic manifestations of this disease after 12 mg. per kilogram daily of the drug. It would be of interest to follow up this observation by using

the drug in an attempt to induce tolerance of small amounts of CNS tissue in adult rabbits as Paterson (1959b) has done in rats.

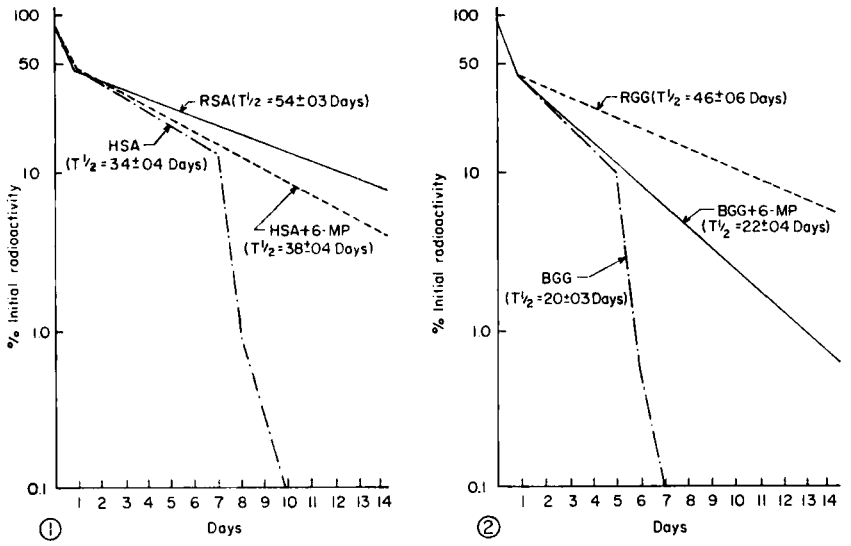

FIG. 6. Comparison of rates of elimination of heterologous and homologous albumins and globulins in rabbits treated with 6-MP. (1) Elimination of albumins; (2) elimination of globulins. (From Schwartz *et al.*, 1959b.)

III. Analysis of the Various Experimental Models and Common Features of Each

The foregoing body of available data has been examined critically only for intrinsic validity in each case. Table IX summarizes these data, comparing certain features for each species and antigen. The gaps in available knowledge were emphasized in the foregoing section and are quite apparent in this table. These should serve as a challenge to further investigation and emphasize the marked limitations of drawing firm conclusions regarding general mechanisms of the tolerant states.

The first question which must be asked is whether sufficient similarities exist in the various experimental models to assume, as has been suggested in several instances, that similar mechanisms may operate in each.

This question may be answered affirmatively to a degree (see also Chase, 1959b). In review, it may be seen that certain features of the various models are consistent (a) for different antigens within the species, (b) for the same antigen in most species studied, and (c) to a

lesser extent, for the tolerant state in general. General consistencies justifying an assumption of similar mechanisms will be examined, but detailed considerations of mechanism will be reserved for the next section.

Tolerance through neonatal injection of heterologous RBC, certain tissue components, and serum proteins occurs regularly in those species having young which are relatively immature at the time of first contact with its extra-uterine and extra-oval environment (i.e., rabbit, mouse, rat, chick, and possibly human). The guinea pig, mature at birth, appears to vary in susceptibility to the induction of tolerance, depending on the antigen. Comparison of the response to antigen injected shortly after birth in domestic ungulates, which are relatively mature at birth, and in opossums which are extremely immature at birth would be of interest in further testing this generalization.

The tolerant state appears qualitatively similar in those species characterized in detail with regard to (a) the period of susceptibility to induction, (b) the types of antigens effective, (c) the dose–duration of tolerance relationship, and (d) the requirement for continuous antigen. Quantitatively, however, variations are quite evident, such as the dose required for the induction of tolerance on a weight basis. Approximately 5 mg. BSA per gram of body weight is required to induce tolerance of 6–7 weeks duration in the mouse injected at birth and in the chick embryo injected at 14 days. Only 0.001 mg. BSA per gram of body weight of this antigen induces tolerance of a comparable duration in the neonatal rabbit. Critical quantitative data on the amounts of more closely related antigens in the chick (i.e., duck albumin) and mouse (i.e., rat albumin) are necessary in order to evaluate this difference further. In each species, however, amounts of antigen less than those inducing tolerance have no demonstrable effect on the subsequent immune response. Immunological paralysis to pneumococcal polysaccharide as was brought out earlier, can be induced in mouse, rabbit, and man. In each case, available data indicate that a dose of antigen, several orders of magnitude larger than the immunizing dose, is required for induction.

Whereas tolerance of proteins, certain carbohydrates, and tissue may be produced in several classes of animals, the effectiveness of feeding simple chemicals in preventing all phases of the immune response in adult guinea pigs appears unique to that species. The unresponsive state produced in this way seems to be comparable to other types of tolerance, in specificity, in affecting all phases of the immune response, in having a dose threshold for induction, and in the results of serum or cell transfer. On the other hand, some features suggest that the relation of the antigen

TABLE IX

COMPARATIVE SUMMARY OF DATA ON TOLERANCE OF NONLIVING ANTIGENS IN VARIOUS SPECIES

Experimental model, by species and antigen	Induction successful in				Degree of tolerance usually	
	Perinatal period	Normal mature	X-irradiated mature	6-MP mature	Complete in a high proportion of animals	Partial tolerance, reduction of response
1. Rabbit						
a. Heterologous proteins	+	0q[1]	+	+	+	
b. Azoproteins	+	0			+q[2]	
c. Polysaccharides		+			+	
d. Heterologous tissue	+	0				+q[3]
e. Heterologous RBC	+	0			+	
f. Homologous RBC	+	0			+	
g. Staphylococcus toxoid	+	0				
h. Homografts	0	0	0			
i. Yeast enzyme	+	0				
2. Chicken						
a. Heterologous proteins	+	0			+q[4]	
b. Heterologous RBC	+	0				+
c. Homologous RBC	+	0			+	
d. Homografts	+	0	0		+q[6]	+
e. *Shigella paradysenteriae*	+	0				+
f. *Salmonella pullorum*	+	0				+
3. Guinea Pig						
a. Simple chemicals		+			+	
b. Tuberculin	+	0	0	0		
c. Heterologous proteins	+	0		0	+	
4. Mouse						
a. Heterologous proteins	+	0			+	
b. Homologous RBC	+	0	+			
c. LCM virus	+	0			+	
d. Polysaccharides		+			+	
e. Homografts	+	0	+			
5. Rat						
a. Heterologous RBC	+	0			+	
b. Homografts	+	0	+		+	
c. Rous sarcoma virus	+	0				+q[6]
d. Brain tissue	+	0			+	
6. Cow						
a. *Trichomonas foetus*	+	0				+
7. Human						
a. Heterologous protein	+q[5]	0			+	
b. Homologous RBC	+	0				+
c. Polysaccharides		+			+	

NOTE: + = Feature present or affirmative answer. 0 = Feature absent or negative answer. q[1] = Exception—Model of Dixon and Maurer (1955a) and Johnson *et al.* (1955), may not be equivalent to other model of tolerance—see text. q[2] = No definite tolerance has been induced to the hapten group, only to the conjugated protein.

TABLE IX (*Continued*)

All known phases of immune response affected	Evidence for relation between dose and induction	Evidence for requirement of continuous antigen supply	Effect of various manipulations on tolerant state: Effect of immune serum on tolerant state	Effect of immune cells on tolerant state	Effect of X-ray treatment
+	+	+	0	0	0
	+	+			
	+				
	+				
	+	+	+		
	+	+			
	+				
	+	+			
	+	+			
	+	+	+		
			0	+	
	+				
+				0	
+					
	+	+			
	+		0		
	+			+	
	+	+	0		+
	+		0	+	

q^3 = Tolerance of some components, immune response to others of mixture of antigen. Over-all reduction of response. q^4 = Only in model of Stevens *et al.* (1958). q^5 = Assumed tolerance, no attempt made to challenge. q^6 = Not a high proportion, but significant numbers.

to a component closely related to "self" is critically involved in the induction of this type of tolerance. Immunization of the fed animals with complexes of the chemical with a heterologous protein, in contrast to the result when homologous proteins were used, resulted in elimination of the tolerant state. Intensive stimulation of unresponsive guinea pigs with the chemical antigen in Freund's adjuvants terminated the unresponsive state quite rapidly. These results are different from the ones obtained in the rabbit in which only slight shortening of the tolerant state resulted (Smith and Bridges, 1958).

In summary, comparison of features of the models in which the response to simple antigens has been most extensively investigated shows evidence of many similarities of the tolerant state *once induced.* Therefore, it appears that features of each can, with merit, be examined for tentative generalizations about the tolerant state. Models involving more complex antigens and particularly protozoan or bacterial cells and their products (Kerr and Robertson, 1954; Buxton, 1954; Lindorfer and Subramanyam, 1959; Weiss, 1958) do not justify conclusions at present. These latter data in final analysis may be as pertinent to the phenomena of enhancement or of antigen loading (Dixon and Maurer, 1955a; Johnson *et al.,* 1955; Condie *et al.,* 1957) as to the phenomenon of tolerance.

IV. The Nature of the Tolerant State

Firm conclusions as to mechanisms of tolerance are wholly unwarranted at this time. Data derived primarily from the more completely characterized experimental models of tolerance of nonliving antigens, however, appear to provide grounds for some tentative statements regarding the nature of the tolerant state, particularly as regards the role of antigen in its induction and maintenance and the conditions which permit induction of tolerance rather than of an immune response.

A. Response of the Tolerant Animal to the Tolerated Antigen

As the tolerant state is detectable only by the absence of an expected response to antigenic challenge, it cannot be characterized after the fashion of other immunological phenomena. In several ways, however, tolerance has been examined as critically as the immune response, particularly with respect to specificity and phases of immune response involved.

1. Specificity

Tolerance of an antigen appears to be as specific as the immune response. By this statement it is inferred that tolerance reveals the same

close orientation to the determinant groups of the antigen as that on an antibody molecule which occurs as a result of an unimpeded immune response. Antibody specificity and cross reactions occurring between antigens sharing determinant groups may be demonstrated in a positive fashion by a variety of familiar *in vitro* techniques such as absorption,

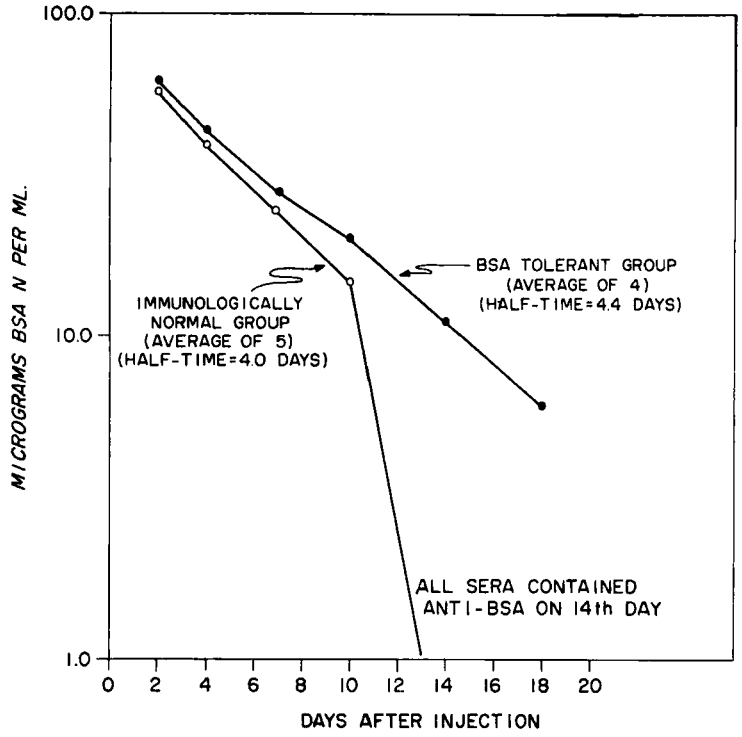

FIG. 7. Immunochemical measurement of BSA in serum of groups of BSA-tolerant and normal animals of the same age following intravenous injection of 25 mg. BSA per kilogram. On the fourteenth day all of the normal group had cleared antigen and had detectable anti-BSA in their sera. Note that the rate of clearance in tolerant animals is approximately the same as the preimmune phase of elimination in the normal groups. (From Smith, 1960a.)

band patterns in gels, hapten inhibition. Tolerance is revealed, however, by the *failure* in the appearance of an expected product of the immune response or by a nonimmune pattern of clearance of an injected antigen.

The pattern of clearance of antigen from the circulation in the completely tolerant animal shows no phase of accelerated or immune elimination. In the example shown (Fig. 7) and in other reported studies (Weigle, 1960), the rate of elimination of heterologous protein in the

tolerant animal is the same as the nonimmune phase of eliminating that antigen in the nontolerant animal. This rate of elimination of antigen from the serum is not necessarily related to the specificity of the tolerant state, however. For example, BSA-tolerant rabbits eliminate lightly iodinated BSA (Smith, 1960a; Freeman *et al.*, 1958) exponentially, as they eliminate BSA, but the rate appears to increase as iodination increases.

Light iodination of the antigen had no demonstrable effect on the state of tolerance to BSA. Animals initially made tolerant of the lightly iodinated protein, on the other hand, were found to show the same rate of elimination measured as radioactivity or antibody content.

Crampton *et al.* (1959a), however, showed that heavily iodinated albumin has a rapid clearance time in the specifically tolerant rabbit, even though no antibody was produced. It would appear, then, that iodine labeling while producing no change in the protein detectable by standard immune techniques, effects subtle structural changes which can be detected by the tolerant animal, but not on the same basis as the tolerant state.

It appears that the mechanisms responsible for clearance of an antigen from the circulation are not always the same as those which recognize and deal with them as antigenically foreign. Particle size, charge, and related factors known to affect clearance from the circulation would have to be controlled in any experiments in which rate of elimination only was used as an indicator of the presence of tolerance.

2. *Phases of the Immune Response Affected in Tolerance*

In general it can be said that all the many manifestations of the immune response to a single antigen appear to be suppressed in the completely tolerant animal. The immune response to a single antigen results rarely, if ever, in the production of a single homogeneous species of antibody protein—an assumption commonly made or tacitly implied in considering the nature of the immune response or tolerance. Rather, the imprint of specificity or complementariness may be the property of cell surfaces (delayed hypersensitivity) or of several proteins secreted by cells which have differentiated along distinctive lines. This product may take one of a number of known forms: 7S γ-globulin; 19S γ-globulin; heat-labile components; antibody with skin-sensitizing properties; and probably others yet to be characterized.

Furthermore, each of the major categories of antibody is probably inhomogeneous biologically and chemically, variability being demonstrable at various phases of the immune response, in number of combining sites, avidity, and other properties.

Antigen injected into the tolerant animal fails to elicit the formation of *any* of the carriers of immune specificity. The complete validity of this conclusion would depend on infinite sensitivity of techniques of detecting antibody. However, search in the serum of completely BSA-tolerant rabbits for nonprecipitating antibody by several different techniques (tannic acid-treated RBC, antigen binding, complement fixation, etc.) has failed to reveal any evidence of an immune response. Nor have attempts in rabbits to elicit hypersensitivity by injecting specific BSA and anti-BSA precipitates shown a delayed response in an otherwise tolerant animal (Smith and Bridges, 1958, unpublished data). Knowledge of the varieties and qualities of antibody produced in the *partially* tolerant animal would be of considerable interest, for it is possible that all phases of the immune response are not uniformly affected under these circumstances.

Chase (1946) demonstrated that unresponsiveness to picryl chloride induced by feeding the antigen extends both to delayed hypersensitivity and antibody formation. A possible exception to this may be found in preliminary experiments reported by Chase (1959a) in which he injected picrylated heterologous proteins into picryl-fed, unresponsive guinea pigs. He demonstrated antibody formation in the face of sustained inhibition of the delayed response to the antigen. Similar findings with phthalic anhydride were also mentioned. These exceptions, if confirmed, will be of considerable importance and require revision of the generalizations just mentioned.

A tentative conclusion which can be derived from the foregoing considerations is that, under most circumstances, none of the later or "productive" phases[5] of the immune response occur in the tolerant animal.

Alternative interpretations are also possible, i.e., that antibody is being constantly produced but bound to antigen and thereby not released, or that the antibody-producing cell is poised, but in an unfavorable environment. These two explanations have no convincing support. Experiments which employ cell transfer techniques appear critical in this connection. In the case of protein tolerance in rabbits, amply confirmed data show that the tolerant animal supports vicarious antibody production by transferred immune cells as well as the normal recipient. This effect is short-lived because of the operation of homograft immunity and has no permanent effect on the underlying tolerant state. Inhibitors of synthesis of the specific antibody protein, probably then, do not exist

[5] This division is used because of considerable evidence suggesting the separability of an early recognition or "inductive" phase of the immunological response from a later or "productive" phase, on qualitative grounds (reviewed by Sterzl, 1960).

in the tolerant animal. Spleen and lymph node cells of the BSA-tolerant animal placed in the normal recipient show no evidence of immediate antibody production. Tolerant cells, therefore, if they exist, are not immunologically activated and poised to produce antibody.

The phase of the immune response subverted in the tolerant animal, therefore, must be the initial recognition or inductive phases. Burnet (1960a, b), Medawar (1960), and others (Chase, 1959b) have suggested this in other ways—that tolerance represents "essential nonreactivity" or "central failure of the immune mechanism." This conclusion would require that the once tolerant animal show no evidence of prior experience with the antigen after tolerance no longer was present. The response to BSA in once BSA-tolerant rabbits in which the tolerant state was allowed an appropriate period of time to lapse was, indeed, found to have the characteristics of a primary response rather than a secondary response (Smith and Bridges, 1958).

The only data usually interpreted as contrary to this conclusion are those indicating accelerated disappearance of passive antibody in the pneumococcal polysaccharide paralyzed mouse (Stark, 1959). However, failure by several recent investigations to demonstrate any evidence of active antibody formation in the polysaccharide paralyzed mouse greatly weakens this argument.

Definition of the nature of block of the inductive phase in the tolerant animal cannot be made at this time on the basis of any substantial data regarding tolerance or immunity. Any working hypothesis to explain tolerance will require some assumption in regard to the site of such a block.

B. Role of Antigen in the Tolerant State

For some time it has been recognized that the mechanism whereby the specific steric configurations complementary of an antigenic determinant are built into an antibody globulin must, in some way and at some time, involve interaction of the host cell with the antigen molecule. However, the manner in which this interaction comes about and particularly the importance of the continuous availability of antigen to the immune response still has not been resolved experimentally. Some indication of the state of knowledge is found in the varying theoretical interpretations of available data which have been suggested (reviewed by Burnet, 1960a). Investigation of the significance of the antigen molecule in the tolerant state, on the other hand, has been favored by the very nature of the experimental model, with the result that certain tentative conclusions appear possible at this time.

1. *Relation of Antigen Dose to Duration of Tolerant State*

Central to understanding the mechanism of tolerance is the question whether this state once established is a permanent acquired characteristic or self-perpetuating state or whether it is of a finite duration. Most available data from experiments with appropriate design indicate that the tolerant state is not a permanent acquired characteristic of the experimental animal resulting from an early exposure with antigen; rather, it is of a finite, predictable duration which depends on the amount of antigen given at birth. After this interval, the animal can respond normally to the previously tolerated antigen.

Support for this conclusion is most compelling in experiments involving injections of heterologous protein into the newborn rabbit (Dubert and Paraf, 1957; Smith and Bridges, 1958; Humphrey, 1960), homologous RBC into the chicken (Mitchison and Dresser, 1960) or into the rat (Nossal, 1958). So consistent has been this relationship in the system employing BSA in the rabbit, that predictions on the duration of complete tolerance to a given amount injected at birth may be regularly confirmed experimentally. Humphrey (1960) has pointed out, however, that by using partial tolerance as a criterion, the outcome of such experiments may be modified considerably.

All protein antigens do not appear to give rise to tolerance of the same duration for an equivalent dose. Though data are meager it appears, for example, that Ea tolerance in the rabbit is shorter, and that HGG tolerance is considerably longer than BSA tolerance. It is perhaps significant that *in vivo* disappearance rates of these three antigens have a similar time relationship (approximate half-lives in tolerant animals by immunochemical methods are Ea = 2 days; BSA = 5 days; HGG = 8 days). In the absence of comparable data for pneumococcal polysaccharide paralysis in rabbits or mice or for simple chemical unresponsiveness in guinea pigs, extension of these conclusions is not possible. The slow rate of degradation of pneumococcal polysaccharide would lead to predicting a long duration of unresponsiveness to this antigen from a small dose.

2. *Antigen Role in Sustaining Tolerance*

Experiments contradicting the establishment of the dose–duration relationship have for the most part depended on the demonstration of prolonged tolerance, surviving multiple antigenic challenges for up to 3 years in some instances. The explanation for this prolonged tolerant state was found in similar experiments in which but a single challenge of the tolerant state was made at varying ages. It then became clear that the

antigenic challenges by themselves had provided increments of antigen which sustained the tolerant state as long as injections were continued. The amount of antigen required to sustain the tolerant state has not been precisely established. In one set of experiments, approximately 10 μg. BSA (see Table II) was sufficient to sustain tolerance in rabbits for at least 30 days. The amount required to keep the tolerant state going, once induced, is therefore less than that required for initial induction. Another demonstration of the requirement for a continuous source of antigen to sustain tolerance is in the experiments of Mitchison and Dresser (1960). In this case tolerance continued as long as small increments of antigen were injected, but if 25 days elapsed, after the disappearance of antigen from the circulation of the host, the tolerant state was lost.

Evidence that homograft tolerance depends on a continuous supply of antigen is more elusive and indirect, since the transplantation antigens responsible have not as yet been isolated and precisely defined and the chimeric feature of the model itself prevents certain critical experiments. Mitchison (1953) demonstrated that homograft rejection and simultaneous permanent reversal of tolerance in mice followed the injection of normal or immune isologous lymphoid cells. This experimental result is conceivably the outcome of elimination of the source of the tolerated lymphoid progeny of the orginally injected cells, at the same time as the graft itself is rejected. Removal of the graft alone does not affect the tolerant state. By this line of reasoning, originally injected cells might be thought of as a continuous source of transplantation antigens to the tolerant host. Data on homologous tissue and RBC tolerance, in general, suggest that the duration of tolerance following a given dose of homologous antigen is much shorter than that observed with heterologous soluble ones. Therefore a permissible interval between the increments of antigen required to sustain the tolerant state would be expected to be much shorter than with heterologous antigens. Further clarification of this point in the models of transplantation tolerance should be possible when transplantation antigens are isolated and defined more exactly.

In apparent contradiction to the conclusion that tolerance is sustained by available antigen is the recent demonstration by Billingham and Silvers (1960) that tolerance of isologous Y-chromosome (Eichwald-Slimser effect) in female mice may in some cases survive after the chimeric state (male donor cells) can no longer be demonstrated. Data on the catabolic rates of the antigens determined by the Y-chromosome will be necessary to interpret this finding. It is possible, at least, that this histoincompatibility antigen is slowly metabolized as are polysaccharides.

3. *Site of Antigen Sustaining Tolerance*

If the foregoing conclusions are valid, as they appear to be for the experimental models discussed, it is of interest to ask where the antigen sustaining tolerance is located, in what form it exists, and how it gets there. Certain data appear pertinent to each of these questions.

When a defined antigen is injected into the tolerant host, the best measure of its fate has been the characteristic pattern and rate of elimination from the serum. It has been tacitly assumed that this rate extrapolated beyond measurable levels reflects the fate of all of the antigen intra- or extracellular in location. As has already been pointed out, however, this is not necessarily true for antigens which have been altered in such a way as to affect the rate of clearance by the reticulo-endothelial system. However, extrapolation of data on antigens, such as BSA in which rates of serum clearance in the tolerant rabbit are similar to homologous albumin, may be assumed to reflect more accurately the metabolic fate of the antigen in all sites. The number of molecules remaining at the end of tolerance is, then, a function of the number injected and the rate of degradation. Calculations based on these assumptions (Smith, 1960a;

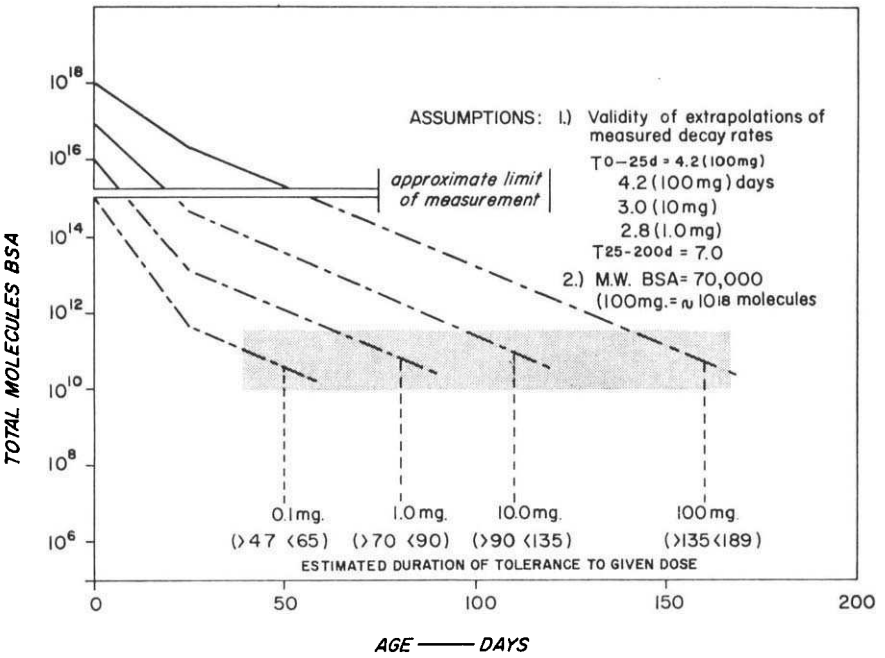

FIG. 8. Theoretical degradation of BSA in rabbits given varying amounts of this antigen at birth. For further explanation, see text. (From Smith, 1960a.)

Humphrey, 1960) would suggest that in the ranges of neonatal dose examined, 10^{10}–10^{12} molecules BSA of the antigen remain at the end of the tolerant state. These calculations are depicted graphically in Fig. 8. Preliminary confirmation of this theoretical prediction has been found in data such as those presented in Table II, in which an amount calculated to extend tolerance at least 30 days, did so.

These considerations render it probable, therefore, that persistence of the tolerant state depends on a very small proportion of the originally injected amount of antigen and, therefore, must be located in a limited number of critical sites. A similar conclusion was reached by Chase (1959b) with respect to unresponsiveness induced by oral antigen administration.

The evidence that the antigen significant to the tolerant state is located intracellularly is indirect but consistent. For the most part, it is based upon the results of experiments in which specific antibody has been injected into or produced vicariously by immune or hypersensitive cells in the tolerant host. Here the antibody combines with and clears the extracellular space of all remaining antigen, and the animal remains tolerant. Though suggesting the intracellular localization of critical antigen, these experiments do not imply that the cleared antigen is no longer available to sustain the tolerant state. If this were true, it would be possible to reverse tolerance rapidly by this operation. Experiments, in which antigen to sustain tolerance already induced with soluble BSA was provided in the form of insoluble specific precipitates, prolonged the tolerant state as regularly as with soluble antigen (Smith and Eitzman, 1959, unpublished data).

The precise intracellular location of critical antigen has not been determined. The distribution of labeled BSA in homogenates was mainly in particulate fractions in the case of iodoproteins (Crampton *et al.*, 1959a), and in the soluble fraction with lightly labeled azoproteins (Garvey *et al.*, 1960). Selective concentration of the latter antigen in liver cell nuclei of tolerant rabbits gives the only available clue to the localization of the critical antigen. Conclusions based upon distributions of the large amounts of antigen necessary for methodological requirements in such experiments, however, may not be pertinent to the distribution of that small proportion responsible for the tolerant state. The same objection may be made to analogous studies of polysaccharide paralysis in mice (Stark, 1959).

An important principle relative to the nature of tolerance is implicit in the data elucidated in this section, viz., as long as tolerance exists, the

antigen specifically tolerated has the same relation to the tolerant cell as existed at the time tolerance was induced. The length of time over which this relationship is possible strongly implies that it is not limited to previously tolerant cells but includes their progeny as well. (Alternative theoretical explanations postulating cell or ribosomal annihilation will be covered in a later section.) An extension of this principle would be: failure of the specific recognition mechanisms which permit tolerance, also allows intracellular access of that antigen required to sustain the state. It is also implied that substances recognized as antigenic are denied comparable access. Whether this access is to a whole cell, a part of a cell, a component of a cell, or to a collection of components is not clear. An experimental approach to this problem may be possible through the use of animals tolerant of one component, but producing antibody to another determinant of an antigen, such as reported by Cinader and Dubert (1956).

C. Conditions Permitting Induction of Tolerance

The general similarities of tolerant states induced by various experimental manipulations, particularly as regards the role of antigen, has been emphasized in the foregoing sections. The same central question about each example remains essentially unanswered—precise definition of the conditions which permit induction of tolerance in the newborn or altered mature host, but result in an immune response in the mature or normal one. No direct pertinent experimental evidence exists; therefore, indirect sources must be sought for insight bearing upon the problem. The special conditions existing particularly in the immature host, but also in the X-irradiated or 6-MP-treated host, which might conceivably affect its response to antigen, will now be considered.

1. The Perinatal Period

Much evidence demonstrates that the fetal and newborn mammal and the avian embryo have limited capacity to develop an immune response. They are not, however, as often assumed or stated, immunologically inert. Sufficient data have now accumulated to allow tentative description of some of the qualitative as well as the quantitative aspects of the ontogeny of immunity (see reviews by Smith, 1960c; Good *et al.*, 1960; Ebert and DeLanney, 1960; Billingham, 1958; and Egdahl, 1958). Marked species variation and difference in the response to various antigens within the same species dictate that these variables be evaluated carefully in making any generalizations.

The capacity for homograft rejection apparently develops in the pre-

natal period in some species, such as the ungulates (Schinckel and Ferguson, 1953; Billingham *et al.*, 1956) and chick embryos (Ebert and DeLanney, 1960), which may be sensitized so that a secondary type homograft rejection can be elicited on the fourteenth day after hatching. Homograft rejection occurs regularly in the newborn rabbit (Sterzl and Trnka, 1959; Egdahl, 1957), and the newborn calf rejects a homograft vigorously (Anderson *et al.*, 1951). Rats, mice, hamsters, and humans appear not to have this mechanism so well developed shortly after birth, as homograft rejection occurs but is prolonged or sluggish as compared with the mature host. In the rat and mouse, transplantation tolerance may be induced at birth by appropriate injection of homologous lymphoid cells.

Delayed hypersensitivity to tuberculin may be elicited by testing infants vaccinated with Bacillus Calmette-Guérin vaccine at birth as early as the third week of life (Gaisford, 1955). On the other hand, their capacity to receive passively transferred hypersensitivity appears depressed or absent during this period (Warwick *et al.*, 1960). The capacity to make specific antibody to bacteria (Sterzl and Trnka, 1957; Smith, 1960b) is developed to some extent at birth in most species although it is imperfect in many respects and cannot be necessarily equated with resistance. For example, antibody titers in the human infant responding to injected *Salmonella* flagellar antigens may measure as high as the adult, but infections with these organisms have been observed to be overwhelming. The antibodies stimulated by certain bacterial antigens in the neonatal period appear to be macroglobulins, in contrast to mature individuals in whom 7S globulins are the predominant product of an immune response (Smith, 1960b).

In contrast, the newborn of some species are unable to develop antibody or other evidence of an immune response to heterologous proteins and similar antigens (Smith and Bridges, 1958; Osborn *et al.*, 1952, 1953; Bridges *et al.*, 1959). Most studies of the basis of this immunological inadequacy have concerned the newborn rabbit. Quantitative and qualitative aspects of the developing immune response to BSA in rabbits were illustrated in Table III. No evidence of any immunological reaction to BSA appears to have occurred under 15 days of age, since reinjection later resulted in a typical primary response. However, when injected after the fifteenth day of life, even in animals not developing detectable antibody, a primary response had occurred, as shown by the results of subsequent challenge. It may be tentatively concluded from such experiments that a primary failure of recognition of this antigen as foreign occurred upon first contact in the neonatal period.

The characteristics of the perinatal response to antigenic exposure, as presently understood, are now listed and discussed briefly as they are pertinent to the question posed at the beginning of this section.

a. Cell Permeability. Increased tissue permeability to BSA in neonatal rabbits may be demonstrated in several types of experiments (Smith, 1960a). Nearly quantitative absorption of BSA through the intestine correlated well in time with susceptibility to the induction of tolerance and ceased roughly when the capacity for antibody production began (Table III). Antigen passed intact through cells which later selectively prevented such penetration. When tolerance-inducing amounts of BSA were injected into newborn rabbits and the amount in the serum measured periodically, it was found that elimination was exponential (Fig. 1) and but slightly faster than mature tolerant animals when appropriate corrections are made for growth. Calculated values of the space of distribution of this antigen injected at different intervals after birth were markedly different. The apparent "BSA space" of newborn animals varied between 20 and 35% of weight, whereas in mature tolerant animals this was about 5%. Lastly, by using several indicators (dyes, radioactivity), it has been found that injected antigen diffuses from the skin of the newborn rabbit much more rapidly and completely than that of the mature animal (Smith, unpublished data).

These studies imply that the capacity to recognize as foreign to itself and to mobilize the means for localizing or excluding an antigenic protein are relatively less effective in the newborn than in the mature animal. They infer further that antigen may perhaps intrude into components or cells which are immunologically inviolate in the mature host.

b. Solubility. One of the possible explanations of failure of bacterial antigens to induce tolerance is that all cell sites necessary to induce tolerance are not reached and saturated. A corollary of this reasoning is that soluble antigens are effective because they are widely accessible. A test of this concept was made in studies presented in Table IV, in which protein antigen presented as an insoluble complex with specific antibody failed to induce tolerance even though, as already shown, such precipitates could sustain an existent tolerant state. It may be pertinent in this regard that Medawar (1960) has pointed out that, whereas only a small proportion of the immunologically competent cells are required to express an immune response, *all* of the cells must be affected for tolerance to exist. Therefore, it would appear that a condition of neonatal tolerance induction is that the antigen be in sufficient quantity and in a soluble form so that it has access to all significant cellular sites.

c. Phylogenetic Relationships of the Antigen to the Host. In general

it can be stated that tolerance is most easily induced to antigenic substances with which the host species shares the maximum number of determinant groups. Cross reactions of antibody to BSA, HSA, and Ea with RSA (rabbit serum albumin) and BGG antibodies with RGG (rabbit γ-globulin) show that determinant groups are shared by these particular antigens most frequently used to induce tolerance in the rabbit. No concerted attempt has been made, however, to compare quantitatively on a phylogenetic basis the regularity, duration, and completeness of tolerance induced with a series of proteins, such as albumins. Such studies would add considerably to the understanding of tolerance. On the other hand, tolerance regularly fails to develop in response to similarly injected antigens which are quite foreign to the host, such as some bacterial products. Exceptions to this may be the *Shigella*-soluble antigen and the staphylococcal exotoxin. Nor do soluble chemical groupings, such as benzene derivatives, induce tolerance upon neonatal exposure (Cinader and Pearce, 1956). A productive type of experiment might be to examine the immune response to bacterial elements which share determinants with a more closely related antigen to which tolerance can be regularly induced; for example, the response to certain bacterial somatic antigens in animals tolerant of a related blood group antigen. Several possibilities for this are suggested by the studies of Springer and colleagues (1958).

d. Complexity of Antigens. From all data it appears that the result of neonatal exposure to a complex mixture of antigenic determinants is determined by the nature, amount, and form of individual determinants present in the mixture. If all are soluble heterologous antigens with determinants not too different from the host and all in sufficient amounts, tolerance of the whole antigen or mixture should result. If one is not in sufficient quantity or insoluble, then antibody production to this component will result upon challenge with the mixture. A number of examples of this principle have been brought out (Dixon and Maurer, 1955a; Wyttenbach, 1960; Smith and Bridges, 1958).

e. Metabolic Requirements for Antibody Production. Dixon and Weigle (1957a, b), Holub (1958; Holub and Riha, 1960), Sterzl and Trnka (1959), and Smith (1960a, and unpublished experiments) and colleagues have sought to use the method of cell transfer to study the capacity of the newborn to express hypersensitivity or to support antibody formation. While species differences and techniques are clearly important (see Nossal, 1959), these studies have consistently demonstrated that the newborn rabbit and mouse, for example, can support nearly as well as can an adult the production of antibody to bacterial antigens by immune cells that have been restimulated with the antigen

prior to injection. However, the primary response elicited by transfer of adult cells incubated *in vitro* with antigen before injection in rabbits and mice was less regular and quantitatively inferior to that of the adult recipient. The chick embryo, on the other hand, appears better able to support cell-transferred antibody formation (Trnka, 1958) than the newborn rabbit.

A potentially illuminating finding was that large excess of nonimmune lymphoid cells, but not nutrient materials (vitamins, etc.), injected in the newborn rabbit (Dixon and Weigle, 1957a, b) enhanced the primary response with incubated adult cells. Newborn spleen cells, incubated *in vitro* with antigen, elicited a good antibody response in adult recipients but the response was quantitatively inferior to the result obtained when mature cells were transferred. Studies with protein antigens have been more difficult because of an induction phase which is longer than the duration of homograft survival in the rabbit. The result with these antigens is similar, however, when the cells are placed in a diffusion chamber protecting them from rejection (Holub and Riha, 1960).

A preliminary report of Sterzl and Hrubesova (1957) that antibody production could be transferred into 5-day-old rabbits by RNA (ribonucleic acid) from stimulated adult lymphoid cells, has not been confirmed by other workers as yet, with negative findings being reported by some (Van Dormaalen, unpublished, quoted by Ebert and DeLanney, 1960; Dixon, 1960, personal communication; Janovic *et al.*, 1959; and Stavitsky, 1958). One possible interpretation of this experiment supported by some data (Hrubesova *et al.*, 1959) is that the RNA extract enhanced the primary response of the newborn rabbit to residual antigen in an effect analogous to the lymphoid cells in the experiments of Dixon and Weigle. Various substances, including nucleic acid derivatives that restore the induction of the immune process in hemolysin formation in mature irradiated rabbits have been found by Jaroslow and Taliaferro (1956) and Taliaferro and Jaroslow (1960). Liberation of nucleic acid derivatives by cytotoxic action may also account for the above normal production of antibody such as when large doses of X-rays are given to the exteriorized spleen (Taliaferro and Taliaferro, 1956) or to the total body (Taliaferro *et al.*, 1952; Taliaferro and Taliaferro, 1954a and b) at strategic times with respect to the injection of antigen. Work with this model should clarify further the nature of immunological inadequacy in the newborn animal and thereby the conditions which allow tolerance. This type of experiment, however, indicates that a defect may exist in the productive phase as well as the initial or inductive phase.

f. The Role of Environment of the Newborn. The mammalian fetus is ordinarily protected until the time of birth from antigenic stimulation of other than those of maternal origin except possibly some viruses. At birth, it then encounters myriad antigens, bacteria, and viruses with which it must cope successfully. The very existence of a normal bacterial flora appears to have a definite effect on the response to attempted immunization in the newborn period. Studies on germ-free animals (Thorbecke, 1960; Sterzl, 1960) and those which compare the importance of age and maturity in humans (Osborn *et al.*, 1953) provide evidence for this with regard to antibody formation. No experimental bacterial infection or exposure to bacterial products has been shown to affect the induction of tolerance, however.

Pregnancy itself represents a violation of homograft immunity with respect to the mother and conceivably, at least, an example of immunological tolerance (see Thomas, 1959). Intrauterine existence might then affect the immune capacity of the newborn through the influence of a hypothetical inhibitor of immunity, related to the circumstance of pregnancy. Although many aspects of the maternal immune response appear intact during pregnancy, some evidence has been presented (Heslop *et al.*, 1954) that the pregnant rabbit shows delayed rejection of homografts. No other evidence is known of any depression of immunological reactivity during pregnancy. In unpublished experiments, Smith and Bridges found that neither placental extracts or maternal serum factors had any demonstrable effects on the immune response of mature rabbits. Whether such factors might delay the appearance of normal immunological capacity if given repeatedly during the early days of life, was not examined. At present, therefore, immunological inhibitors of maternal origin do not appear to provide an acceptable explanation for neonatal immune deficiencies, but this is a subject worthy of further investigation.

g. Nonspecific Resistance Mechanisms. Largely ignored in considerations of specific immunity is the fact that all immune responses begin with the nonspecifically elicited inflammatory mechanism. It seems possible that initial recognition of foreignness occurs through this mechanism, particularly with respect to most bacterial antigens. The neonatal period is characterized in humans, at least, by a nonspecific inflammatory response which differs qualitatively from more mature individuals (Eitzman and Smith, 1959a). Other striking, but poorly studied, differences in response to noxious stimuli, as compared with their mature counterparts, are known (Baumgartner, 1934). The recent work of Fishman (1959) suggests that preliminary interaction with certain cellular components of nonspecific inflammation may permit antibody synthesis

to occur entirely *in vitro*. This observation suggests that further examination of the deficiencies in the neonatal inflammatory mechanism may be extremely important in explaining the induction of tolerance.

h. General Metabolic Status of the Newborn. The mammalian newborn undergoes a rapid and vital series of changes in energy metabolism and enzymatic activities of many types. For example, from a strong dependence on glycolysis and extreme resistance to deficiency of molecular oxygen, the newborn, over the first few days of life, synthesizes the enzymatic means to depend primarily on aerobic energy metabolism (reviewed by McCance, 1959a, b). This synthetic activity in newborn rabbits is reflected in extremely high rates of turnover of RNA and DNA (deoxyribonucleic acid), which are as much as 10-fold higher than in mature rabbits (Smellie, 1955). In the chick embryo, these events appear to occur much earlier. For example, DNA and RNA turnovers reach a high peak between the thirteenth and fourteenth day, after which DNA turnover is reduced to a low level (Stevens, 1952). Antibody protein synthesis, like all protein syntheses, depends on active energy-yielding mechanisms and on initial high rates of DNA and RNA turnovers. Therefore, competition with, alterations of, or interference with any of the precursors or intermediates in this process could profoundly affect the ability of the newborn to mobilize an immune response. No definite evidence can be adduced to support this intriguing possibility as yet. It appears to be a most promising though difficult hypothesis to test.

2. *Irradiation*

Very little is known of the effects of irradiation which subvert the normal immune response to homologous tissues or defined heterologous antigens, inducing what appears to be a tolerant state. Irradiation appears primarily to effect a profound but temporary interference with the earliest phases of the immune response (Taliaferro, 1957a; Makinodan and Gengozian, 1960). The interference is probably brought about through disruption of active DNA synthesis in the cell populations responsible for the immune response (Chargaff and Davidson, 1955) since incorporation of P^{32} into DNA ceases abruptly with heavy irradiation. The work of Potter (1959) and Bollum *et al.* (1960) indicated that X-ray inhibition of nucleic acid synthesis is largely localized to the earlier stages. This result is consistent with the findings of Taliaferro and Jaroslow (1960) that partial depolymerized nucleic acids will restore the antibody-forming capacity in previously X-rayed rabbits, whereas the purines, pyrimidines, nucleosides, and nucleotides are ineffective. Whether the effects are primary, perhaps inhibition of proliferation of

clones of antigen-stimulated cells, or secondary, as a result of general inhibition of protein synthesis, is not known. Studies of the fate of antigen analogous to those performed in the neonatal period are necessary before similarities in mechanism can be inferred.

3. *6-Mercaptopurine Treatment and Related Mechanisms*

The mechanism of action of 6-MP and related antagonists in creating conditions resulting in general repression of the immune response is not established. It is assumed that these drugs interfere with the orderly procession of intracellular events which lead to antibody production and antigen elimination. This interference could be interposed at any time, from the first rapid increase in DNA synthesis occurring soon after antigenic stimulation (Andreini *et al.*, 1955) to the increase in cytoplasmic RNA as antibody production occurs (Ehrich *et al.*, 1949). Inasmuch as the tolerant states probably represents failure of the inductive rather than the productive phase of antibody synthesis, it is most logical to look to an early effect on DNA synthesis for the 6-MP effects which results in specific tolerance. Such a working hypothesis appears justified at this time, even though this drug has been found to have other effects on energy-requiring systems *in vivo* and *in vitro* (Biesele, 1955; Elion and Hitchings, 1957; Hochstein, 1957).

Other agents which are known to interfere with antibody production, such as amino acid analogs (β-thiethylalanine, Wissler *et al.*, 1956), vitamin inhibitors (deoxypyridoxine, Eisen and John, 1957), steroids, and nitrogen mustards, have not been used in attempting to create the conditions in which a tolerant state might follow antigen administration. Further exploration of the effects of various other antagonists and competitors of intermediary steps in nucleoprotein and protein synthesis should be rewarding.

V. Theoretical Aspects of Immunological Tolerance

During recent years it has become evident that the phenomena of immunological tolerance are intimately tied to those of the immune mechanism and will have to be accounted for in any general theoretical explanation of the biological and biochemical nature of immunity. It is equally apparent that the available information does not permit any completely consistent integrated theory of immunity *or* tolerance. Medawar has stated (1960) that all we can do now is to classify our ignorance. The many recent as well as earlier attempts to construct meaningful hypotheses have nonetheless been extremely important in provoking ex-

perimental efforts along new and productive lines. The reader is referred to the papers of Jerne (1955); Talmadge (1959); Burnet (1960a); Lederberg (1959); Crampton *et al.* (1959b); Medawar (1960), and Szilard (1960) for recent discussions of the theoretical aspects of immunity, which for the most part attempt to tie the data of tolerance to those of the immune response. No critique of these various theories will be attempted on any systematic basis here nor is such an evaluation likely to be useful.

It may be of value, however, to reiterate some of the critical points which appear to provide the general framework on which any theory of tolerance must be built. These statements are made positively, but should be construed as approximations with varying support and degrees of certainty.

(a) Tolerance is the expression of and the result of failure of the induction or recognition phase of the immune response. All manifestations of the productive phase of the immune mechanism are equally inhibited.

(b) The tolerant state depends on the continued presence of antigenic determinants in critical sites, probably intracellular. In this sense it is an "instructive" phenomenon (see Lederberg, 1960).

(c) As long as tolerance exists the determinants of the antigen have the same relation to or access to these sites as upon initial exposure.

(d) Tolerance is specific to each determinant site of an antigen, not necessarily to the entire antigen.

(e) All cells having immunological potential with respect to a determinant must be tolerant, as must their progeny, for the host to remain completely tolerant.

(f) Restoration of immunological reactivity occurs upon elimination of the antigen from critical sites, and the reaction to the antigen after an appropriate interval appears to be a primary response.

These premises, which appear to be well supported at least with respect to the models of protein tolerance, provide the basis for discussion of hypothetical mechanisms of tolerance.

As a special case of protein synthesis, it is difficult to conceive of any mechanism of antibody formation in which genetic control was not directly or indirectly exerted in determining the amino acid sequence and, consequently, the secondary structures of the antibody globulin. Current immunological theory would suggest that this control is effected through selective stimulation of clones of cells or, perhaps, ribosomes within cells, which have a genetically determined affinity for the antigen. These stimulated cells are thought to proliferate and differentiate along specialized lines which fit them for antibody synthesis. The induction of

immunological tolerance, according to this line of reasoning, would be associated with inhibition rather than stimulation of cellular proliferation and no development of clones or ribosomes with the capacity for production of antibody with complementarity for the specific antigen. Several explanations of this inhibitory mechanism appear at least plausible at the present time.

One explanation of immunological dormancy with respect to the tolerated antigen might be stated as follows: the initial interaction of the antigenic determinant with a cell or cell component with which it has a genetically determined specific affinity results in the death and elimination of the cell.

Such a mechanism has been proposed by Burnet (1960a) and by Lederberg (1959, 1960) but it has not accumulated specific experimental support as yet. Mitchison and Dresser (1960) have recently discussed and evaluated these ideas briefly. This annihilation theory has the multiple attractive features of explaining why immunity is not promoted toward self-components (such clones are continuously annihilated), the specificity of tolerance, the requirement for a continuous source of antigen, and the finite duration of tolerance. To be sound on quantitative grounds, however, this mechanism requires the assumption that each immunologically competent cell differentiates with a high specific affinity for a single antigen or very few antigens at the most. If multiple specificities are possible for each stem cell, physical elimination of any significant proportion of such competent cells would probably affect the response to other antigens—which does not happen as far as is known. This reduction also would narrow severely, as Burnet has pointed out, the possibilities of having a population of cells exclusively tolerant of self-components. Another defect in the annihilation theory is that it does not account adequately for tolerant states induced in mature animals or in those receiving metabolic antagonists.

Such objections as these have led to proposals that tolerance involves a similar elimination of subcellular recognition units (Burnet, Lederberg, 1960 *et ante;* Crampton *et al.,* 1959b). While this idea has merit in meeting quantitative objections to whole cell elimination, it has no more experimental support. In all, the annihilation theories seem incompatible with the interpretation that intracellular antigen is necessary for the tolerant cell to maintain the tolerant state.

Another plausible and equally compatible explanation would be that initial contact with antigen *inhibits* the cell metabolically rather than killing it, preventing division, proliferation, and differentiation. This might be thought of as functional elimination of the competent cell. Con-

tinued access of a tolerated determinant to the specific critical site would be accounted for by assuming that each newly differentiated stem cell with affinity for the determinant goes briefly through the same embryonic condition which existed in all such cells at the time of birth. The role of antigen in sustaining tolerance, therefore, would be to be available in sufficient concentration to combine with and inhibit or inactivate each new cell before it matures and becomes susceptible to the proliferation stimulating effect of antigen. This hypothesis, implying specific arrest of differentiation, also accounts for the capacity of mature animals to marshal an immune response; i.e., many of their immunologically competent cells or subcellular fractions will have differentiated beyond susceptibility to tolerance at any given time, whereas none have at birth (Mitchison and Dresser, 1960).

Finally, it appears appropriate to consider the possible biochemical nature of the "critical site," the hypothetical location of antigen postulated to inhibit cell proliferation and differentiation in the tolerant animal. One possibility can be suggested. This would depend simply upon the well-demonstrated steric complementarity of antigenic determinants and combining sites on antibody (Pauling, 1954). Most evidence suggests that the specific site on an antibody molecule has a structure complementary to the RNA template on which it was made. During proliferation and differentiation of cells in preparation for antibody production, the basic source of genetic information in the cell, DNA, must in some way produce or cause the production of the template cytoplasmic RNA. Whether this occurs directly or through "messenger" RNA, the template upon which cytoplasmic RNA is constructed could, therefore, be complementary of *antigen*. Further, it could be located so that this affinity for the antigenic determinants could be expressed. Such access to a nucleic acid molecule might be the common feature of neonatal life, X-irradiation, or chemical treatment. Antigen . . . combining with "messenger" RNA . . . might conceivably inhibit rather than stimulate proliferation and differentiation. The only justification for such speculation is that it invites direct experimental testing.

The foregoing hypothetical suggestions represent but a few of the possible lines of investigation which are inherent in the use of models of tolerance as a means of inquiring into the mechanisms of immunity. In a sense, they provide *in vivo* parallels to immunochemistry, and it seems probable that in the next few years the phenomena of tolerance will become more and more important to the biological approach to immunity.

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Functions of the Complement System[1]

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There are two major trends in current investigations of the complement system which offer material promise for continued progress. The isolation and characterization of the individual complement (C′) components and the clarification of their reaction mechanisms comprise one of these areas. A recent review by Mayer (1958) deals with these problems. The second general area, which is as yet much less advanced, pertains to the growing awareness of the possible participation of C′ in processes of presumed pathological significance other than immune lysis of erythrocytes. Following a restatement of the current status of problems relating to immune hemolysis, this article will attempt to evaluate the nature and validity of the evidence which seeks to relate C′ activity with other biological and immunological phenomena. Schmidt (1959) has also summarized the work in this field. Certain aspects of C′ fixation reactions in highly dilute systems have been discussed previously (Osler, 1958) and will not be considered here.

I. Mechanism of Immune Hemolysis

It has now been confirmed that the family of four C′ components has been augmented by the inclusion of a fifth member. Almost thirty years ago, da Costa Cruz and de Azevedo Penna (1932a, b) described experiments indicating the activity of the third component of C′, C′3, could be inactivated by yeast, formaldehyde, or sodium hydrosulfite. The effects of the several methods of inactivation were not identical since the function destroyed by formaldehyde was present in the hydrosulfite-treated guinea pig serum. More recently Rapp (1958), Rapp and Sims (1958), and Amiraian *et al.* (1958) substantiated this finding through the use of different techniques and independent approaches. As a consequence of this and other developments, the reaction sequence for immune hemolysis outlined by Mayer (1958) can now be slightly modified as follows:

$$E + A \longrightarrow EA \quad (1)$$

$$EA + C'1 \xrightarrow{Ca^{++}} EAC'_1 \quad (2)$$

$$EAC'_1 + C'4 \longrightarrow EAC'_{1,4} \quad (3)$$

$$EAC'_{1,4} + C'2 \xrightarrow{Mg^{++}} EAC'_{1,4,2} \quad (4)$$

$$EAC'_{1,4,2} + C'3b \longrightarrow EAC'_{1,4,2,3b} \quad (5)$$

$$EAC'_{1,4,2,3b} + C'3a \longrightarrow E^* \quad (6)$$

$$E^* \longrightarrow \text{ghost} + \text{hemoglobin} \quad (7)$$

where E represents erythrocyte, A, antibody and C′ with the appropriate number, one of the five presently known C′ components.

Before discussing certain aspects of the individual reaction steps in this sequence, a few general statements might be in order. Until recently, most of the studies with C′ components utilized the reagents R1, R2, etc., which comprised serum preparations devoid of the function ascribed to one or more of the C′ components. Thus R3 represented serum treated with zymosan for the preferential destruction of C′3, etc.

Three considerations were implicit in the use of these reagents:

1. The hemolytic titer of a serum was determined by the component present in least quantity, the principle proposed by Hegedüs and Greiner (1938).

2. The reagent used for the estimation of a component, e.g., R3, was in itself completely lacking C′3 activity but did provide an excess of the remaining components.

3. This reagent had neither lytic nor anticomplementary properties at the concentrations employed.

As techniques became available for the isolation of some of the intermediate compounds in the hemolytic process through the work of Levine and Mayer and their collaborators (Levine and Mayer, 1954; Levine *et al.*, 1954), it became apparent that the notion of Hegedüs and Greiner was no longer tenable. Interpretations of results based on this principle with the use of the conventional component preparations lacked theoretical validity and could, therefore, be misleading. The major difficulties associated with the use of the component reagents emerge from the fact that the threshold quantities required for the experimental situation often border much too closely on their hemolytic or anticomplementary potencies. In consequence, definitive and valid interpretations are difficult to extract from the results. Furthermore, titrations obtained under such restricted experimental conditions are not adequately reproducible and vary with the source of reagents used for these estimations. The magnitude of the latter problem may be illustrated by data taken from studies by Jonsen (1955a, b) and Jonsen *et al.* (1951) who with Rice (1950a) and Rice and Crowson (1950) sought methods for more uniform component titrations. The data in Table I (Jonsen *et al.*, 1951) illustrate the unpredictable titer variations obtained when guinea pig and swine sera were titrated for the four component activities with reagents prepared from guinea pig, human, or pig sera. Rice and Crowson (1950) have carried out extensive studies on the interchangeability of C′ components and have noted the unpredictable variations in titer and reproducibility, even within a single laboratory. Their explicit warning

that the numerical values assigned to the component titrations were not reliable because of fluctuations in stability and inhibitory properties might well have been heeded by some later investigators.

TABLE I

VARIATIONS IN COMPONENT TITERS AS A FUNCTION OF THE SOURCE OF REAGENTS USED IN THE TITER ESTIMATIONS[a]

Component reagents prepared from	100% Lytic titer			
	C'1	C'2	C'3	C'4
		Guinea pig C'		
Guinea pig serum	3500	400	350	7000
Human serum	2500	350	250	anticomp.
Pig serum	2000	1000	350	4000
		Pig C'		
Guinea pig serum	3000	100	3500	200
Human serum	2500	100	not given	not given
Pig serum	2000	200	3500	3000

[a] Data taken from Jonsen *et al.* (1951).

The interpretation of these titers is even more obscure in the light of the results obtained by Heidelberger and his associates (1951). These investigators, as did Silverstein (1954a, b), confirmed earlier impressions of Osler and Heidelberger (1948a, b) and others that after fixation by

TABLE II

ALTERATIONS IN HEMOLYTIC AND COMPONENT ACTIVITIES AFTER REACTION OF GUINEA PIG C' WITH A SPECIFIC PRECIPITATE[a]

$C'H_{50}$ initially available	$C'H_{50}$ remaining		100% Hemolytic unit titers of components			
			C'1	C'2	C'3	C'4
140	Prefixation	140	850	500	320	2000
140	Postfixation	81[b]	500	500	270	600
135	Prefixation	135	600	400	250	4000
135	Postfixation	11[c]	100	800	200	10
270	Postfixation	60[c]	250	250	250	250

[a] Modified from Heidelberger *et al.*, 1951.

[b] Fixation with 0.32 μg. S III + 2 μg. N homologous rabbit antibody.

[c] Fixation with 80 μg. N washed specific precipitate of egg albumin and homologous rabbit antibody.

immune aggregates, the residual hemolytic activities of the serum could not be related in any simple or direct manner to the disappearance of component activities. The data in Table II are given by way of illustration. The situation is yet more complex when attempts are made to compare the fixation of C' components in sera of different species

(Cavallo and associates, 1958a, b; Manski and Kielczewska-Roultowska, 1957, and many others).

Initial steps have been taken toward the eventual resolution of these procedural difficulties and interpretational uncertainties with the application of ion-exchange chromatographic methods as reviewed by Sober and Peterson (1958) and by Fahey and associates (Fahey *et al.*, 1958; Fahey, 1960). The former report includes previously unpublished data of Becker describing successful separation of individual component activities. Further substantial progress has been reported by Rapp and his colleagues (1959), by Borsos and Rapp (1959) and Borsos and associates (1961a, b, c), by Becker (1960), and by Hoffmann *et al.* (1959). The combined use of these mild, nondestructive techniques with the kinetic assay procedures described by Mayer (1958) has substantially clarified present understanding of the individual reaction steps in the immune hemolytic process which will now be discussed.

A. The Nature of the Hemolytic Antibody and Its Mode of Action

The apparently simple reaction between the surface components of a sheep erythrocyte and homologous rabbit antibody has been subjected to intensive study. Renewed attention has been focused on older observations that the hemolytic antibody is heterogeneous with respect to its molecular structure, the specificity of the inciting antigen, and the number of antibody molecules required for the lysis of a single erythrocyte.

Data obtained by Rapp (1953), Talmage *et al.* (1956; Talmage, 1957), Weinrach and associates (1958), and Weinrach and Talmage (1958), Koyama and Nashimoto (1959), reviewed in Stelos and Taliaferro (1959a, b) have established that the major hemolytic antibody function is performed by two species of molecules, a γ_2-globulin with a molecular weight of about 160,000 and the heavier γ_1-globulin whose molecular weight approximates 1,000,000. All the investigators agree that the heavier γ_1-globulin (18S) is produced in greater quantity during the earlier stages of immunization with a variety of Forssman-type antigens. The lack of serologic identity between the Forssman antigens in sheep erythrocytes, guinea pig kidney, and human type A red blood cells has been shown by Stelos and Taliaferro (1959b). Rapp (1953) has stressed the advisability of immunizing rabbits with sheep erythrocyte stromata which have been washed thoroughly and heated at 100° C. to destroy the isophile antigen. Immune sera of high hemolytic potency may be obtained from rabbits injected with these preparations and bled within 5–7 days after the last of about 10 injections. Antibody obtained under these conditions is

entirely anti-Forssman. With continued immunization, the resulting antibody is somewhat deviant in its behavior with respect to the sensitization of erythrocytes for studies of C′ function (cf. Osler and associates, 1952). Goodman and Masaitis (1960) have confirmed the findings of Bowman *et al.* (1951) that the hemolytic antibody is dissociable. They also traced the extent of transfer from one red cell to another as a property of the γ_2-globulin which was formed more frequently and in greater abundance following multiple injections of sheep erythrocytes. Comparative studies of hemolytic antibody N uptake and inhibition of hemolysis led Rapp (1953) to suggest that a portion of the antibody might not participate in the hemolytic process. However, the specificity of this nonreactive antibody was not ascertained.

On the basis of antibody weight estimates with purified Forssman antigen and hemolytic activity measurements, it was calculated that optimal sensitization of sheep erythrocytes entails about 1000 molecules of this antibody per erythrocyte. Possibly greater theoretical interest attaches to the determination of the minimal number of antibody molecules required for the lysis of a single erythrocyte. Weinrach and Talmage (1958) and Weinrach and associates (1958) have approached this problem through kinetic studies with Cr^{51} labeled red blood cells in a reaction system to which C′ is added prior to hemolytic antibody. Under these conditions a long lag phase is observed before the onset of lysis, which in the opinion of these investigators cannot be accounted for entirely on the basis of the time required for the absorption of hemolysin and C′. The presumption is that part of this interval is required to form a critical complex of at least two antibody molecules on adjoining antigenic sites of a single cell as an essential prelude to the involvement of C′ in the lytic process. It has been further concluded that when a large fraction of the hemolytic activity of a serum resides in the γ_1 or 18S fraction, the rate of hemolysis is proportional to a square function of the antibody concentration. Koyama and Nashimoto (1959) have also separated the hemolytic activities in rabbit anti-sheep erythrocyte serum by the combined use of electrophoretic and chromatographic techniques and confirmed the observation that the γ_1-globulin was more efficient in its cytotoxic activity. With the low-molecular-weight antibody, the lytic rate varies with the fourth power of the antibody concentration. At the present writing there is no substantial evidence which may be offered to question the conclusion that a small number of antibody molecules, a number exceeding one, is required to initiate the release of hemoglobin from an erythrocyte in the presence of C′. This problem arises again in the discussion of the role of C′ and antibody aggregation in hypersensi-

tivity phenomena. Further evidence along these lines might emerge from comparative studies with highly purified antibody and γ-globulin fragments obtainable by enzymatic digestion as described by Porter (1959) and Nisonoff and Woernley (1959).

The findings of Bier *et al.* (1956) and those of Goodman (1958a, b; 1959a, b) are of some relevance to the present issue. Goodman (1959b) compared the hemolytic efficacy of rabbit anti-sheep erythrocyte serum with the C′-fixing potency of the same antibody in its reaction with formalin-treated red blood cells. The number of C′-fixing units increased as immunization was prolonged. A lack of correlation was also observed between the C′-fixing ability of the serum and its hemolytic potency. Discrepancies of this type had been noted previously in studies of the following antisera; human anti-A, rabbit anti-human erythrocytes, and rabbit anti-sheep erythrocytes, in agglutination, C′ fixation, and hemolytic titrations with the appropriate antigens (Goodman, 1958b). The interpretations which can be drawn from these experiments are limited by the lack of data relative to the number of antigenic components operating in each system and the weight of antibody required for each immune reaction and species of erythrocyte. In addition, kinetic studies of hemolytic antibody by Mayer (1958) and by Rapp (1953) have pointed out the serious limitations of interpretations drawn from dilution end-point titers of this antibody.

Bier and co-workers (1956) used the passive or indirect hemolysis method of Fisher (1950) and Fisher and Keogh (1950) and of Adler (1950) to demonstrate the lack of parallelism between the hemolytic and C′-fixing antibody activities. They found that whereas horse antibody possessed only one-fifth of the C′-fixing potency of rabbit antibody, the former was twice as effective in provoking lysis of erythrocytes which had adsorbed the polysaccharide antigen homologous for both of the antisera. The apparent discrepancy may perhaps be related to the fact that diminution of the hemolytic potency of C′, the index of C′ fixation, results from the fixation of C′1, C′4, and C′2 in one of many possible pattern variations. The loss in C′3 activity is not required for the demonstration of C′ fixation but is, of course, essential for cell lysis. Further, since cell lysis occurs at only a single site, fixation of C′ components to other sites of the same cell is inconsequential in terms of the lytic process. Lastly, since dilution end-point estimates of hemolytic potency are subject to considerable uncertainty further studies with component titrations may be required to settle this problem.

The characterization of the hemolytic antibody as just discussed sheds little light on its mode of action. Some aspects of the cell damage asso-

ciated with C′ action are considered in the following, but it is pertinent to inquire in what manner antibodies of different specificities, acting on cellular substrates ranging from treponemata to Ehrlich ascites cells, prepare these cells for the lytic action of C′. Research efforts in this area remain sparse.

B. The C′ Components: Some Aspects of Their Sequential Reactions

There are as yet no definitive data as to the chemical nature of the C′ components. A clearer understanding of their mode of action and improved methods of assay have been provided recently through the development of methods for the isolation of the intermediate reaction products in the hemolytic reaction sequences.

1. C′1

Investigators are now generally agreed upon the major aspects of reaction (2).

$$EA + C'1 \xrightarrow{Ca^{++}} EAC'_1 \qquad (2)$$

Studies by Mayer (1958) and by Levine and Mayer (1954; Levine *et al.*, 1954) and Levine and co-workers (1953a, b, c) demonstrated that the first steps in the fixation of C′ required Ca^{++}, but it was not known whether this divalent cation participated in the fixation of C′1, C′4, or both components. Partial resolution of this problem has now been achieved in studies initiated by Pillemer *et al.* (1953a, b) and by Lepow *et al.* (1953, 1954) with respect to the sparing effect of Ca^{++} on the loss of hemolytic activity in serum maintained at 37° C. These observations were greatly extended by the detailed experiments with guinea pig and human C′ of Laporte and his group who were among the first to prepare EAC'_1 (Laporte *et al.* 1955, 1956, 1957) and who supplied evidence showing that Ca^{++} exerts a protective action against the thermal inactivation which occurs at 37° C. They then noted that inhibition of the destructive effect by Ca^{++} is specific and reversible. Other divalent cations like Ba^{++}, Sr^{++}, Ni^{++}, Co^{++}, Fe^{++}, and Mg^{++} could not be substituted for Ca^{++}, a finding similar to that of Levine *et al.* (1953b, c) for C′ fixation. The effect of this cation probably involves a reversible interaction with C′1 only prior to the combination of the latter with the immune complex. The magnitude of the protective effect by Ca^{++} against inactivation at 37° C. may be gauged by the following data taken from Laporte *et al.* (1955). The half-life of guinea

pig serum activity at a dilution of 1:280 and incubated at 37° C. was 75 minutes. In the presence of 1.5×10^{-4} *M* Ca^{++}, the corresponding time was 27 hours. In a continuation of these studies, the authors concluded that the function of Ca^{++} in the fixation process involves the rapid uptake of C'1 by EA and that this complex may be dissociated by ethylenediaminetetraacetic acid (EDTA) with the release of the individual elements of the complex EAC'_1. Becker (1960) confirmed this observation and found that the EA, after uptake and removal of C'1, were unchanged in respect to their reactivity in the later stages of the lytic reaction. Becker also demonstrated the priority of action of C'1 and that removal of Ca^{++} from the complex $EAC'_{1,4}$ resulted in a selective loss of C'1, yielding EAC'_4.

The essential aspects of these observations have been confirmed by Klein (1960) who prepared EAC'_1 with C' that had been pretreated with hydrazine or with ethyl ether. In effect the ether-treated C' presents workers in this field with another form of the reagent R4, a serum preparation presumably devoid of C'4. Unpublished experiments by Hoffmann (1960) have also led to the successful production of the intermediate EAC'_1 with the use of serum fractions obtained from cellulose columns. The fraction containing C'1 is entirely lacking in C'4 activity and was obtained without resort to the usual destructive procedures. The studies by Hoffmann provide, for the first time, an accurate assay for C'1 and a definite confirmation that the order of reaction is C'1 and then C'4. In addition the reaction between C'1 and sensitized sites on the erythrocyte is stoichiometric and proceeds at a rate proportional to the concentration of C'1.

A slightly discordant note with respect to the Ca^{++} requirement for C' uptake may be found in the report by Lepow and Pillemer (1955) in their studies with human C'. These authors concluded that the reaction of antigen–antibody aggregates with C', was not affected by Ca^{++} which probably participated in subsequent steps. On the other hand, Leon (1956a, 1957c) concluded that Ca^{++} does take part in the initial reaction steps of EA with human C'. In the absence of experiments which are in support of, and more definitive than those in Lepow and Pillemer (1955), the weight of present evidence favors the conclusion that Ca^{++} participates in the interaction of human C' with an immune aggregate. Of interest is the finding of Brumfield and Pomeroy (1959) that turkey antisera can fix avian but not guinea pig C', owing to the possibly specific requirement for avian C'1. Note is also taken of the observation that a cation-independent reaction may precede the uptake of C'1 (Müller-Eberhard and Kunkel, 1960).

2. C'4

$$EAC'_1 + C'4 \longrightarrow EAC'_{1,4} \qquad (3)$$

This reaction has been studied in some detail by Hoffmann (1960). Presently available data do not indicate a cation requirement in the reaction between C'4 and a site on the sensitized erythrocyte which has reacted with C'1. This step proceeds stoichiometrically at 0°C. but at slower rates than at 37°C., and C'4 activity was shown to disappear from the supernatant fluid. These experiments were carried out with a serum fraction, isolated by chromatography on a diethylaminoethyl (DEAE) cellulose column, which lacks C'1 as judged by the criterion of $EAC'_{1,4}$ formation. Studies with these functionally pure components indicate that step (3) proceeds without a lag, an observation of considerable significance with respect to the enzymatic nature of C', as discussed later. Further, it has been calculated that there are approximately from 2 to 5×10^{12} effective molecules of C'4 per milliliter of guinea pig serum and that the formation of a single reactive site suffices to prepare the now converted erythrocyte to the product $EAC'_{1,4}$ which serves as a precursor for the reaction with C'2.

The availability of the reagents C'1 and C'4 in a form virtually devoid of other C'-component activities presages successful efforts for activity estimations in many important areas. The application of new C'1 fixation procedures should provide a discriminating analytical tool for the study of C' involvement in many immune phenomena, to mention autoimmune disease as one example. Further experiments bearing on the enzymatic nature of C'1 may also be approached with somewhat greater confidence. With the exception of the weli-established observations that C'4 may be inactivated by primary amines, nothing is known as to the chemical properties of this component. Cavallo and Cavallo (1958) have shown that C'4 may be destroyed by the addition of acetic acid to pH = 3.8 and by metaperiodate at 0.02 *M* at room temperatures (Shojiro and Hiroshi, 1959). However, as is also the case with other destructive treatments, these methods are probably not specific for C'4.

3. C'2

The reaction step involving the conversion of $EAC'_{1,4}$ to $EAC'_{1,4,2}$ has received considerable attention in the past few years. The most recent advances by Borsos and Rapp (1959) and Borsos and associates (1961a, b, c) are attributable to the availability of precise assay techniques for the characterization of the intermediate products of immune hemolysis and to the successful chromatographic isolation of reagents with but a single component activity.

It has been established that C'2 reagents of this type are stable at 37°C. in the purified state and complete the forward reaction

$$EAC'_{1,4} + C'2 \xrightarrow{+\ Mg^{++}} EAC'_{1,4,2} \qquad (4)$$

in the presence of Mg^{++} without any induction period, indicating, as was the case for reaction (3), that the conversion of a single site at this stage suffices to carry the sensitized erythrocyte toward eventual destruction. The step involving C'2 is somewhat complicated by the fact that the reaction sites, $SAC'_{1,4,2}$ are subject to decay, reverting to the form $SAC'_{1,4}$, although active C'2 does not seem to be released.

Borsos and Rapp (1959) have demonstrated that the decay of the site $SAC'_{1,4,2}$ to $SAC'_{1,4}$ does not lead to an inactive product, in a series of experiments which involved repeated decay of $EAC'_{1,4,2}$ and conversion to the original activity levels with C'2. These observations lead to the conclusion that the extent of hemolysis in a given experimental situation does not necessarily depend on the component present in lowest concentration (cf. Hegedüs and Greiner, 1938) but also on the comparative rates of the forward and decay reactions. Thus Borsos found that the time at which maximal reactivity of C'2 occurs varies only with the number of sites in the state of $SAC'_{1,4}$. However, the extent of the reaction shows a concentration dependence for C'2 and is influenced by the comparative rates of the decay of $SAC'_{1,4,2}$ and its reaction with C'3. It would be of interest in this connection to consider whether the inhibitors of C' fixation in guinea pig, human, and pig C' postulated by Cavallo and associates (1958b) might not be sought in this reaction step.

Measurements of C'2 activity have been made by Borsos and associates (1961a, b, c) who concluded that a minimum estimate of C'2 concentration in guinea pig serum approximates 2.5×10^{12} molecules per milliliter. The values for C'2 and C'4 mentioned in the foregoing are derived from calculations based in part on the experimental demonstration that the lysis of an erythrocyte by antibody and C' requires the participation of only a single effective molecule of components C'4 and C'2.

Leon has conducted extensive kinetic studies on human C' with results that parallel those described in the foregoing for guinea pig serum (Leon, 1956a, 1957c, 1959; Leon and Norden, 1959). Despite some differences of a relatively minor nature, such as those associated with levels of Ca^{++} and optimal conditions of temperature and time for maximal lysis, the essential steps outlined, including the process of decay, have been confirmed. Verification of this type is of some importance since

species differences in other systems often defy direct extrapolation. Leon has cautiously refrained from use of the term $EAC'_{1,4,2}$ in describing the intermediate complex otherwise analogous to that obtained in the guinea pig system. However the term, $EAhuC'_A$ used as the operational equivalent, might well be discontinued in view of the many properties common to the immune complexes which have reacted with either human or guinea pig C'.

4. C'3

As already indicated, the existence of at least two steps in the reactions involving guinea pig C'3 has been deduced by several groups of investigators. Amiraian *et al.* (1958) drew this conclusion from experiments with pig serum reagents eluted from powdered cellulose columns. They found a fraction associated with the β-globulins which did not lyse $EAC'_{1,4,2}$ in the presence of EDTA (thus excluding C'1, C'4 and C'2) but which potentiated the lytic activity of guinea pig serum under these conditions. Independent studies by Rapp (1958) and by Rapp and Sims (1958) led these authors to similar conclusions. Several years earlier Rapp (1955) developed a kinetic theory for the reaction step involving C'3 and demonstrated that the rate and extent of the conversion from $EAC'_{1,4,2}$ to E^* varied as the square of the C'3 concentration. This observation and the finding that a sigmoidal relationship characterized the kinetics of conversion to E^* (Rapp, 1958) indicated the possibly multiple nature of C'3 interaction. Rapp succeeded in preparing two fractions both precipitated from guinea pig sera, one (C'3b) at pH = 5.0 and the other (C'3a) by methanol. Each precipitate, when redissolved in isotonic solution was far less active than a mixture of the two. Independent confirmation for the existence of at least two substances also emerged from chromatographic studies (Rapp *et al.*, 1959). On the basis of further mathematical analysis. Rapp (1960) has now proposed that the reaction step $EAC'_{1,4,2} \rightarrow E^*$ comprises at least three steps. The precise correspondence between the components isolated from guinea pig serum, the pig serum fractions, their human counterparts as described by Leon *et al.* (1955, 1956) and by Taylor and Leon (1959a, b) has not yet been established.

However these studies have demonstrated yet another limitation in the Hegedüs and Greiner concept that the over-all hemolytic activity of fresh serum is determined by the component present in lowest titer. It is apparent that the complex $EAC'_{1,4,2}$ may either revert to $EAC'_{1,4}$ or progress through the subsequent reactions to the state of E^*. The rate and extent of lysis will therefore be influenced by such factors as temper-

ature and the relative concentrations of the intermediate $EAC'_{1,4,2}$ and C'3, i.e., the competing forward and degradative reactions.

Preparation of fractions with increased C'3 activity has also been recorded for human C' by Arday and Pillemer (1958) and by Arday *et al.* (1959) through application of Cohn fractionation procedures. Since these preparations were but slightly lytic for $EAC'_{1,4,2}$, it may be presumed that they had preferentially concentrated either 3a or 3b. Similar considerations apply to the finding of Taylor and Leon (1959a) regarding the destructive effect of hydrazine on the C'3 complex.

The disclosure of a fifth C' component has led to some confusion in terminology. It would therefore be advisable for the investigators in this area to adopt a uniform system of nomenclature to avoid further complications. As matters stand now, it is Rapp's opinion that the sequence of reaction involves C'3b as a prior step to that requiring C'3a.

The need to systematize present knowledge emerges even more sharply in the light of the recent findings of Müller-Eberhard and his colleagues (1960) and Müller-Eberhard and Nilsson (1960). Careful analyses of immunoelectrophoretic patterns of fresh and aged normal human sera revealed that the former contained a β_{1C}-globulin which was not found in the same specimen of serum stored at 1°C. for 4 to 6 weeks or at 37°C. for 6 days. In sharp contrast to the β_{1C}-globulin present in the fresh specimen, the aged serum contained a β_{1A}-globulin. The two proteins, isolated by means of preparative electrophoresis and chromatography, each contained similar amounts of carbohydrate and were lipid-free. Evidence of a close antigenic relationship was shown on Ouchterlony plates. The β_{1C}-globulin had a sedimentation constant of 9.5 S while that of the converted product was 6.9 S. When fresh human serum was treated with zymosan or immune aggregates, the β_{1C}-globulin was removed by conversion to the β_{1A}-globulin. However, the isolated preparation did not react with these immune aggregates. Since this protein did interact with $EAC'_{1,4,2}$, as indicated by suppression of its decay to $EAC_{1,4}$, but did not lyse cells of this type, it may be asked whether β_{1C}-globulin is not equivalent to Rapp's C'3b. The action of these proteins is not markedly influenced by 0.01 *M* EDTA, a finding also in accord with this speculation. Collaborative studies between the two laboratories should readily determine whether C'3b is β_{1C}-globulin and thus achieve an unequivocal chemical characterization of a C' component for the first time. Becker and Wirtz (1959) have recently reported that the interaction of C'3 (a or b?) is impeded in hypertonic NaCl solutions, thus confirming Pillemer *et al.* (1953a).

a. Inhibitors of C'3. Two laboratories have independently reported

that the reaction between $EAC'_{1,4,2}$ and C'3 may be inhibited. Mills and Levine (1959) showed that salicylaldoxime is a moderately potent inhibitor (approximately 50% inhibition of 2 $C'H_{50}$ at 5×10^{-4} *M*) of immune hemolysis, immune adherence, neutralization of T_2 coliphage by normal human serum, and conversion of *Salmonella typhosa* to protoplasts. Rodriguez and Osler (1958, 1960) found phlorizin to inhibit immune hemolysis at equivalent concentrations. Phloretin, the aglucone of phlorizin, was somewhat more efficient. Interest in both of these inhibitors attaches in part to the fact that *ortho*-substituted compounds are more potent. It was not possible to ascertain whether the inhibitory action of phlorizin was competitive in nature. Phlorizin has also been shown to depress phagocytosis (Puchkov, 1955; Ludany *et al.*, 1957) and to inhibit anaphylactic-type reactions (see Section VII, B).

5. E^*

The release of hemoglobin from an erythrocyte in the condition $EAC'_{1,4,2,3b,3a}$ is a time-consuming process which proceeds more rapidly at 37°C. than at lower temperatures. The constituents of the reaction medium do not influence the inevitable release of hemoglobin from an erythrocyte in this state, called E^*.

This reaction step has as yet received comparatively little study, perhaps too little. There has been so much concern with the events occurring in the fluid phase of the antibody–complement system that the resulting cell damage has possibly not been placed in proper prospective. Some aspects of this problem are discussed in Section II.

6. *Miscellany*

The possibility that there are C' components other than those discussed in the foregoing has not been overlooked. Thus, studies dealing with the inhibition of immune hemolysis by substances such as polyanetholesulfonate, heparin, and germanin (Wecker and Klein, 1955 and Klein and Wecker, 1955) have led Klein (1956a, b) and Klein and Lange (1956) to postulate the presence of an additional component, C'm in guinea pig serum. The C'm is reported to be heat-labile, ammonia resistant, and located in the mid-piece of C'. According to Klein (1956b), it is not C'4 and is required for the fixation of C'2. This component has not yet been identified in current studies with chromatographic C' fractions. Its possible existence has been challenged by Inoue *et al.* in their study of the anticomplementary action of polyvinyl alcohol sulfonate (1959b). These and other anticoagulants have been characterized as destructive of C'3 (Osler *et al.*, 1959a).

Brumfield (1954) has described a new component in C′ that is inactivated by histamine and ethylenediamine, but the validity of these findings is uncertain because of possibly inadequate control for the hemolytic and anticomplementary effects of the reagents.

Inhibition of C′ fixation has been described by Mountford (1953) and by Cavallo *et al.* (1958b). More detailed studies by Adler (1960) of an inhibitor in rabbit serum, associated with γ-globulin, show that the effect is primarily on the rate of fixation. The observation that this inhibiting substance may be absorbed by unrelated antigen–antibody precipitates is reminiscent of the rheumatoid factor.

McVickar and Weinfeld (1959) have initiated a detailed and careful study of immune hemolysis at 0°C. The reaction is much slower at this temperature even with C′ levels about one hundred times as high as those required at 37°C. In contrast, antibody concentrations ten times beyond those considered optimal at higher temperatures will lead to rapid and complete lysis. By extending the time course of the reaction through lowered temperatures, some unknown aspects of the hemolytic reaction may be brought to light, although caution may be advisable in drawing conclusions that identical mechanisms are operative at the two temperatures.

Cavallo *et al.* (1957) and Plescia with his associates (1957a, b) analyzed the effect of varying temperatures and concentrations of antibody and C′ on the outcome of immune hemolysis. The results produced by these apparently simple manipulations are fairly complex since each change may lead to simultaneous and unknown alterations of several steps in the hemolytic reaction sequence. The ultimate effect is, therefore, a composite of several factors which must be unraveled for careful analysis. The experiments of Becker and Wirtz (1959) may be illustrative in this connection. These authors reported that an increase in ionic strength markedly decreases the lytic efficiency of guinea pig C′ at 37°C. in confirmation of older observations (cf. Mayer *et al.*, 1946). However, their conclusion that this inhibition occurs at the reaction step involving C′3 requires further study with respect to at least three other variables, namely, the concentration of C′3a, C′3b, and the decay of $EAC'_{1,4,2}$ to $EAC'_{1,4}$. Levine *et al.* (1961) have also studied the inhibition of C′ fixation by hypertonic solutions of NaCl. They attribute the effect to several other possibilities, such as a change in the nature of the antigen–antibody aggregate or an increased rate of C′1 utilization. A further illustration may be drawn from the studies of Gordon (1951, 1953) and those of Gordon and Turner (1955, 1956a, b) on the inactivation of C′ by ammonia and by exposure to elevated temperatures. It would appear from

these studies that glycine, glucose, galactose, mannose, sorbose, and inositol, at hypertonic concentrations, protect the hemolytic potency of guinea pig serum from inactivation at temperatures of 56°C. or higher. The protective effect is somewhat specific and cannot be accounted for solely on the basis of hypertonicity. Schwarz (1955) has reported similar effects with glycocoll.

Haurowitz and Hawkins (1960) have studied the uptake of C′ components from the sera of guinea pigs injected with C^{14}- or S^{35}-labeled amino acids. They concluded that not all of the components are bound irreversibly. However, their failure to observe uptake of C′4 by radioactivity assays may merely reflect an inadequate sensitivity limit of the method for the experimental conditions used by these authors. The apparent lack of specificity in C′ uptake experiments with isotope-labeled proteins is also apparent in the earlier study of Penn *et al.* (1957).

The past few years have seen revived interest in C′ activities of sera from species other than the guinea pig. Reference has already been made to studies with human and pig serum. Leon and Norden (1959) have carried out kinetic studies with horse C′ and reported that guinea pig C′–EDTA mixtures could lyse EA that were pretreated with equine C′ in sublytic amounts. However, human, equine, or swine serum could not be substituted. The rapid decay of the equine intermediate may prove to be pertinent with respect to the conglutination phenomenon and studies of inhibitors of immune hemolysis.

Mouse C′ continues to be of interest because C′2 has been difficult to demonstrate in hemolytic or bactericidal tests. The findings of Brown (1943) confirming much earlier work cannot be considered as conclusive evidence for the presence or absence of C′2 because of the possible presence of citrate in undisclosed quantities in the preparations. Rice and Crowson (1950) found very low titers of C′2 and C′4 in fresh mouse sera, and Marcus *et al.* (1954) were unable to demonstrate bactericidal activity at low dilutions, a finding verified more recently by Muschel and Muto (1956). These reports are contradicted to some extent by that of McGhee (1952) who showed that the sera from 8 of 12 mice lysed a dilute suspension of sensitized erythrocytes and that the lytic factor is heat labile. Clarification of this problem has been achieved by Borsos and Cooper (1961).

Low levels of C′-fixing ability in equine anti-polysaccharide sera have been shown by Bier *et al.* (1956) and by Osler *et al.* (1957). Similarly, the ability of avian antibody to fix C′, long a matter of some controversy, has been demonstrated in more recent years by several investigators including Rice (1947, 1948), Rice *et al.* (1960), Benedict and

McFarland (1958), and Brumfield and Pomeroy (1957, 1959). Direct C′-fixation tests with turkey antiserum were obtained if normal, fresh chicken serum was incorporated in the diluent. The nature of this factor remains undisclosed, although C′1 is suspected.

It may be anticipated that many of the perplexing problems mentioned briefly in this section and elaborated upon in greater detail in the following will be amenable to solution with the use of purified C′-component reagents. Application and extension of the techniques developed by Mayer and by Rapp should prove fruitful in resolving many of the present ambiguities, in that they provide the technological means for detailed exploration of individual phases of the hemolytic system.

7. *Anti-C′ Antibody*

This term refers to an antibody to C′, generally measured by its ability to block immune hemolysis. At least two obvious difficulties present themselves in any attempt to evaluate the present status of work along these lines. The first concerns the matter of antigenic homogeneity. Experiments with preparations of anti-C′ obtained by immunization of rabbits with whole guinea pig serum or mid-piece and endpiece are not readily interpretable in terms of specific antibody to C′ (Haba, 1957a, b). A more refined approach was taken by McKee and Jeter (1956), Jeter and McKee (1957), and Jeter (1960) who immunized rabbits with suspensions of specific precipitates formed in the presence of various normal sera. The sera of the immunized rabbits precipitated with the homologous fresh sera and were also anticomplementary. Unequivocal evidence for a specific anti-C′ still eludes the efforts of these workers since the sera of these rabbits might also contain antibody to a non-C′ constituent which would also precipitate with fresh serum and be anticomplementary as a consequence of this immune reaction. The possibility of incorporating antigenic materials other than C′ in procedures such as these has been stressed by Schmidt (1959) and is shown clearly in the experiments of Ellis and Gell (1958). These investigators found antibody to a minimum of four proteins in the serum of a rabbit immunized with a rabbit-specific precipitate formed in the presence of normal human serum. These data should be considered in evaluating the interpretations drawn by Goldwasser and Shepard (1958) and by Klein and Burkholder (1959) that the increased fluorescence of a rickettsial immune aggregate exposed to guinea pig serum and tested with anti-guinea-pig globulin, is attributable entirely to anti-C′. During the past year Klein and Burkholder (1960a, b) attempted to produce anti-C′ by immunizing rabbits with sensitized sheep erythrocyte stromata–C′ complex. These sera ag-

glutinated EAC′ even after preliminary treatment of the antibody with EA. This agglutination reaction seemed to bear a close relationship to the presence of C′4 in the EAC′ complex. The authors considered that C′1 and C′2 played no part in these reactions and reported that an agglutinogen other than a C′ component was also detected. Since a decomplemented guinea pig serum was reactive with the antibody and removed its agglutinating potency for alexinized erythrocytes, the evidence for an anti-C′ remains dubious. Use of the well-defined intermediates, such as $EAC'_{1,4}$ and functionally purified C′ components may be expected to expedite the preparation and characterization of an antiserum to one of the several C′ components. Reagents of this type would find many interesting applications in studies of C′ participation in diverse immunologic phenomena.

II. Cytotoxic Phenomena Other Than Immune Hemolysis

A. Mammalian Tissue Culture Cells

Much of the effort in this area has centered about studies on the destruction of neoplastic cells by antibody and C′, with the view toward possible therapeutic application. The accumulated data leave little room for doubt that specific antiserum may agglutinate and alter the morphological and physiological properties of tissue culture cells derived from a variety of sources (Mountain, 1955; Liu and Heyl, 1957; and Green and Lorincz, 1957). Virtually all of the studies demonstrate that addition of fresh serum induces a more rapid and profound alteration of the cells leading to their eventual destruction.

Use of the indirect fluorescent antibody procedure by Hiramoto and associates (1960) demonstrated destruction of HeLa cells in the presence of antibody and fresh serum; the initial reaction occurring on the cell surface. The *in vitro* and *in vivo* effects of antibody and fresh serum on HeLa cells have also been described by McAllister *et al.* (1958). Most of the interest has been directed toward a description of the ultimate effect on the cell so that definitive data regarding the C′ requirement or mechanism are still lacking. Several examples may be mentioned. Kalfayan and Kidd (1953) described structural changes in Brown-Pierce rabbit carcinoma cells which occurred only in the presence of fresh serum. These include imbibition of fluid with swelling of the cytoplasm, loss of basophilism and marked thinning, without rupture, of the cell membranes. Interesting studies with the aid of phase and electron microscopic techniques led Latta (1952, 1959a, b) to describe a number of changes which followed the addition of antibody and C′ to chick-embryo

heart tissue cell cultures. Latta postulated an increase in intermolecular forces at the cell surface accompanied by permeability changes and consequent increase in intracellular osmotic pressures. These changes were observed only in the presence of fresh guinea pig serum. On the basis of these earlier studies the possible requirement for C′ was given more substantial experimental support in the findings that the cytotoxic activity was virtually abolished by heating the serum or by specific decomplementation of the immune serum (Latta, 1959). Lumsden (1959) has also described the lethal effect of fresh guinea pig serum on explants of adult cockerel heart, findings echoed by Defendi *et al.* (1958) for two sublines of amnion cells, and also confirmed by Bickis and co-workers (1959), by Flax (1956), and by Bolande and McClain (1960). Interesting studies on the kinetics and specificity of the cytotoxic reaction are described by Defendi and Colter (1959). Other aspects of these cytotoxic reactions are presented by Goldstein and Myrvik (1958) and by Goldstein *et al.* (1959). Ainis and collaborators (1958) showed that fresh, adult guinea pig serum on admixture with Murphy-Sturm lymphosarcoma protected recipient Wistar rats, but their conclusion that C′3 does not participate in the protective action of guinea pig serum is not adequately supported by the experimental data since zymosan may reduce other component activities in addition to C′3 (see Section V). Wissler and Flax (1957) and Flax (1956) have described inhibition of glycine-C^{14} incorporation and depression of the rates of glycolysis and respiration during the cytotoxic process. Ellem (1957, 1958) equated fresh, normal rabbit serum with C′ and noted an immediate rapid swelling and permeability changes involving increased diffusion of inorganic P and loss of organically bound P as changes attributable to both C′ and antibody.

Goldberg and Green (1959) as well as Green and colleagues (1959b; Green and Goldberg, 1960) have conducted detailed studies of the events immediately preceding the release of hemoglobin from erythrocytes and the lysis of Krebs ascites tumor cells exposed to rabbit antibody and C′. Their findings, based on electron microscopic studies, estimates of electrolyte exchange, and loss of amino acids and ribonucleotides into the surrounding medium, may be summarized as follows. Antibody alone produced local structural changes in the cell membrane which were attributed to *in situ* formation of antigen–antibody complexes. The addition of C′ (normal rabbit serum) resulted in marked swelling of the mitochondria and endoplasmic reticulum. Within a few minutes there was a profound cellular loss of potassium, free amino acids, cellular proteins, and ribonucleotides. It is believed that there are at least two phases, the first step involving production of holes in the cell membrane,

large enough to permit the rapid exchange of inorganic cations and small molecules. The subsequent derangement of the regulatory mechanisms leads to secondary swelling with release of macromolecules such as hemoglobin and other proteins. The type of cell damage described in these reports may be functionally similar to the fine cracks on the erythrocyte surfaces described by Latta (1952).

The participation of C′ in the destruction of transplants of lymphoma cells has been reported by Winn (1960a). These data indicate that the role of humoral factors cannot be ignored entirely in problems of homotransplantation, as stressed by Stetson and Demopoulos (1958) and by Stetson and Jensen (1960).

Roizman and Roane (1961) have recently devised an interesting method for differentiation of viable from injured or killed cells. Human epidermoid carcinoma cells (HEp-2) are infected with herpes simplex virus and then treated with fresh guinea pig serum and rabbit antiserum to the HEp-2 cells. Those tissue culture cells rendered nonviable through the immune cytolytic process do not support replication of the virus and consequently fail to form plaques. The diminution of the plaque count as compared to the controls provides a quantitative estimate of the activity of the anti-HEp-2 cell antibody. Other recent studies dealing with the cytotoxic activity of fresh serum on mammalian cells are described by Winn (1960b). See also comments by Mayer (1961) and by Osler (1961).

A careful sifting of the combined evidence provided by this group of studies leaves little doubt that the cytotoxic effect of fresh serum on these mammalian cells is probably due to the C′ system as it is operative in immune hemolysis. However, this conclusion does not emerge clearly from any single study nor has the possible role of non-C′ factors been excluded in any rigorous fashion.

B. Other Mammalian Cells

Waksman (1958, 1959) has written extremely comprehensive reviews of work in this area in which "cell lysis" has been defined in the most general terms as any form of cell damage resulting from an antigen–antibody reaction at the cell surface. From the point of view of C′ involvement there would seem to be at least two different types of situations.

1. Lysis Involving an Antigenic Constituent of the Mammalian Cell

Aside from the Forssman system, the evidence for a C′ requirement in the lysis of other cells of mammalian origin remains incomplete. In

some instances it has been assumed that an effect resulting from fresh serum may be ascribed to C′. In others, different criteria have been used but systematic studies correlated with hemolytic activity measurements remain to be done. This state of affairs is partly due to the recent spurt of interest in "immunologic disease" and much effort is still devoted to descriptions of the various phenomena, their association with clinical status, and, in a few instances, to studies of the nature of the offending cell or antigenic constituent. Further, as in some of the autoimmune diseases, it has not been clearly established whether the presence of circulating antibody is causally or coincidentally related to the characteristic tissue damage. In the opinion of some, these antibodies may appear only as a result of the underlying disease process. Lastly, immunological activity of the delayed hypersensitivity type may provide a crucial segment in the total picture and the involvement of C′ in these events can only be a matter for speculation. In the light of these considerations, a discussion of the C′ problem in systemic lupus, thyroiditis, aspermatogenesis, and diseases considered associated with leucocyte antibodies will be omitted.

One other aspect of this problem may be mentioned. The preponderance of work on immune hemolysis should not preclude the possibility that the combined action of C′ and antibody on some tissue or blood cells may terminate in a stage prior to extrusion of the intracellular contents, a stage analogous to that of E* discussed earlier. These effects should be sought, since a subtle disorganization of the cell economy may also prove destructive to certain normal physiological processes even in the absence of cell dissolution. Lastly, studies such as those by Cruz (1953) involving destruction of platelets by C′ and antibody may prove informative in contemplated approaches to the human situation.

2. *Lysis Involving Antigen–Antibody Systems Not Related to the Cell Serving as Indicator*

Studies involving polysaccharide or protein antigens adsorbed onto erythrocyte surfaces emphasize two factors of importance. One of these is the possible parallelism between this type of reaction and those ordinarily considered restricted to hypersensitivity reactions. As discussed in the section on hypersensitivity, allergic tissue reactions can be elicited when either antigen or antibody is fixed to the appropriate tissue site. The ability of soluble antigenic material to adhere to cell surfaces in sufficient proximity to lead to cell alteration or destruction invites caution in the interpretation of cell lysis as a reflection of antibody fixation only.

The mechanism involving antigen fixation has been recognized with respect to the hemoglobinuria associated with some infectious processes. The possibility might also be considered that lysis results from the interaction of C′ and antibody with an antigen associated with the erythrocyte surface but derived as a consequence of injury to other tissues. The participation of C′ in the Donath-Landsteiner reaction in paroxysmal cold hemoglobinuria has been reviewed and studied further by Jordan *et al.* (1951, 1952) as well as by Baumann-Grace and Tomcsik (1959). The report that lysis of cells from patients with this disease occurred in the absence of C′1 and C′3 (Jordan *et al.*, 1951) was not confirmed. Nor can the nocturnal and cold forms of this syndrome be distinguished on the basis of a Mg^{++} requirement (Baumann-Grace and Tomcsik, 1959; Harris *et al.*, 1951). With respect to the nature of the antibody, it is of interest to note the following experiment carried out in the author's laboratory. A single specimen of serum from a patient with paroxysmal cold hemoglobinuria preserved with 0.25% phenol became opalescent and developed marked anticomplementary properties on exposure to 0°C. for 2 days. These changes were not seen in a portion of the same specimen maintained at room temperatures approximating 25°C. for the same period. Centrifugation of the cooled serum at high speed yielded a pellet in which resided the anticomplementary activity (cf. also Fudenberg and Kunkel, 1957). Jonsen and Kass (1959) have also noted a fall in levels of hemolytic activity presumably resulting from a diminution of C′1 and C′2 titers in a case of cold hemoglobinuria. These decrements were observed after exposure of the patient or his serum to low temperature. The relationship of these findings to the problem of aggregated γ-globulin and hypersensitivity reactions will be discussed below. It may suffice at this point to indicate that cell lysis can perhaps be initiated without the addition of antigen provided the γ-globulin is induced to aggregate, as may be the case in paroxysmal cold or nocturnal hemoglobinuria (cf. Van Loghem *et al.*, 1952). The observation that at least a portion of the Wassermann antibody is of high molecular weight (Davis *et al.*, 1944, 1945) may, therefore, be pertinent toward understanding the mechanism of lysis in hemoglobinuria associated with syphilis. Hinz *et al.* (1961) have recently confirmed the C′ requirement in hemolysis associated with paroxysmal nocturnal hemoglobinuria and demonstrated the interaction of C′1 at low temperatures.

The possible effect of antibody and C′ on lymphocytes and other leucocytes pretreated with tuberculin or other antigenic components of the tubercle bacillus is still unresolved. It may be suggested that the product of this interaction should also be sought in terms of release

from the cell of a specific intracellular constituent such as an enzymatic activity or a nucleotide. Preliminary explorations along these lines have been initiated by Dr. L. Levine but have not yet been brought to a definitive stage.

There is yet another example of C′ utilization by a formed blood element acting in conjunction with antibody and an extraneous antigenic substance. Reference is made to the interesting studies of Shulman (1958a, b, c) in which are elucidated several aspects of C′ fixation, as related to the mechanism of quinidine-induced thrombocytopenic purpura. Certain technical facets of this investigation have been discussed previously (Osler, 1958). Of pertinent interest is the observation that destruction of the platelets follows only when a platelet–quinidine complex serves as antigen. Neither the platelets nor the hapten alone induced the fixation of C′, a process leading to platelet destruction. On the basis of correlated quantitative *in vitro* studies and induced thrombocytopenia, Shulman concluded that the quantity of antibody attached to the platelets during the natural disease process is insufficient to cause fixation of C′ or platelet agglutination, but it is sufficient to increase the susceptibility of platelets to the usual processes of sequestration and phagocytosis. This deduction may be questioned with respect to several factors. In immune hemolysis, lysis is demonstrable with fewer antibody molecules per erythrocyte than the number present per platelet as calculated in Shulman's study. Further, this conclusion rests on the inability to detect *in vitro* fixation of C′, a criterion that may be altered by variation in the C′ available for the reaction (Osler, 1958). Finally, there is evidence that C′ enhances phagocytosis including erythrophagocytosis (Bonnin and Schwartz, 1954).

Shulman's interpretation is perhaps at variance with the observations of Ackroyd in sedormid purpura (1951) as well as with those of Reader (1955), Dacie (1955), Gardner and Harris (1950), Jordan (1957), and Van Loghem *et al.* (1952) in acquired hemolytic anemia.

C. Bactericidal Reactions

Substantial progress may be recorded in this area due largely to the studies of Muschel and Treffers (1956a, b, c) and to those of Amano and his group (1954, 1955) and of Inoue *et al.* (1959a, b). As is often the case, technological developments preceded the acquisition of new facts relating to the mechanism of action of antibody and C′ on bacteria. The former group of investigators developed a photometric assay procedure which circumvents the more laborious plate count methods and yields results reproducible to about 10%. The organisms under study are in-

cubated with C′ and antibody and the relative number of survivors estimated by photometric analyses of subcultures prepared from test and control reaction mixtures. Serum titers are expressed as the volume required to kill 50% of the initial inoculum. Application of this procedure demonstrated a Mg^{++} requirement for the bactericidal process and an estimate of about 700 antibody molecules for killing a single *Salmonella typhosa*. It may be safely predicted that this value will decrease with further refinements of technique since the lethal action of antibody for immune hemolysis approximates less than 10 molecules on the assumption of a similar mechanism of operation. These authors, as did Osler and Knipp (1957a, b) in another situation, observed that immune human sera lost considerable antibody activity during the customary thermal inactivation conditions of 56°C. for 30 minutes, a finding confirmed by Maurer and Thorpe (1960) and of considerable pertinence to the properdin problem. The Neisser-Wechsberg inhibition effect was attributed to the presence of antibody in excess rather than to ill-defined anticomplementary activity.

Studies of convalescent and hyperimmune human sera also yielded findings of considerable interest. As expected there was a significant variation in the ratio of agglutinin and bactericidal titers. This fluctuation may be ascribed to different levels of reproducibility of the two methods as well as to the fact that the bactericidal reaction may not be as sensitive to changes in combining ratio of the antibody as is agglutination. However increased lethal efficiency was noted in sera as a function of immunization, another correlation of interest with respect to C′ fixation (Wallace *et al.*, 1950). Further, the antigens involved in the fixation of C′ may play roles of varying and unequal importance with respect to the bactericidal process. In fact, the authors report the greater effectiveness of anti-Vi antibody than anti-O in the killing of *S. typhosa*. An interesting application of the turbidimetric assay by Treffers and Muschel (1954) showed that increased resistance of *S. typhosa* to chloramphenicol did not alter its lytic susceptibility to antibody and C′.

Some of these findings extended those reported earlier by Adler (1953a, b) who also found that fewer than 2000 molecules of antibody sufficed for the destruction of a single organism. This value as well as that of Muschel and Treffers cannot be accepted as the final verdict in view of their observations as well as those of Nagington (1956a, b) with regard to the multiplicity of antigens participating in this phenomenon. Nagington adopted the precautionary step of presensitizing the bacterial suspension to minimize agglutination, as well as the inhibitory and competing action of soluble antigens for the available antibody. The use of

tetrazolium reduction as an index of the number of surviving organisms permitted a sharp decrease in the number of test organisms, a desirable feature on several counts. It was concluded in agreement with Adler, Muschel, and co-workers that the antigenic surface architecture was an important determinant in the bactericidal effect of R, O, or Vi antibody, since the reaction of each of these antigens with C′ and homologous antibody could result in cell death (cf. Section II, B, 2). This notion had been proposed previously by Adler (1952) in his demonstration of the so-called "passive bactericidal" reaction wherein organisms of the genus *Salmonella* could be killed by antibody to *Escherichia coli* and C′, provided the former were coated with *Escherichia* antigens. The flagellar antigens are not at all concerned in the bactericidal reaction (Amano *et al.*, 1954). The studies of Carey *et al.* (1960) and Landy and Webster (1952), however, infer that antibody to Vi may be the major factor in protection against infection. Thus, mice injected with as little as 0.2 μg. of a purified Vi preparation reacted to a challenge of living virulent *Salmonella typhosa* with rapid conversion of the organisms to protoplasts (Muschel *et al.*, 1959). The heightened response elicited in immunized mice would seem to indicate involvement of C′ so that earlier findings (Muschel and Muto, 1956) relative to a lack of bactericidal antibody in normal mouse serum may be ascribed to different considerations.

Amano and his associates have published an extensive series of papers dealing with the bactericidal action of antibody and C′. One of their findings may be of fundamental importance with respect to the mechanism of C′ action, namely that the cell substrate is prepared by the action of antibody and C′ for the terminal action of lysozyme in producing cell lysis (Amano *et al.*, 1958, Inoue *et al.*, 1959a). Although cells of *E. coli* B could be rendered nonviable through the interaction of antibody and C′ alone, the addition of lysozyme to a concentration of 0.02 μg./ml. markedly enhanced the rate and extent of lysis. The presence of the enzyme was indispensible for spheroplast formation, a finding confirmed independently by Muschel *et al.* (1959). Earlier studies by the Japanese group showed that lysozyme derived from a variety of mammalian sources acted in a similar fashion when tested with bacteria of several genera (Amano *et al.*, 1955). These exciting results bear repetition in the light of the report by Baumann-Grace and Tomcsik (1959) who found that spores of *Bacillus megaterium* adsorbed lysozyme from a meat infusion broth so that 4 out of 8 rabbit antisera to *B. megaterium* contained precipitable antilysozyme. The experiments of Amano and his co-workers might, therefore, involve lysozyme–anti-lysozyme as an additional immune system which potentiates the action of the specific anti-

bacterial serum through adsorption of the enzyme on the cell surface and subsequent interaction with anti-lysozyme. Although lysozyme activity was removed from the antisera with bentonite as a precautionary measure, tests for the possible presence of anti-lysozyme were not reported.

The mechanism of action of mucin is of some pertinence to *in vivo* bactericidal events. Interest in this substance has dwindled in recent years, possibly owing to a lack of clarity with regard to its mode of action. However, the recent studies by DeWitt (1958) and by Lambert and Richley (1952) suggest that the infection-promoting activity of mucin may be referable to its anticomplementary properties. This implication is in accord with the findings of Pillemer *et al.* (1955) and others that many polysaccharides of high molecular weight may interact with C'3. Further studies would seem indicated.

The suggestion that the C' system involved in bactericidal reactions differs qualitatively from that in the hemolytic system has been renewed by Jordan (1957) on evidence that is untenable.

D. Lethal Action of Antibody and C' on Other Microorganisms

1. Viruses

The participation of C' in virucidal activity may be considered as a generally accepted impression which has not yet received rigorous experimental verification. Many studies like those of Howitt (1950) and of Bang *et al.* (1951) indicate that the addition of fresh, but not heated, normal serum will enhance the neutralizing activity of specific immune serum. In fact, Sabin (1950) and Smithburn (1954) have adopted the addition of fresh, normal serum or the use of fresh, convalescent, or immune serum as a routine procedure in neutralization tests with arthropod-borne viruses. Leymaster and Ward (1949) also demonstrated that the enhancing activity for mumps was removed by treatment of fresh, normal monkey or immune human serum with an ovalbumin-specific precipitate. These experiments have not been confirmed with other viruses. Some of the issues and complexities involved in the identification of the heat-labile virus inactivation system present in fresh serum have been discussed by Karzon (1956). In the case of Rous sarcoma virus, Rubin (1956) and Borsos (1958) showed that the presence of anti-tissue antibody could simulate virus-neutralizing activity through interference with host cell multiplication. The consideration that individual virus particles may be rendered incapable of cell attachment through combination with a single molecule of antibody is of obvious pertinence to this question and is supported by many reports including those of Jerne and

Avegno (1956), Jerne (1956), Rubin and Franklin (1957), and Mandel (1960). The reaction of individual virus particles with single molecules of antibody might be influenced by the presence of C′ through a depression of the rate of dissociation of these complexes. The possible role of other heat-labile substances must also be considered. Finally, the formation of nonviral antigen–antibody aggregates with the nonspecific inclusion of a considerable number of virus particles may also lead to reduction in infectivity. Since C′ enhances aggregation in some systems (see Section III), the addition of fresh serum would tend to increase the apparently specific neutralizing activity as would also the possible presence of virus-neutralizing activity in the serum used as a source of C′.

2. *Protozoa*

Warren and Borsos (1959) have fulfilled somewhat more exacting criteria for C′ participation in the demonstration that unheated chicken antisera will lyse *Trypanosoma cruzi* in the crithidial stage of development. The lytic property is lost on heating but restored with fresh, normal chicken serum. The heat-labile factor required divalent cations for its action and could be found in sera of chickens reared in a normal or germ-free environment.

Sinclair (1958) correlated the immobilizing and hemolytic activities of normal guinea pig serum and specific antibody on *Paramecium aurelia* and *Tetrahymena pyriformis*. It was shown that lysis followed agglutination of these organisms in the presence of antibody and C′, provided all of the C′ components were present. Nevertheless, C′ fixation experiments with the external antigens of *P. aurelia* and homologous antiserum were negative. These observations are reconcilable on the basis that immobilization reactions may be detected with 1% or less of the antibody required to fix C′ under the conditions employed in this study.

3. *Spirochetes*

The observation of Lawrence (1955) to the effect that heat-inactivated antiserum alone causes lysis of leptospires is rather unique. Addition of fresh guinea pig serum exerted no apparent effect. It would be of considerable interest to obtain an estimate of the number of antibody molecules required per leptospire and to ascertain whether this figure can be reduced by the addition of guinea pig serum of proven hemolytic activity. In the treponemal immobilization reaction of Nelson and Mayer (1949), titration of the reaction mixtures for residual C′ has been of great help in the interpretation of test results in which the essential role of C′ has been thoroughly established. Experiments by Seldeen (1952) had

indicated that these organisms might possess a coating of hyaluronic acid. Recent studies by Metzger and co-workers (1961) have now demonstrated that this reaction may be intensified and accelerated by the incorporation of lysozyme in the reaction mixtures. The mechanism of action of these enzymes with respect to the surface structure of *Treponema pallidum* has not yet been explored. Treponemal immobilizing titers may also be increased by the addition of Ca^{++} (0.0014 *M*) to the reaction medium (Chorpenning and Beers, 1960).

E. Phagocytosis

There have been several approaches to the study of phagocytosis, each with a somewhat different orientation. The studies of Wood and his associates, as summarized in Wood (1953) and described more recently in Smith and Wood (1958), have placed primary stress on the physicochemical properties of the reaction medium as a vital factor in the phagocytic process during the acute phases of infection prior to the formation of specific antibody at the usual levels of detection. Extensive reviews from the standpoint of parasite-cell interaction have been written by Suter (1956) and by Hirsch (1959). Robineaux and Pinet (1960) have emphasized the value of morphological studies, and methodological advances have also emerged from the kinetic studies of Benacerraf, Biozzi, Halpern, and their associates as reviewed in Benacerraf *et al.* (1957) and applied by Benacerraf *et al.* (1959a, b). Application of these techniques may well clarify the role of humoral factors, including C′, in the ingestion and fate of particulate antigens. This issue is still a matter of dispute since Gelzer and Suter (1959) have recently questioned the participation of C′ in phagocytosis. It may be suggested that the Krebs-Ringer buffer solution used in these experiments precluded the possibility of C′ action in this study, designed primarily for studying other aspects of phagocytosis. Contrary evidence is supplied by Benacerraf and Miescher (1960) who noted that the addition of fresh serum led to immediate clearance of sensitized rat erythrocytes by the reticuloendothelial system of the intact mouse. In the absence of fresh serum a significant lag phase was evident. Enhancement of human and rabbit erythrophagocytosis by C′ has also been reported by Mabry *et al.* (1956) in confirmation of studies by Maaloe for *Salmonella typhimurium* (1946, 1948a, b) and by Nelson (1956) who used a slight modification of Maaloe's technique for the phagocytosis of starch granules. In this study, the investigators extended earlier findings of Delaunay and Pages (1946) and the observations of Tullis and Surgenor (1956). Concurrent hemolytic and phagocytic studies showed a clear correlation

between C′ activity and efficacy of starch granule phagocytosis. It is of some interest that Ludany *et al.* (1957) found that phlorizin, an inhibitor of C′3, also suppressed phagocytosis.

In 1943, Mudd *et al.* concluded that the interaction of specific antibody and C′ with encapsulated pneumococci, as visualized in the electron microscope, produced alterations which were not found on deleting the fresh serum from the reaction mixture.

Slopek *et al.* (1959a, b, c) and Skurski *et al.* (1959a, b, c) utilized washed equine leucocytes and fresh, normal horse serum to study ingestion of various bacteria in an interesting application of phagocytosis for the study of various problems. The importance of antigenic composition in the resistance to the action of phagocytes has been underscored by Wood (1953) and has been studied in some detail by the Polish group for the Gram-negative bacteria. They report that in the absence of specific antibody the Vi antigen may be the chief inhibitor, so that Vi-containing organisms can be phagocyted only after destruction of this surface antigen by heat. A normal serum requirement, probably C′, was shown for the phagocytosis of the smooth colony types whereas the R organisms were ingested without the addition of serum, presumably by the cells alone. The C′ was considered as the phagocytic-enhancing system in normal serum. However the absence of antibody in "normal" sera can only be considered in relative rather than absolute terms as discussed in Section V (cf. also Muschel, 1960).

III. The Participation of C′ in Aggregation Phenomena

Several aspects pertaining to the enhancement of immune aggregation by the C′ system convey implications of potential significance in a number of disease processes. These implications may not have been considered in some of the studies designed to elucidate the role of C′ in the aggregation reactions which are discussed in this section.

A. Incorporation of Extraneous Particles into Antigen–Antibody–C′ Aggregates

Since the present article is oriented solely toward an evaluation of C′ participation, the associated semantic and historical controversy reviewed by Lamanna (1957) with regard to the Rieckenberg reaction needs no further elaboration. The fundamental observation which dates back some 60 years refers to the adhesion of extraneous particles, such as platelets, bacteria, collodion particles, starch granules, and erythrocytes, to the complex formed through the interaction of antigen, antibody, and

C′ (Lamanna and Hollander, 1956). Applications of this reaction to studies of staphylococcal disease in humans have been described by Kourilsky *et al.* (1955, 1956, 1957; Kourilsky and Pieron, 1957).

The requirement of C′ in this reaction is manifest through the work of several investigators. Lamanna and Hollander (1956) showed the inhibitory effects of a chelating agent and the restorative properties of Ca^{++} and Mg^{++}, for the adhesion of *T. pallidum* to streptococci. A similar cation requirement was demonstrated for the adhesion of guinea pig platelets to sensitized sheep erythrocytes by Nelson and Nelson (1959a, b). These authors concluded that mixed aggregates of two sensitized particulate antigens may occur in the presence of C′.

The essential role of C′ has been stressed by many investigators and received renewed attention by Turk (1959a) who found that fresh rabbit and mouse sera were less efficient than those of the human, horse, pig, or guinea pig. This parallelism to hemolytic C′ was extended with the observations that horse and pig C′ showed very weak reactions in combination with rabbit antibody (cf. also Rice and Crowson, 1950) and that mouse serum was deficient or lacking in C′2 (Turk, 1959b).

Nelson, whose studies with rickettsiae, starch granules, zymosan, etc. revived interest in this phenomenon, suggested that the order of fixation for the components was the same as for other reactions involving C′ (Nelson, 1953, 1956). It was also demonstrated that the reaction could be employed with primate erythrocytes as the indicator for the detection of antibodies to a variety of microorganisms including *T. pallidum* (Nelson, 1953). The sensitivity of this reaction was established at approximately 0.01 μg. of antibody N, a level approaching that of hemagglutination and passive cutaneous anaphylaxis (Nelson, 1956; and Nelson and Nelson, 1959b). Evidence was offered to differentiate this reaction from conglutination and the adhesion of particles induced at low pH levels.

Taverne (1957) described a hemagglutination reaction with bacteriophage T_2, antibody, C′, and human erythrocytes. She demonstrated the presence of antibody to this virus in normal guinea pig and human sera and indicated that the fixation of C′ to the sensitized phage particle was the rate-limiting reaction.

On the basis of classic C′ component studies, Turk (1959a) has postulated the requirement of an additional serum cofactor which is independent of C′4. The evidence for the presence of this factor rests on the observation that the immune-adherence reaction cannot be restored when fresh guinea pig serum is replaced by a mixture of R3 (zymosan-treated guinea pig serum) plus R4 (ammonia-treated guinea pig serum).

The factor thus appears similar to that described by Pensky *et al.* (1958) for the properdin system. Further studies by Turk (1959b) with a variety of bacterial antigens and normal human, rabbit, and guinea pig sera led to the conclusion that this phenomenon involves a specific antibody and the participation of a nonspecific substance resembling properdin. This deduction was based, in part, on the finding that the addition of one of several polysaccharides markedly reduced the titers for salmonellae, vibrio, and staphylococci but not for *Erysipelothrix rhusiopathiae* (see Section V). In an earlier report, Turk (1958) studied this reaction with several soluble protein and polysaccharide antigens. The observed failure with streptococcal group-A antigen and diphtheria toxoid may be ascribed to technical difficulties such as the unavailability of C'2, to possible decay of one of the intermediates, or to other "anti-complementary" activities in reaction mixtures containing only a single unit of C'. Studies with such low levels of C' are highly subject to uncontrolled fluctuations.

B. Enhancement of Specific Aggregation by C'

The previous section has shown a rather surprising unanimity of opinion with respect to the involvement of C' in the formation of mixed aggregates. When viewed from a slightly different context, the additional conclusion may be drawn that C' enhances aggregation phenomena involving an extraneous particle (e.g., erythrocyte, platelet, starch granule) in the complex which also contains antigen and antibody. In view of the definitive studies by Heidelberger and his associates (reviewed in Kabat and Mayer, 1961) on the addition of C' to specific precipitates, it might be anticipated that the corollary demonstration of enhancement of aggregation by C' would be at hand in an equally conclusive fashion. Such is not the case and the reason may be sought in part on technical grounds. However, the available evidence points to the conclusion that the presence of C' intensifies immune aggregation. Thus, Liu and Heyl (1957) reported that factors in human and guinea pig serum, indistinguishable from C', enhanced the fluorescence which was obtained when fluorescein-labeled rabbit anti-human globulin was added to complexes of primary atypical pneumonia virus particles and convalescent human serum. These observations were considerably extended by Goldwasser and Shepard in their studies with *Rickettsia mooseri* and convalescent human typhus fever serum (Goldwasser and Shepard, 1958). It is of interest to note that the augmentation of fluorescence involved only C'1, C'4, and C'2 as judged from experiments with the classic component reagents. Subsequently, Liu (1961) demonstrated that the addition of normal serum

enhances the specific antibody activity to primary atypical pneumonia virus. Attempts to identify the factor led to the tentative conclusion that it was indistinguishable from serum C′, notably C′1, C′2, and C′4. The use of C′-component reagents, such as R1, may have accounted for the difficulties in reaching a more definitive statement. These observations may then be considered as an example where the effect attributable to C′, which does not involve cell lysis, may progress in the absence of C′3. These findings recall the demonstration by Heidelberger that heat-inactivated C′, present at the time of specific precipitation, invariably added N to the aggregate. However, this increment has never been equated with C′4 or C′3 but may well be due to other substances. Gorrill and Hobson have also indicated that a heat-stable factor in rabbit, human, and guinea pig sera which resembles C′4 potentiates agglutination of sensitized sheep erythrocytes (Gorrill and Hobson, 1952). Stohlman (1953) considered that the C′ of dogs but not that of other species enhanced the agglutination of canine erythrocytes by isoantibody. This finding is in accord with that of Lalezari and Spaet (1960) but is not in agreement with the reports of Aubel-Lesure (1950, 1951) for the human isohemagglutination system. The inhibition of bacterial agglutination in the presence of fresh, but not heated, serum was observed by Thjotta and Jonsen (1949) who also carried out correlated hemolytic studies.

An effort to clarify this problem was undertaken by Maurer and Talmage (1953a, b) who found that heated normal rabbit serum augmented the N values of specific precipitates, that this effect was most marked in the region of antigen excess, and that rabbit serum stored at 0°C. for 3–5 months still contributed coprecipitable N. When fresh rabbit serum was pretreated with an unrelated immune aggregate, the capacity for coprecipitation was diminished. Further, specific precipitates formed with horse antibody failed to incorporate any N from rabbit C′ (Maurer and Weigle, 1953). These observations were confirmed in some measure by Morton and Deutsch (1956) with rabbit antibody to several different protein antigens and with human, bovine, or rabbit C′. Contributions of N to specific precipitates were obtained from euglobulin preparations that correspond to C′ mid-piece but not from other plasma or milk proteins. The identification of this nitrogenous material has been pursued further by Weigle and Maurer (1957a, b, c, d) who found no relationship between the addition of N to aggregates containing protein or polysaccharide antigens and the hemolytic potency or component activity of the human or guinea pig serum under study. These observations lacked a clear interpretation. Later studies by Weigle and Maurer (1957c, e) with radio-iodinated serum proteins indicated that the C′ uptake by specific

precipitates was partly reversible, as noted previously by Osler and Hill (1955) and Hill and Osler (1955). In addition, it was concluded that substances other than C′ might be taken up by immune aggregates, thus emphasizing the need for more than a single criterion in the characterization of the C′ system.

It may be inquired whether the findings of Weigle and Maurer might not be referable to the presence of macroglobulins in these rabbit sera, capable of adding on to specific precipitates much in the same fashion as those in sera of humans with rheumatoid arthritis or those recently identified in normal chicken sera (Makinodan *et al.*, 1960). The possible interplay of such factors as the aggregated state of γ-globulin in the anti-O (Thjotta and Jonsen, 1949) and isohemagglutinin (Aubel-Lesure, 1950) must also be considered in view of the reports that these proteins may be of the 19S variety. The effects of Liquoid in the enhancement of hemagglutination of tuberculin-sensitized erythrocytes has been noted by Popp (1957).

C. Conglutinin and Immunoconglutinin

The incorporation of C′ and C′-like substances into immune aggregates has long been associated with the phenomenon of conglutination. The definition offered by Marks and Coombs (1957) may serve as a guide for this section of the discussion. Immunoconglutinin has been defined as "An immune substance produced in the serum of an animal following an antigenic stimulation and which has the property of reacting with C′ adsorbed on an antigen–antibody complex or other surface, causing a marked clumping or flocculation of the reactants *in vitro*." Conglutinin was used initially by Bordet to denote the substance in normal bovine (or horse, cat, pig) sera which agglutinates sensitized erythrocytes which have absorbed C′ (see review by Hole and Coombs, 1947a, b, c). The technique for the detection of conglutinin, by means of the conglutinating complement absorption test and its applications have been described in detail by the British investigators, who have stressed the importance of selecting the proper source of C′ for the immune system under study (Blomfield *et al.*, 1949, 1950). The effects of variation in antibody or in C′ on the outcome of these reactions have also been reported by Rice (1950a, b, 1953) and Rice and Avery (1950).

In line with older studies, Berman (1950) and Rice (1953) concluded that all the C′ components were required to demonstrate conglutination. Leon has shown a Ca^{++} requirement for the conglutination of sensitized erythrocytes (Leon, 1957a). Coombs and co-workers (1950) viewed conglutination as the product of a union between conglutinin and C′1, C′4,

and C'2. The concept was then advanced by Coombs and Coombs (1953) that conglutinin acts as an antibody to one of the C' components, possibly C'4. This hypothesis in turn served as a basis for the further assumption that conglutinin (in previously unimmunized animals) provided an example of autostimulation. The host injected with a bacterial suspension presumably formed immunoconglutinin in response to the complex, bacteria–antibody–C' (Coombs, 1954).

Once formed, immunoconglutinin can be removed only with sensitized bacteria pretreated with fresh serum, and not by bacteria alone, sensitized bacteria alone or by sensitized bacteria pretreated with R4, a reagent lacking in C'4. The inference is that immunoconglutinin is an antibody to C'4 (Coombs and Coombs, 1953). Neither conglutinin nor its immune counterpart have been purified to a degree adequate for characterization studies. However, the species distribution and properties of immunoconglutinin have been described by Coombs (1954) and by Marks and Coombs (1957) in an epidemiological study of a human population. Pernis *et al.* (1959) report elevated levels in silicotics. Further, studies by Ingram *et al.* (1959; Ingram, 1959a, b) indicate that following infection with a variety of bacterial, rickettsial, and viral antigens, there is a marked parallelism in the time course between increments of immunoconglutinin and specific antibody. Of interest also is Ingram's report that higher survival rates were observed in mice, challenged with one of several pathogens, provided that an injection of conglutinin preceded the pathogenic agent (Ingram, 1959b).

The direct conglutination reaction has been used by Barber (1955) for the demonstration of antibodies to *Coxiella burneti.* Mollison and Polley (1960) have used the immunoconglutinin reaction to detect a variety of human blood group antibodies.

As already mentioned, immunoconglutinin has been considered an antibody produced by the host to its own C', possibly C'4, altered as a consequence of interaction with antigen and antibody. While there is much experimental evidence consistent with this hypothesis, an additional suggestion might also be considered.

It has been clearly demonstrated in the author's laboratory by Ishizaka and Ishizaka (1959, 1960) and independently by Christian (1959, 1960a, b) that aggregates of γ-globulin will fix C'. The efficacy of these complexes in the fixation of C' is about the same whether aggregation was induced by specific antigen or by a variety of other treatments such as heat and diazotization. With respect to conglutinin, the possibility may be entertained that the agglutination-enhancing activity in normal bovine serum, which supplies both the red cell antibody and the con-

glutinin, may result from a macroglobulin-type of antibody which forms a physical union with C′ components after reacting with the erythrocytes. Several observations are in accord with this notion. Thus kaolin acts in the same fashion as do sensitized particulate antigens in taking up C′ (Coombs and Coombs, 1953), presumably through the aggregating property of kaolin which may resemble dextran, inulin, etc. in this respect (Osler *et al.*, 1959a, b). Furthermore, bovine serum contains the high-molecular-weight γ-globulin of the 19S variety. Finally bovine conglutinin gains in activity after heat inactivation, a procedure which promotes greater aggregation.

Immunoconglutinin in this view might, therefore, also be descriptive of a substance, somewhat analogous to the rheumatoid factor in its capacity to react with the complex, antigen–antibody–C′1, as described by Taranta and Weiss (1959, 1960). These considerations do not exclude the participation of C′4 as the incitant for the formation of immunoconglutinin. The emphasis placed on C′4 in the studies of Coombs may reflect the specific experimental conditions in which decay of C′2 would be quite rapid and the uptake of C′3 undetectable. In view of the reports concerning the greater stability of some of the C′ components (exclusive of C′2) after incorporation into an immune aggregate (Lamanna, 1957; Hoffmann, 1960), the possibility also exists that immunoconglutinin is an antibody to a complex containing C′, or an aggregate of γ-globulin.

These speculations can be subjected presently to experimental verification with the availability of functionally pure C′ components and chromatographic methods for antibody purification.[2]

IV. The Postulated Enzymatic Nature of C′ Activity

Despite the continued attempts over many years to ascribe the hemolytic action of C′ to one or more enzymatic processes, the available experimental evidence does not yet provide for a unique interpretation. The recent observation by Levine (1955) that the esterase inhibitor, diisopropyl fluorophosphate (DFP) suppresses immune hemolysis lent renewed impetus to investigations of the enzymatic activity of C′. Levine localized the site of action of the inhibitor at the step involving the interaction of C′2 with $EAC'_{1,4}$. Becker attributed the inhibition to interaction with C′1 (1955, 1956a) and concluded that C′1 is an esterase which exists in the serum in precursor form. It is noteworthy that in-

[2] Coombs, Coombs, and Ingram (1961) have summarized their experience with conglutinin and immunoconglutinin in a volume not yet available at the time these comments were written.

hibition by DFP is demonstrable only in the presence of sensitized cells indicating that the action is probably associated with one of the intermediate complexes. The data in Becker (1956a) restricting the interpretation of this effect to C'1 may be subject to alternative explanations and certainly bear repetition now with purified C'1 or with preparations of EAC'_1 free of other components. Preliminary data along these lines confirm the original impression linking DFP with C'1 (Becker, 1960).

Becker has estimated enzymatic activity in fresh serum or component preparations in terms of acid liberation with *p*-toluenesulfonyl-L-arginine methyl ester (TAME) as the substrate (Becker, 1956b). Although this investigator considered it likely that several or all of the components of C' might exhibit proteolytic activity as deduced from inhibition studies (Cushman *et al.*, 1957), the esterase activity of C'1 was explored most intensively because of the finding that EAC'_1 hydrolyzed TAME. These observations do not suffice to identify C'1 with the esterase in view of the following areas of unclarity. When the esterase was eluted from preparations containing EAC'_1 and assayed by techniques other than immune hemolysis, an excellent degree of correspondence was noted in the estimates of enzymatic activity. However, the hemolytic activity measurements did not correlate well with the esterase assays. Further, in assays carried out independently of each other, no correlation could be found between those factors influencing the outcome of the hemolytic reaction (e.g., cell or antibody concentration) and TAME esterase activity (Becker, 1957, 1959a, b). These discrepancies imply that the TAME esterase activity associated with EAC'_1 might result from minute quantities of one of the several serum esterases which had been incorporated into the immune complex. Thus, even if C'1 is a TAME esterase, it must be separated and distinguished from other enzymes normally present in serum and erythrocytes.

Later studies in which esterase activity was induced by peptone or antigen–antibody reactions, brought some of these difficulties into sharper focus (Austen *et al.*, 1958, 1959; Austen and Brocklehurst, 1960; Austen, 1960; Becker *et al.*, 1959). Since the activation of esterase activity did not require the participation of C'1, the thought was expressed that further exploration of the enzymatic nature of C'1 is still required (Austen *et al.*, 1959).

In an independent series of studies, Lepow and his associates (1956a, b, 1958) and Lepow (1959) had also concluded that esterase activity was involved in the reactions requiring C'1. Ratnoff and Lepow (1957) and Lepow *et al.* (1958) have described some of the properties of the esterase eluted from specific precipitates formed in the presence of fresh

serum or after treatment with streptokinase. Present speculation as to the significance of these investigations is, of course, influenced by the multiple number of esterases present in normal serum and by the recent conclusions drawn by Becker, as noted in the foregoing. Hoffmann (1960) also disagrees with the interpretation that C'1 is an esterase or precursor since Lepow found that esterase activity may be eluted under conditions wherein C'1 is absorbed. A new and more direct study of this problem with more highly purified C'1 preparations is indicated. The effect exerted by purified esterase preparations on the hemolytic system should also be studied. A word of caution might be in place with respect to the continued use of streptokinase as a reagent for C' studies. Preparations of streptokinase of "high purity" undoubtedly contain antigenic substances reactive with "normal" sera of man or guinea pig, an inference drawn several years ago by Tillett *et al.* (1955) and Wannamaker (1959). The findings of Pillemer and his associates (1953b, c) that the interaction of streptokinase with C' required Ca^{++} and results in the inactivation of C'1, C'4, and C'2 are consistent with this notion. In this light, the activation of plasmin by streptokinase, and the accompanying inactivation of C' cannot be accepted as adequate evidence for assuming a relationship between C' and plasmin (Lepow *et al.*, 1953, 1954). In fact, Austen has offered evidence which differentiates between plasmin and the esterase activity induced by chloroform, peptone, or antigen–antibody aggregates (Austen, 1960).

The attempt to assign a proteolytic function to one or more of the C' components has met with some failures, e.g., Soulier *et al.* (1956) and Jemski *et al.* (1953). However, the use of 0.15 *M* phosphate buffer in the latter studies renders these results questionable. More recently, Kolmen and associates reinvestigated the problem and concluded that the activation of the fibrinolytic system proceeded quite independently of the serum C' activity (Kolmen *et al.*, 1959). These investigators showed that the specific fixation of C' in immune blood, plasma, or serum resulted in a 75 to 83% reduction in hemolytic levels without induction of any fibrinolytic activity. These workers also confirmed the findings of Becker *et al.* (1959) in showing the independence of the fibrinolytic and C' systems in dog blood.

Ungar and his associates have been among the more enthusiastic sponsors of the notion that anaphylactic reactions involve *in vivo* proteolytic events instigated by C' utilization (Ungar *et al.*, 1953; Ungar and Damgaard, 1951, 1955; Ungar, 1956; Ungar and Hayashi, 1958). The conclusions drawn from these studies are subject to serious interpretational difficulties because of technical and quantitative considerations.

One example revolves about the attempt to show that fibrinolysin activation is most pronounced at equivalence zone ratios of the ovalbumin immune system. From the data given by these workers it would appear that the sera of the immunized guinea pigs contained about 4 mg. of anti-Ea N per milliliter, an unusual level of antibody in a guinea pig which had received but a single injection of 5 mg. of ovalbumin 6 weeks prior to bleeding (Tables II and III in Ungar *et al.*, 1953). These experiments merit repetition with rigorously controlled immunologic techniques and reagents (see also Marrack, 1955).

Geiger (1952a, b) approached this problem through a demonstration of protease activation as a consequence of antigen–antibody interaction. However, the increments in proteolytic activity were not very marked. A further attempt to demonstrate a participation of C′ in this system through the parallel action of inhibitors on C′ and protease activity may be brought into question on several grounds, chiefly because of the implicit assumption that the inhibiting compounds exert their effect only on the specific systems under study.

V. Properdin

One of the more dramatic and controversial developments during the past decade was launched with the reports by Pillemer and his colleagues (1954, 1955; Pillemer, 1956) announcing the discovery of properdin. The attractive simplicity of the hypothesis which attributed a major role to properdin in the poorly defined phenomena of natural resistance quickly drew many adherents. More recently, there has been a noticeable and growing concern as to the very existence of this immunologic Pandora's box. Properdin was originally described as a normal serum protein present in human and animal sera in quantities which were unrelated to prior antigenic stimuli. Properdin was considered to act nonspecifically, and, in conjunction with Mg^{++} and the C′ components, was supposed to mediate such diverse phenomena as the destruction of bacteria, neutralization of viruses, lysis of certain red cells, and the protection against the lethal effects of total body irradiation.

The present status of the properdin problem may be described by evaluating the properties considered to be unique for this substance and a scrutiny of the supporting evidence. By way of a preliminary statement, it may be indicated that properdin titrations are carried out in two steps. A stock reagent is prepared which ostensibly contains all the serum components except properdin, and is called RP in analogy with the C′ component reagents, R1, R2, etc. The RP is prepared by treatment of a

reference serum with zymosan at 17°C., conditions designed for the complete removal of properdin without presumably altering the C′-component levels. In the first step the RP is reacted with the serum or body fluid under study in the presence of zymosan and Mg^{++}, and the mixture is then titrated for residual C′3 after incubation at 37°C. for 1 hour. The properdin unit is defined by this zymosan assay as the smallest volume of serum or body fluid that results in the loss of 120 units of C′3 from 1 ml. of RP. The properties assigned to properdin will now be discussed.

1. Properdin combines with zymosan at 17° C. without causing a significant inactivation of C′3 or other C′ components.

Unfortunately the repeated statements to the effect that the formation of the properdin–zymosan (PZ) complex, does not lead to a loss of C′ components (Pillemer, 1956; Wedgwood and Pillemer, 1959; Isliker, 1959) fail to coincide with the observations in other laboratories. Experiments in the author's laboratory clearly indicate that the addition of zymosan to fresh rat, human, or guinea pig serum is accompanied by the fixation of C′. At 0°C. for 1 hour, some 10–20% of the hemolytic activity is lost without any decrement in C′3 activity. At 17°C. there is a greater loss in hemolytic activity (30–40%) as well as a diminution in C′3 potency. At 37°C. there is a marked loss in the activity of all the components (Osler *et al.*, 1959a). Sewell has provided similar data (Sewell, 1959).

Nelson has studied this aspect of properdin action in greater detail and provided independent evidence that the PZ complex also contained C′1, C′4, and C′2 in varying amounts, depending on the reaction time and temperature (Nelson, 1958). It was pointed out in this study that the practice of expressing experimental results on a percentage basis proved to be misleading, since the uptake of considerable C′1 and C′4 activity by the PZ complex might account for only a small percentage difference particularly under experimental conditions in which relatively undiluted serum is used as a source of C′. The purported specific Mg^{++} requirement for properdin activity was also brought into question in this study and by Leon (1958a, b).

These observations are crucial in the sense that the behavior of the PZ complex can thus be considered completely analogous to that of classic antigen–antibody aggregates. To the extent that the latter findings are confirmed, the distinguishing features of properdin become progressively less apparent.

2. Properdin is a normal serum constituent and differs from antibody in its lack of specificity.

The many attempts to establish or disprove this statement have pro-

vided the most significant data in the prolific properdin literature. On one side the proponents of the properdin theory cite the manifold activities of this system, its presence in germ-free animals, its ubiquity in the sera of normal animals, and the requirement for Mg^{++} as some of the characteristics which differentiate the action of properdin from that of specific antibody. Much of this evidence was summarized by Pillemer and his colleagues (1955) and Pillemer (1956). Some repercussions of this concept are also prominent in the several attempts to establish simpler or more generally applicable properdin assays, as for example those of Kent *et al.* (1957), McNall (1957), Wardlaw and Pillemer (1956, 1959; Wardlaw *et al.*, 1958), Barlow *et al.* (1958a, b), and Leon (1956b, 1957a, b). However, the evidence for the lack of specificity of properdin action has been seriously challenged.

One of the first indications of specificity in properdin action may be drawn from the studies of Landy and Pillemer (1956a, b) who noted that a single injection of bacterial lipopolysaccharide, at microgram levels, induced a rise in properdin levels and an increased resistance to infection. Even within the limits of the large experimental error of the two assay procedures, an element of specificity could be discerned and was so noted by the investigators.

At least two other possibilities must be excluded in the interpretation of these findings before the prompt and dramatic increase in resistance can be ascribed to the action of properdin. The first concerns the observations that a single injection of bacterial lipopolysaccharides may stimulate the phagocytic capacity of the reticulo-endothelial system to a degree commensurate with the increased resistance to the Gram-negative pathogens. Recent evidence along these lines has been offered by Biozzi *et al.* (1955), Boehme and Dubos (1958) and Boehme and colleagues (1959), and Benacerraf *et al.* (1959c). The publications of Howard and Wardlaw (1958) and of Howard *et al.* (1958) also emphasize the role of the phagocytic mechanism and point out that while most of the polysaccharides enhance a nonspecific immunity, only some of these lead to higher properdin levels. Subsequently, Rowley concluded that the nonspecific immunity which had been observed could be attributed to greater opsonic capacity of the serum as well as to augmented phagocytic efficiency (Rowley, 1956, 1960). Further evidence is provided by the recent observations by Cutler (1960, 1961) that injections of zymosan enhance antibody production and erythrophagocytosis in the rat.

Still another interpretation has been suggested as a substitute for the properdin hypothesis. This mechanism explains properdin activity on the basis of the combined action of the C′ components with low levels of

antibody, present in varying quantities in the sera of different individuals and species. The activities of this antibody–C′ complex would be manifested under the Mg^{++}, temperature and ionic strength requirements initially set forth as criteria for properdin (Nelson, 1958).

Neter *et al.* (1959), Milgrom and Swiercynska (1955, 1959), Gaines and Landy (1955), Muschel (1960), and Muschel *et al.* (1958) have shown that human sera or γ-globulin preparations contain specific antibody for a wide variety of bacterial antigens including many of the Gram-negative organisms, observations that barely need restatement. As to the extent of possible cross reactions with polysaccharide antigens, it may suffice to recall the current investigations of Heidelberger (1960) and Heidelberger and Cordoba (1956). Further, Gustafsson and Laurell (1960) observed that the properdin content of sera from germ-free rats was lower than that in control animals reared in a conventional environment. They concluded that the level of properdin, like that of γ-globulin was influenced by the normal bacterial flora. Similarly, we have observed a marked variation in the capacity of individual guinea pig sera to fix C′ with Types III and VIII pneumococcal polysaccharides (S). Some of the sera reacted only with S III, others only with S VIII and still others with neither or with both of the capsular carbohydrates. Along these lines may be mentioned Blattberg's clear demonstration, which has been confirmed by Turk (1959b), that the injection of zymosan into rabbits and guinea pigs increases the serum bactericidal titer to *E. coli* (Blattberg, 1956); that the composition of zymosan includes glucans in β-1, 3- and β-1, 6-linkages, mannans, and glucosamine (DiCarlo and Fiore, 1958); and that yeast glucans are capable of combining with properdin (Pillemer *et al.*, 1955). In this light Nelson's finding that some human and animal sera agglutinate zymosan granules is entirely plausible (Nelson, 1958) despite the failure to find this agglutinin in other sera (Lepow *et al.*, 1959). Turk (1959b) has adduced further support in the demonstration that absorption of guinea pig sera with a variety of bacterial antigens, zymosan or polysaccharides such as starch, levan, inulin, and xylan, lowered the immune adherence and bactericidal titers to the homologous as well as to apparently unrelated organisms. There are also the data of Comes (1957) and of Muschel *et al.* (1958) who were able to demonstrate a marked specificity in the bactericidal action of normal sera to specific antigenic constituents of S. *typhi* and *Shigella flexneri*. The latter studies were extended by Osawa and Muschel (1960) in the demonstration that the invocation of a properdin system is inadequate to explain the bactericidal action of serum. The data in Table III taken from this report, summarize the type of evidence which is most difficult to interpret on

TABLE III

DISCREPANCIES IN BACTERICIDAL AND PROPERDIN TITERS OF RABBIT AND HUMAN SERA TESTED UNDER DIFFERENT EXPERIMENTAL CONDITIONS

Experiment	Serum	Treatment	50% Bactericidal titer	Agglutinin titer for Zymosan	Agglutinin titer for *Shigella dysenteriae*	Properdin titer (units/ml.)	Lysozyme content (μg./ml.)
A	Preimmunization, rabbit	None	227	—	10	32	—
B	Preimmunization, rabbit	56°C.—30 min.	204	—	10	Not done	—
C	Postimmunization (with *Shigella dysenteriae*)	None	19,000	—	1280	96	—
D	Postimmunization (with *Shigella dysenteriae*)	56°C.—30 min.	18,000	—	1280	6	—
E	Preimmunization, rabbit	—	69	40	2	32	3.4
F	Postimmunization (with zymosan)	—	294	1280	2	96	23.2
G	Normal human serum	None	248	—	—	8	—
H	Normal human serum	56°C.—30 min.	137	—	—	1	—
I	Normal human serum	Absorbed with zymosan	156	—	—	1	—

the basis of properdin activity. Note must also be taken of the fact that many sera may contain lysozyme, a factor which may be of some importance in this type of study. Reports by Inoue *et al.* (1959a), by Muschel *et al.* (1959), by Carey *et al.* (1960), by Hook and Muschel (1959), and by Hook *et al.* (1960) depict the role of lysozyme as one of the factors in the lysis of various smooth type strains of Gram-negative bacteria by antibody and C′.

The inferences are clear that bactericidal and properdin activities in the same serum are quite independent of each other (experiments C, D and A, E). The former may be more heat-labile in some "normal" sera than in that of a hyperimmunized rabbit (experiments A, B and G, H). In contrast to an earlier report of Wardlaw and Pillemer (1956), the presence of antibody at these levels does not impede the action of properdin. Further, absorption with zymosan leads to greater and preferential loss of properdin than of bactericidal activity (experiments G, I). Other data in this report show that absorption with dysentery organisms reduces the specific bactericidal properties without alteration of the properdin levels.

Similar conclusions as to the immunological specificity associated with properdin action emerge from studies with bacteriophage. The inactivation of this virus by normal human serum has been proposed as a more reproducible properdin assay by Barlow *et al.* (1958a, b). Cowan then showed that this assay might lack validity in the sense that the phage neutralization titers for different types of bacteriophage could not be ranked in the same order for all sera (Cowan, 1958). Nor was there agreement in comparisons of properdin titers as determined by the zymosan and bacteriophage assays (Baltch *et al.*, 1960). Laurell and Reyn (1959a, b) and Toussaint and Muschel (1959) supported these conclusions in the demonstration that phage neutralization and properdin titers were not related to each other and the reduction of neutralizing activity from 16 or 23 units to less than 1 by specific absorption was not accompanied by parallel decrements in properdin activity.

It is quite clear that these considerations as well as the analogies drawn in Nelson's study (1958) create serious doubt as to the very existence of a single substance called properdin endowed with a myriad of immunological properties. The manifold claims for the diverse manifestations attributed to properdin can be considered as reflections of the extensive immunological reactivities resulting from many different antibodies present at low levels in "normal" sera. This point of view has now been adopted in the sense that antibody is considered as a requirement for properdin activity (Wedgwood, 1960), and that the

most recent studies on the Donath-Landsteiner reaction do not involve a properdin requirement (Hinz *et al.*, 1961). In addition, activation of the reticulo-endothelial system by other, as yet ill-defined, mechanisms must also be entertained as a factor in the interpretation of the phenomena. Some indication of the latter is given by Stauch and Johnson (1959). However, successful attempts at purification of properdin (Todd *et al.*, 1959 and Lepow *et al.*, 1959) which will lead to the demonstration of a single substance capable of mediating the many processes supposedly due to this substance, will serve to dispel the present uncertainties as to its existence.

VI. Complement and Normal Physiological Processes

Many studies centering on the role of C′ in disease phenomena have been undertaken despite an inadequate understanding of the normal physiological aspects of the C′ system. Thus, elementary information as to the levels of hemolytic activity ascribable to C′ in tissues and body fluids other than serum is surprisingly sparse. Dr. Elizabeth Maltaner has offered suggestive evidence as to the possible presence of C′4 in guinea pig leucocytes (Maltaner, 1935) and Heidelberger and Muller (1949) demonstrated that cerebrospinal fluid may contain low levels of hemolytic activity associated with the four components of C′ in situations compatible with normal permeability relationships of the meningo-vascular barrier.

According to Torok and Szemere (1959), hemolytic C′ levels are higher during the first 6 months of life than in the second half of the first year. Ellis and Walton (1957) found no age dependency in older subjects partly in agreement with the findings of Hartmann (1958) who reported similar activity levels in age groups 8–21 and 22–35. In the group aged 35–60 years the C′ levels were approximately 30% higher than in the younger groups. Rice and Duhamel (1957) studied the activity levels of C′, conglutinin, and hemolytic antibody in calves during the first year of life. The C′ levels changed little if at all during this period. However, other studies have shown that the sera of young guinea pigs had lower C′ activity than that of older animals. Serum C′ levels of guinea pigs bred and raised in a germ-free environment fall within the same range as normal animals (Newton *et al.*, 1960; Wagner, 1959; Toussaint and Muschel, 1959).

A number of interesting findings that bear repetition with current techniques for component estimations should be recorded. Thus Ecker and co-workers (1947) have reported significantly lower levels of hemo-

lytic activity in the sera of pregnant women, a finding possibly related to the recent report of Gordon and Benditt (1955). In the latter studies hemolytic C′ levels dropped from a mean value of 10.8 ± 2.8 to 3.4 ± 0.6 in a group of rabbits receiving bovine plasma proteins with concurrent injections of cortisone. Growth hormone apparently doubled C′ levels in the absence of immunization. The hormonal control of C′ is also indicated in a report by Ludany *et al.* (1955, 1956) who found a marked decrease in the sera of humans injected with 1 mg. of adrenaline, and in dogs following noradrenaline.

Seasonal variations have been reported by Rice and Annau (1957). They found that guinea pig C′ levels are lower during the months of November and December, although there are also data which indicate that guinea pigs acclimated to low temperatures develop higher levels of hemolytic and phagocytic potencies in their sera (Szemere *et al.*, 1958a, b, c). Perlick (1954, 1955) found a transient increase in hemolytic C′ (20–55%) in dogs following vagotomy. In none of these studies was hemoconcentration or dilution considered although Rice found that total protein, albumin, globulin levels could not be correlated with estimations of C′ (Rice, 1954). Rice and Boulanger (1951) found no change in C′ titers of guinea pigs kept in a dark atmosphere for 5 months.

In contrast to these sporadic reports there have been recurrent efforts over a period of many years to establish a common mechanism for C′ activity and blood coagulation. The link supposedly established between these two phenomena by Felix *et al.* (1949), Maltaner (1948), Mann and Hurn (1948), Maltaner and De Almeida (1949), Marx *et al.* (1949), and Diacono *et al.* (1953) in terms of cation requirements and simultaneous inhibition by common reagents was not based on vigorously controlled experiments.

Rice and Boulanger (1950), Boulanger and Rice (1951), Boulanger *et al.* (1953a, b, 1954) and Rice *et al.* (1951a, b, 1952a, b, c, 1953a, b) and Rice and Duhamel (1955) described parallel changes in hemolytic activities and blood coagulation as a consequence of the following manipulations: variations in dietary vitamin C and protein, and injections of dicumarol, carbon tetrachloride, chloroform, gum acacia, or ethionine. When the treatment was sufficiently intense so as to impair protein synthesis and lead to degenerative liver changes, diminished C′ levels were often accompanied by prolongation of clotting time. Many of these findings are summarized in Rice (1954) and Rice *et al.* (1958). The effect of parenchymal liver damage in lowering C′ levels has been confirmed by Asherson (1960).

Büsing and Beller (1955) and later Büsing (1957) concluded that since

anticoagulants like heparin are also anticomplementary, the coagulation process and the C′ system may reflect the operation of a single mechanism. This conclusion is diametrically opposed to that reached by Sharp (1957) who found no significant differences between C′ levels in normal human sera and in those obtained from cases with severe hemophilia, Christmas disease, or deficiencies in Factors V and VII.

Further pursuit of this problem would not appear promising at this time unless investigations were confined to the individual reaction steps in both processes, and preferably with purified reagents—a prospect offering no immediate promise of achievement in both of these complex situations.

VII. The Influence of Various Disease Processes on Serum C′ Levels

Most of the reports to be discussed in this section have been oriented toward estimations of serum C′ levels in various pathologic situations, in the hope that information of diagnostic or pathogenetic significance might emerge.

A. Rheumatic Fever

In this disease C′ levels have been reported as depressed, unchanged, or elevated (Fischel, 1949; Fischel *et al.*, 1949). However a degree of clarity was attained when closer attention was paid to the stage of the rheumatic process. Fischel and Gajdusek (1952) and Fischel and Pauli (1949) reported the rather surprising finding that hemolytic activity levels were elevated during the acute phase of this disease. Further investigations confirmed this finding (Fischel *et al.*, 1958) not only with respect to rheumatic fever but also to the acute phases of myocardial infarction and other inflammatory reactions (Fischel, 1953, 1957b; Boltax and Fischel, 1956). Therapeutic use of cortisone or salicylates is associated with a decline of C′ levels (Fischel *et al.*, 1958). Careful component assays in these situations should prove fruitful.

The finding of elevated levels of C′ activity as a manifestation of an acute phase response may be of considerable interest as an auxiliary diagnostic aid. Thus, Simonsen has reported elevated C′ titers in dogs following homotransplantation of kidney, nephrectomy, splenectomy, etc. (Simonsen, 1953), confirming Jordan's finding in humans after splenectomy (Jordan, 1950). By way of contrast, the decline in hemolytic activity noted in other situations would appear to assume a more specific significance.

B. Rheumatoid Arthritis

With reference to the objectives of the present review, the chief interest as to the possible involvement of C′ in rheumatoid arthritis is derived from the presence of macro γ-globulin complexes in the sera of patients with this disease. As is known, the reaction of these aggregates with human or other γ-globulins forms the basis for some of the diagnostic procedures in this area. The central question relates to the mechanism of formation of these macroglobulins and their mode of interaction with other substances. Since there is ample evidence that aggregates of γ-globulin react with C′, the demonstration that components of C′ have been found in association with the rheumatoid factor comes as no surprise. Taranta and Weiss (1959, 1960), Taranta *et al.* (1961) and Müller-Eberhard and Kunkel (1960, 1961) have provided the experimental evidence for this possibility. The latter group has shown that fresh normal human serum contains a substance compatible with the properties assigned to C′1, which will precipitate aggregated human γ-globulin. Schubart (1959) demonstrated that C′1 might inhibit agglutination of latex particles coated with γ-globulin by the rheumatoid factor. The inhibiting substance was detected in sera from normal or rheumatoid arthritis patients and also conformed to C′1 in that it was heat-labile, present in mid-piece preparations, and could be absorbed by antigen–antibody precipitates. Preferential destruction of C′3 and C′4 did not influence the inhibitory properties in any marked fashion. The available data are in accord with the notion that inhibition in this instance may be due to the presence of an excess of this substance. It is to be noted that these findings are at variance with those of Brine *et al.* (1958) who considered that C′2 and C′4 were responsible for the inhibition of this reaction. Hobson and Gorrill (1952) claim that C′4 is required in the agglutination tests for this disease but this is disputed by Laurell and Grubb (1958). Laurell (1959) has reported that rheumatoid sera contain a factor which interfered with the zymosan assay of properdin. These data provide no assurance that the interaction of the rheumatoid factor with aggregated γ-globulin is mediated by the C′ system or one or more of the C′ components. If, as stated by Grubb (1958), Kunkel (1958), and others, the rheumatoid factor may be considered as an autoantibody to γ-globulin, the incorporation of C′ might simply represent an incidental event.

Opinions vary as to levels of C′ during the manifestations of the disease. Laurell and Grubb (1958) observed no change, a finding not shared by most other workers. Vaughan and his co-workers were among the first to demonstrate an increase in the hemolytic activity of sera from

patients with this disease (Vaughan *et al.*, 1951a, b) as also did Williams and Law (1958) who attempted to correlate the increase with severity of the disease. The recent data of Ellis and Felix-Davies (1959) also show an increase in hemolytic C′ and C′1 levels as measured by the gel diffusion technique of Ellis and Gell (1958).

The significance of an increased C′ activity in early rheumatoid arthritis is still a speculative matter. It would be of interest to determine whether the increase is due solely to C′1 or whether one of the subsequent reactions might not be involved as well.

C. Systemic Lupus Erythematosus (S.L.E.)

The general agreement about C′ levels in rheumatoid arthritis is duplicated in the reports of diminished hemolytic activity in S.L.E. Recent documentation for this statement may be found in reports by Elliott and Mathieson (1953), Williams and Law (1958), Ellis and Felix-Davies (1959), Asherson (1960), and Lange *et al.* (1960).

Since the findings of lowered C′ levels imply that the rate of destruction or utilization may exceed the synthetic process, it is of interest to record that Formijne and Van Soeren (1958) as well as Seligmann and Robineaux (1958) reported that the formation of the lupus cell requires the participation of fresh human or guinea pig serum. Evidence that the accessory factor in the fresh serum is C′ is meager, as is also the contrary point of view proposed by Aisenberg (1959). Townes *et al.* (1960) have shown serum C′ levels in S.L.E. are lowered during the active disease process and return to normal levels coincident with steroid therapy and remission of symptoms.

D. Renal Disease

A diminution of serum C′ levels in association with acute glomerulonephritis has been recorded by Fischel (1957a), Fischel and Gajdusek (1952), Hoene (1952), Wedgwood and Janeway (1953), and in numerous papers by Lange and co-workers (1951a, b, 1955, 1956, 1957) and Lange and Wenk (1954a, b), as well as by Ellis and Walton (1957, 1958) who also review earlier studies in this field. All investigators are agreed that C′ titers are lowest during the early active stages of acute nephritis, that a tendency to revert to normal levels characterizes the subacute phase of the disease and clinical manifestations of recovery. Low levels were also reported for the nephrotic syndrome by Hoene (1952), by Asherson (1960), and by Lange *et al.* (1951a, b, 1955) but Ellis and Walton conclude that this type of renal damage does not affect C′ levels, all other symptoms being equal.

Lange and his colleagues (1955, 1956, 1957) showed that the use of steroid therapy in the nephrotic syndrome is associated with increased hemolytic activity of the serum. Lange and Wenk (1954b) as did Ellis and Walton (1958) attribute the early, low C′ levels to a deficiency in C′2 and C′4. This is of some interest in the light of recent observations by Beeson and Rowley (1959) who compared the anticomplementary properties of various tissue extracts by means of bactericidal and hemolytic assay techniques. The interesting inference was drawn that the accumulation of ammonia in the renal medulla through glutaminase action rendered this portion of the kidney susceptible to infection through destruction of C′4 and consequent suppression of host defense mechanisms. Although pathologic studies are consistent with this hypothesis, some of the technical aspects of the C′ assays require more rigorous confirmation. Particular reference is made to the statement that C′ titrations in the presence of 0.005 *M* Mg^{++} were unchanged when 0.15 *M* PO_4 was added to activate the glutaminase enzyme. Moreover, the quantitative relationships between C′4 destruction under these conditions and the levels of C′ activity required to destroy the small numbers of bacteria that might initiate an infection require additional study. If confirmed, this finding offers a dramatic and relatively rare demonstration of the *in vivo* action of C′. Klein and Burkholder (1959) report the presence of C′ in the walls of the glomerular capillaries in rat kidneys after injection of nephrotoxic serum, but the identification of the incorporated material as C′ also merits confirmation. Freedman and Markowitz (1959) consider that C′ may potentiate the affinity of antibody globulin for specific kidney tissue thereby enhancing tissue damage. This mode of action of C′ may be of some importance as illustrated by the detection of streptococcal antigens in the glomeruli of human kidneys removed from patients with glomerulonephritis (Seegal *et al.*, 1959). Nastuk *et al.* (1960) reported unusually low as well as high C′ levels in sera obtained from patients with myasthenia gravis. In an accompanying study, Strauss and co-workers (1960) detected a plasma protein in the sera of patients with this disease which bound to muscle and fixed guinea pig C′.

E. Nutritional Disturbances

Wertman *et al.* (1954) and Axelrod and Pruzansky (1955) reported diminished C′ activity in rats rendered deficient in niacin and tryptophan, and in pyridoxine and riboflavin, respectively. Similarly, Trnka (1956) found lowered C′ levels in rats maintained for 4 months on a protein-deficient diet. The role of an adequate diet in maintaining normal C′

levels has also been stressed by Fekete *et al.* (1957). The exact significance of these data is not readily apparent, except that the depressions in C′ activity may reflect a more generalized disturbance of protein synthesis.

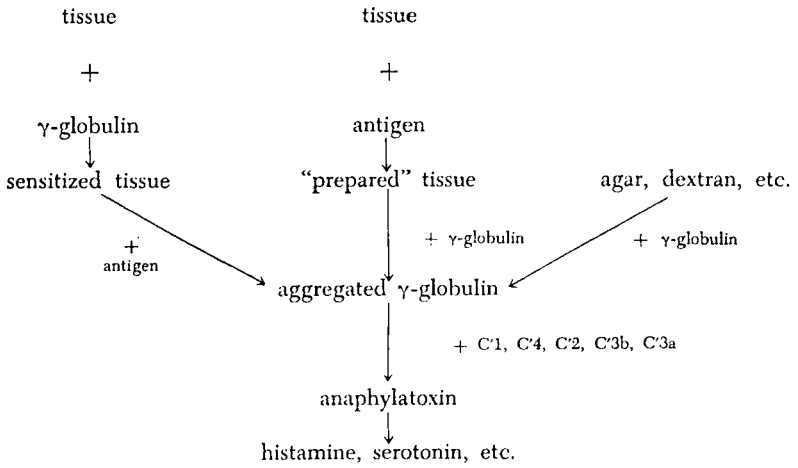
Rice (1954) and Rice *et al.* (1958) found that antibody production and C′ activity could be influenced independently in guinea pigs with fatty degeneration of the liver as a consequence of repeated administration of ethionine or carbon tetrachloride. Injections of gum acacia lowered both antibody and C′. Rabbits did not always respond in the same fashion as did guinea pigs (Rice, 1954). The effect of carbon tetrachloride in lowering guinea pig C′ levels has been confirmed by Rangam *et al.* (1956, 1957) and by Formal *et al.* (1959), but not by Dulaney *et al.* (1948) in humans, although lower values in patients with naturally acquired or induced malaria were recorded in the latter study. Further relationships between liver damage and C′ functions are described by Rice and her colleagues (1952c, 1955a, b), Rice and Duhamel (1955), and Boulanger *et al.* (1953a, b).

F. Miscellany

Following the observation of Boursnell *et al.* (1946) and Watkins and Wormall (1948) that nitrogen mustard had a fairly potent anticomplementary action *in vitro*, these authors (Watkins and Wormall, 1952) and Southam and Goldsmith (1951) failed to observe similar effects upon injection of methylating agents into guinea pigs or humans. It may be presumed that these reagents were consumed in reactions with other plasma or tissue proteins present in much greater quantities than those associated with the C′ system. A summary of alterations in C′ as associated with various diseases is given by Bruckel *et al.* (1957). These authors as well as Jonsen and Kass (1957) and Good (1959) noted no remarkable changes in levels of C′ or its components in patients with agammaglobulinemia. Jordan (1950) reported a correlation between the presence of enlarged spleens and an anticomplementary property of serum from bone marrow in a varied group of diseases. Gernand and Schabinski (1957) reported that prolonged administration of various antibiotic agents leads to a destruction of *E. coli* in the intestinal tract, a diminution of vitamin K, and a marked drop in C′ levels resulting from the vitamin deficiency.

VIII. The Participation of C′ in Manifestations of Hypersensitivity Reactions of the Immediate Type

This section of the discussion will evaluate the evidence bearing on the validity of the following sequence of events as they concern the role of C′ in the immediate types of allergic phenomena.



Before developing the evidence, several preliminary comments may be appropriate. The first is to record a changing orientation toward the concept of allergy. Conventionally, the phenomena encompassed in this term emphasize the disproportionate and dramatic nature of the tissue response to repeated antigenic stimulation, as witness the terms hypergy, hypersensitivity, and allergy. Moreover, much of the present body of data in this area is concerned with the nature and mode of action of the elusive reagin or skin-sensitizing antibody. Within the recent past increasing attention has been given to the varied and cumulative manifestations of tissue damage that may result from recurrent interaction of antigenic substances with the precipitating type of antibody, demonstrable by the usual *in vitro* techniques. Inferences that a similar mechanism is operative in the hay fever type of allergy remain highly speculative.

Secondly, the action of C′ is known almost entirely through studies of its hemolytic functions. In consequence, all judgments relating to its identification must refer back to the lysis of sensitized erythrocytes. In view of this, the validity of correlating *in vitro* with *in vivo* events and the justification for extrapolating from one situation to another must be subjected to critical scrutiny. Moreover, this type of

correlative data cannot be construed as definitive evidence. These considerations dictate the need for a multifaceted approach to the problem of C′ involvement in anaphylactic phenomena so that sufficient data may be accumulated to provide a valid basis for a working hypothesis.

Finally, the discussion of the scheme outlined on page 181 will not consider the cytotoxic action of the Forssman antigen–antibody system. The *in vivo* toxicity of this antibody acting in conjunction with C′, for mammalian and avian tissues containing the Forssman-type antigen, has been well documented and widely accepted (Redfern, 1926; Witebsky and Neter, 1935; Bier and Seiler, 1936). Spear also adduced convincing data in the demonstration that desensitization of the guinea pig by repeated injections of Forssman antibody, simultaneously rendered the animals resistant to the toxic effect of the antibody and depressed C′ activity to very low levels (Spear, 1955). No attempt was made to determine whether the resistance of the guinea pigs was attributable to lack of antigen, lack of C′, or a combination of the two.

A. Fixation of Antibody or Antigen to Tissues

Although the phenomenon of antibody fixation is well documented, the mechanism of interaction between the antibody and the tissue is but poorly understood. With respect to the C′ question, it may be noted that antibody derived from bovine, equine, ovine, and avian sources does not fix well, if at all, to guinea pig tissues, although horse antibody is retained by the rat skin for several hours. Possibly unrelated to this fact is the accompanying observation that anticarbohydrate antibody in the horse and antibody derived from cattle or sheep also fix guinea pig and rat C′ very poorly. The suggestion may be offered that the failure of these γ-globulins to fix to guinea pig tissues may be traced to their high-molecular-weight characteristics as indicated recently by Ovary *et al.* (1960). The dynamics of the retention process are also quite obscure except for the observation that following passive transfer, human reaginic antibody is retained in the human skin for a longer time than is the so-called heat-stable blocking antibody. Ovary and Bier have provided some data for rabbit antibody in the guinea pig, rat, and mouse skin (reviewed in Ovary, 1958). The findings of Halpern *et al.* (1959) and of Nielsen *et al.* (1959) that rabbit antibody fixed to guinea pig ileum may be detected after many hours and multiple washings also indicate a firm retention by the tissues. Furthermore, there are scattered reports to the effect that a variety of protein and polysaccharide antigens may also be fixed to tissues in sufficient concentration to induce anaphylactic phenomena. Although the fixation of antigen to the tissues

may not be as important or consistent a finding as antibody fixation, it may, nevertheless, help clarify certain puzzling phenomena, such as the ability of horse antibody to evoke Arthus reactions in the guinea pig. It is also pertinent to mention that Seegal *et al.* (1959) and Kaplan *et al.* (1961) have offered evidence, based on immunofluorescent studies, for the presence of antigens in human tissues, reactive with bacterial blood group and Wassermann antibodies, as well as in autoimmune situations (Kaplan and Dallenbach, 1961).

B. Aggregation of γ-Globulin, C′ Fixation, and Anaphylactic Reactions

1. Induction of Aggregation by Antigen

a. In Passive Cutaneous Anaphylaxis (PCA). The correspondence between the degree of aggregate formation by antigen and antibody and the efficiency of C′ fixation has long been established (evidence reviewed in Osler, 1958). With the development of improved technical procedures, the parallelisms between the aggregating capacity of the antibody and the efficiency of C′ utilization were placed on a firmer and more rational basis. Supporting evidence for this relationship was drawn from quantitative C′-fixation studies with different antigen–antibody ratios (Osler *et al.*, 1948; and Osler and Knipp, 1957a, b); from comparisons of homologous and cross-reacting immune systems (Osler and Heidelberger, 1948a, b); in comparative studies of the C′-fixing potencies of different immune sera (Wallace *et al.*, 1950); and in the demonstration of the varying reactivities of antibody with a single specificity in individual antisera (Osler and Hill, 1955; Hill and Osler, 1955). When allergic reactions were investigated similar relationships were observed between the ability of the antibody to aggregate and induce the tissue response.

In a study of PCA the writer and his colleagues (Osler *et al.*, 1957) concluded that the intensity of this anaphylactic reaction as well as that of C′ fixation could be influenced by the same parameters, namely, level of antigen, quantity of antibody, and the amount of C′ available for the reaction. Rats rendered deficient in circulating serum C′ activity showed diminished cutaneous reactions which were partly restored by supplementation with guinea pig or rat sera containing among other components, C′3. The skin responses in normal rats were also augmented by the same reagents. The concurrent variations in C′ utilization and PCA were applicable to cross-reacting systems and to the interactions of ribonuclease with homologous rabbit antiserum. Acetylation of the enzyme used as antigen led to suppression of C′ fixation and of PCA reac-

tions, despite the demonstration that the acetylated molecule is fully reactive with antibody to the native enzyme.

b. In Reverse PCA. Ovary (1960a, b) has reserved judgment as to the essential nature of the C′ system in PCA reactions of the guinea pig. The basis for this position emerges from his observations on the lack of parallelism between the ability of certain human, chicken, and horse antisera to rabbit γ-globulin to provoke reverse PCA (RPCA) reactions in the guinea pig and the relative inability of these immune reactants to fix guinea pig C′ in the test tube. The technique for eliciting RPCA reactions involves intradermal inoculation of the antigen (in this instance, rabbit γ-globulin) and intravenous injection of the antiserum plus Evans blue several hours later.

In one set of experiments, Ovary used 20 μg. of chicken antibody N for the animal experiments and found that half of this amount of antibody fixed little or no C′ on admixture with equivalent levels of antigen in the presence of 100 $C'H_{50}$ units, in a reaction volume of 10 ml. This apparent lack of correlation between PCA and C′ utilization was reinvestigated under slightly altered experimental conditions, designed to simulate more closely the conditions that might obtain in the animal body (Osler *et al.*, 1960).

Three observations were noted in these experiments with one of the chicken antisera kindly supplied by Dr. Ovary.

1. The unabsorbed serum, upon addition to undiluted guinea pig serum was highly anticomplementary, accounting for the loss of about 30% of the total hemolytic activity of the guinea pig C′. After absorption with guinea pig serum, the anticomplementary activity was noticeably reduced and slight fixation of C′ could then be observed. Estimates of C′3 utilization have not yet been undertaken and may prove critical in the resolution of this problem, as shown in Osler *et al.* (1959b).

2. The chicken antisera to rabbit γ-globulin gave substantial precipitin reactions with whole guinea pig serum. Following multiple absorptions of the chicken antiserum, the precipitable antibody content for the homologous antigen rabbit γ-globulin was decreased to about 60% of the initial level. It will be necessary to evaluate the role of this cross reaction in the interaction with C′ and in the induction of PCA.

3. The chicken antiserum was highly reactive with sheep erythrocytes. In one case, 14 successive absorptions were required to eliminate the sheep cell agglutinins. The implications of this observation with respect to immediate allergic reactions may be of great significance in view of the reports by Tanaka and Leduc (1956) and by Hawes and Coombs (1960) that Forssman antigen is present in the guinea pig vas-

cular endothelium. The recent demonstration of a macroglobulin in normal chicken sera, which is coprecipitated by the interaction of antibody and antigen (Makinodan *et al.*, 1960), provides still another factor whose role in C′ fixation and RPCA requires elucidation.

c. In Anaphylatoxin Formation. The initial studies on anaphylatoxin (summarized in Friedberger and Weissfeiler, 1933; see also Hahn and Giertz, 1960) and their recent continuation (Osler *et al.*, 1959a, b) established that the fixation of C′ in the presence of fresh rat serum may result in the formation of a substance, anaphylatoxin, capable of enhancing capillary permeability and contracting smooth muscle.

With respect to aggregation, a clear demonstration was provided that inhibition of anaphylatoxin formation and of C′ fixation was associated with inhibition of precipitation by antigen in large excess. The C′ requirement for anaphylatoxin was demonstrated in several different ways. At 0°C., C′1, C′4, and C′2 can be removed from rat serum without producing anaphylatoxin. At 37°C., the extent of anaphylatoxic activity and of C′3 utilization closely parallel each other. The presence of chelating agents suppresses C′ fixation and anaphylatoxin formation. Both are restored upon the addition of Ca^{++} and Mg^{++}. Phlorizin, an inhibitor of C′3 interaction in immune hemolysis (Mills and Levine, 1959; Rodriguez and Osler, 1958, 1960) also suppresses anaphylatoxin formation. Salicylaldoxime, another inhibitor of C′3 (Mills and Levine, 1959) as well as phlorizin reduced fatal reactions in passively sensitized guinea pigs from 73% in 30 controls to approximately 30% in 40 animals pretreated with these inhibitors (Mills *et al.*, 1959).

Becker reported that DFP, an inhibitor of C′1, did not suppress anaphylatoxin production in rat plasma treated with agar (Becker, 1959a). The available data are not sufficiently extensive to provide for an explanation of this finding. If, for example, inhibition of C′1 was incomplete, further progression of the reaction could still occur. Caution must also be taken in this type of experiment to remove all traces of agar, since these may continue to generate anaphylatoxin. Lastly, the report does not indicate the quantitative aspects of the C′1 inhibition and of anaphylatoxin production.

d. In Relation to the Mode of Action of Soluble Complexes. The resurgence of interest in the role of soluble complexes in the serum sickness type of hypersensitivity reactions was initiated by the findings of Germuth and McKinnon (1957). These authors reported that the intravenous injections of supernates of precipitin reactions prepared in the region of antigen excess caused anaphylactic symptoms and death in guinea pigs. In common with the experiments of Friedberger and those of Braune

(1955), fairly large quantities of soluble complexes were required, and it is, therefore, plausible to attribute these reactions to the *in vivo* generation of anaphylatoxin. However, soluble complexes were soon shown to induce diverse other effects. Tokuda and Weiser confirmed the foregoing findings in mice (1958a, b); while Ishizaka and Campbell (1958, 1959) and Rosenberg *et al.* (1958) demonstrated the capacity of these complexes to enhance capillary permeability in the guinea pig skin. Trapani *et al.* (1958) found that the stimulating action of these complexes on smooth muscle contraction was limited to aggregates formed in the region of moderate but not marked antigen excess, while McCluskey and coworkers (1960) and Benacerraf *et al.* (1960) added the important observation that complexes of various specificities produced lesions of glomerulonephritis in mice and rats. Much larger concentrations of soluble complexes could also produce cutaneous reactions in rabbits (Cochrane and Weigle, 1958).

The mode of action of these complexes has been studied in some detail. With respect to serum sickness in rabbits, the data and conclusions presented by Weigle (1958) and by Weigle and Dixon (1958) are somewhat equivocal as they pertain to the role of C′, but the studies of the Ishizakas in Dr. Campbell's laboratory are more definitive. They observed that the permeability enhancing property of the complexes is markedly dependent on two factors, the species origin of the antibody (Ishizaka *et al.*, 1959a) and the molecular ratio of antigen and antibody in the complex (Ishizaka *et al.*, 1959b). Horse and chicken antibody complexes did not produce cutaneous effects in the guinea pig because of the lack of tissue fixation by these antibodies, nor did complexes at the limit ratio of Ag_2Ab. In these respects as well as in their quantitative expressions, a consistently positive correlation was observed between the C′-fixing capacities of the immune aggregates and their ability to augment capillary permeability. Experimental verification was thus provided for an earlier prediction that soluble complexes prepared in the region of moderate antigen excess can fix C′ (Osler and Heidelberger, 1948a). Of further pertinence to the role of C′ in the biological activity of soluble complexes may be the findings that their ability to enhance capillary permeability is suppressed by phlorizin and also in rats rendered deficient in serum C′ (Ishizaka and Osler, 1959).

2. *Induction of γ-Globulin Aggregation by Other Means*

Immunologists have long been aware of the anticomplementary properties of γ-globulin, especially since the reports by Davis *et al.* (1944, 1945), and the extensive studies of Nørgaard (1950a, b, 1954a, b, 1955a,

b, 1956). Other reports such as that by Pitner and Smith (1952) have commented on the highly significant correlation between elevated γ-globulin levels and lowered serum C′. Even earlier, Jersild concluded, perhaps too broadly, that the demonstration of anticomplementary activity in a patient's serum could be regarded as a diagnostic sign of multiple myeloma (Jersild, 1936, 1939). These workers (Jersild and Pedersen, 1938) reported, as did Olhagen (1945) and Nørgaard (1955a), that heating at temperatures approaching 60°C. markedly enhanced the ability of sera to bind C′ in the absence of added antigen. Further, Nørgaard (1955a) associated this property of the sera with the γ-globulin component. Similar inferences had also been drawn by Engboek and Simonsen (1953) although Cavallo and Plescia (1958) also localized anticomplementary activity with the β-globulins.

Considerable clarification of this question has been achieved in the last 2 years through the experiments conducted by the Ishizakas (1959, 1960) in the author's laboratory and by the studies of Christian (1959, 1960a, b). The former showed that, indeed, the addition of antigen was not essential for inducing aggregates of γ-globulin endowed with the capacity to fix C′ and enhance capillary permeability. The earlier work as to the effects of heat was confirmed. Moreover, aggregation achieved by other means of denaturation, such as through the use of concentrated urea, rupture of disulfide bonds, or diazotization with a multivalent hapten were all effective in rendering these preparations highly anticomplementary and skin reactive. To elicit this property the aggregates had to originate from rabbit, human, or guinea pig γ-globulins. As in PCA, preparations from horse or chicken serum did not bind to the guinea pig skin. The other essential condition, or at least an inseparable counterpart, was the anticomplementary property involving all the components, a requirement previously shown for PCA reactions and for anaphylatoxin formation discussed previously. In a companion, as yet unpublished study, the author with Dr. Leighton Cluff found that heat aggregated human γ-globulin in microgram quantities could evoke an inflammatory reaction with wheal and flare in the skin of normal or allergic human volunteers.

While these studies were in progress, Christian independently arrived at very similar conclusions and provided the striking demonstration that injection of 5 mg. of this aggregated protein into guinea pigs reduced C′ levels to less than 5% of the initial hemolytic activity present in these sera.

Marcus (1960) has compared the similarities in the mechanism of C′ fixation by antigen–antibody complexes and by γ-globulin aggregated by

reaction with bentonite. His failure to find a diminution of C'2 in the latter instance is not in accord with the observations of the Ishizakas.

3. *The Aggregating Capacity of the Antibody in Relation to Allergic Phenomena*

The preceding sections have emphasized the sequential, and possibly causal, relationships among aggregation of γ-globulin, the fixation of C' and manifestations of hypersensitivity. Implicit in this scheme is the notion that the participating antibody must be multivalent, i.e., capable of aggregation by antigen and of participation in the fixation of C'. The failure of sera from patients with allergic diseases due to inhalant or ingestant incitants to aggregate on addition of the provocative antigenic extracts provides an obvious and important contradiction which demands clarification. Speculations in this area might best be delayed until better characterization of the immune reactants is available.

Another apparent exception refers to an earlier report of Kabat and Benacerraf (1949) that the nonprecipitating portion of anti-hen ovalbumin and the precipitating antibody were equally effective in sensitizing guinea pigs to fatal systemic anaphylactic shock with the homologous antigen. The pertinence of this observation in the present discussion concerns the lowered C'-fixing capacity of the "univalent" or coprecipitating antibody (cf. Osler and Hill, 1955; Hill and Osler, 1959). However, it would seem that the so-called nonprecipitating antiovalbumin used in the experiments of Kabat and Benacerraf (1949) also fixed C' as well as did the precipitating antibody (p. 319 in Becker, 1957). These observations would seem to require further study especially since reports are available on the inhibition of systemic anaphylactic shock by haptens (Tillett *et al.*, 1929); the inability of monovalent haptens to produce γ-globulin aggregates capable of fixing C' and irritating guinea pig skin (Ishizaka *et al.*, 1959a, b), or to induce PCA reactions with antibody to a protein hapten conjugate (Ovary and Karush, 1960); the failure of photooxidized antisera to induce systemic passive anaphylaxis in guinea pigs or contractions of smooth muscle in the Schultz-Dale type of experiment (Tyler, 1945); the failure of antisera exposed to ultraviolet irradiation to form aggregates or to sensitize guinea pigs passively to anaphylactic shock (Hanan, 1952).

Maurer and Thorpe (1960) also concluded that rabbit anti-BSA sera heated at 65°C. to 80°C. for 10 to 30 minutes yielded discordant results in a comparison of C' fixation and PCA induction on a weight basis. The capacity to precipitate with BSA apparently declined at a more rapid rate than did the C' fixation or anaphylactogenic potency in some of the

preparations. Unfortunately, the data are not sufficiently detailed for a careful evaluation but it would seem that certain extraneous factors were not excluded. The latter include the C′-fixing ability of the γ-globulin aggregates induced by heating and the cross reactions frequently observed between rabbit anti-BSA sera and albumins of other species. Further, some of the data indicate that PCA reactions were obtained at levels exceeding the sensitivity limits described by Ovary (1958). Finally, the possibility of *in vivo* aggregation of these nonprecipitable aggregates containing heated antisera must be considered (Maurer and Talmage, 1953a, b), as discussed recently by Terres and Wolins (1959).

C. C′ Levels and Allergic Responses

Numerous investigators have attempted to define the role of C′ in allergic reactions through concurrent studies of hemolytic activity levels and susceptibility to hypersensitivity reactions (e.g., Bier *et al.*, 1955). The complexities associated with this approach and the pertinent literature have been given in Osler *et al.* (1957, 1959a, b). This type of study is limited by the inability to obtain data other than those of a correlative nature. Nevertheless, current interest in this problem continues. Szabo (1956) has reviewed some of the literature and assigned an essential role to the C′ system in the initiation of fatal symptoms. A number of workers have reported a sharp decline (about 30–50%) in serum C′ activity shortly after challenge of sensitized guinea pigs (Rice, 1955; Rice and Boulanger, 1954; Rice *et al.*, 1953b, 1954a, b; Morikawa *et al.*, 1957). Wilhelm (1960) observed that injection of guinea pigs with zymosan conferred a marked protection to otherwise fatal anaphylactic shock. This treatment has been shown to be associated with lowered C′ values (Osler *et al.*, 1957). Interestingly, depressions of similar magnitude occurred during tuberculin shock but only after several hours (Rice *et al.*, 1954b; Morikawa *et al.*, 1957). Furlanetto (1959) found only slight reduction in hemolytic activity (6–8%) in sera taken from sensitized guinea pigs immediately after injection of antigen in a quantity which produced severe or fatal shock. In contrast, the simultaneous injection of large quantities of immune reagents resulted in a more dramatic fall in C′ without producing anaphylactic shock. Compare also Weigle *et al.* (1960). These data demonstrate clearly that the intervention of a latent period following the intravenous injection of antibody increases the sensitivity of the guinea pig to fatal shock. Further, they denote that cell-fixed antibody may be more anaphylactogenic than soluble complexes. For these reasons they shed little light on the C′ problem. Another consideration refers to the present lack of knowledge as

to the quantitative relationships and nature of the intermediate steps between C′ utilization and fatal anaphylaxis.

Pruzansky *et al.* (1959) and Feinberg and Pruzansky (1958) have opposed the notion that C′ may participate in these reactions, but the data offered in support of this position are subject to alternative interpretations. To cite one example, the injection of fairly large volumes of heterologous or even homologous serum may diminish the severity of the anaphylactic reaction from causes quite independent of serum C′. Similar considerations apply to the study of Bier and Siqueira (1959) who indicate some of the difficulties in attempting to assign or exclude a role to C′ in a complex reaction without adequate control of all other known parameters. Among these may be mentioned the fact that over-all C′ titers do not necessarily reflect the interaction of the components which may be vital to an analysis of the specific problem.

A further factor of possible importance in eliciting the manifestations of the Arthus reaction, as pointed out by the authors, is the intravascular agglutination of platelets. This reaction which may precede the hemorrhagic necrosis characterizing the Arthus phenomenon, in itself seems to require the active participation of all the C′ components (Siqueira and Nelson, 1961).

It may be concluded, therefore, that the aforementioned studies, oriented primarily toward attempts to correlate C′ levels and allergic manifestations, suffer from a technical inability, perhaps insurmountable at the present time, to encompass all the known variables in this complex process. More direct approaches with more circumscribed objectives should be entertained.

D. C′ and Anaphylactoid Phenomena

Although several aspects of this question have been dealt with in some detail in Osler *et al.* (1959a), the essential conclusions may be outlined briefly. A study of the literature with respect to anaphylatoxin formation and the release of histamine from various tissues by such substances as dextran, indicates many observations that are in accord with, but not conclusive for, the notion of C′ participation. This work, exclusive of the C′ problem, has been summarized in two recent volumes (Wolstenholme and O'Connor, 1956; Rocha e Silva, 1955). One of the serious difficulties that hampers unequivocal interpretation of the data concerns the complexity of the reaction systems under study. The study of Middleton and Sherman (1960) provides a fitting illustration. These workers conclude that C′ is probably not involved in the release of histamine from whole blood of allergic individuals to which the

incriminated allergen was added. However, the reaction medium in these studies contained Ca^{++} in a concentration of 0.001 *M*, a level which is inhibitory for hemolytic action of human and possibly guinea pig C′ (Fife, 1960) but perhaps of value in maintaining the integrity of the leucocytes. Therefore the conditions for the study of histamine release are not necessarily optimal for the investigation of C′ activity. Similarly the addition of inhibitory substances does not permit the investigator to localize the effect of the inhibitor with respect to the several reactions that are taking place, such as the formation of a mediator, the action of the mediator, or the responsiveness of the cell. Under such conditions, the choice of any interpretation, even the correct one, would be premature. Similar considerations apply to the studies of Mongar (1958) and Mongar and Schild (1957a, b, c, 1958), Schild (1959) and Austen and Brocklehurst (1960), who concluded that C′ might be involved in the release of histamine from chopped guinea pig lung fragments. This deduction was drawn, in part, from evidence which included a comparison of the hemolytic activity of undiluted guinea pig serum after exposure at 45°C. with the capacity of the tissue mince to release histamine following similar thermal treatment. The choice of this experimental model for ascertaining a C′ requirement may not permit a resolution of this issue because of the inability to separate the effects of the various treatments on the histamine depots from those on the C′ system.

Of pertinent interest are the studies of Audia and Noah (1960), Noah and Brand (1959), and Waalkes and Coburn (1959), all of whom report a requirement for Ca^{++} in the release of histamine from leucocytes or platelets. The last-named authors showed the similarities in this regard among an antigen–antibody reaction, soluble complexes, and glycogen.

In an effort to further explore these uncertainties, the author and his associates carried out systematic investigation of anaphylatoxin formation in a cell-free medium (Osler *et al.*, 1959a, b). It was concluded that the formation of this substance(s) can be represented as a product of C′ fixation and requires the interaction of all known C′ components. Further, various other "anaphylactoid" reagents such as agar, dextran, heparin, Liquoid (polyanetholesulfonate), and inulin act in a similar fashion with the participation of C′. The state of aggregation of substances like dextran proved an important determinant of anaphylatoxin formation. On the basis of the present data, it cannot be ascertained whether substances like dextran fix C′ through interaction with antibody present in pooled normal rat serum, as has been demonstrated for human serum by Jacobsson (1958) and Jacobsson and Zsiga (1958), or whether the mechanism

resembles the aggregation of γ-globulin by bentonite (Marcus, 1960). Kabat and co-workers reported significant correlations between precipitating antidextran in the sera of humans and their cutaneous and systemic allergic reactions to dextran (Kabat *et al.*, 1957). Of further interest is the anaphylactoid reaction reported by Shofer and Marlow (1959) following administration of an iron plus dextran preparation. Dextran has also been reported to inhibit lysis when added to the blood of a patient with paroxysmal nocturnal hemoglobinuria possibly through a preliminary fixation of C′ (Crosby and Benjamin, 1958).

The contraction of smooth muscle following contact with rat serum pretreated with *E. coli* lipopolysaccharide has been reported by Greisman (1960). The experimental conditions outlined in that report are associated with marked C′ fixation (Osler and Hill, 1959). However, the observations of Jaques on the inhibition of systemic anaphylaxis by minute quantities of endotoxin (Meier *et al.*, 1957; Jaques *et al.*, 1959) do not appear explicable on the basis of C′ utilization.

The role of anaphylatoxin as a histamine-releasing agent has been amply recorded by Rocha e Silva (1955), by Keller (1957), by Mota (1959), and Hahn and Giertz (1960), among many others. Halpern *et al.* (1956) found that pretreatment with anaphylatoxin protected passively sensitized guinea pigs against an otherwise lethal shock. The mechanism of this protective effect is quite unclear, although the use of normal serum as a control might have been indicated. Seelich and Stockinger (1954, 1956) have blocked the cytotoxic activity of antibody in tissue culture with heparin, Liquoid, or dextran and restored it with fresh serum.

Archer (1958, 1960) has reported that the addition of dextran or zymosan to normal rat serum results in the production of a substance which liberates histamine from rat mast cells. It was concluded that C′ was required for this process but the identification of the histamine-releasing mediator as anaphylatoxin remained somewhat in doubt since both heat-labile and heat-stable products were found. Most investigators have found that anaphylatoxin is relatively heat-stable.

Adamkiewicz and colleagues have studied the anaphylactoid swelling of the rat paw as induced by dextran, promoted by insulin and inhibited by alloxan (Adamkiewicz and Langlois, 1957, 1958; Adamkiewicz *et al.*, 1958; Adamkiewicz and Adamkiewicz, 1959a, b). Goth (1959) and Goth and co-workers (1959) found that 2-deoxyglucose also inhibited the dextran-induced edema. The pertinence of these observations to C′ remains problematical.

In his study of smooth muscle contraction due to anaphylatoxin and to antigen–antibody interaction, Greisman (1958) has concluded that

the two are causally unrelated. The pertinent observations deal with the undiminished response to anaphylatoxin following desensitization to specific antigen. Initial experiments in the author's laboratory have yielded similar findings (Neu *et al.*, 1961). However, an alternative interpretation may be offered which includes anaphylatoxin in the physiological mechanism resulting in the release of capillary poisons. The process of desensitization with antigen may involve exhaustion of antibody or a tissue constituent required for the production of anaphylatoxin. Since the latter acts at a subsequent step, the state of tachyphylaxis (refractoriness to anaphylatoxin) may simply reflect suboptimal conditions for elaboration of anaphylatoxin precursors, but not for its action.

IX. Concluding Comments

It may be appropriate in concluding this survey, to comment briefly on the meaning of a C′ titer in terms of hemolytic activity. Elucidation of the reaction sequence described in Section I leads to the conclusion that the conditions associated with the utilization of C′2 constitute important determinants of immune hemolysis. The concentration of C′2 with respect to the speed of formation of cell sites in the state $C'_{1,4,2}$, the resulting influences on the forward and degradative reactions, all participate in determining the eventual fate of the sensitized erythrocyte. In addition, the effects of volume, temperature, ionic strength, cation concentration, and pH must also be considered in the estimation of C′ activity for the system composed of guinea pig serum and sheep red blood cells sensitized with rabbit anti-Forssman antibody (Kabat and Mayer, 1961). Until these interrelationships are clarified for the many situations and species in which C′ studies have been conducted, it is difficult to interpret the precise meaning of C′ titer estimations. This restriction should be kept in mind particularly in studies of human diseases, since some of the parameters of human C′ remain to be clarified.

Another property deserving comment refers to the relative lack of specificity of the C′ system. As is well known, C′ components react with γ-globulins derived from numerous species and with a galaxy of antigens differing vastly in their immunochemical behavior. Two problems emerge from this situation. Since C′ may interact with so many different immune systems, the investigator who seeks to affirm or deny an essential role to this system in allergic or other phenomena, is compelled to design experiments which differentiate between a nonmaterial and indispensable utilization of C′ in the events under investigation.

The second problem concerns the fact that the same set of C′ com-

ponents apparently exercises a lethal action on many types of cells. A generally acceptable explanation for the apparent lack of specificity at the cellular level is not immediately at hand. As initially postulated by Heidelberger and colleagues (1941), C'1 may form a loose, readily dissociable union with γ-globulin. The relative lack of species specificity which characterizes this interaction will be explained when chemically purified reagents will be available for studies at the molecular level. The reversibility of antigen–antibody–C'1 complex and its dependence on Ca^{++} suggest an interaction based on the ionic properties of the reactants rather than on covalent bond formation.

The aggregation of γ-globulin by antigen, or possibly by other means, may also serve to render the antibody–C'1 complex less easily dissociable, and thus set off the chain of events involving the remaining components. The terminal step of this series includes a derangement of some vital cell surface function, such as the maintenance of optimal osmotic equilibrium relationships. This reaction step may mark the final action of the C' system on a cell, in which the surface disorganization may then lead to the release of hemoglobin as from an erythrocyte, or possibly, histamine from a mast cell.

Detailed studies of the reactions immediately preceding the release of these indicators may serve to clarify further the mechanism of action of the C' components in immune hemolysis as well as in the many other functions currently associated with the C' system.

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In Vitro Studies of the Antibody Response

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I. Introduction

A. Scope

There are an impressive volume and variety of data concerning the antibody response which, beginning with the entrance of antigen into certain cells, are considered here to include all the cellular events which culminate in the synthesis of circulating antibody. Nevertheless, certain phases of this response are poorly or inadequately understood. The purpose of this review is to describe and to appraise the available observations so that certain promising areas may be selected for exploration and discussion. Recent *in vitro* studies are emphasized. Since *in vitro* studies do not cover all aspects of the antibody response and may include artifacts of the *in vitro* circumstances, these data are complemented and compared with data derived in studies of the intact animal. Several important aspects have been excluded for lack of space. These have been reviewed elsewhere as follows: sites (McMaster, 1953; Harris and Harris, 1956; Speirs, 1960), nutritional factors (Axelrod and Pruzansky, 1955), characterization of γ-globulins (Porter, 1960), inhibitors (Taliaferro, 1957), morphological aspects (Fagraeus, 1948a; Marshall and White, 1950; Fitch *et al.*, 1953; Coons *et al.*, 1955; Speirs, 1960), secondary response (System of Bacteriology, 1931; Freund, 1953), theories (Burnet and Fenner, 1953; Schweet and Owen, 1958;

Burnet, 1959; Lederberg, 1959; Talmage, 1959), and general view (Wilson and Miles, 1957).

B. Historical

The discovery, application, and study of the process of antibody formation is a fascinating story. The early chapters contain the names of Jenner, Pasteur, and Behring and deal mainly with the application of the principles of immunization to the prevention and alleviation of human suffering. However, investigators soon became interested in the analogy between antibody synthesis and other biological phenomena such as adaptive enzyme formation in microorganisms (Dienert, 1900). The first comprehensive theory, the side-chain theory, of antibody formation was proposed at the turn of the century (Ehrlich, 1900), and the recent clonal selection theory (Burnet, 1959; Talmage, 1959; Lederberg, 1959) bears a striking resemblance to Ehrlich's hypothesis. Studies of the antibody response then proceeded along many lines. The basic distinctions between the primary and secondary antibody responses were recognized early (Solomonsen and Madsen, 1895; Glenny and Sudmerson, 1921). Antibody production was studied *in vitro,* utilizing the tissues of immunized animals (reviewed in Salle and McOmie, 1937; Fagraeus, 1948a). Cells were transplanted from antibody-forming to normal animals and evidence adduced that the antibody which appeared in the recipients was synthesized by the transferred cells (Chase, 1951). A similar phenomenon had been observed previously (Deutsch, 1899), but clear-cut evidence for the function of the transplanted cells was not presented at that time. Participation of the lymph node in antibody formation was established (McMaster and Hudack, 1935; Harris and Harris, 1956). The role of plasma cells in antibody synthesis was suggested by Scandinavian workers (Bjorneboe and Gormsen, 1943; Bing *et al.,* 1945; Fagraeus, 1948a) and put on a firmer basis by the immunofluorescent studies of Coons (1959). The sensitive hemagglutination (Boyden, 1951) and isotope incorporation (Ranney and London, 1951; Keston and Katchen, 1956) assays for antibody were valuable in the solution of many questions.

During the past 10 years there have been a quickening of the pace and a change in the character of research on the antibody response. Many lines of investigation have been converging. The process of antibody synthesis has been studied rather extensively *in vitro* isolated from many complicating factors which exist in the intact animal. Antibody synthesis has been employed as a model for the study of the *in vitro* biosynthesis and secretion of a specific protein. With the advent of new

tools and methods, such as isotopes and the preparation of subcellular components, the antibody synthetic process is being analyzed at the cellular and subcellular level (Askonas, 1958; Kern *et al.*, 1959). The serologically functional fragments of antigen (Lapresle *et al.*, 1959) and antibody (Porter, 1959) are being characterized. Attention is shifting from the study of the synthesis of antibody to the analysis of the more obscure, early phases of the antibody response. Even a casual perusal of the data summarized in these pages will reveal that substantial progress is being made in elucidating the fundamental mechanisms of this process.

II. Newer Methods for *in Vitro* Study of Antibody Response

A. Maintenance of Function of Antibody-Forming Cells

Spleen, lymph nodes, lung, bone marrow, and liver from immunized animals were cut into small fragments or slices with scissors or scalpel and incubated in suitable media. Provided the tissue was not badly macerated during preparation, antibody was synthesized (Fagraeus, 1948a; Thorbecke and Keuning, 1953; Stavitsky, 1955; and Askonas and White, 1956). Treatment or disruption of antibody-forming tissues by heating, freezing and thawing, autolysis, incubation in distilled water, or homogenization resulted in the loss of much if not all ability to synthesize antibody *in vitro* (Fagraeus, 1948a; Stavitsky, 1955; Askonas and Humphrey, 1958a). Cells also were isolated from lymph nodes, but some antibody synthetic activity was lost during the procedure (Stavitsky, 1958a, b). Methods for isolating single (Attardi *et al.*, 1959; Nossal, 1958) or multiple (Vaughan *et al.*, 1960) cell preparations which synthesize antibody *in vitro* have been described.

Tissue fragments and cells from immunized animals have been cultured in suspension or embedded in plasma clots or agar in tubes containing suitable media. Antibody production is equivalent under these conditions during 24-48 hours of culture. Synthesis is maintained for 1 to 3 weeks in embedded preparations (Michaelides, 1957; Askonas and Stavitsky, 1959; Grabar and Corvazier, 1960; Bauer and Stavitsky, 1960). The agar seems preferable because, unlike the clots, it is not digested and need not, therefore, be repaired or replaced. Various types of chambers for the maintenance of antibody-forming cells (Steiner and Anker, 1956; Ainis, 1960) and tissues (Trowell, 1959; La Via *et al.*, 1960b) have been described.

In vitro and *in vivo* studies show a positive correlation between the weight of the spleen and its antibody-forming capacity in chickens

(Norton and Wolfe, 1949; Grabar and Corvazier, 1960) and rabbits (Mountain, 1955b). Rabbit lymph node fragments synthesize antibody for 1 to 3 weeks in plasma clot or agar preparations, but similar preparations of rabbit spleen and bone marrow are active for only 2 to 4 days (Askonas and Stavitsky, 1959; Bauer and Stavitsky, 1960). An agar mount of chicken spleen synthesizes antibody for 6 to 8 days (Grabar and Corvazier, 1960).

The presence of optimal concentrations of certain amino acids in the medium is the most important factor affecting antibody synthesis in 24- to 48-hour cultures of antibody-forming organs (Mountain, 1955a; Wolf and Stavitsky, 1958; Vaughan *et al.*, 1960). Addition to the medium of serum, serum ultrafiltrate, chick embryo extract, purines and pyrimidines, certain lipids, carbohydrates, and coenzymes did not promote antibody synthesis in these cultures (Wolf and Stavitsky, 1958). Addition to the medium of purines and pyrimidines, chick embryo and liver extract, certain vitamins, and sucrose did not enhance antibody formation (Mountain, 1955a). In this study higher concentrations of sucrose, cysteine, cystine, and reduced glutathione inhibited antibody production. Vaughan *et al.* (1960) added ribonucleosides, glucose, and ascorbic acid to the medium without affecting antibody synthesis, but higher concentrations of ascorbic acid, glutamine, and nucleosides inhibited antibody formation. The nutritional requirements for successful long-term cultures are less well defined. It appears that the presence of serum is advantageous. Coons (1960) has accumulated evidence that the addition to the medium of inositol, estradiol, linoleic and linolenic acids and vitamins A, K, B_{12} and tocopherol promote the synthesis of antibody in long-term plasma clot cultures of rabbit lymph nodes.

Askonas and Humphrey (1958b) studied antibody synthesis in perfused lungs from hyperimmunized rabbits. The preparation was maintained for 5 to 6 hr. The large amounts of antibody synthesized seemed to approximate more closely the amounts made in intact animals than the *in vitro* slice preparations did. The large amounts of tissue and the yield of large volumes of perfusate facilitated the analysis of the kinetics of antibody synthesis and secretion.

B. Assays of Antibody and Antigen

The passive hemagglutination procedure of Boyden (1951) as modified in this (Stavitsky, 1954b) and other laboratories (reviewed in Stavitsky and Arquilla, 1958) may detect 0.01 μg. of antibody and 0.001 μg. of antigen. However, in view of the sensitivity of the reaction it

must be established that it is specific for the antigen–antibody system in question rather than for contaminating systems. Many precautions and tests must be employed to insure the specificity of the reaction for the antigen(s) adsorbed onto the tannic acid-treated erythrocyte (Boyden, 1951; Stavitsky and Arquilla, 1958).

The incorporation of radioactive amino acids into antibody has been utilized as an assay of antibody synthesis (Ranney and London, 1951; Dixon *et al.*, 1956; Green and Anker, 1954; Keston and Katchen, 1956; Steiner and Anker, 1956; Askonas and Humphrey, 1958a; Stavitsky and Wolf, 1958). Following treatment of the antiserum or tissue culture medium with a heterologous antigen–antibody aggregate to remove nonspecific radioactive substances, the radioactive antibody in the supernatant was isolated by direct precipitation with antigen or by coprecipitation on a homologous antigen–antibody aggregate. When large amounts of antibody were synthesized *in vivo* or *in vitro* by the by the tissues of hyperimmunized animals, the incorporation assay seemed to furnish a reliable and reproducible measure of antibody which is about as sensitive as the hemagglutination method (Stavitsky, 1958b, 1960c). However, when the isotope assay was employed for the measure of small amounts of antibody, the presence of radioactive proteins other than antibody sometimes has interfered with the isolation and specific assay of antibody (Keston and Katchen, 1956; Stavitsky, 1958b, 1960a). Similar difficulties have been encountered in attempts to isolate specific radioactive serum albumin from contaminating radioactive substances (Campbell and Kernot, 1959). The utility of the incorporation assay as an index of active antibody synthesis was demonstrated in long-term cultures. When the medium containing radioactive amino acids was changed every 2 to 3 days, the lymph node fragments from immunized rabbits continued to produce radioactive antibody as well as hemagglutinating antibody over a period to 1 to 3 weeks (Askonas and Stavitsky, 1959; Bauer and Stavitsky, 1960).

III. Sites and Mechanisms of the Antibody Response

A. Uptake and Fate of Antigen[1]

1. Mechanisms of Uptake of Antigens

Particulate antigens such as bacterial cells are taken into mammalian cells by the process of *phagocytosis.* The mechanisms whereby antigens

[1] The phase of antigen uptake and metabolism is assumed to merge into the inductive phase in which the antibody-forming system is developed and the latter into the synthetic phase during which antibody synthesis occurs.

in solution are taken up by these cells are not established, but proteins may be incorporated into certain mammalian cells by the process of *pinocytosis*, the drinking of large fluid vacuoles containing the proteins in solution. Chapman-Andresen (1957) observed the formation of intracellular vacuoles when guinea pig and rabbit peritoneal exudate cells were incubated *in vitro* with bovine γ-globulin, human and bovine serum albumin, and β-lactoglobulin. Vacuole formation was complete within a few minutes after the cells were incubated with the proteins. The globulins were more effective inducers of vacuole formation than the albumins. Little or no vacuole formation occurred in the absence of proteins. Holter and Holtzer (1959) noted the uptake of fluorescein-labeled proteins by isolated mammalian cells *in vitro*. Comparison of their observations with those reported for macrophages and amoebae led them to conclude that *pinocytosis* had occurred. They also found that the fluorescein-labeled protein solution functioned as a cytoplasmic and nuclear "stain" when applied to dead and injured cells, perhaps because of an increase in the selective permeability of these cells. They, therefore, recommended that observations on protein uptake by fluorescent techniques should be confirmed by other methods before being considered conclusive.

The factors which influence phagocytosis *in vivo* and *in vitro* have been studied extensively (Wilson and Miles, 1957). It has been observed that phagocytosis requires actively glycolytic phagocytic cells and that increased cellular metabolism is involved in this process (Sbarra and Karnovsky, 1959). Relatively little is known about the factors which modify the uptake of soluble proteins by mammalian cells (Holter, 1959). Recently the cell membrane and endoplasmic reticulum have been implicated in the transport of substances from the outside to the inside of the cell by pinocytosis (Palade, 1956; Bennett, 1956).

2. *Cells Involved in the Initiation of the Antibody Response*

a. In Vitro Uptake of Antigen Followed by Antibody Synthesis in Vivo. In these studies antigen was incubated with cells for a few minutes *in vitro* after which the cells were transplanted and apparently synthesized antibody in their new hosts. Presumably little more than a combination of antigen with certain cellular components occurred *in vitro*. Antibody was synthesized more rapidly and in greater amounts when bacterial antigens rather than proteins were added to the cells. It is possible that the greater antibody response to bacteria represented a secondary rather than a primary response, that the animals had been

exposed to small amounts of these antigens previously by absorption from intestinal microorganisms. The transplanted cells which were stimulated by proteins probably were rejected by a homograft reaction before they synthesized antibody, whereas the more rapid immune response to bacterial antigens preceded rejection of the cells.

One series of studies implicates an *in vitro* reaction between soluble *Shigella* antigen and rabbit lymphocytes in the initiation of the antibody response (Harris and Harris, 1958; Harris *et al.*, 1958). Antigen was mixed with lymph node cells (98% lymphocytes), and was removed by washing or by antibody; the cells were then transplanted into X-irradiated rabbits. Antibody appeared in the recipients within several days. The antigen-cell reaction occurred within 5 minutes at 37° C. since antiserum added 5 minutes after mixture of the reagents did not reduce the titer of antibody in the recipients. A few hundred molecules of antigen per cell were required to initiate the antibody response (Harris and Harris, 1958). When incubation was at 4° C. rather than at 37° C., titers were lower. If the cells were X-irradiated *in vitro* with 150–400 r (Harris *et al.*, 1959) or heated at 56° C. for 30 minutes (Harris *et al.*, 1958) after reaction with the antigen, antibody titers were reduced. The recipients were X-irradiated to abolish their capacity to synthesize antibody against any antigen occluded on the cells. Strong evidence was thus provided that the cells which had taken up antigen *in vitro* later had synthesized antibody within the recipients. In similar studies with *Salmonella* (Sterzl, 1957; Sterzl and Trnka, 1959; Trnka and Riha, 1959; Trnka and Sterzl, 1960) optimal amounts of soluble antigen were required since large amounts inhibited the antibody response after transplantation. Similar results were obtained with fowl splenic cells which were antigenically stimulated *in vitro* and then injected into neonatal chicks. Nossal (1959a) incubated mouse and rat splenic cells with isolated flagella from *Salmonella* for 30 minutes at 37° C., neutralized free antigen with antibody, and transplanted the cells to normal and sublethally X-irradiated animals of the homologous species. Antibody appeared in the recipients in several days. Aliquots of the cells which were heated (at 56° C. for 30 min.) after exposure to and removal of antigen failed to cause the appearance of agglutinins in recipients.

The primary response to proteins also has been elicited by incubating cells with antigen *in vitro* and then transplanting the cells. The results have been less consistent and the titers of antibody lower than when bacterial antigens were employed. Lymphoid cells from the cisterna chyli or lymph nodes of young rabbits plus 0.05–0.1 mg. of ovalbumin or bovine serum albumin were placed in diffusion chambers

which permit the passage of protein but not of cells (Holub and Riha, 1960). The chambers were then placed in the peritoneal cavity of 3- to 5-day-old rabbits which cannot make antibody. In 9 to 12 days variable titers of hemagglutinating antibody (Boyden, 1951) were detected in the chamber fluids. In another study (Trnka and Riha, 1959) fowl spleen cells were incubated *in vitro* with bovine serum albumin and injected into 18-day chick embryos. Several days after hatching antibody appeared in the sera of the chicks. When such cells were inoculated into newly hatched chicks rather than chick embryos, antibody did not appear. The neonatal chicks presumably rejected the spleen cells before antibody was synthesized, whereas the embryos developed tolerance to the transplanted cells which survived and completed the antibody response.

Information is also available regarding the types of cells which may be involved in the early phases of the antibody response. Thus far most consistent results have been obtained when rabbit lymphocytes have been incubated with antigen and transplanted (Harris *et al.*, 1956; Holub and Riha, 1960). Similar treatment of predominantly neutrophilic or monocytic peritoneal exudates has not resulted in antibody synthesis (Harris *et al.*, 1956). Similar handling of rabbit blood leucocytes, which contain 65–90% lymphocytes, has resulted in low antibody titers.

Walsh and Smith (1951) observed the phagocytosis of *Salmonella* by a rabbit pleural exudate consisting mainly of polymorphonuclear leucocytes. After *in vitro* incubation for 6 hours at 37° C., these phagocytes were washed and injected intravenously or into the footpads of rabbits. The antibody response was impaired by this treatment. Phagocytosis of the bacteria by peritoneal macrophages did not consistently result in reduction of antigenicity of the organisms upon subinoculation of the macrophages into rabbits.

b. In Vitro Uptake of Antigens. *In vivo* studies by the immunofluorescent technique provide a basis for judging the validity of *in vitro* studies on antigen uptake by cells. The injection of various proteins (Coons *et al.*, 1951) and bacterial polysaccharides (Hill *et al.*, 1950) into rabbits, rats, and mice soon resulted in the appearance of immunologically reactive antigen within reticular cells, macrophages, lymphocytes, plasma cells in lymph nodes, spleen, and bone marrow, fibroblasts, and cells of the renal tubules, liver parenchyma, adrenal cortex, and vascular endothelium. The injection of variously labeled proteins yielded essentially similar results (Sabin, 1939; Smetana, 1947; Kruse and McMaster, 1949; Schiller *et al.*, 1952; Gavosto and Ficq, 1953;

Cheng *et al.*, 1958). Holter and Holtzer (1959) studied the *in vitro* uptake of fluorescein-labeled proteins by various mammalian cell and tissue preparations. The proteins were taken up by cells from reticuloendothelial organs (reticular cells, lymphocytes, macrophages), from liver (Kupffer cells), and from peritoneal exudate cells. Other cells from a variety of organs did not take up the proteins. The similarity between the *in vivo* and *in vitro* observations indicates that the *in vitro* methods provide a reasonably accurate index of the ability of various cells to incorporate antigens. Moreover, the results imply that a rather restricted group of cells (macrophages, reticular cells, lymphocytes) is specialized for the uptake of antigen and could participate in the early phases of the antibody response.

Sorkin and Boyden (1959) investigated the uptake of I^{131}-labeled human serum albumin by guinea pig monocytes. After incubation with the protein for 2 hours at 37° C. and washing off of excess antigen, about 0.1% of the albumin was associated with the cells. Prots *et al.* (1960) mixed rabbit splenic and lymph node cells with S^{35}-ovalbumin *p*-azophenyl sulfonate. After incubation for 2 hours at 37° C. and removal of excess antigen, about 0.1% of the antigen was associated with the cells. Autoradiography revealed antigen within the cytoplasm of lymphocytes, macrophages, and reticular cells.

c. Subcellular Sites of Antigen Localization. Discussion is restricted to subcellular localization of antigen within cells which may participate in the antibody response. The causal relationship of even these observations to the antibody response is not known.

Antigens have been observed within the cytoplasm of reticular cells, macrophages, lymphocytes, and plasma cells minutes or hours after the injection of viruses (Gavosto and Ficq, 1953), proteins (Coons *et al.*, 1951), labeled proteins (Sabin, 1939; Smetana, 1947; Kruse and McMaster, 1949; Cheng *et al.*, 1958), and bacterial polysaccharides (Hill *et al.*, 1950). Corresponding to the foregoing observations antigen has been detected in various subcellular fractions of spleen shortly after inoculation (Haurowitz, 1959). Antigen has not been observed consistently within the nucleus of cells. On the one hand, by immunofluorescent techniques, proteins and bacterial carbohydrates were detected in the nucleus of splenic reticular cells, lymphatic sinus cells, and lymphocytes, often in higher concentration than in the cytoplasm (Coons *et al.*, 1951). Antigen appeared to be in solution in the nuclear sap rather than adsorbed to chromatin or nucleoli, and some antigen was localized around the nuclear membrane. On the other hand, Schiller *et al.* (1952) did not note the localization of fluorescein-labeled bovine

serum albumin in the nucleus of cells after injection. They cited a personal communication from A. H. Coons who considered the possibility that the discrepancy in results of nuclear localization of antigen in the two studies was because of differences in the sensitivity of methods for detecting antigen. Schiller *et al.* (1952) emphasized that antigen was introduced into the living animal in their experiments, whereas Coons reacted intracellular antigen with labeled antibody *in vitro* (Coons *et. al.*, 1951). They suggested that the permeability to antigen of the nuclear membrane of viable cells was greater than the permeability to antibody of this membrane *in vitro*. They also wondered whether antigens might redistribute themselves within the cell during the *in vitro* steps of the immunofluorescent procedure before the labeled antibody is applied. The discrepancy between these two sets of observations should be resolved because the finding of antigen in the nucleus has set off considerable speculation regarding the role of the nucleus in the initiation of the antibody response (Schweet *et al.*, 1958).

After inoculation antigen apparently is rapidly bound to cellular components so that free antigen is not readily detectable by serological methods. Stavitsky (1960c) injected diphtheria toxoid into rabbits by various routes. Lymph nodes and spleen were removed 15 minutes later. According to a dose versus secondary response curve (Stavitsky, 1954a, 1960c), these extracts contained 0.02–0.1 μg. of toxoid which elicited a typical secondary response upon injection into mice which had received toxoid some weeks before. Nevertheless, toxoid was not detected in these extracts by the sensitive hemagglutination-inhibition procedure (Boyden, 1951; Stavitsky and Arquilla, 1958) which can detect 0.001–0.005 μg. of toxoid. Presumably, antigen was released from its bound form within the mice. Recent reports suggest that by more extensive fractionation of tissue extracts, serologically active antigen can be detected (Garvey and Campbell, 1954, 1956, 1957).

d. Degradation and Persistence of Antigen. Proteins labeled with I^{131}, S^{35}, or C^{14} may persist in the tissues after injection. It is not clear in some studies whether the protein per se, the label per se, or both label and protein persisted. Even after injection of proteins internally labeled with S^{35} or C^{14}, the proteins may be degraded and the liberated S^{35}- or C^{14}-labeled amino acids reutilized for the formation of new radioactive protein (Haurowitz, 1959). It thus becomes impossible to decide whether any of the protein-bound radioactivity is associated with any of the originally injected protein. These difficulties were circumvented by demonstrating the antigenicity or allergenicity of the persisting material in the tissues. McMaster *et al.* (1954) detected bovine

γ-globulin in rabbit liver for 6 weeks by anaphylactically shocking mice previously injected with this antigen. Stavitsky (1954a) detected diphtheria toxoid in lymph node and spleen cells 3 weeks after inoculation by eliciting with cell extracts a secondary antibody response in mice previously sensitized to toxoid. Garvey and Campbell (1957) demonstrated bovine serum albumin in rabbit liver 3 weeks after injection by utilizing the tissue extract to anaphylactically sensitize guinea pigs. Radioactive antigen also was identified in tissue extracts by coprecipitation with unlabeled homologous antigen and antibody. Antigens of tobacco mosaic virus were thus found in mouse liver for up to 15 days after injection—also confirmed by electron-microscopic observations of intracellular virus (Erickson *et al.*, 1957). Hemocyanin (Garvey and Campbell, 1956) and bovine serum albumin (Garvey and Campbell, 1957) which precipitated with antibody as well as degraded fragments which inhibited specific precipitation with antibody were also found in tissue extracts.

There is little information regarding the sites where antigen persists. By immunofluorescent methods proteins and bacterial carbohydrates were seen in reticulo-endothelial cells for 1 to 2 days (Coons *et al.*, 1951). Stavitsky (1954a) found diphtheria toxoid associated with lymph node and splenic cells 3 weeks after the first injection of antigen. Garvey and Campbell (1956, 1957) detected S^{35}-labeled hemocyanin and bovine serum albumin in ribonucleic acid-rich fractions of rabbit liver. There was some evidence that these antigens were present in the form of a soluble antigen–antibody complex (see also Garvey and Campbell, 1959). Hawkins and Haurowitz (1959) obtained similar localization of S^{35}-labeled bovine serum albumin or globulin in rat spleen.

The available data suggest that in reticulo-endothelial cells bacterial antigens are degraded and rendered nonantigenic very rapidly (Walsh and Smith, 1951; K. B. Roberts, 1955). However, microorganisms which produce chronic infections (*Mycobacterium tuberculosis, Brucella abortus*) may persist and even multiply in the tissues for weeks or months and thus evoke a prolonged antibody response.

B. Inductive Phase

The inductive phase is considered to include those processes which follow the uptake of antigen and result in the development or appearance of the antibody-forming system within certain cells. This phase cannot be demarcated sharply from the preceding or succeeding phases. Moreover, part of antigen which is injected a second time or which persists from the primary injection might initiate the inductive phase

in a new group of cells, while the remainder stimulates the secondary antibody response in cells previously primed with that antigen.

1. Induction of Primary Antibody Response in Vitro

There have been some attempts to induce the primary antibody response *in vitro* by adding antigen to cells, but there are only a few reports of success (summarized in Salle and McOmie, 1937; Stevens and McKenna, 1958). Moreover, the specificity of the antibody produced is questionable (Carrel and Ingebrigsten, 1912) and in general not enough detailed data are presented for critical appraisal. In one study (Salle and McOmie, 1937) antigen was added *in vitro* to chick embryonic tissue, but antibody was not produced. It is now known that even the intact embryo cannot synthesize antibody (Good *et al.*, 1960). Recently, however, several claims of *in vitro* initiation of antibody synthesis which merit careful appraisal have appeared.

Stevens and McKenna (1958) added bovine γ-globulin *in vitro* to splenic fragments from rabbits which had been injected with *Salmonella typhosa* endotoxin 24 hours previously. After removal of excess antigen, the tissues were placed in a synthetic medium. Small amounts of hemagglutinating substances appeared in the medium after only 1 hour's incubation but larger amounts appeared with longer incubation. The hemagglutinating material was considered specific antibody to bovine γ-globulin because it agglutinated erythrocytes treated with this protein but not untreated cells or cells coated with casein. Moreover, the agglutination reaction between this material and the bovine γ-globulin cells was inhibited by the homologous antigen but not by casein. In low dilution this material fixed complement in the presence of bovine γ-globulin. In a subsequent study (McKenna and Stevens, 1960) similar results were obtained when bovine γ-globulin or egg albumin was added *in vitro* to a rabbit peritoneal exudate consisting predominantly of monocytic cells.

Attempts were made to confirm these experiments by essentially the same procedures and employing the incorporation assay (Stavitsky, 1960a,b,c). In several experiments substances appeared which agglutinated bovine γ-globulin-coated red cells to a titer of 1:80, but these reactions were not inhibited by the antigen. Moreover, these media contained radioactive substance(s) which were coprecipitated with an antigen–antibody system (ovalbumin–anti-ovalbumin) unrelated to bovine γ-globulin (BGG). As shown in Table I it appeared that these substances were produced by unstimulated spleen fragments, but their synthesis was stimulated by the addition of BGG to the tissues and by

longer (22 hours) incubation. This nonspecific coprecipitating material was found to be nondialyzable, precipitable by Rivanol® (2-ethoxy-6,9-diaminoacridine lactate) which precipitates serum proteins other than γ-globulins (Hořejši and Smetana, 1956) and diminished in amount when the spleen was damaged or incubated in the absence of amino acids (Stavitsky, 1960c). The nature of this nonspecific material, apparently protein, which is synthesized under the conditions of these experiments, is unknown. It could be complement or other substances[2] which are nonspecifically bound to antigen–antibody aggregates. The relationship of this material to that which appeared in the experiments of

TABLE I

PRODUCTION OF NONSPECIFIC SUBSTANCES BY SPLEEN FRAGMENTS *In Vitro*

Antigen[a] (mg.)	Incubation[b] (hours)	Specific activity[c] (c.p.m. mg. ppt.)	
		EA	BGG
—	10	157	106
—	22	268	213
10	10	163	105
10	22	354	240
25	10	162	112
25	22	517	604

[a] Bovine γ-globulin was added to spleen fragments from a rabbit which had received 10 μg. *Salmonella typhi* endotoxin intravenously 24 hours before. Fragments were incubated for 1 hour at 37° C. and antigen was then washed off.

[b] After removal of antigen, fragments were incubated in W medium (Wolf and Stavitsky, 1958) containing 2 μc. C^{14}-glycine for the designated times.

[c] Figures refer to specific activity of EA (ovalbumin)–anti-EA and BGG (bovine γ-globulin)–anti-BGG precipitates isolated from the medium after incubation.

Stevens and McKenna (1958) and McKenna and Stevens (1960) has not been established. It is not even certain that the substance(s) detected in their experiments is actively synthesized rather than preformed in and released from the splenic fragments; the inhibitory effect of nitrogen mustards and corticosteroids suggests that active synthesis had occurred. The lability at –20° C. of the hemagglutinating substance in their experiments certainly is not a property shared by most antibodies which have been described. In view of the uncertainty of the nature of the substances appearing in the media in the experiments of Stevens and McKenna (1958), Stevens and McKenna (1960), and

[2] Makinodan *et al.* (1959) found a macroglobulin in normal chicken serum which coprecipitated with the BSA (bovine serum albumin)–anti-BSA aggregate.

Stavitsky (1960a, c), it is desirable that the material(s) be isolated and characterized by more rigorous physicochemical and immunochemical methods—rather than by hemagglutination alone—such as have been employed by Campbell and Kernot (1959) and Peters (1959) for serum albumins.

Fishman (1959) incubated hemocyanin or T_2 bacteriophage of *Escherichia coli* with rat peritoneal macrophages for 30 minutes at 37° C. The supernatant was then incubated with rat lymph node cells in a synthetic medium plus serum for many days. Substances which neutralized T_2 but not T_1 phage appeared in low titer on day 5 and increased in titer until day 11 when the cells began to degenerate. Substances which hemagglutinated red cells coated with hemocyanin to a titer of 1:10 appeared in the medium after 7 days and to a titer of 1:80–1:160 after 11 days of culture. Although the times for these substances to appear in the medium generally agree with the times required for antibody to appear in the blood or in cultures prepared from animals after injection of antigen (see Section III, B, 2), the nature and specificity of the substances which appeared in the medium must be more rigorously established. Moreover, since lymph node cells from immunized rabbits have not synthesized antibody for more than 3 days *in vitro* (Vaughan *et al.*, 1960; Stavitsky, 1960c) the apparent continued antibody synthesis by rat lymph node cells for 11 days in Fishman's study seems surprising. Again there really is no conclusive evidence that active synthesis rather than release of preformed material occurred.

Fishman's data have been interpreted to suggest that more than one type of cell was required for the initiation and completion of the antibody response. However, even accepting his data as indicating *de novo* induction of antibody synthesis, his observations do not exclude the possibility that the cells which take up antigen *in vivo* are then converted to another type of cell which actually produces the antibody. This conversion may require more than one day and/or may not occur under the *in vitro* conditions provided in various studies to date. It would, consequently, be necessary to furnish the two types of cells at the same time *in vitro* in order to duplicate completely the *in vivo* process.

In contrast with the difficulty in carrying out the complete primary antibody response *in vitro*, the secondary antibody response can be consummated completely *in vitro* by the addition of antigen to primarily stimulated lymphoid tissue. The evidence for this statement will be considered in another section (III, C, 4).

2. *Length of Induction Period before Antibody Appears*

The precise determination of the length of the induction period is complicated by the possibility that antibody may first be synthesized bound to subcellular particles (Askonas, 1958; Kern *et al.*, 1959), may exist intracellularly bound to antigen (Garvey and Campbell, 1959), or may not be present in sufficient concentration to be detected by present methods. Some of the data described in the previous section suggest that a distinct induction period does not exist and that antibody appears immediately after antigenic stimulation of tissue (Stevens and McKenna, 1958). These *in vitro* experiments were based on earlier ones (McKenna and Stevens, 1957) in which rabbits were injected with endotoxin and 24 hours later with BGG. Splenic fragments prepared 2 hours later and cultured led to the appearance of hemagglutinating substances for erythrocytes treated with this antigen. Since the assay and results employed were similar to those in the later study (Stevens and McKenna, 1958), the reservations previously expressed apply equally well to the earlier study. Moreover, as is indicated in Table II and in the following discussion, efforts (Stavitsky, 1960c) to confirm the data of the earlier study (McKenna and Stevens, 1957) thus far have been unsuccessful.

Table II summarizes the results of studies in which antigen was injected *in vivo* and antibody synthesis measured either *in vivo* or *in vitro* by sensitive antibody assays. The dilution of antibody by extracellular fluids which is unavoidable with *in vivo* systems was sometimes minimized by utilizing an *in vitro* antibody-synthesizing system. Antibody assays as sensitive as (isotope incorporation) or more sensitive (bacteriophage neutralization[3]) than the hemagglutination method used by Stevens and McKenna (1958) were employed. The neutralization and incorporation procedures may detect nonprecipitating antibody (Stavitsky, 1960c) which probably is not detectable by the hemagglutination technique (Stavitsky and Arquilla, 1958). Since the antibody which is synthesized early may be qualitatively distinct from that made later (Heidelberger and Kendall, 1935; Barr, 1951; Farr, 1958), it might be desirable to utilize methods which detect nonavid as well as nonprecipitating antibodies. Despite these efforts to detect antibody as early as possible by present methods the data in Table II clearly indicate that at least 2–4 days must elapse after antigenic stimulation be-

[3] The bacteriophage neutralization assay is calculated to require only about 1/100,000 the number of antibody molecules that is required for the hemagglutination assay for a positive reaction (Mathies and Stavitsky, 1960).

TABLE II

LENGTH OF INDUCTION PERIOD IN RABBIT

Experimental material[a]	Assay[b]	Interval (days)[c]	References
Serum after intraven. KLH	Precipitin	4	Ainis, 1959
Serum after foot pad KLH	Hemagglutin.	3	Stavitsky, 1960c
Serum after foot pad T_2	Neutralization	4	Mathies and Stavitsky, 1960
Serum after intraven. BGG	Hemagglutin.	8	Ward *et al.*, 1959
Serum after intraven. or foot pad diph. toxoid	Hemagglutin.	17	Stavitsky, 1960c
Efferent lymph from regional node after foot pad *Shigella*	Agglutination	2–4	Ehrich and Harris, 1942
Lymph node cells after foot pad diph. toxoid	Immunofluorescent	4	Leduc *et al.*, 1955
Medium from cultures of lymph nodes after foot pad injections of	Hemagglutin. Isotope incorp.		Bauer and Stavitsky, 1960; Stavitsky, 1960c
KLH		2 + 4	
BGG		4 + 3	
diph. toxoid		5 + 8	

[a] Experimental material used for assay; KLH = key hole limpet hemocyanin; T_2 = T_2 bacteriophage of *E. coli*; BGG = bovine γ-globulin; diph. toxoid = diphtheria toxoid; intraven. = intravenous; foot pad = injection into hind foot pads.

[b] Hemagglutin. = passive hemagglutination (Boyden, 1951); isotope incorporation = method of Stavitsky (1960a).

[c] Interval between injection of antigen and first appearance of antibody in experimental material; 2 + 4—first figure gives number of days tissues remained *in vivo*, the second the number of days in culture.

fore antibody is detectable in the serum or before tissues can be removed to form antibody *in vitro*.

Sterzl (1959b, 1960) made extensive but unsuccessful attempts to induce antibody synthesis *in vitro*. Bacterial antigens were added to rabbit or chicken spleen cells *in vitro*, and the cells were transplanted to homologous neonatal animals with the appearance of antibody in the recipients. The cells evidently took up enough antigen to initiate the antibody response *in vivo*. Moreover, the cells took up antigen and after 48 hours *in vitro* remained viable and competent for antibody synthesis upon transplantation. Nevertheless, when the antigen-stimulated cells were maintained *in vitro* antibody was not produced. Endotoxin was injected into the rabbits before removal of the cells for addition of antigen, as was done by Stevens and McKenna (1958), but without effect. In order to test the possibility that antibody was synthesized immediately under these conditions but in indetectable concentrations, Sterzl (1960) concentrated the medium 250–400 times but still did not detect antibody.

3. *Sites and Mechanisms of Induction of Antibody Synthesis*

The foregoing data suggest that there is a distinct inductive phase of the antibody response. In an attempt to define the inductive phase more clearly additional observations and data on the sites and basic mechanisms are presented.

a. Sites. Morphological, cytochemical, and immunofluorescent studies implicate primitive and "activated" reticulum cells, plasmablasts, immature plasma cells, lymphocytes, eosinophiles, and macrophages in the inductive phase (Marshall and White, 1950; Makinodan *et al.*, 1954; Leduc *et al.*, 1955; Wissler *et al.*, 1957; Coons, 1959; Speirs, 1960). Within a day or two after the injection, antigen has been detected within each of these cells, with the exception of eosinophiles (Fagraeus, 1948a; Coons *et al.*, 1951; Schiller *et al.*, 1952; Gavosto and Ficq, 1953; La Via *et al.*, 1960a). Several days later antibody appeared in certain large, primitive cells which have been called hemocytoblasts (Leduc *et al.*, 1955). A few days later antibody appeared in immature and mature plasma cells (Leduc *et al.*, 1955). In another study (Holub and Riha, 1960) rabbit lymphocytes and egg albumin were placed in a diffusion chamber in the peritoneal cavity of a neonatal rabbit. By immunofluorescent technique, antigen was detected during the next 2 days in large lymphocytes, monocytes, newly developed macrophages, and traces in small lymphocytes. Eight days later antibody was detected by this

method in cells of the plasma cell series and in stress lymphocytes. Little or no mitosis occurred within the chamber.

These two studies (Leduc *et al.*, 1955; Holub and Riha, 1960) more clearly implicate lymphocytes, macrophages, monocytes, and various primitive lymphoid and plasmacytoid cells in the early phases of the antibody response. The conversion of one cell, which at one time contains antigen, to another cell type which synthesizes antibody is also indicated (J. C. Roberts *et al.*, 1957). At present, however, induction cannot be definitely attributed to either of these cells. Nor can the role of mitosis in the inductive phase be decided. The results of Holub and Riha (1960) suggest that certain cells can participate in the initiation and induction of the antibody response without undergoing mitosis.

Upon finding antigen in the nucleus, Coons (1959) speculated that the antibody response may begin in or be associated with this structure. However, the finding of antigen in the nucleus has been considered an artifact of the experimental method (Schiller *et al.*, 1952). On the basis of the finding of antigen in the cytoplasm (Coons *et al.*, 1951), the association of increased ribonucleic acid metabolism with protein synthesis (Brachet, 1957), and the association of antibody with microsomes (Askonas, 1958; Kern *et al.*, 1959), it may be postulated that these subcellular particles are the site of the inductive phase.

There is some indication of the number of cells which must be employed to study the inductive phase. In the diffusion chamber study (Holub and Riha, 1960) about 50×10^6 cells were employed. From 5×10^5 to 50×10^6 cells sufficed when bacterial antigen was added *in vitro*, and the cells subsequently synthesized antibody upon injection into chick embryos, neonatal rabbits, and chicks (Trnka and Sterzl, 1960). Since only a small percentage of cells is involved in antibody synthesis (Attardi et al., 1959; Nossal, 1958), perhaps only 5–20% of these cells actually participate in the inductive phase.

b. Basic Mechanisms. A prominent feature of the early days of the antibody response is hypertrophy of antibody-forming organs. While cellular infiltration may cause some of the hypertrophy, microscopic examination reveals much proliferation of reticulo-endothelial and lymphoid elements (Marshall and White, 1950; Makinodan *et al.*, 1954; Wissler *et al.*, 1957). In the previous section evidence was presented that conversions of one cell type to another also occur during this period. Enhanced mitosis, cellular differentiation, and enhanced deoxyribonucleic (DNA) and ribonucleic acid (RNA) metabolism, which are associated with heightened cellular activity (Brachet, 1957), have therefore, been implicated in the early phases of the antibody response.

X-ray inhibits a critical early phase of the antibody response: rabbits injected with antigen 6 hours after X-irradiation produced normal amounts of antibody, whereas those given antigen 12 hours after irradiation formed little or no antibody (Taliaferro *et al.*, 1952). Irradiation of lymph node cells *in vitro* after addition of antigen apparently abolished their ability to initiate the antibody response because antibody did not appear upon transplantation of these cells (Harris *et al.*, 1959). There is evidence (Strauch *et al.*, 1959) that X-ray may affect certain stem cells which are stimulated by antigen to proliferate and differentiate into the cells which synthesize antibody. If rats were given carbon 5–10 days before X-irradiation, they synthesized antibody in response to *Brucella,* whereas controls not given carbon did not respond to the bacteria. The carbon apparently initiated proliferation of reticulum cells which may be the predecessors of the antibody-producing cells. Endotoxin, which counteracts the inhibitory effect of X-ray on the antibody response (Kind and Johnson, 1959) and shortens the induction period in nonirradiated, immunized animals (Ward *et al.*, 1959) seems to enhance mitotic activity and cellular differentiation in the spleen (Ward *et al.*, 1959). The X-ray inhibition of the inductive phase of antibody synthesis is counteracted by the injection of various nucleic acid derivatives (Taliaferro and Jaroslow, 1960).

Cortisone inhibits antibody synthesis *in vivo* and appears to affect an early phase of the response (Fagraeus, 1952). *In vitro* effects of this hormone on the process have been inconsistent. Fagraeus (1952) was unable to inhibit antibody synthesis by splenic fragments upon addition of 70 μg. cortisone per millileter of medium. This result is consistent with the suggestion that this steroid affects a phase of the antibody response before the antibody-synthesizing system is developed. However, by the use of 320 μg. per milliliter, Mountain (1955a) did succeed in inhibiting antibody synthesis *in vitro.* Vitamin deficiency also appears to interfere with the primary antibody response and perhaps with the inductive phase (Axelrod and Pruzansky, 1955). Splenic cells from pantothenic acid-deficient rats which were given antigen did not synthesize antibody when cultured *in vitro* (Stavitsky and Axelrod, 1957) or when transferred to normal rats (Axelrod and Pruzansky, 1955). Evidence was obtained that the mean DNA content of isolated splenic nuclei from pantothenic acid-deficient rats which were given antigen was lower than that of comparable controls (experiments with Dr. C. Leuchtenberger, cited in Axelrod and Pruzansky, 1955). It was postulated that the vitamin deficiency interfered with the increased mitotic activity which presumably precedes or accompanies antibody production. Since

cell division is always preceded by an increase in DNA content, a direct role for pantothenic acid in DNA synthesis was considered a possibility (Axelrod and Pruzansky, 1955). The fact that pantothenic acid-deficient rats produced normal amounts of so-called "normal" γ-globulin but did not produce antibody (Axelrod and Pruzansky, 1955) also suggests that the defect in these animals is associated with the inductive phase during which the antibody-synthesizing system is evolved.

Thus far all efforts to find agents other than X-ray which will inhibit the inductive and not the synthetic phase of antibody synthesis have been unsuccessful (Dutton *et al.*, 1960a; Stavitsky, 1960c).

Enhancement of the synthesis of proteins other than antibody γ-globulin may be a property of the inductive phase (Stavitsky, 1960a,c; Utchitel and Konikova, 1960). The nature of these nonspecific proteins and the manner in which their synthesis is linked to the synthesis of specific antibody is not clear.

C. Antibody Synthesis during Primary and Secondary Antibody Responses

1. Anatomical Structures Involved

a. Organs. In accord with the results of *in vivo* and cell transfer studies (McMaster, 1953; J. C. Roberts and Dixon, 1955; Harris and Harris, 1956; Stavitsky, 1954a, 1957a,b), organ sites of *in vitro* antibody synthesis are determined by the type, amount, and physical form of antigen, route of injection, and the presence of adjuvants in the inoculum. Large amounts of soluble antigen stimulated antibody synthesis in organs remote from the site of inoculation. For instance, 100 Lf units of diphtheria toxoid injected intravenously stimulated not only the spleen but the mesenteric lymph nodes as well (Stavitsky, 1957a). Small amounts of particulate or precipitated antigen injected into an area evoked antibody synthesis mainly in the regional lymph node (S. Roberts, *et al.*, 1949; Thorbecke and Keuning, 1953; Stavitsky, 1955; Askonas and Humphrey, 1958a). However, the injection of antigen in Freund's adjuvants into the leg stimulated not only the homolateral lymph node but evoked an even greater antibody response in the contralateral lymph node (Askonas and White, 1956). Whether this was a consequence of the drainage of excessive amounts of antigen into the homolateral node with resultant "paralysis" of that node is not known. Particulate antigens injected intravenously stimulated the spleen and bone marrow to form antibody (Thorbecke and Keuning, 1953; Stavitsky, 1955). The hemolysin response varied considerably, as compared to the intravenous route, when red cells were injected into

various sites in intact and splenectomized rabbits (Draper and Süssdorf, 1957). When large concentrations of soluble antigen are given repeatedly, organs, which usually are inactive or slightly active in response to minimal concentrations of antigen, may synthesize larger amounts of antibody. For instance, large and repeated injections of egg albumin cause the lung and bone marrow to become major sites of antibody synthesis in the rabbit (Askonas and Humphrey, 1958a).

An interesting feature of the antibody response is its persistent localization in the organ which received the initial antigenic stimulus. When suitably small amounts of aggregated antigens are injected into a local area, the bulk of the primary and secondary antibody response may occur in the regional lymph node (S. Roberts *et al.*, 1949; Stavitsky, 1954a; Coons *et al.*, 1955; Harris and Harris, 1956; R. G. White, 1960). Even when the interval between injections was 7 months, *in vitro* studies of the antibody-producing capacity of the organs indicated that the draining lymph node made the bulk of the antibody (Stavitsky, 1960c). White (1960) injected rabbits with diphtheria toxoid into the left foot pad and ovalbumin into the other. Eighteen days later both feet were injected with both antigens. By immunofluorescent technique antibody was always found within cells in the node which received the primary stimulus with the homologous antigen (diphtheria antitoxin in the left node and anti-ovalbumin in the right).

The thymus, liver, lungs, kidney, and appendix of immunized animals did not synthesize antibody *in vitro* (Fagraeus, 1948a; Thorbecke and Keuning, 1953; Stavitsky, 1955; Askonas and White, 1956; Askonas and Humphrey, 1958a), but the thymus of animals immunized with tetanus toxoid did produce antitoxin following transplantation (Stoner and Hale, 1955). Some of these organs possess some ability to synthesize antibody as shown by the finding of small numbers of antibody-containing cells in liver, lungs, kidney, and thymus by immunofluorescent methods (Askonas and White, 1956) and the demonstration of some antibody in the appendix (Humphrey and Sulitzeanu, 1957). The granuloma formed at the site of injection of antigen in Freund's adjuvants contributes an appreciable amount of antibody in the rabbit (Askonas and Humphrey, 1958a) but very little antibody in the guinea pig (Askonas and White, 1956).

Certain tissues from normal rabbits synthesize γ-globulin *in vitro* (Askonas and Humphrey, 1958a; Thorbecke, 1960). It is not known how much, if any, of this γ-globulin is antibody. In neonatal rabbits 1 week or older most γ-globulin synthesis is limited to the appendix (Thorbecke, 1960). In adult animals peripheral lymph nodes, bone

marrow, spleen, and lung produce appreciable amounts of γ-globulin, whereas mesenteric lymph nodes, appendix, and thymus form less and kidney cortex and liver form none (Thorbecke, 1960). Immunization enhances formation of this protein in spleen, lung, and lymph nodes (Askonas and Humphrey, 1958a; Thorbecke, 1960). *In vitro* synthesis of other γ-globulin may even exceed that of specific antibody by various tissues of rabbits hyperimmunized with pneumococci or ovalbumin (Askonas and Humphrey, 1958a).

These data agree with findings of studies with perfused rat livers and perfused rat carcasses that liver synthesizes plasma albumin, α-globulin, and β-globulin (including fibrinogen) and that the extrahepatic tissues synthesize the γ-globulins (Miller and Bale, 1954; Miller *et al.*, 1954).

The relative capacity of various organs to synthesize antibody has been studied *in vitro* and *in vivo*. In incubations for 6 to 12 hours so as to insure the maintenance of maximal synthetic capacity during the period of observation, it was found that per unit weight of tissue rabbit lymph nodes synthesized more antibody than was synthesized by the spleen when diphtheria toxoid was injected so as to stimulate both organs maximally (Stavitsky, 1960c). Studies of hemolysin production in normal and splenectomized rabbits led to the postulate that the spleen participates in the early, short-lived but rapid antibody synthesis, whereas nonsplenic sites are responsible for the persistent formation of antibody (Taliaferro and Taliaferro, 1952). Other data suggest that the lymph nodes and/or bone marrow are active in antibody production in the earliest stages of immunization and that the spleen becomes a relatively more important site of synthesis as immunization continues (Askonas *et al.*, 1956).

b. Cells. Precise definition of the cells involved in the synthesis of antibody has been hampered by the lack of adequate morphological and cytochemical criteria. Recently, however, some criteria have been provided (see, for example, Fagraeus, 1948a; Marshall and White, 1950). By combining these criteria with various other techniques considerable information about the types of cells which synthesize antibody has been acquired. Many types of evidence implicate plasma cells of varying states of maturity in this process. The immunofluorescent technique (Coons *et al.*, 1955; Leduc *et al.*, 1955) has been valuable in providing direct evidence for the role of this cell type. Following the primary injection of proteins, antibody was detected first in cells which were termed "primitive leucocytes or hematocytoblasts," according to Marshall and White (1950), and later immature and mature plasma

cells (Leduc *et al.*, 1955; Coons, 1959). Following the insertion of proteins and lymphoid cells in a diffusion chamber and placing the chamber in the peritoneal cavity, antibody was observed in plasmacytoid cells (Holub and Riha, 1960). Also following secondary antigenic stimulation antibody was observed in immature and mature plasma cells (Coons *et al.*, 1955; Leduc *et al.*, 1955; Askonas and White, 1956). Antibody-forming organs synthesized antibody maximally when they contained large numbers of immature plasma cells (Fagraeus, 1948a,b; Keuning and van der Slikke, 1950; Askonas and White, 1956), and antibody was observed within these cells by the fluorescent technique (Askonas and White, 1956). *In vitro* studies, employing single cells from immunized animals, indicate that individual immature plasma cells synthesize antibodies against bacteriophage (Attardi *et al.*, 1959) and against flagellar *Salmonella* antigens (Nossal, 1959c). Electron micrographs of these cells reveal a highly organized endoplasmic reticulum which is characteristic of cells that actively synthesize and secrete proteins, such as pancreatic acinar cells (Palade, 1956).

Whereas it is generally agreed that immature plasma cells synthesize antibody, the role of other cells is not established. Eosinophiles have been implicated in the inductive phase, but not in the synthetic phase (Speirs, 1960). The cells which must be seriously considered include lymphocytes, macrophages, and various more primitive cells which are difficult to distinguish such as hemocytoblasts and "activated" reticulum cells (Marshall and White, 1950; Coons *et al.*, 1955; Leduc *et al.*, 1955). Shortly after the role of the lymph node in the synthesis of antibody was established (McMaster and Hudack, 1935; Harris and Harris, 1956), a controversy began regarding which cells in the node synthesize antibody. Lymph node cells from immunized animals, consisting predominantly of lymphocytes, and a few per cent of mononuclear and immature plasma cells, were transplanted into normal animals with the appearance of antibody in the latter (J. C. Roberts and Dixon, 1955; Harris and Harris, 1956; Stavitsky, 1958a). Further observations suggested, however, that following transplantation the lymphocytes were converted into other cell types, eventually into immature plasma cells which synthesized the antibody in the recipient (J. C. Roberts *et al.*, 1957; Neil and Dixon, 1959). The finding by immunofluorescent technique of antibody in developing plasma cells within the recipient supported this suggestion (Neil and Dixon, 1959). Attempts to detect antibody within isolated lymphocytes have yielded equivocal results (Erslev, 1951; McMaster, 1953; Harris and Harris, 1956). Bacteria have been observed stuck to the surface of lymphocytes of loose connective

tissue (Hayes and Dougherty, 1954) or lymph nodes (Reiss *et al.*, 1950) of animals immunized with these microorganisms, but in other experiments, bacteria were adherent to plasma cells (Moeschlin and Demiral, 1952). However, the specificity of these reactions was not tested with antigenically unrelated bacteria. Such a control is necessary because the specific agglutination of bacteria by washed cells requires the presence of antibody in the cell wall or the action of long-range specific forces between extracellular organisms and intracellular antibody. Although antibody has been demonstrated on microsomes (Askonas, 1958, 1960; Kern *et al.*, 1959), it has not so far been shown in the mammalian cell wall. There is convincing evidence against the existence of long-range specific forces between antigen and antibody (Karush and Siegel, 1948). Nossal (1959c) studied the primary and secondary antibody responses of rats to *Salmonella* flagellar antigens. During the primary response of 601 cells observed in microdroplets, 93 formed antibody and 91 of these were plasmablasts or plasma cells. In another series of 306 cells, only 1 lymphocyte synthesized antibody.

Recent evidence does, however, suggest that lymphocytes or cells which are difficult to distinguish morphologically from lymphocytes synthesize antibody. In immunofluorescent studies (Coons *et al.*, 1955), small amounts of antibody occasionally were observed in the lymphoid follicle cells of the spleen and lymph nodes. In more detailed studies by this method (R. G. White, 1960), antibody was detected in large and medium lymphocytes within germinal centers of lymph nodes during the secondary response to diphtheria toxoid. Ortega and Mellors (1957) detected human γ-globulin in the germinal centers of lymph nodes draining the sites of human carcinomata. Attardi *et al.* (1959) isolated lymph node cells from rabbits immunized with bacteriophage. When these cells were prepared in microdroplets, 14% of cells of the lymphocyte series and 27% of cells of the plasma cell series produced neutralizing antibody. Ancillary evidence was presented that active synthesis of antibody rather than passive adsorption of antibody on the cells and release occurred. Wesslen (1952) immunized rabbits with *Salmonella* typhi. Two to three weeks later the bacteria were injected intravenously, followed in 3 days by the collection of thoracic duct lymph cells. This type of cell suspension, which usually does not contain reticulum or plasma cells but may contain some large immature lymphoid cells in addition to lymphocytes (Gowans, 1959), was washed and cultivated in a medium. Specific agglutinins were produced in low titer.

Historically, reticulo-endothelial cells or macrophages were the

earliest implicated in antibody synthesis (Sabin, 1939; McMaster, 1953). Interest in these cells temporarily went into eclipse while attention was concentrated upon lymphocytes and plasma cells, but recent studies again have brought the macrophages back into consideration. Peritoneal exudate cells from rabbits immunized with bovine serum albumin were capable of a secondary antibody response after transplantation into X-irradiated rabbits and subsequent antigenic stimulation (Dixon *et al.*, 1957b). Evidence was presented that the macrophages which comprised 70% of the population rather than the lymphocytes which consisted of 11% were responsible for the antibody response. However, it appeared that following transplantation the macrophages developed into plasma cells which then synthesized antibody (J. C. Roberts *et al.*, 1957). Similar studies with bacterial antigens thus far have been negative. Nossal (1959c) did not observe antibody synthesis by single macrophages *in vitro,* but he looked at relatively few of this type of cell compared to the numbers of plasma cells and lymphocytes examined. K. B. Roberts (1955) and Harris and Harris (1956) did not detect antibody in peritoneal macrophages of rabbits immunized intraperitoneally with *Shigella* and *Salmonella,* respectively. McKenna and Stevens (1960) and Stavitsky (1960c) detected antibody-like substances in cultures of peritoneal cells from rabbits immunized with proteins. McKenna and Stevens maintained the cells for several weeks during which cell types other than macrophages were eliminated. However, in both studies the hemagglutination titers were low and the specificity of these substances must be questioned (cf. Section III, B, 1).

Evidence suggests that the different types of γ-globulin which are distinguishable by chromatography are synthesized by different cells which may be unevenly distributed in the different organs (Askonas *et al.*, 1956). Depending on whether rabbit spleen, lymph nodes, or bone marrow were used, *in vitro* experiments showed that each tissue incorporated radioactive amino acids into a different γ-globulin fraction at a different rate. Similar studies with the tissues of immunized animals indicated that these organs also differed in the fraction of antibody γ-globulin produced. These observations suggested an explanation for the change in the chromatographic type of antibody upon continued intravenous immunization. After a preliminary series of injections, the lymph nodes were the principal antibody producers *in vitro,* whereas after months of immunization the spleen was most active.

c. Subcellular Particles. Thus far evidence of antibody or γ-globulin synthesis by subcellular particles has not been obtained (Askonas, 1958, 1960). Possible explanations (Askonas, 1960) include damage to

the particles incidental to rupture of the cells or by cathepsin and protease activity of antibody-forming tissues. The fact that only a small percentage of the cells in such tissues synthesize antibody means, moreover, that most of the subcellular particles isolated are inactive. Nevertheless, much recent evidence implicates nuclei, mitochondrial and microsomal fractions, in the synthesis of proteins (Zamecnik, 1960), including the formation of hemoglobin by ribonucleoprotein particles from reticulocytes (Schweet *et al.*, 1958) and the production of albumin by liver microsomes (Campbell and Kernot, 1959).

Askonas (1958, 1960) showed that the microsomal fraction from spleen and bone marrow of a hyperimmunized rabbit contained bound antibody which was released upon disruption of the particles by ultrasonication or incubation at 37° C. with ribonuclease, trypsin, chymotrypsin, deoxycholate, and alkali methods known to release protein and ribonucleic acid. The other cell fractions had smaller amounts of antibody, but were contaminated with cytoplasmic and microsomal debris. It was, therefore, not possible to compare the antibody content of the various subcellular particles. Askonas (1958, 1960) speculated that the antibody was synthesized bound to subcellular particles such as microsomes where it was retained until it mixed with the intracellular pool from which secretion into the medium occurred.

Kern *et al.* (1959) injected 2,4-dinitrophenyl-BGG into guinea pigs and weeks later found that the microsomes derived from the draining lymph node cells contained antibody against the haptenic group. This antibody specifically bound the haptene to the microsomes. Convincing evidence was presented that this antibody was not merely adsorbed on the microsomes from soluble antibodies in the nodes. They could not conclude that the microsomes had synthesized the antibody because the antibody could have been synthesized elsewhere and then translocated to the microsomes. Feldman *et al.* (1960) detected antibodies to sheep red cells and horse serum globulin in a soluble protein fraction derived from microsomes of splenic and lymph node cells from rabbits immunized with these antigens. The microsomes were disrupted with deoxycholate and the resultant ribonucleoprotein particles were isolated by centrifugation. These intact particles showed no antibody activity, but the soluble proteins derived from them by treatment with ribonuclease contained such activity.

2. *Pathways of Antibody Synthesis*

In vitro and *in vivo* investigations have yielded much information regarding the precursors utilized by the cell for the synthesis of anti-

body. The earliest view (Pauling, 1940) was that a normal γ-globulin or polypeptide precursor was converted to antibody under the influence of antigen. In its unmodified form this view no longer is tenable. Preferential utilization of normal γ-globulin for antibody synthesis did not occur when labeled normal γ-globulin was injected into an immunized animal (Gros *et al.*, 1952; Askonas *et al.*, 1956) or when cells containing labeled γ-globulin were injected into normal animals (Taliaferro and Talmage, 1955; Stavitsky, 1958a) or cultured *in vitro* (Stavitsky, 1958b). A recent report of the existence of a nucleoprotein cellular precursor of antibody in the organs of immunized animals (Sterzl and Hrubesova, 1956) has not been confirmed (Stavitsky, 1958a; Hrubesova *et al.*, 1959).

Overwhelming evidence now indicates that antibody is formed from the free amino acid pool of the cell. Radioactive amino acids are incorporated into antibody by certain cells of immunized animals *in vivo* and *in vitro*. Incorporation and antibody synthesis require a full complement of amino acids (Mountain, 1955a; Stavitsky, 1955; Wolf and Stavitsky, 1958; Vaughan *et al.*, 1960), are energy-dependent (S. Roberts *et al.*, 1949; Stavitsky, 1955; Ogata *et al.*, 1956), and require intact cells (Askonas and Humphrey, 1958a; Stavitsky, 1958a,b). The rapid incorporation of radioactive amino acids into antibody does not involve exchange or turnover of antibody (Heidelberger *et al.*, 1942; Taliaferro and Talmage, 1955; Askonas and Humphrey, 1958a). However, over a period of days substantial breakdown of labeled protein and reincorporation of the liberated amino acids into various proteins, including antibody, may occur *in vivo* (Taliaferro and Talmage, 1955; Dixon *et al.*, 1956; Taliaferro and Taliaferro, 1957) and *in vitro* (Stavitsky, 1960c). There is ample evidence that the antibody synthesized *in vitro* has the same general properties of the antibody synthesized *in vivo*. This antibody can be fractionated by partition chromatography in the same way and with the same results as the antibody produced in the animal (Askonas *et al.*, 1956). This antibody is precipitated by specific antigen (see, for example, Taliaferro and Talmage, 1955; Dixon *et al.*, 1956; Askonas and Humphrey, 1958b) or coprecipitated by a homologous antigen–antibody precipitate (Keston and Katchen, 1956; Askonas and Humphrey, 1958a; Stavitsky, 1958b). The most persuasive evidence for the role of amino acids in the synthesis of the antibody molecule was derived from studies of the distribution and concentration of radioactivity in Fractions I and II (combining fractions) and Fraction III (antigenic fraction) prepared by papain digestion of rabbit antibody. Porter (1959)) observed an equal rate of

incorporation of radioactive amino acids into the three fractions which suggests a simultaneous synthesis of the whole antibody molecule from the same pool of amino acids.

3. *Quantitative Aspects of Antibody Synthesis and Secretion*

This section deals with the measurement of the rates of antibody synthesis and secretion, chiefly by isotopic methods. Data obtained during the secondary or hyperimmune antibody responses are emphasized because they are less subject than the primary response to certain errors which limit their accuracy. Isotope which is injected early in the primary response may upon turnover of the initially labeled protein be incorporated into antibody and yield falsely high values (Dixon *et al.*, 1956). If antibody assays are made prior to elimination of circulating antigen, the antibody formed may combine with antigen, be rapidly catabolized, and tend to give falsely low values. These difficulties have been minimized in short-term *in vitro* synthetic experiments. Even in these experiments, however, only relative rates of synthesis have been established because it has been difficult to make accurate estimations of the turnover times of intracellular antibody. Such estimations are subject to the cumulative error of many measurements and closely limited by the validity of many critical assumptions regarding the rapidity and completeness of mixing of extracellular and intracellular labeled constituents and the sizes of various intracellular pools. Estimations of the turnover of proteins are complicated by lack of information about the kinetics of intermediate reactions and the magnitude of the breakdown of labeled proteins and reincorporation of the liberated labeled amino acids into other proteins. Calculations of the turnover of antibody require some unique assumptions of which the validity is questionable (Humphrey and Sulitzeanu, 1957; Askonas and Humphrey, 1958b). It must be assumed that estimates of the specific radioactivity and concentrations of intracellular free amino acids made in organs containing many cell types apply to the particular cells which synthesize antibody. It is assumed that all of the antibody-forming cells are continuously synthesizing and secreting antibody, whereas there may be cells which secrete but do not synthesize antibody; under these circumstances any labeled antibody is gradually diluted with unlabeled antibody. There may also be antibody which is not recently synthesized, but is stabilized in the tissue as an antigen–antibody complex and is not, therefore, in equilibrium with circulating antibody (Garvey and Campbell, 1959). By making reasonable assumptions, the turnover time of antibody has been estimated to be 2–3

hours in two different systems, in the intact immunized animal (Humphrey and Sulitzeanu, 1957) and in spleen slices synthesizing antibody *in vitro* (Askonas and Humphrey, 1958a).

Little is known of the detailed mechanisms involved in the turnover of antibody, especially the relationships among synthesis and secretion and degradation. It is not known where antibody is degraded. Askonas and Humphrey (1958b) found that hyperimmunized, perfused lungs were able to catabolize γ-globulin but estimated their contribution to the over-all catabolism of γ-globulin to be only 7% a day. Such lungs contained many macrophages, and it was considered that they might be responsible for the degradation of the globulin. Humphrey and McFarlane (1954) found that transfused rabbit antibody was removed from the circulation by cells in the liver, kidney, spleen, lung, bone marrow, and gut. Antibody then apparently was rapidly converted into other kinds of intracellular protein. The intracellular, free amino acids derived from the breakdown of antibody apparently never went into complete equilibrium with the plasma, free amino acid pool.

a. Rates of Antibody Synthesis at Various Times after Antigenic Stimulation. The rates of antibody synthesis are influenced by a number of factors relating both to the antigen and the host. Of these only the length of time elapsing between the second or succeeding injections of antigen and the removal of tissue has been studied in detail. Table III summarizes the results of a number of studies. These data, obtained with various antigens, tissues *in vivo* and *in vitro* systems, and assays, indicate that several days after the second injection of antigen the maximal rate of synthesis is attained. Tissues removed earlier show little (first day) or less (second day) synthetic capacity. After the peak is reached, the tissues rapidly lose their ability to synthesize antibody so that by the sixth to eighth day only a small, persistent synthesis can usually be detected *in vitro* (Bauer and Stavitsky, 1960; Boyden *et al.*, 1960).

There is a difference between the secondary antibody response as it occurs *in vitro* or *in vivo*. *In vivo* the peak of the response may be on the third day and the low point on the sixth or seventh day after antigenic stimulation; however, if an antibody-forming organ is removed at the peak of the response (third day) and then cultivated *in vitro*, it will continue to produce antibody at only a slowly declining rate during the next 1–3 weeks (Bauer and Stavitsky, 1960). It appears that some factor(s) which control the rate of antibody formation *in vivo* are absent or modified under *in vitro* conditions. Two factors are suggested: degradation of antigen and/or conversion of one cell (immature plasma

TABLE III

PERIOD OF MAXIMAL RATE OF ANTIBODY SYNTHESIS DURING SECONDARY RESPONSE IN THE RABBIT

Antigen[a]	System[b]	Assays[c]	Maximal response (days)[d]	References
S. typhi	Spleen cultures	Agglutin.	4–5	Fagraeus, 1948a
BGG	Spleen *in vivo*	Isotope incorp.	4–6	Dixon *et al.*, 1956
BSA	Spleen transpl.	Precipitin	2.5	Taliaferro and Taliaferro, 1957
Diph. tox.	Node transpl.	Hemagglutin.	3–4	Stavitsky, 1960c
Diph. tox.	Node cultures	Hemagglutin.	3–4	Stavitsky and Wolf, 1958
Diph. tox.	Node cultures	Isotope incorp.	3–4	Stavitsky, 1960c
HSA	Spleen cultures	Isotope incorp.	3–5	Boyden *et al.*, 1960

[a] BGG = bovine γ-globulin; BSA = bovine serum albumin; Diph. tox. = diphtheria toxoid; HSA = human serum albumin.

[b] Cultures, tissues removed for culture on various days after the second or subsequent injections of antigen; *in vivo* refers to intravenous injection of antigen followed by assay of antibody in serum; transpl. = cells transplanted at various times after second injection of antigen.

[c] Hemagglutin. = passive hemagglutination (Boyden, 1951).

[d] Days of maximal response after last injection of antigen.

cell) which synthesizes large amounts of antibody into another type (mature plasma cell) which synthesizes smaller amounts of antibody. The latter conversion has been observed to occur *in vivo* (Coons *et al.*, 1955; Wissler *et al.*, 1957) but not *in vitro* (Stavitsky, 1960c).

It has been found that rabbit tissues removed at the peak of the secondary antibody response synthesize the following amounts of antibody *in vitro:* 1.9 mg. anti-ovalbumin per gram of spleen in 24 hours (Steiner and Anker, 1956); 400 μg. antidiphtheria toxoid per gram lymph node in 24 hours (Stavitsky and Wolf, 1958); 32 μg. anti-ovalbumin per gram of spleen in 3 hours (Askonas and Humphrey, 1958a); 400 μg. anti-ovalbumin per gram of spleen in 24 hours (Vaughan *et al.*, 1960). Substantially larger amounts of antibody (10 mg. per gram of lymph node in 24 hours) were made by transplanted lymph nodes in man (Martin *et al.*, 1957).

There is considerable evidence of the continued production of antibody for many months or years after the peak of the response (reviewed in Burnet and Fenner, 1953). Antibody has been detected in the blood for this long after the last injection of antigen (Barr and Glenny, 1947; Richter and Haurowitz, 1960, Stavitsky, 1960c). In addition, antibody may be stored in the tissues for many months or perhaps years. There is some question whether this antibody is being continually synthesized or is metabolically inert. In some of their perfusion experiments with the lungs of immunized animals, Askonas and Humphrey (1958b) observed that the specific radioactivity of intracellular antibody was much less than that of secreted antibody. They suggested that metabolically inert antibody must have been present and sections of lung from such rabbits, prepared by the immunofluorescent technique, showed many cells which contained antibody in granular and presumably inert form. Using the binding of radioactive antigen as a criterion, antibody has been detected in the spleens and lymph nodes but not in the blood of rabbits 10 months after the second injection of alum-precipitated diphtheria toxoid (Bauer and Stavitsky, 1960).

b. Rapidity of Synthesis and Secretion of Antibody. In vitro studies permit detailed analyses of the kinetics and mechanisms of antibody synthesis and secretion. Figure 1 illustrates the typical results of an *in vitro* study employing spleen slices from a rabbit hyperimmunized with ovalbumin which were incubated in the presence of C^{14}-labeled amino acids, and incorporation into intracellular and extracellular antibody determined. Similar results were obtained with lungs of hyperimmunized rabbits which were perfused with C^{14}-amino acids for 5 to 6 hours. After 10–30 minutes radioactivity was present in intracellular

antibody, but no radioactive antibody had been secreted into the medium. Thereafter, the radioactivity of the intracellular antibody gradually leveled off, whereas that of the secreted antibody continued to rise at a constant rate for 4 hours. Similar results were obtained *in vivo:* radioactive antibody was extracted from the tissues of immunized animals within 15 minutes after the injection of labeled amino acids (Humphrey and Sulitzeanu, 1957), whereas radioactive antibody appeared in the blood only after 25–60 minutes (Humphrey and

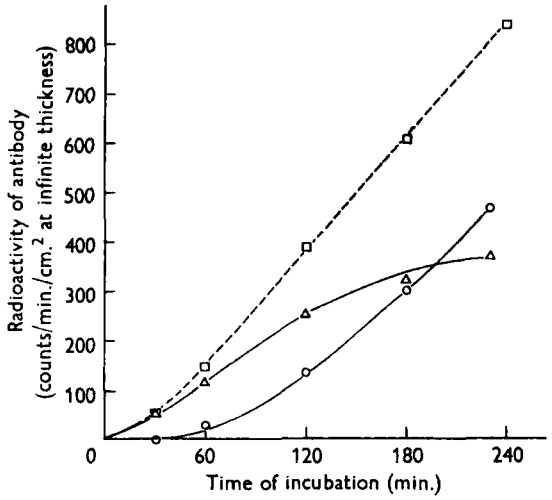

FIG. 1. Incorporation of C^{14}-glycine into intracellular and secreted anti-ovalbumin by spleen slices. ○, Secreted antibody isolated from medium; △, intracellular antibody extracted from spleen slices after homogenization; □, combined intracellular and extracellular antibody. Slices of spleen (0.5 gm.) from a rabbit immunized intravenously were incubated in the usual medium containing 3 μc. of C^{14}-glycine. Antiovalbumin carrier (6 mg.) was added before isolation of each antibody fraction. (Reproduced with permission from Askonas and Humphrey, 1958a.)

Sulitzeanu, 1957; Taliaferro and Taliaferro, 1957). Similar results were obtained with perfused lungs (Askonas and Humphrey, 1958b) and with lymph node cells *in vitro* (Helmreich et al., 1960). After about 5 hours of perfusion, the specific radioactivity of the extracellular antibody sometimes approached that of the intracellular antibody, which indicated that the newly synthesized antibody molecules mixed with the intracellular antibody pool from which a steady secretion into the surrounding medium occurred. In some experiments the specific activity of intracellular antibody was much less than that of secreted antibody at the end of perfusion and, therefore, metabolically inert intracellular antibody must have been present (Askonas and Humphrey, 1958b).

Since antibody was bound to microsomes, by analogy with other systems (Peters, 1957; Siekevitz and Palade, 1958), Askonas (1958, 1960) proposed that antibody was synthesized in bound form and then secreted. By analogy with other findings Askonas (1960) suggested that secretion is an energy-requiring process. Helmreich *et al.* (1960) have shown that intracellular antibody is secreted more slowly under anaerobic than under aerobic conditions. The process of release of antibody from the cell is too rapid to be attributed to dissolution of cells. Moreover, it has been found that in the disruption of antibody-forming cells with X-irradiation (Craddock *et al.*, 1949) or nitrogen mustard (Marshall and White, 1950), with adrenal cortical hormones (Craddock *et al.*, 1949) or colchicine (Fagraeus and Gormsen, 1953), appreciable amounts of antibody are not liberated.

Antibody synthesis occurred at a constant rate for about 4 hours when spleen slices from hyperimmunized rabbits were incubated *in vitro* (Askonas and Humphrey, 1958a). In studies with spleens from hyperimmunized rabbits (Vaughan *et al.*, 1960) the cells removed 2 days after the last injection of antigen showed a lag of 1 day *in vitro* before beginning to synthesize antibody at a constant rate. The cells removed 3 days after antigenic stimulation showed a lag of about 1 hour before attaining a constant rate of synthesis. Once the constant rate was attained both the "2 day" and "3 day" cells maintained this rate up to 48 hours. The spleen cell preparations would, therefore, seem to have many advantages for a detailed analysis of antibody synthesis.

The data on the rapidity of synthesis of antibody are in accord with the results of *in vitro* and *in vivo* studies with other proteins in mammals. The time required for antibody synthesis approaches even the short interval of a few minutes indicated for the synthesis of enzymes in bacteria (Hogness *et al.*, 1955). Loftfield and Eigner (1958) found that, *in vivo,* ferritin became radioactive in rat liver within 2 minutes after intravenous injection of C^{14}-amino acids. It is possible that *in vitro* the process of formation and release is slower.

4. Distinction between Primary and Secondary Antibody Responses

Many distinctions have been noted between the antibody response to a primary injection of antigen (primary response) and the type of response elicited by succeeding injections (secondary response) (Glenny and Sudmerson, 1921; Burnet and Fenner, 1953; Wilson and Miles, 1957). Recently, some light has been shed on possibly distinctive mechanisms or rates of reactions in the two responses.

Leduc *et al.* (1955) by immunofluorescent techniques observed that many more cells contained antibody and fluoresced more brightly during the secondary as compared to the primary response. Single-cell *in vitro* experiments also revealed that during the secondary response many more cells are engaged in antibody synthesis and that each cell synthesizes more antibody than during the primary reaction (Nossal, 1959b). The histological and cytochemical picture of the primary antibody response is qualitatively similar but quantitatively distinct from that observed during the secondary response: there are fewer immature plasma cells with antibody and increased RNA content in the cytoplasm, fewer primitive cells in mitosis and less evidence of the metamorphosis of primitive cells into immature plasma cells (Leduc *et al.*, 1955). During an apparent primary response of the rat to bacterial antigens (Wissler *et al.*, 1957) its tissues may resemble those of the rabbit secondary reaction, but it may be questioned whether the reaction in the rat is not a secondary response. *In vitro* cultures of antibody-forming organs, whether derived from the animal during the height of the primary or secondary responses, show very few cells in mitosis (Fagraeus, 1948a; La Via *et al.*, 1960a, b; Stavitsky, 1960c). Transplantation of cells from immunized to normal animals also results in the synthesis of antibody without the obvious occurrence of mitoses (Neil and Dixon, 1959).

Although it has been difficult to detect mitosis among the cells of antibody-forming organs *in vitro* or even *in vivo*, it is likely that it occurs, perhaps earlier in the antibody response. That mitosis is stimulated more by the secondary than primary injection of antigen is suggested by the data of Nossal (1960). He took spleen cells from a rat primarily immunized with *Salmonella* flagellar antigens, stimulated these cells *in vitro*, and put them into neonatal rats. Two weeks later spleen cells were removed from the rats, stimulated with antigen *in vitro*, and again transplanted. In this manner it was possible to maintain antibody-synthesizing cells through 5 transfers as manifested by the appearance of antibody in the rats. If the cells were not restimulated with antigen, antibody synthesis was maintained through only 2–3 passages.

Perhaps, the most clear-cut distinction between the primary and secondary type of response is their inducibility *in vitro*. The inducibility of the primary one is doubtful (see Section III, B, 1). The secondary response to diphtheria toxoid has been induced *in vitro* by the addition of this antigen to lymph node (Michaelides, 1957; Stavitsky, 1960a,b) or spleen fragments (Stavitsky, 1960a). Continued culture of these fragments resulted in the synthesis of hemagglutinating antibody

(Michaelides, 1957) or the detection of antibody by the incorporation assay (Stavitsky, 1960a, c).

5. *Nature of the Antibody-Forming System*

Various types of evidence from studies of protein synthesis (Zamecnik, 1960) and antibody formation implicate nucleic acids, particularly RNA, in the synthesis of antibody. Increased amounts of RNA have been found in lymphoid cells of antibody-forming organs (Harris and Harris, 1956; Makinodan *et al.*, 1954; Dutton *et al.*, 1960b). Recently it has been observed that crude nucleic acid digests restore the ability of X-irradiated rabbits to produce antibody (Taliaferro and Jaroslow,

TABLE IV
INHIBITION OF *In Vitro* ANTIBODY SYNTHESIS BY RABBIT TISSUES

System[a]	Inhibitor[b]	Conc. (m*M*)	% Inhibition	References
Tox.–LN	DL-Eth	1.0	82	Stavitsky and Wolf, 1958
	p-FløAl	1.0	85	Stavitsky and Wolf, 1958
	γ-EAG	1.0	80	Stavitsky and Wolf, 1958
	8-AG	2.5	60	Stavitsky, 1960c
	TRB	0.3	90	Prots *et al.*, 1960
	Puro	0.3	95	Prots *et al.*, 1960
	5-BRUD	0.2	80	Prots *et al.*, 1960
EA–spleen	DL-Eth	8.0	93	Vaughan *et al.*, 1960
	p-FløAl	5.5	93	Vaughan *et al.*, 1960
	8-AG	1.7	40	Dutton *et al.*, 1958
		5.0	80	Dutton *et al.*, 1958
	5-BRUD	0.02	60	Dutton *et al.*, 1960a

[a] Tox. = diphtheria toxoid; LN = lymph node fragments; EA = ovalbumin; spleen refers to spleen cells.

[b] DL-Eth = DL-ethionine; *p*-FløAl = *p*-fluorophenylalanine; γ-EAG = γ-ethylamidoglutamic acid; 8-AG = 8-azaguanine; TRB = 4,5,6-trichloro-1 = (β-D-ribofuranosyl)benzimidazole (Tamm *et al.*, 1960); Puro = puromycin; 5-BRUD = 5-bromouracil deoxyriboside.

1960). Table IV lists a number of substances which inhibit antibody synthesis *in vitro* and which presumably, therefore, act indirectly or directly upon the antibody-forming system. Unfortunately, the biochemical mechanisms whereby inhibition occurs are only beginning to be studied. It is not known whether any of these substances is acting directly upon the antibody-forming template. There is in fact evidence that several of these substances do inhibit antibody synthesis indirectly. For instance, 8-azaguanine in addition to inhibiting antibody synthesis also reduced the uptake of P^{32} into the acid-soluble RNA and DNA

fractions of spleen cells (Dutton *et al.*, 1958). It is, however, possible that 8-azaguanine suppressed P^{32} uptake into cells in the population which were not engaged in antibody synthesis and suppressed antibody formation in other cells by still another mechanism. Puromycin inhibits amino acid incorporation into proteins from a cell-free preparation from rat liver (Yarmolinsky and de la Haba, 1959). This substance inhibits antibody synthesis *in vitro*, but only at concentrations which completely inhibit acid production by these cells *in vitro* (Prots *et al.*, 1960). Of special interest are 5-BRUD (5-bromouracil deoxyriboside) which apparently inhibits DNA metabolism (Dutton *et al.*, 1960a) and TRB [4,5,6-trichloro-1-(β-D-ribofuranosyl)benzimidazole] which interferes with RNA synthesis (Tamm *et al.*, 1960). The 5-BRUD (Dutton, 1960; Dutton *et al.*, 1960a) and TRB (Prots *et al.*, 1960) inhibit antibody synthesis *in vitro*.

IV. Discussion

This section resorts more extensively to inference, analogy, generalization, and speculation from the data than the foregoing. Although much is oversimplified or fallacious, I agree with Poincaré: "that it is far better to foresee without certainty than not to foresee at all."

Certain general factors may affect all aspects of the antibody response and therefore influence interpretation of experimental data. These include the chemical and physical nature and dosage of antigen; the age, strain, and species of experimental animal; the amounts and type of antibody produced; the methods utilized for the assay of antigen and antibody. For instance, antibody may not appear upon injection into a certain species. It is, however, entirely possible that because of the antigen (diphtheria toxoid) or the animal (mouse) the antibody produced either was inadequate in amount or of a nonprecipitating type which is not detectable by the methods employed. It is well known that different amounts of different antigens may be required to elicit an antibody response and that amounts of different antigens which "paralyze" the antibody response may vary by a factor of 10–100 or even more (Stavitsky, 1960c). The length of the induction period for various antigens may also vary (Table II). The ability of an individual animal to respond to a given antigen may be determined by its genotype (Sang and Sobey, 1954). By analogy with the differences in types of antibodies (precipitating, sensitizing, blocking) produced by different humans upon exposure to an antigen (Wilson and Miles, 1957), it may be postulated that not only the specificity but also the type of antibody produced may depend on the genotype of the individual.

A. Present Status

1. Sites of Antibody Response

Cell types could be linked to various phases of the antibody response with more precision if the various cell types, particularly the stem cells of the lymphoid and plasmacytoid series, and the cells into which they seem to metamorphose could be identified with greater certainty (Sundberg, 1947; Taliaferro, 1949; Marshall and White, 1950; Downey, 1955; Trowell, 1958; A. White, 1958). Many more cells seem to take up antigen than make antibody. How many cells participate in the antibody response is not known exactly because some cells may contain amounts of antigen too small to detect but adequate to trigger this response; or amounts of antibody too meager for detection. A restricted number of cell types seem capable of taking up antigen. Macrophages phagocytose aggregated antigens, and reticular cells and lymphocytes appear to incorporate soluble antigens. Although macrophages may contain both bacteria and eosinophiles (Speirs, 1960), there is no evidence that the eosinophiles contain antigen. Further evidence is required to link the eosinophiles to the antibody response. Although there is no evidence that macrophages synthesize antibody, these cells might participate in the inductive phase. Primitive cells of the lymphoid and plasmacytoid series also are promising candidates for this task. Macrophages, lymphocytes, and reticular cells seem to be converted into immature plasma cells which synthesize antibody (Fagraeus, 1948a; Leduc *et al.*, 1955; Neil and Dixon, 1959). Cells other than immature plasma cells apparently synthesize antibody (large lymphocytes, see R. G. White, 1960; stress lymphocytes, see Holub and Riba, 1960; hemocytoblasts, see Leduc *et al.*, 1955; White, 1960), but it is impossible at present to distinguish among these cells. R. T. Smith (1960) demonstrated an anti-*Salmonella* flagellar macroglobulin antibody in the blood of neonatal infants immunized with *Salmonella*. Since plasma cells were not found in the tissues of these infants, it was suggested that other types of cells synthesized this antibody. This finding reinforces the suggestion from other studies (Askonas *et al.*, 1956) that the heterogeneity of antibody molecules produced against a single antigen may depend on the participation of different types of cells in the synthesis of these antibodies.

The mechanisms whereby the secondary antibody response may be restricted to that lymph node involved in the primary response (Stavitsky, 1960c) is not clear. In normal rats it appears that large numbers of predominantly small lymphocytes are continually being recirculated from lymph nodes through thoracic duct and blood back into the nodes

(Gowans, 1959). However, there is no direct evidence that the small lymphocytes are involved in the antibody response; it seems more likely that large lymphocytes or even more primitive cells with a propensity to proliferate and remain in the lymph node are involved in this localized response.

2. *Mechanisms of Antibody Response*

Antigens probably must be split into fragments to initiate the antibody response. Antibodies reacting with antigenic fragments have been detected in antisera against human and bovine serum albumin (Lapresle *et al.*, 1959; Ishizaka *et al.*, 1960) or produced by injection of fragments derived by degradation of human serum albumin with a splenic protease (Lapresle *et al.*, 1959). Antisera against these fragments may contain antibodies to new antigenic groups which apparently were not present in the native protein (Lapresle *et al.*, 1959). Further combined *in vitro* and *in vivo* studies of this type will be necessary to determine exactly how antigen is metabolized *in vivo* as part of the antibody response. It seems reasonable to assume that subunits of protein and carbohydrates corresponding in size to the dimensions of the antibody-combining sites function in the antibody response.

The initial reaction between antigen and cells occurs within a few minutes (Harris and Harris, 1958; Prots *et al.*, 1960) and seems to result in a firm union because antiserum cannot reverse the reaction (Harris and Harris, 1958), bound labeled antigen is not released by the addition of unlabeled homologous antigen (Prots *et al.*, 1960); and it is difficult to detect free antigen *in vivo* in extracts prepared soon after injection of antigen, although it is known that active antigen is present (Stavitsky, 1954a, 1960c). If the initial reaction is specific, pinocytosis alone would not explain it. Specificity might be introduced by the reaction of antigen with specific receptors on the cell membrane, followed by pinocytosis which, according to Palade (1956) and Bennett (1956), involves the infolding of the cell membrane. According to recent theories (Lederberg, 1959; Talmage, 1959) the cell receptor could be specific antibody which is being synthesized by certain cells even in the absence of antigenic stimulation. There is no evidence for the presence of a specific receptor in the cell wall or elsewhere or of the presence of antibody in cells without antigenic stimulation. Study of the reaction between antigen and cells under various circumstances (varying antigen concentration, X-irradiation, etc.) may yield unexpected dividends in terms of understanding phenomena such as acquired tolerance and the difference between the primary and secondary antibody responses.

About 10^6–10^8 cells are required to initiate the antibody response (Holub and Riha, 1960; Trnka and Sterzl, 1960). A few hundred molecules of soluble *Shigella* antigen (Harris and Harris, 1958) to a few thousand molecules of hemocyanin (Prots *et al.*, 1960) per cell will induce this response, but this is an average figure and individual cells which participate in the response may contain fewer or more molecules. Excessive amounts of antigen may block the antibody response; whether by complexing with newly synthesized antibody and thus preventing secretion of antibody from the cell or by interfering with the induction or synthesis is not known. Inability to detect antibody within the cell following the injection of large amounts of antigen (Stavitsky, 1960c) favors the second explanation.

It seems likely that more than one step is involved in translocating antigen from the cell membrane to the intracellular site(s) where induction occurs. This would necessitate another receptor within the cell. By analogy with the induced synthesis of enzymes in bacteria (Monod, 1958), this receptor could be a precursor of antibody having the same primary but different secondary or tertiary structures than antibody. Although it is unlikely that the mysteries of the inductive phase will be solved through this information, it might be valuable to know whether antigen regularly gains entrance to the nucleus as well as to the cytoplasm of the intact cell.

According to recent theories, the inductive phase may consist of either the activation by antigen of a pre-existing template or the stimulation of synthesis of a new template. The clonal selection theory (Burnet, 1959; Lederberg, 1959; Talmage, 1959) predicts that antibody is always being synthesized within cells possessing a certain genotype so that these cells would contain the specific templates. Antigen might activate a precusor of antibody on the template, converting it to active antibody (cf. Monod, 1958) or remove a repressor of antibody synthesis (cf. Pardee *et al.*, 1959). There are few data which permit even an intelligent choice between these alternatives, although recent genetic studies (Lederberg, 1960) suggest that the first alternative is more likely. By analogy with recent studies of enzymatic induction in bacteria (Monod, 1956, 1958), information on the rapidity and kinetics of induction by antigen and how it is affected by antigen concentration would permit a more enlightened choice between the alternatives just posed. Further information of many types is required before the basic mechanisms of the inductive phase can begin to be discerned. Pertinent questions include: (1) What is the role, if any, of X-ray, endotoxin, cortisone, and vitamin deficiency in modifying the inductive phase? Do these

agents affect mitosis or exert more subtle effects, perhaps on DNA and RNA metabolism? Is mitosis an obligatory event for induction? (2) What is the nature of the link between the synthesis of antibody and of so-called "normal" γ-globulin? Why should the former but not the latter be blocked by vitamin deficiency (Axelrod and Pruzansky, 1955). (3) Why cannot the antibody response be initiated readily *in vitro*? Why must organs remain *in vivo* for a few days after the injection of antigen before they will produce antibody *in vitro*? Is it merely a problem of finding proper conditions for cellular proliferation and differentiation *in vitro* (Sterzl, 1960)? (4) How is information for antibody synthesis transferred from cell to cell? Does it require mitosis or involve the transfer of DNA, RNA particles from cell to cell (Trowell, 1957)? Since this information was not transferred following homotransplation of antibody-forming cells (Stavitsky, 1957a, b), does transfer require genetically compatible recipient cells?

There is some evidence (Section III, B, 1) of the presence in spleen and lymph nodes of a system which synthesizes small amounts of antibody-like proteins and which in the presence of certain antigens is stimulated to produce larger amounts of these substances (see Section III, B, 1 and Table I). The latter system is reminiscent of the rapidly induced, relatively nonspecific immunity which is induced in invertebrates by bacterial antigens (Steinhaus, 1946). This type of immunity seems to be associated with the presence of a protein which is not γ-globulin (Favour, 1958). Numerous cells must possess this system because detectable amounts of antibody may appear within a day. Perhaps this relatively nonspecific primitive system has been carried over into the mammalian realm (McKenna and Stevens, 1960).

Antibody is derived from the free amino acid pool—like bacterial (Hogness *et al.*, 1955) and other mammalian (Loftfield and Harris, 1956; Zamecnik, 1960) proteins. Compounds more complex than amino acids, but derived from amino acids, must somehow intermediate between the amino acids and proteins (Kruh *et al.*, 1960). The available data preclude the accumulation of appreciable amounts of this type of compound (Taliaferro and Talmage, 1955; Taliaferro and Taliaferro, 1957; Stavitsky, 1958a, b) and it must be assumed that, if it exists, it turns over very rapidly in antibody-forming cells. Antibody synthesis is essentially an irreversible reaction (Taliaferro and Talmage, 1955; Askonas and Humphrey, 1958a).

By analogy with other protein-synthesizing systems (Brachet, 1957; Monod, 1958; Zamecnik, 1960), the antibody-forming system probably is associated with one of the subcellular fractions such as the microsomes

(Askonas, 1958, 1960; Kern *et al.*, 1959). It may also be assumed that the information for the synthesis of the specific combining site of antibody is encoded in the sequence of nucleotides in RNA and DNA of the antibody-forming cells (Lederberg, 1960). Since different antibodies have the same over-all amino acid composition (E. L. Smith *et al.*, 1955), the differences between different antibodies must reside either in the sequence of amino acids in the combining site or in a subtle difference in the folding of the molecule. The antibody-forming template seems quite labile; it has not been possible to achieve antibody synthesis with broken cell preparations *in vitro* (Askonas, 1960) or *in vivo* (Stavitsky, 1957a; Hrubesova *et al.*, 1959) thus far. There have been a number of observations on altered physiology and metabolism of antibody-forming organs, particularly during the secondary response. In addition to enhanced mitosis (Leduc *et al.*, 1955) and transformations of one cell type into another (Marshall and White, 1950), these changes include an increase in rate of incorporation of P^{32} into phosphoprotein (Kern and Eisen, 1959), into acid-soluble fraction, RNA, and phospholipids (Stavitsky, 1957b; Dutton *et al.*, 1960b). Some of these alterations (mitosis, differentiation, enhanced RNA metabolism) definitely are associated with antibody-forming cells, but it is not known whether the other changes are. Recent studies on the function of phospholipids in the transport of amino acids (Silberman and Gaby, 1960), proteins, and other substances (Hokin and Hokin, 1956) through the cell wall suggest that enhanced phospholipid metabolism might be advantageous in bringing increased concentrations of amino acids into the cell and in promoting secretion of antibody from the cell.

Although mitosis does not seem essential for antibody synthesis (J. C. Roberts *et al.*, 1957; Holub and Riha, 1960), it might enhance this synthesis by furnishing larger numbers of potential antibody-forming cells. Although antibody synthesis may go on for weeks in the absence of mitosis (Michaelides, 1957; Bauer and Stavitsky, 1960), the ability to inhibit this synthesis with various inhibitors of DNA and RNA metabolism (Table IV) suggests that DNA and RNA metabolism is going on in these apparently "resting" cells (cf. Lindner, 1959).

The factors which influence the rates of antibody synthesis at various times after the second or subsequent injections of antigen (Table III) are unknown. Two factors may be suggested: the drop of antigen to a level inadequate to maintain synthesis and the conversion of one cell type (immature plasma cell) which synthesizes large amounts of antibody into another type (mature plasma cell) which produces smaller amounts. The second factor seems to provide the more likely explana-

tion. On this basis the discrepancy between *in vivo* and *in vitro* results (Section III, C, 3, a) could be attributed to the failure of one cell type to be converted into another *in vitro*. Attempts to boost the declining rate of antibody synthesis during the secondary response *in vivo* and *in vitro* thus far have been without success (Stavitsky, 1960c), possibly because the additional antigen was quickly complexed with circulating antibody and promptly catabolized.

The basic mechanisms of the secondary antibody responses are beginning to be discernible. This response is largely owing to the *de novo* synthesis of antibody from amino acids after the injection of antigen rather than to the release of "stored" antibody. However, recent evidence suggests that under some conditions some of the antibody, which appears soon after the second or subsequent injections of antigen, represents antibody which had been stored in the form of an antigen–antibody complex in the liver and perhaps elsewhere (Garvey and Campbell, 1959). Since some weeks must elapse between the primary injection and the elicitation of a secondary type of response by the injection of antigen (Glenny, 1931; Stavitsky, 1954a), it is a reasonable assumption that this time is needed for an increase in the number of antibody-forming units, whether these be cells or subcellular templates. The fact that the secondary response can be completed *in vitro* (Michaelides, 1957; Stavitsky, 1960a), whereas the primary cannot, is also in accord with the pre-existence of many more antibody-forming units which, upon second reaction with antigen, somehow result in antibody synthesis. Why it still takes 2 or more days for antibody to appear during the secondary response (Leduc *et al.*, 1955; Mathies and Stavitsky, 1960) is not clear, unless excess antigen temporarily inhibits antibody synthesis until the concentration of antigen is decreased.

The inducibility of the secondary response with smaller amounts of antigen than for the primary response has been attributed to the more efficient capture of antigen by the primed cells (Haurowitz *et al.*, 1959). Attempts to obtain evidence in support of this explanation have been unsuccessful. Large amounts of antibody are required to demonstrate increased uptake of antigen by mononuclear (Sorkin and Boyden, 1959) and lymphoid (Boyden *et al.*, 1960; Prots *et al.*, 1960) cells *in vitro*. Such large amounts of antibody have not been detected in the lymphoid cells or tissue extracts at a time when antigen will elicit a striking booster response (Boyden *et al.*, 1960; Bauer and Stavitsky, 1960), even when methods which detect nonprecipitating antibody have been employed. In fact, it has been shown that enhanced uptake of antigen by splenic cells occurs for only about a week after, but not before, the booster

response is elicited (Boyden *et al.*, 1960). It is conceivable that the relatively few cells which are involved in the secondary antibody response take up antigen more efficiently; but a more reasonable working hypothesis seems to be that the secondary response depends upon the more efficient utilization of antigen at the subcellular sites of antibody synthesis.

The nature of the γ-globulin which seems to lack serological activity and which appears in the course of the antibody response is not known. It is possible that it all represents antibody of one specificity or another. Inability to detect antibody activity may be explained partially by the recent finding that antisera against bovine serum albumin may contain some antibody directed against internal antigenic determinants which are not in the native protein available for reaction with antibody (Ishizaka *et al.*, 1960). However, Askonas and Humphrey (1958b) made extensive but unsuccessful efforts to demonstrate that some of this "normal" γ-globulin in hyperimmune pneumococcus antisera reacted with constituents of the organisms other than the capsular polysaccharides. Attempts to demonstrate nonprecipitating antibodies to ovalbumin or antibodies to contaminating proteins (Conalbumin, ovomucoid) in hyperimmune sera were also unsuccessful. Sterzl *et al.* (1960) obtained some evidence of the formation of non-antibody γ-globulin during the early days of ontogeny, at a time when the animals were unable to fabricate antibody. The fact that germ-free animals make less γ-globulin than normal animals (Thorbecke, 1960) suggests that contact with antigen is an important factor governing the formation of γ-globulin. That it is not the only factor is indicated by the finding that vitamin-deficient animals make normal amounts of γ-globulin, but do not make antibody (Axelrod and Pruzansky, 1955).

The function of persisting antigen in the tissues is not understood. Whether this antigen contributes significantly to the maintenance of low antibody levels by inducing or boosting antibody synthesis is not known. That the answers to these questions may partly be tied up with the nature of the antigen is suggested by certain observations. A persistent antibody response to BGG was elicited by repeated injections of antigen (Dixon *et al.*, 1956), but prolonged synthesis of antibody to sheep red cells was not achieved by repeated administration of antigen (Taliaferro and Taliaferro, 1952). The form in which antigen persists, whether complexed with antibody or not, and in which cells it persists must also influence the course of events.

B. Outlook for the Future

A challenging and fascinating array of problems await the investigator of the antibody response. The goal clearly is to reproduce *in vitro* the entire antibody response from uptake of antigen to the synthesis of antibody. It is likely, however, that we shall have to be content with more modest accomplishments for some time to come. Table V summarizes some of the more glaring requirements and urgent questions which must be answered in order to gain further understanding of the basic mecha-

TABLE V

Outline of Techniques and Basic Information Required for Study of Antibody Response

I.	Techniques
	A. Detection and characterization of small amounts of intracellular, bound, and/or free antigen within single cells
	B. Detection and characterization of small amounts of intracellular, bound, and/or free antibody within single cells
	C. Development of methods for production of large amounts and detection of small amounts of antibody to haptenes
	D. Isolation of clones of immunologically competent cells against single antigens
	E. Methods for increasing the number of immunologically competent cells against single antigens
	F. Better methods for maintaining antibody-forming organs and cells for a longer time *in vitro*
	G. Finding agents which specifically inhibit the inductive and/or specfic phases of the antibody response
	H. Development of subcellular preparations for synthesis of antibody
II.	Basic Information
	A. About protein, RNA, and DNA metabolism of antibody-forming cells
	B. About action of drugs on metabolism and antibody response of antibody-forming cells

nisms of the antibody response, including the possible *de novo in vitro* induction of this process.

Possible solutions to some of these problems already are at hand. More specific and sensitive detection of antigen and antibody in cells may be achieved through the combination of autoradiography (Berenbaum, 1958, 1959) and tritiated antigens and antibodies (Humphrey, 1960). The use of ferritin–antibody complexes which are electron dense may even permit the use of the electron microscope for this purpose (Singer, 1959). Isolation of clones of cells may be feasible by a combination of *in vivo* methods (Nossal, 1960) and improved methods for culture of antibody-forming tissues or cells. The numbers of active cells may be enhanced by endotoxin (Ward *et al.*, 1959). Potential antibody-forming

cells may be separated from the general population by the sucrose density procedure (Makinodan *et al.*, 1960).

A necessarily impressionistic picture of the over-all antibody response is gradually emerging. With heightened interest, better tools, more incisive questions and bold, stimulating theories it is certain that many of the details will be filled in at an ever accelerating pace. We may even anticipate some surprises. Just as the study of human hemoglobins recently has contributed significantly to the understanding of gene action when bacteria and viruses seemed more promising experimental materials, studies of the antibody response may make contributions in unexpected areas such as cellular differentiation and the transfer of information for protein synthesis from cell to cell.

It is hoped that the data, synthesis, and questions presented will at least stimulate the search for more basic information. I concur with Mark Twain that "supposing is good, but knowing is better."

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Duration of Immunity in Virus Diseases

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I. Introduction

Basic principles of immunity and resistance apply to viral diseases just as they do to bacterial diseases and other fields of biology. In fact, the classic example of the resistance which followed an outbreak of smallpox was the basis for a great deal of subsequent immunological research. One outstanding feature in the virological field is that, following either clinical or subclinical attacks by certain viruses, a lifelong immunity is usually conferred upon the individual. Examples of this are found in smallpox, measles, mumps, poliomyelitis, and yellow fever. Persistence of resistance or immunity is, however, not the invariable rule; with infections such as influenza and the common cold, any resistance resulting from an infection is transient and repeated attacks are commonplace.

II. Mechanics of Immunity

A. Humoral Immunity

When an individual had recovered from a virus infection it was possible to show that the serum contained substances capable of rendering harmless the virus concerned. In the early days when virus could only be studied experimentally by its effect on animals, these immune sera were demonstrated by their ability to protect the animal against symptoms or death. Such sera were therefore described as containing neutral-

izing antibodies. Some of the earliest experiments demonstrating the importance of these circulating antibodies are those by Andrewes (1929). He was able to show that Virus III would grow and produce inclusion bodies in tissue cultures from immune hosts in the presence of normal serum but would not do so in cells from nonimmune hosts in the presence of immune serum.

This protective antibody which renders a virus noninfective is adsorbed to the virus particle. Clear demonstration of this is afforded by the finding that the adsorption of the immune sera by the homologous virus will remove the protective effect. This was demonstrated by Friedewald (1944) and Wiener *et al.* (1946) in the case of influenza virus antisera and for vaccinia antisera by Salaman (1937). Presumably antisera adsorbed to the virus particle in some way prevents its entry into susceptible host cells. This interference may be with the capacity of the virus to make union with specific elements of the host cell the so-called "receptors"; a mechanism that appears to be important in diseases such as influenza. Antibody adsorbed to virus may also prevent its union to intracellular structures with which combination is essential before intracellular multiplication can commence. Immunity will, therefore, depend on a sufficient concentration of antibody being available at the right site to combine with any invading virus before it can make contact with susceptible cells. There is no evidence that antibody can alter the behavior of virus once it is established in the host cell. It might conceivably prevent spread of the virus produced in the infected cells to distant sites but will be ineffective against spread to and infection of contiguous cells. A very similar picture is present in immunity against toxins. Antitoxin is only effective if it is able to combine with toxin before it unites with susceptible cells. Glenny and Hopkins (1925) found that a 0.001 unit of antitoxin was sufficient to prevent a Schick dose of diphtheria toxin from producing a skin reaction on intradermal inoculation, providing the antitoxin and toxin are mixed before inoculation. If, however, the toxin is injected first then neither 10 units of antitoxin injected intravenously 10 minutes later nor 1000 units injected 30 minutes later are sufficient to prevent a small reaction at the site of the injection of the toxin.

A further example of the inability of antibody to influence the behavior of virus already established in the host cell is seen in individuals subject to herpes simplex eruptions. These individuals possess circulating neutralizing antibodies (Burnet and Lush, 1939; Shaffer and Enders, 1939). Nevertheless the virus must remain latent in the epithelial tissues between eruptions. The form in which the virus exists is uncertain but it is the infective form that initiates the herpes simplex lesion. Quite

clearly the circulating antibody is not capable of preventing the initiation of the lesion or the spread of the virus to contiguous cells.

B. Cellular Immunity

The possibility of tissue immunity has been raised in the past but little in support of this type of immunity has been produced. Several workers have shown that leucocytes will phagocytose virus particles, rinderpest (Todd and White, 1914), ephemeral fever of cattle (Mackerras *et al.* (1940), and generalized vaccinia in the rabbit (Douglas and Smith, 1930). Sabin (1935) studied the part played by leucocytes in immunity against vaccinia. He found that although the virus was taken up by rabbit leucocytes it remained fully effective and also that immune sera did not give rise to an opsonic effect. All available evidence suggests that the virus is taken up as is any other foreign matter by the phagocytes which play no part in immunity mechanisms. The phagocytes may actually act as vehicles of virus dissemination as the virus particles, when phagocytosed, remain fully infective. In line with this conclusion of the unimportance of phagocytes in protection against virus infections is the finding that these infections are associated with a leucopenia in which both the polymorphonuclear leucocytes and lymphocytes are reduced.

A group of children have been recognized who are particularly prone to recurrent bacterial infections—generally pneumococcal, streptococcal, hemophilus, or staphylococcal infections. These children have no circulating γ-globulin, and the abnormality is apparently due to failure of γ-globulin and antibody synthesis (Good and Zak 1956; Martin *et al.*, 1956; Owen *et al.*, 1956; Gitlin *et al.*, 1959; Zak and Good 1959). There are apparently two forms, one a sex-linked genetic anomaly seen in males and the other an acquired form. The production of lymphocytes, neutrophiles, eosinophiles, and even red blood cells as well as plasma cells may be deficient in certain patients which indicates that the basic disturbance involves undifferentiated cells of the hemopoietic reticulum (Good and Varco, 1955; Good and Zak, 1956).

Although these agammaglobulinemic individuals are prone to recurrent bacterial infections they seem to be no more troubled by virus diseases than the normal individual. Although Bruton (1952) has described three attacks of mumps in one agammaglobulinemic patient, generally they show resistance against recurrent attacks of measles, chicken pox, mumps, and poliomyelitis. As Burnet (1959) points out there is the serious possibility that two (or three) of the attacks reported by Bruton could well result from subacute bacterial parotitis. Good (1959) reports that several of his patients with the most complete immunological defect,

as reflected in the lowest level of γ-globulin observed, had been intimately re-exposed to measles and chicken pox on numerous occasions over a period of years following the first attack without manifesting clinical signs. One of the agammaglobulinemic children had chicken pox at age 8 and on re-exposure at the age of 12 years suffered a typical herpes zoster infection. In general, Jennerian vaccination runs a normal course in these patients. It should not be concluded that agammaglobulinemic patients handle all virus diseases well; three fatal cases of clinical hepatitis due to hepatitis virus B have been reported (Good and Page, 1960).

Despite this apparent normal resistance to certain virus infections following the primary attack repeated attempts have failed to show antibody responses to poliomyelitis, western equine encephalitis, mumps, and rickettsial antigens. It should be realized, however, that Barnett *et al.* (1960) have shown plaque-neutralizing capacity with properties of pure antibody for most of eight enteroviruses in the serum from two congenital hypogammaglobulinemias and five acquired hypogammaglobulinemia patients. In a personal communication Baron (1960) says that this neutralizing fraction is present in the γ-globulin fraction. The antibody response to polio vaccine of four hypogammaglobulinemia patients was followed, and some rise in neutralizing capacity was observed although titers were always low. The γ-globulin level in the patients studied ranged from 70 to 300 mg.%, but sera from patients with γ-globulin levels of 0.20 mg.% also showed some neutralizing capacity. This resistance might depend on either minute amounts of antibody that cannot be detected by our present methods or some specific or nonspecific mechanism that is independent of circulating antibody.

Although the normal immunological mechanisms may not be found in agammaglobulinemia, Kulneff *et al.* (1955) and Porter (1955) showed that persons with this defect can be immunized with BCG and would develop delayed skin sensitivity to tuberculin. They could also be sensitized to chemical compounds such as 2,4-dinitrofluorobenzene. This sensitivity could be transferred to normal individuals by subcutaneous injection of the agammaglobulinemic leucocytes but not by large inocula of their sera. Conversely nonsensitized agammaglobulinemics could develop a long lasting type of sensitivity to tuberculin following subcutaneous inoculation of cells from highly sensitive immunologically normal individuals. These findings suggest that although the ability to produce classic circulating antibody is lacking or very deficient, they have the normal capacity to develop delayed allergy. In view of the resistance to certain virus conditions shown by these individuals, despite the absence or relative deficiency of detectable circulating antibody, it is pos-

sible that this resistance is mediated by a mechanism of the delayed sensitivity type.

Certain evidence obtained during studies made with attenuated poliomyelitis vaccine suggests that resistance without detectable antibodies may also be operative in immunologically normal individuals. Sabin (1959) shows one person who though devoid of type 1 antibody failed to excrete type 1 virus when the attenuated type 1 strain was fed by mouth. Two years later a refeeding of the same strain led to normal excretion in the feces. As there was no evidence of the presence of any interfering virus in the alimentary tract at the time of the first feeding it might indicate that the resistance at that time in the absence of antibody was due to the mechanism already suggested.

III. Virus Infections Associated with Immunity of Short Duration

Certain virus infections result in a relatively short period of immunity and recurrent attacks are the rule; influenza, the common cold, and trachoma are classic examples. The feature of these conditions immediately apparent is that they are localized infections, the virus gains access to an area of the body directly and multiplies locally. There is no systemic invasion, the short incubation period indicates that symptoms are not preceded by a period of general dissemination of the virus.

Influenza is a surface infection of the mucous membrane of the respiratory tract, effected by extension over the surfaces exposed to the air. These surfaces are constantly washed by a film of fluid transuded from the blood stream. Francis (1940) demonstrated that in human beings this fluid contained antibody, and Fazekas de St. Groth and Donnelley (1950) studied this phenomenon in mice. As long as the antibody titer in the blood stream is high, the level in the fluid that covers the mucous membranes will presumably be sufficient to neutralize virus particles that gain access and prevent infection. As the multiplication of virus is local it might be that antigenic stimulation is limited to antibody-forming cells in the vicinity of the infection. Therefore serum antibodies may not reach a high level and might be expected to decline quickly, a phenomenon which could explain the transient type of immunity. This conclusion is, however, refuted by the recent observations of Mulder and Masurel (1958). Following the isolation of Influenza A/Asian/Singapore 1/57 (Lim *et al.*, 1957) the former workers managed to collect sera from as many people as possible, aged 50–100 years, before the Asian strain was established in Europe. With sera already held in the laboratory from younger age groups, 1256 were collected. Fifty-two of these sera were

found to give a positive hemagglutination-inhibition test against the Asian strain, no positives were found in adults under 30 and the greatest incidence of positive tests, 8/21, was in persons aged 87. The authors suggested that these antibodies were probably the result of exposure to influenza in the pandemic of 1889–1890 which also originated in Asia. Similar findings were reported for Italians in the age group 78–85 by Davoli and Corsi (1957). Davenport and Hennessey (1956) proposed the doctrine of "original immunological sin." Following an attack of influenza or the administration of an influenza vaccine the serological response obtained will depend mainly on the type of influenza A virus first encountered. Individuals in their fifties would give a response to the Swine virus which is believed to be responsible for the 1918–1919 pandemic whereas younger individuals would yield antibody against the PR8 strain current in the 1930's.

All this evidence points to the duration of circulating antibodies against influenza or to the continued existence of antibody-forming cells capable of giving rise to that protective antibody. Reasons for repeated attacks of influenza are, therefore, more likely to reside in changes in the virus rather than in the loss of immunity in the host. Smith *et al.* (1933) isolated the WS strain of influenza virus in ferrets, and since then strains have been isolated from epidemics and major outbreaks throughout the world. Serological comparison of these strains have shown a constantly changing antigenic pattern. The PR8 strain was isolated in 1934, but most workers consider that this strain has not been responsible for outbreaks since 1939 when the FM1 or A-prime was isolated, to be followed in 1951 by the Liverpool L strains of Isaacs *et al.* (1951). Soon after the isolation of a new type it rapidly becomes dominant and responsible for epidemics in all parts of the world. Recently the A/Asian/Singapore 1/57 strain appeared in Asia and very shortly epidemics from this strain were reported in all parts of the world. These antigenic changes may not be so discontinuous as they appear since each year minor alterations of antigenic nature may be taking place. Thus it is quite easy to realize that one attack of influenza will give very little immunity against future strains of different antigenic constitution. It has not been so easy to demonstrate this type of antigenic drift in the influenza B strains, the prototypes of which are LEE (U.S.A. 1940) and BON (Australia 1943), but Jordan and Gaylin's (1953) work points to a similar type of change even in these strains.

Similarly with the common cold, failure to demonstrate immunity following attacks may be due to the fact that there are multiple antigenic types of the common cold virus, that the virus undergoes a

rapid antigenic shift with the passage of time, or it may be that the common cold is a clinical syndrome elicited by many different viruses. Tyrell (1960) of the Common Cold Centre, Salisbury, has evidence that there are a number of distinct viruses causing the common cold. It has been impossible to demonstrate any immunity in people who have recently recovered from a common cold experimentally induced even when they were challenged with the same strain of virus (Commission on Acute Respiratory Diseases, 1947). Nevertheless it is a well-known phenomenon that people who have been in isolation away from civilization (in the Antarctic, for example) usually suffer from very severe colds on their return. This fact suggests that a partial immunity exists in communities where colds are common so that infections from virus attack are associated with mild symptomatology.

Examination of the reported cases of dengue show that, in general, epidemics of this disease have occurred every 10 years. This would indicate that either the immunity that follows the disease wanes in a period of 10 years or a lasting immunity is conferred by an attack but that the dengue virus shows something of the antigenic shift seen with the influenza virus. It is difficult to believe that the first hypothesis is valid. Mosquitoes once infected with dengue virus remain so for their life and, although there is no known extra human reservoir of dengue, sufficient susceptible children are born each year to ensure that virus circulation continues. Therefore the population would be constantly increasing its resistant members, and, even if an individual's immunity only lasted 10 years, it is difficult to understand how the herd immunity could fall below the threshold that would allow an epidemic to break out. The most likely explanation would appear to be the appearance of a new antigenic variety of the dengue virus which enables it to sweep through the population. Very little experimental work has been conducted on these problems but with the increase in technical methods now available for studying the group of viruses to which dengue belongs we can expect advances that may elucidate this problem. In this respect it may be significant that already we are aware of two strains of dengue virus, the so-called Hawaiian and New Guinea strains.

IV. Virus Infections Associated with Long Lasting Immunity

The virus diseases associated with long lasting and permanent immunity following the primary attack are those characterized by an incubation period of a week or longer. This has led to the suggestion that it is a rapid secondary antibody response that may be the factor concerned

in failure of second attacks. Although reinfection might take place, the secondary antibody response would be elicited within the incubation period so that clinical symptoms do not become manifest. This mechanism is certainly a possibility but in these diseases there is evidence that following an attack antibodies are present for very long periods. Persistence of antibodies is very easily understood in populations where the viruses are endemic and might result from repeated infection with antibody stimulation. There is considerable evidence, however, that antibodies persist in the absence of repeated antigenic stimulation. Individuals who after recovery from yellow fever have then resided in areas where yellow fever virus is nonexistent show persistence of neutralizing antibodies. Paul *et al.* (1951) conducted a survey for poliomyelitis antibodies among eskimos in North Alaska. They were able to show that the three types were introduced on three distinct and well-spaced occasions and that after its introduction the virus did not persist in the population. Nevertheless individuals who were young enough to experience infection with the first virus type that was introduced retained their antibodies against this type for a very long period although, as already stated, this virus did not continue to circulate in the community.

Two different theories have been advanced to account for the persistence of antibody. On the one hand it is suggested that the continued presence of antigen is not essential for continued antibody production, and Burnet (1959) exemplifies this in his clonal selection theory of immunity. Others, notably Rivers and Horsfall (1959), are inclined to interpret this persistence of immunity as a result of continued presence (probably in some latent form) of the infecting agent.

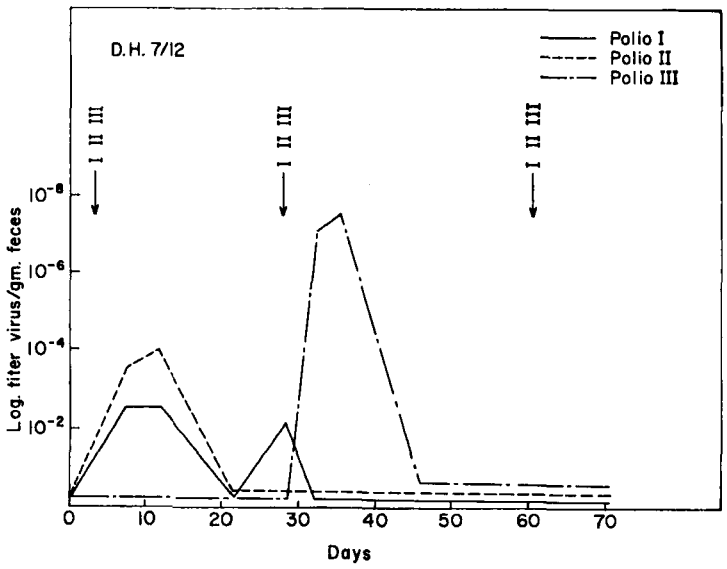
Many examples can be cited in favor of this latter possibility. Probably the best clinical example of persistence of infection is Brills-Zinsser disease, the recurrent form of epidemic typhus that develops long after the initial attack. Herpes simplex infections are another excellent example of latent infections and virus persistence in the body. Repeated herpetic attacks follow a primary infection, usually an aphthous stomatitis that occurs in infancy or early childhood. Following the primary infection the virus must remain latent in the deep layers of the epithelium, and, as the cells multiply to maintain the epithelial structure, daughter cells must also contain the virus. Certain triggering mechanisms, fever, exposure to wind, sunlight, or sometimes psychogenic stimuli may provoke an outbreak which is usually self-limited as all these individuals possess circulating antibodies. Meyer (1942) showed that budgereegahs were infected with psittacosis virus as nestlings by ingesting material contaminated by droppings of the parent birds. Some died but others

recovered and, although virus was no longer excreted, it remained in the spleen and kidney. Presumably it remained latent or multiplied slowly, but in a proportion of such birds certain stimuli might result in activation and virus excretion. Burnet (1935) suggested that this mechanism accounted for the persistence of psittacosis as an enzootic disease of wild parrots and cockatoos in Australia. There is a reported case (Meyer and Eddie, 1951) of the recovery of psittacosis virus from the sputum of a patient 8 years after recovery from a symptomatic illness. Adenoviruses have been recovered from tissue cultures of tonsillar and adenoid tissue which seems to indicate that the virus has remained latent in these tissues. We, ourselves (Gardner, Wright, and Hale, 1961), following the excretion of viruses in feces of children in a residential nursery have observed excretion of adenovirus type 5 by a child for a period of over 4 months. Thus there is ample evidence that certain viruses can remain latent in the tissues. In the experimental field, Maniere and Galasso (1959) have shown persistent infection of HeLa cell cultures with the meningopneumonitis virus, Fernandez (1960) has shown persistence of herpes simplex in HeLa cells, and Medina and Sachs (1960) have established a line of mouse cells in which a stable cell and polyoma virus association existed.

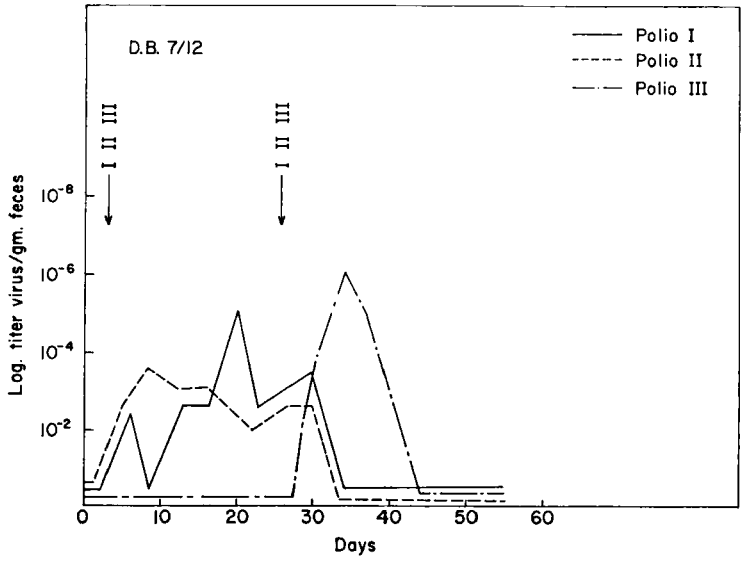
Although the persistence of virus undoubtedly occurs in some infections, it is dangerous to assume that it is the cause of the lasting immunity in certain diseases. In all the situations cited, the virus has been demonstrated by experimental methods or the virus has become self-evident by flaring into limited activity. Measles has attacked isolated communities, and Panum's (1847) study in the Faroe Islands showed successive measles epidemics separated by 65 and 31 years. If the lasting immunity in this disease was due to persistent virus, then one would have assumed that there was sufficient time for the liberation of the virus carried by one of these immune individuals. A sufficient number of new susceptible subjects, e.g., young children, would exist to act as indicators so that some cases of measles should have occurred. Also there are no reports of the isolation of poliomyelitis or yellow fever virus several years after the recovery from an attack although this problem may not have been investigated with great thoroughness. Burnet (1955) makes a very pertinent observation that following virus infection it is possible to demonstrate complement-fixing and neutralizing antibodies. The latter persist for years but the former only a matter of months; so much so that complement-fixing antibodies have come to be regarded as evidence of recent infection. If lasting immunity resulted from the presence of virus, then one would expect per-

sistence of complement-fixing antibodies but this is not seen in poliomyelitis, yellow fever, or measles. Accepting this view, the alternative hypothesis is a host unit modified by interaction with antigen that is self-replicating without further antigenic contact, a theory that is highly developed by Burnet (1959) in his book to which readers should refer.

The hypotheses advanced explain the long-term immunity seen in certain virus diseases in terms of circulating antibodies. There is, however, as already suggested, evidence of an immunity as seen in hypogammaglobulinemia that is not associated with circulating antibodies and is apparently dependent on a local tissue immunity or delayed sensitivity type of reaction. Although this phenomenon has been studied in hypogammaglobulinemia, this form of local tissue immunity is probably also operative in the normal individual. Following administration of an adequate dose of Salk type formalinized polio vaccine, a triply negative child will develop circulating antibodies to all three types. In exactly the same way, antibodies appear in the circulation of a child fed the three types of attenuated polio vaccine. Here the similarity ends. If attenuated vaccine is fed to the Salk-vaccinated children, poliovirus multiplies and is excreted in the feces whereas, if it is fed to those vaccinated with the attenuated virus, the virus does not establish itself in the intestine. This obviously points to some local difference in the



(a)



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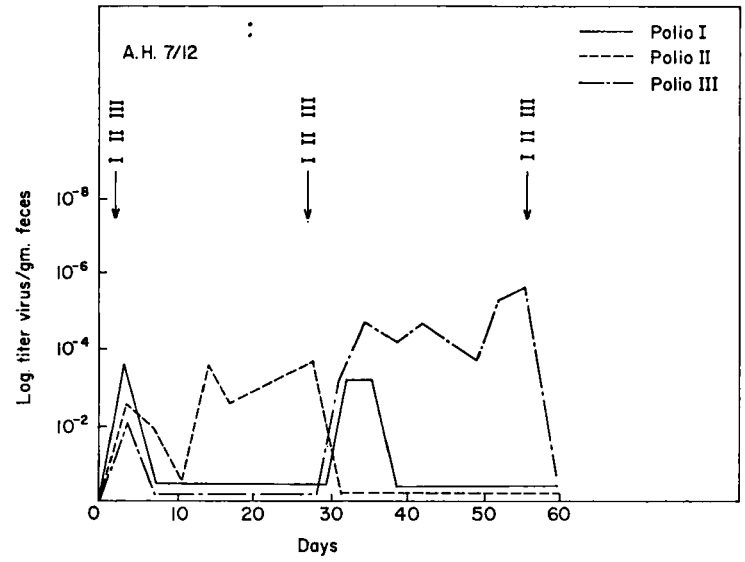

(c)

FIG. 1. Fecal excretion of poliovirus in young children following the administration of the Sabin attenuated poliovirus types 1, 2, and 3. Each type was fed simultaneously in 10×10^6 TCD_{50} doses at monthly intervals.

susceptible cells as both children could well have approximately similar serum antibody titers. Burnet (1955) has made the suggestion that this "local" immunity phenomenon may result from the susceptible cells of the intestinal tract being bathed with antibody-containing fluids that combine with the virus and thus prevent its incorporation with the cells. If this is accepted, then we are forced to the conclusion that following parental administration of vaccine, although antibody appears in the blood stream, none or very little of the antibody leaks into the gut and affords protection there, whereas following growth of the virus, antibody cells in the region of the intestine are stimulated and antibody appears both in the gut and in the blood stream. It seems more likely that the actual multiplication of virus results in a local cellular immunity that may be of the delayed sensitivity type. This would mean that in normal individuals the same type of protection exists as seen in the hypogammaglobulinemic in addition to that afforded by the presence of circulating antibodies.

From studies in poliomyelitis there is considerable evidence that, in order to achieve such a local immunity, it is essential for the virus to have undergone adequate multiplication over some prolonged period. In some cases after feeding attenuated vaccine, examination of the stools may show virus excretion of low titer and for short periods. Although this may result in antibody production, refeeding vaccine at a later date usually results in virus excretion again although the titer is usually low for only a very few days. On the other hand, where feeding resulted in a prolonged virus excretion subsequent refeeding shows no virus excretion. This phenomenon is shown in our own work (Hale, Wright, and Gardner) in which young children aged from 6 months to 1 year were fed a mixture of all three types of Sabin-attenuated poliovirus simultaneously. A dose of 1×10^6 TCD_{50} of each type was administered by mouth, fecal excretion of virus was checked, and refeeding of the same mixture repeated on two occasions at intervals of 1 month. Several examples of what may be expected are shown in Fig. 1. It is quite obvious that interference between the strains results from triple feeding, and one strain usually dominates and is excreted for a much longer period and in greater amounts. When the three strains are fed again a month later it can be seen that the gut has become resistant to the strain that was dominant and only multiplication of the other strains results. Where two strains multiplied to good titer after the first feeding, then only the third is manifest after the second feeding. The third refeeding only allows multiplication of a strain that had shown insufficient propagation up to that point.

V. Resistance to Virus Infection Usually from Interference

A. Experimentally Produced Interference

Dalldorf, Douglass, and Robinson (1938) showed that monkeys infected with the lymphocytic choriomeningitis virus failed to become paralyzed when challenged by poliovirus, the resistance lasting about 2 weeks. Schlesinger *et al.* (1943) demonstrated an interference between Theiler's virus and western equine encephalomyelitis in mice. Domok (1959) found that mice inoculated subcutaneously or intracerebrally with Coxsackie B were resistant to challenge with the Lansing strain of poliomyelitis for a period of 60 days.

Examples of interference between antigenically related strains have also been reported. Burnet and Lind (1951) showed that allantoic fluid infected with the neurotropic strain of the influenza virus NWS killed mice up to dilution of 10^{-6} when inoculated intracerebrally, whereas the normal MEL strain of the influenza virus did not cause death in mice by this inoculation route. When MEL-infected allantoic fluid was added to the NWS-infected allantoic fluid to give a final concentration of 1/10 then the intracerebral infective titer of this mixture was reduced to 10^{-2} or less. An interesting manifestation of interference arises sometimes during adaptation of a virus to a new host or new route of inoculation. By repeated passages it is hoped to select mutants possessing survival advantages in the new host. Often a stage is reached during the adaptation process when it is found that animals inoculated with small doses of the infective material show a higher mortality rate than those injected with larger doses. This results when the virus inoculated consists of mixtures of adapted and nonadapted particles, i.e., virulent and nonvirulent particles, and is due to the latter blocking the former at high concentrations but not at low concentrations. This type of phenomenon is referred to as autointerference (von Magnus, 1951).

With the introduction of tissue culture techniques into the field of virology, it has been possible to show interference between viruses in this system. Jungeblut and Kodza (1959) showed that Col-SK virus does not produce cytopathic effects in HeLa cells although it is adsorbed and undergoes at least one cycle of multiplication. Large doses of Col-SK virus (about 10^7 mouse infective doses) in a strain of HeLa cells were found to interfere with several strains of poliovirus and with Coxsackie B. This interference depended on the growth of the Col-SK virus as it was not manifest until after 4 hours' incubation of the cells with the virus and it was prevented if the adsorption of Col-SK virus to the cell surface

was inhibited. Heating suspensions of Col-SK virus destroyed its interfering capacity and infectivity at the same rate.

B. Interference Phenomena Occurring in Nature

The procedures described are all experimental and involve large doses of virus by unnatural routes and allowance is made to give carefully planned advantage to the interfering strain. It may, therefore, be questioned whether this type of mechanism is operative under normal conditions in man and to what extent. When attenuated yellow fever virus and dengue virus were inoculated into human volunteers, the resulting attack of dengue was much milder than when the dengue virus was inoculated alone. The immunogenic effectiveness of attenuated vaccines of dengue and yellow fever are diminished when inoculated together; antibody to one may be produced to the exclusion of the other. Their antigenic potency depends on their ability to multiply in the tissues so that failure is an example of interference (Schlesinger *et al.*, 1956). It should be noted in passing that similar interference between viruses has been demonstrated in the mosquito vector *Aedes egypti*. The geographic spread of arthropod-borne diseases could well be determined by such mechanisms when it is realized that insect vectors once infected remain so for life. With the introduction of more and more attenuated viruses for immunization procedures, the possibility of interference is of great importance.

Sabin (1959) reveals several instances of interference that occurred during the administration of his attenuated poliomyelitis virus vaccines. A young adult possessing no poliomyelitis antibodies was fed the three serological types simultaneously. The three types showed a limited multiplication for 6 days and thereafter type 2 continued to be excreted for 6 weeks. Tests on sera taken at 4 weeks revealed the presence of types 1 and 2 but not of type 3 antibodies. It would at first appear that interference with type 3 alone had taken place but retesting of the sera 2 months later revealed only the presence of type 2 antibodies. Quite obviously there had been interference with the type 1 strain which multiplied only on a limited scale and gave transitory antibody production. As a result of this and other very similar experiments, Sabin recommended administration of the three types of virus used in his vaccine separately in the order 1, 3, and 2 at 4-week intervals or longer in order to obviate possible interference. In Singapore during the latter part of 1958, Hale *et al.* (1959) faced with an epidemic outbreak of poliomyelitis resulting from the type 1 virus, decided to attempt to elicit this interference under field conditions. Children 3 months to 10 years of age were

fed the attenuated type 2 strain in the hope that alimentary tracts colonized with the type 2 strain might resist subsequent colonization with the virulent type 1 strain. Approximately 200,000 out of a total of 500,000 children under the age of 10 years were administered the vaccine strain, and Fig. 2 shows the time after administration of the vaccine that type 1 cases occurred in vaccinees. Cases that occurred within 8 days of administration could be shown to be individuals in the incubation stage of type 1 poliomyelitis when fed vaccine. The significant finding was that no cases in vaccinees occurred between 8 to 35 days when any interference effect could be expected to be maximal. This clear-cut result was quite remarkable because obviously not all the children vaccinated were type 2 susceptible and therefore type 2 excreters. This did, however, in-

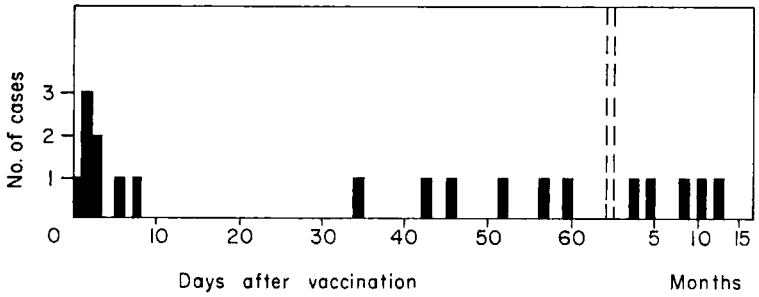

FIG. 2. Cases of poliomyelitis due to type 1 virus that occurred in children who had received Sabin type 2 vaccine.

dicate that a long lasting effect, due to the type 2 antibody's heterologous protection, was present. The absence of cases between 8 to 35 days was, therefore, due to two causes; to interference in those in which the virus was capable of multiplying and to some heterologous immunity in those already immune to type 2 virus.

Use of attenuated poliomyelitis vaccine has also revealed interference with the poliovirus in individuals infected with enteric viruses. Sabin stated that abnormal patterns of multiplication of the orally administered vaccine strains were invariably associated with concurrent spontaneous infection with various enteric viruses, ECHO, Coxsackie A and B, and adenovirus. This finding has been substantiated by most workers who have used attenuated polio vaccine either on a large or small scale.

C. Mechanics of Interference

This type of suppression or interference probably results from a substance known as interferon (Isaacs and Lindenmann, 1957; Lindenmann

et al., 1957; Isaacs *et al.*, 1958; Isaacs and Westwood, 1959) produced by the infected cells. This substance which can be transferred to normal cells prevents them becoming infected. It appears to penetrate the cells and its action is not merely adsorption to the surface with consequent alteration of surface characteristics of the cell because virus adsorbs quite normally to treated cells but fails to multiply. Interferon is non-sedimentable and from its properties it could well be a basic protein of the histone variety.

There are many cited examples of persistent infection of tissue cultures by viruses (Bergs *et al.*, 1958; Deinhardt *et al.*, 1958; G. Henle *et al.*, 1958). W. Henle *et al.* (1959), and Ho and Enders (1959) have shown that interferon is produced by infected tissue cultures. It could well be that these examples of persistent infection represent a nicely adjusted balance between virus multiplication and interferon production, a theory advanced by Wagner (1960). In the culture, infected cells are replaced continually by the growth of susceptible uninfected cells at a reduced rate. If virus production outstrips that of inteferon all susceptible cells will become infected and die. On the other hand, if interferon production is great then infection will be cured.

D. Interference and Possible Long-Term Resistance to Virus Infection

From the foregoing it seems justifiable to speculate whether interference may not offer a long-term protection or immunity against certain virus diseases. During a study of fecal virus excretion in residential nurseries, a number of children excreted adenovirus type 5. This virus is regarded by many authorities as relatively nonpathogenic (Huebner *et al.*, 1954), but it was found that several children excreted this virus for periods of several months. Coupled with the fact that adenovirus type 5 has been frequently isolated from tonsillar material taken at autopsy, this seems to suggest that a condition of permanent carriage might well exist.

During the study it became apparent that it was far easier to make isolations from feces than from throat swabs. The feces must have contained a fair quantity of virus as the minimal amount detectable in the 10% fecal suspension that was used for tissue culture inoculated in 0.1-ml. amounts must be 100 TCD_{50} virus per gram feces. These findings rather indicated that feces were not contaminated by virus swallowed from tonsillar and nasopharyngeal regions but that virus multiplication must be taking place in tissues in close proximity to the alimentary canal. In this respect it is interesting that Gardner *et al.* (1960) have reported

adenovirus infection associated with diarrhea. It could well be that multiplication of the virus takes place in the same type of cells that the poliomyelitis virus infects. If that is so, then with chronic infection with adenovirus these cells could be producing interferon that could prevent subsequent infection with poliovirus. Although nonspecific this would afford a long-term protection or immunity. It must be realized that this is presented as a pure speculation which requires experimental corroboration that we are in the process of attempting.

VI. Summary

Virus diseases fall into two main categories in respect of the duration of immunity that results from a previous attack. Those diseases that afford negligible or very short duration immunity are generally of localized character, with short incubation periods. Two main factors are often responsible, first, antigenic stimulus is probably limited to relatively few of the antibody-forming cells of the body and second, the responsible viruses usually demonstrate shifts in antigenic constitution. Diseases characterized by long lasting immunity are those with longer incubation periods associated with general dissemination of the infecting agent. Under these conditions there is ample opportunity for extensive antigenic stimulation and should the agent invade a second time then the longer incubation period before symptoms develop allows for a secondary antibody response that might well result in the infection being overcome before clinical illness develops. Another outstanding feature is that the infecting agents are of uniform antigenic make-up and show little tendency to mutate with passage of time.

On balance it is probably this stability of antigenic constitution which is the most important feature in determining the duration of immunity. There is now abundant evidence that antibodies or the capacity to produce antibodies is retained for very long periods even in the case of the disease associated with short-term immunities such as influenza. Unfortunately in the latter such antibodies may exert little effect on the viruses of the future when antigenic structure will be modified.

A specific immunity to certain virus infections is found in agammaglobulinemic children. This resistance may be owing to what is termed a local tissue immunity that is of a delayed sensitivity type. Certain evidence (Barnett *et al.*, 1960; Baron, 1960) points to the capacity of these hypogammaglobulinemics to produce viral-neutralizing bodies. Thus protection in these cases could be due to the action of an agent such as interferon (this might result in diminution of infective virus particles) and

the small amount of neutralizing bodies that might be present in these patients' sera. Future research may demonstrate which of the mechanisms discussed is operative in these hypogammaglobulinemia patients. Nevertheless there can be little doubt that such a mechanism would also be called into play in normal individuals following viral infection, although it is impossible to decide whether this or the presence of circulating antibodies are of greater importance. It does, however, clearly indicate that thinking in regard to prophylaxis in viral diseases should be orientated to the live attenuated type of vaccine which should prove more effective than killed vaccine. With the attenuated vaccine, virus growth in the body can result in widespread antigenic stimulation but what seems more important is that when such an attenuated vaccine can be administered by the route by which natural infection occurs then not only will antibodies result but any "localized" immunity would be evoked. If such attenuated viruses are not available killed vaccines must be such that sufficient antigenic material is available in the vaccine to give adequate antibody responses.

Although not of specific character, a viral infection may confer immunity of a nonspecific character which protects against infection by a second virus. This type of phenomenon might well interfere with vaccination campaigns in which attenuated type vaccines are being administered by the channels through which natural infection takes place. Thus it could well be that the use of attenuated poliovirus vaccine campaigns might give more satisfactory results in temperate zones than in tropical zones where enterovirus circulation is more common.

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Fate and Biological Action of Antigen–Antibody Complexes[1]

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I. Introduction

It has been shown that numerous pathological reactions occur as a result of the interaction between antigen and antibody both *in vivo* and in isolated tissue. Not only are these reactions produced when actively and passively sensitized animals or tissues are challenged with specific antigen, but also when preformed soluble antigen–antibody complexes are injected into normal animals or applied to normal isolated tissues and cells. It has been demonstrated that certain soluble antigen–antibody complexes prepared with excess antigen will produce anaphylaxis (Germuth and McKinnon, 1957; Tokuda and Weiser, 1958a, b; Treadwell *et al.*, 1960; Weigle *et al.*, 1960), smooth muscle contraction (Kulka,

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1942; Trapani *et al.*, 1958), lesions of serum sickness (Germuth and McKinnon, 1957; McCluskey and Benacerraf, 1959), and cutaneous reactions (Ishizaka and Campbell, 1958; Ishizaka *et al.*, 1959a; Cochrane and Weigle, 1958). Most of these investigations were performed with serum protein antigens which permitted the quantitative determinations of the antigen and antibody involved, the *in vivo* behavior of the complexes, and the physical-chemical properties of the complexes. Thus, it has been possible to relate the biological activity of soluble antigen–antibody complexes with their antigen–antibody composition, physical-chemical properties, ability to fix complement, and *in vivo* fate. The correlations which can be made among the various *in vitro* and *in vivo* properties of preformed antigen–antibody complexes have given considerable insight into the mechanisms involved in the experimental disease of hypersensitivity.

II. *In Vivo* Fate of Antigen–Antibody Complexes

The fate of antigen–antibody complexes in animals depends on both the nature of the antigen involved and the immune state of the animal. Although particulate antigens, such as bacteria, are removed from the circulation more rapidly after antibodies appear, they are removed relatively rapidly even in the absence of antibody. Soluble protein antigens, such as hemocyanin, ferritin, egg albumin, and denatured and chemically altered serum proteins, are also eliminated rapidly from the circulation before antibody synthesis begins. Thus, studies concerning the effect of antibody on the *in vivo* behavior of these antigens are difficult. In contrast, in the absence of antibody native serum protein antigens are slowly eliminated from the circulation similarly to homologous serum protein antigens. Since serum protein antigens circulate in the blood for relatively long periods of time, their *in vivo* behavior can be followed by isotopic techniques (Talmage *et al.*, 1951; Dixon, 1953). Following injection into animals, I^{131}-trace-labeled homologous serum proteins are eliminated in two phases. The first phase is the *equilibration phase* which results from the equilibration of the protein between intra- and extravascular fluid spaces (Fig. 1). In the second phase, the *exponential phase,* which begins when equilibration is complete, the protein is eliminated at a logarithmic rate. This latter phase is the result of normal catabolism by the host. The rate of elimination during the exponential phase varies considerably and depends on the physical-chemical properties of the protein and the metabolic activity of the host (Weigle, 1957). With heterologous serum

proteins, there may also be a rapid elimination of the protein which results from its combination with antibody. This phase of elimination is called the *immune phase* and is followed by the appearance of free antibody in the serum. The time and rate of the immune phase also varies and depends on the protein employed and on both the species and immune state of the host.

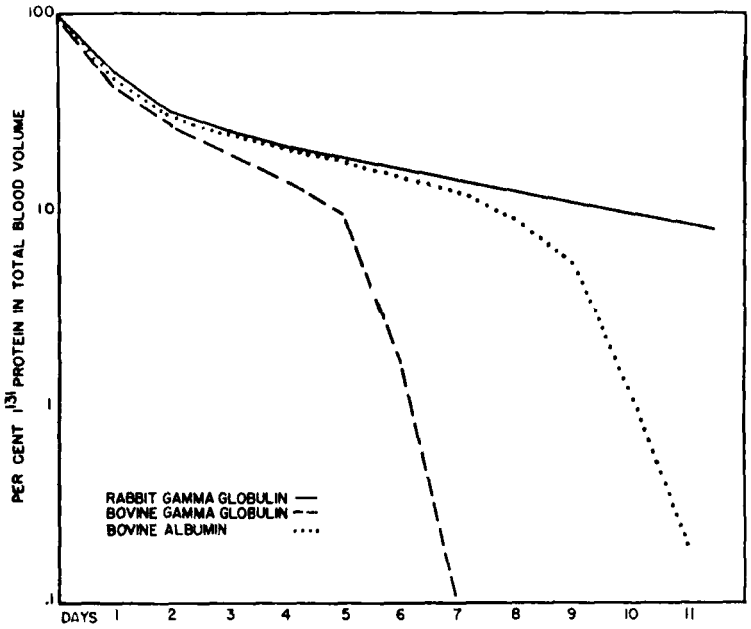

FIG. 1. Elimination of homologous and heterologous serum proteins from the blood of rabbits.

The following discussion will deal only with complexes composed of soluble serum protein antigens and their specific antibodies, since a correlation between the *in vivo* behavior of complexes and their biological activity can be made only with these systems.

A. ANTIGEN–ANTIBODY COMPLEXES FORMED DURING AN IMMUNE RESPONSE

In rabbits, circulating antigen–antibody complexes can be demonstrated during the early phase of an immune response to a serum protein antigen. Since these complexes are probably similar to the *in vitro* prepared complexes which produce anaphylactogenic and phlogogenic reactions, knowledge concerning their behavior is important. Studies on the dynamics of the immune response in rabbits to

serum protein antigens indicate that as antibody is synthesized, it reacts with the circulating antigen and the resulting antigen–antibody complexes are rapidly eliminated from the circulation (Talmage *et al.*, 1951). With bovine serum albumin (BSA) immune elimination takes place between the ninth and fifteenth day following injection of the antigen. Immediately prior to and during the immune elimination of antigen, circulating antigen–antibody complexes are present (Weigle, 1958a; Weigle and Dixon, 1958). These complexes were detected by virtue of the differential solubility of the albumin antigen and the globulin antibody in 50% ammonium sulfate saturation (Farr, 1958). Rabbits injected with I^{131}-trace-labeled BSA (I*BSA) were bled periodically and the sera fractionated at 50% saturation with ammonium sulfate. The globulin as well as any I*BSA bound to antibody (globulin) was precipitated from the sera. The globulin-bound I*BSA was then determined by measuring the I* activity in the precipitate. The I*BSA activity not bound to antibody remained in the supernatant. When 40 mg. of BSA were injected per kilogram body weight, complexes appeared in the circulation from 1 to 2 days prior to the initiation of immune elimination. On the other hand, when the injection dose was 250 mg. per kilogram, complexes appeared in the sera 3 to 8 days prior to immune elimination. In some of the animals receiving the large dose of antigen, immune elimination failed to occur although over 50% of the circulating antigen was bound to antibody. In previously sensitized rabbits which contained little or no circulating antibody, an anamnestic immune elimination of serum protein antigens occurred between the fourth and fifth days, and circulating antigen–antibody complexes appeared on the third day following injection. Soluble antigen–antibody complexes have also been demonstrated in guinea pigs during a primary antibody response to heterologous serum albumins (Weigle and Dixon, 1957).

The presence of antigen–antibody complexes in the sera of rabbits during an immune respone has also been demonstrated by starch block electrophoresis (Weigle and Deichmiller, 1960). Sera taken from rabbits at various times after they were injected with 250 mg. I*BSA per kilogram body weight were analyzed by starch block electrophoresis and the I*BSA migrating with globulin determined. A comparison of the results observed with electrophoresis and with ammonium sulfate is given in Fig. 2. With both methods, a parallel rise in globulin-bound I*BSA was observed on the ninth day. The level of globulin-bound I*BSA then dropped, paralleling the immune elimination of the antigen. With most rabbits studied the globulin-bound I*BSA was less when

determined by electrophoresis than when determined by precipitation with ammonium sulfate, probably as a result of a dissociation of the antigen–antibody complexes during electrophoresis. The globulin-bound I*BSA activity migrated with the γ-, β-, and α-globulins. This diversified electrophoretic distribution of globulin-bound I*BSA activity was also probably the result of dissociation and variability of antibody/antigen ratios of the complexes.

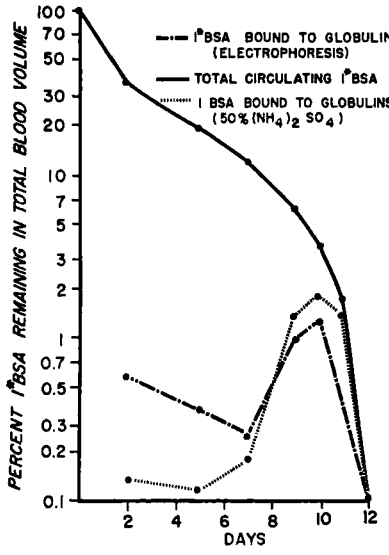

FIG. 2. Behavior of I*BSA in a rabbit injected with 250 mg. I*BSA per kilogram body weight. Reprinted from *J. Immunol.* **84**, 434 (1960).

As would be expected, circulating antigen–antibody complexes also appear when serum protein antigens are injected into sensitized rabbits containing circulating antibody. In experiments where passively sensitized rabbits (Dixon and Maurer, 1953) and actively sensitized rabbits (Dixon and Maurer, 1953; Francis *et al.*, 1957) were injected with either an equivalent or less than equivalent amount of antigen, the resulting antigen–antibody complexes were rapidly eliminated from the circulation, deposited in the lung, liver, or spleen (Francis *et al.*, 1957), and quickly catabolized (Dixon and Maurer, 1953). The fate of complexes formed by intravenous injection of soluble antigens into sensitized animals probably depends on the quantitative relationship between the antigen and antibody in the circulation at the time of reaction. When an extreme excess of the antigen is injected, soluble complexes which circulate in the blood are probably formed. Miescher *et al.* (1954) in-

jected an excess amount of I* human serum albumin (I*HSA) into sensitized rabbits and found complexes present in the circulation 20 and 60 min. later. When the sera were fractionated by the Cohn's alcohol method (method 10), the I*HSA present in the sera after 20 minutes was in Fractions I and III, and to a lesser extent in Fraction II. After 60 minutes, I*HSA remained only in Fraction II. The diversified distribution of the I*HSA activity in the fractions probably was the result of the presence of I*HSA–anti-HSA complexes of varying composition.

Sternberger and co-workers (1953) reported the presence of a material in the sera of rabbits immunized with serum protein antigens which precipitated upon treatment of the sera with alkali. It was postulated that this material was a soluble complex of an antigenlike substance and specific antibody. Whatever its nature, this material is quite different from the circulating complexes formed between the native antigen and the newly synthesized antibody (Weigle, 1958a). The I*BSA–anti-BSA complexes were detected in rabbits injected with I*BSA just prior to and during the rapid immune elimination of the antigen. However, alkali-precipitable material which was also present in some of the rabbits at this time did not contain the I*BSA. Whether the alkali-precipitable material contains a host synthesized antigenlike substance, as has been suggested (Sternberger, 1957), remains to be seen. At present no pathogenic significance has been attributed to alkali-precipitable material.

That leucocytes can take up antigen–antibody complexes formed *in vivo* and subsequently break these complexes down has been demonstrated by Cochrane *et al.* (1959). In the Arthus phenomenon there occurred vascular damage, deposition of antigen and antibody in the damaged walls of the vessels, and infiltration of polymorphs into the walls. Since vascular damage and polymorph infiltration were negligible despite the deposition of antigen–antibody complexes in the vessel walls in animals depleted of polymorphs, it appears that polymorphs play an essential role in the initiation of the Arthus phenomenon. The polymorphs which were found in the vessel walls during the early phases of the reaction contained antigen and probably antibody. Later, both the polymorphs and the antigen–antibody deposition in the vessel walls disappeared and healing of the vessels took place. In polymorph-depleted rabbits, the antigen remained in the vessel walls and did not disappear until restoration of the polymorphs occurred. It appears that the polymorphs not only initiate the Arthus reaction, but also cause cessation of the reaction by removing the offending antigen–antibody

complexes. That polymorphs are capable of degrading at least the antigen portion of the complexes has been shown by Cochrane *et al.* (1959). Following the injections of I*BSA into the spleens of BSA-sensitized rabbits, there occurred a massive influx of polymorphs, accompanied by changes in the sinusoids, resembling the necrotizing vascular reactions seen in a cutaneous Arthus reaction. These polymorphs contained I*BSA and rabbit γ-globulin (antibody). When these cells were removed from the spleen and incubated *in vitro,* the I* antigen was degraded and nonprotein-bound I* liberated in the form of monoiodotyrosine, diiodotyrosine, and free iodide. Sorkin and Boyden (1959) demonstrated that I*HSA in the form of I*HSA–anti-HSA complexes was both taken up and degraded by mononuclear cells obtained from peritoneal exudates of normal guinea pigs. In these latter experiments the nonprotein I* which was liberated was identified as a peptide. The extent of I*HSA degradation depended on the quantitative relationship between the antigen and the antibody in the complexes. Whether the antibody portion of antigen–antibody complexes is similarly degraded by leucocytes has not been studied.

B. Antigen–Antibody Complexes Prepared *In Vitro*

Studies concerned with the *in vivo* fate of antigen–antibody complexes prepared *in vitro* have permitted a correlation between the *in vivo* behavior of antigen–antibody complexes and their composition. When antigen–antibody precipitates formed at equivalence were injected into rabbits, both the antigen and antibody components were rapidly eliminated from the blood (Weigle, 1958b; Walter and Zipper, 1959) and the antigen portion at least deposited in the lung, spleen, and liver. Antigen–antibody precipitates were more rapidly eliminated from the blood of previously sensitized rabbits than from the blood of normal rabbits (Walter and Zipper, 1959). However, the organ distribution of the antigen portion was the same in both normal and sensitized animals. As previously mentioned, a similar rapid elimination and deposition of antigen–antibody complexes occur in actively or passively sensitized rabbits injected with antigen.

In contrast to antigen–antibody precipitates formed at equivalence or with excess antibody, certain soluble antigen–antibody complexes prepared with excess antigen persist in the circulation of rabbits in a manner similar to the complexes formed early during a primary antibody response (Weigle, 1958b). These soluble complexes were prepared with an I*-labeled γ-globulin fraction of rabbit anti-BSA and unlabeled BSA. An equivalent amount of BSA was added to the labeled

γ-globulin fraction and the precipitates which formed were washed until free of nonantibody I*-activity and then dissolved by the addition of BSA in amounts ranging from 2 to 50 times the amount necessary to precipitate all of the antibody at equivalence (2 to 50 times equivalence). The complexes were then injected intravenously into rabbits which had been X-rayed to abolish an immune response to the antigen, and followed in the blood with the I*-labeled antibody. A portion of these complexes was rapidly removed from the blood within several

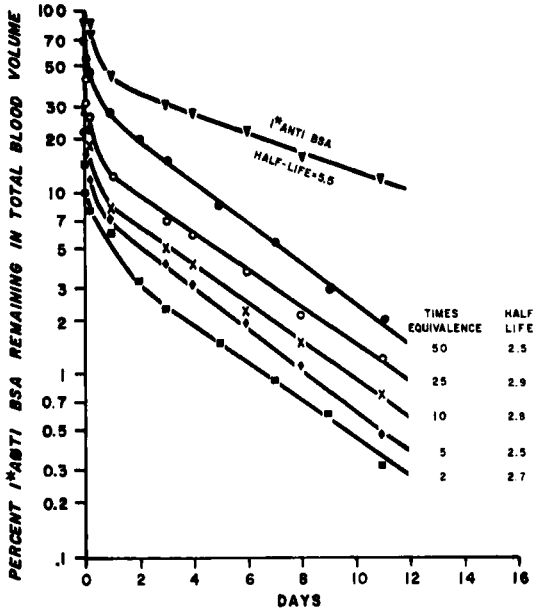

Fig. 3. Elimination of BSA–I*BSA complexes from the blood of rabbits. Reprinted from the *J. Immunol.* **81**, 204 (1958).

hours following injection, and the remaining portion was eliminated more slowly at an exponential rate with a half-life of 2.7 days, which is shorter than the half-life of either the antigen or antibody when injected alone. The amount of the complexes rapidly eliminated from the blood was greater when the complexes were prepared near equivalence than when they were prepared with a large excess of antigen (Fig. 3). Similar results were observed when the fate of I*BSA–unlabeled anti-BSA complexes was followed in the blood of rabbits by determining the globulin-bound I* antigen in the circulation. In view of the large number of reports demonstrating that the average size of soluble antigen–antibody complexes prepared with excess antigen decreases as

more antigen is added (Heidelberger and Pedersen, 1937; Pappenheimer *et al.*, 1940; Eisen and Karush, 1949; Marrack *et al.*, 1951; Singer and Campbell, 1951, 1952; Oncley *et al.*, 1952; Becker, 1953; Singer and Campbell, 1955; Karush, 1956; Weigle and Maurer, 1957b), it appears that the larger complexes predominating near equivalence were rapidly removed from the circulation while the smaller complexes predominating in the region of extreme antigen excess remained in the circulation longer. The smaller complexes predominating in large excess antigen have an electrophoretic mobility which is similar to that of β-globulin, whereas the larger complexes formed near equivalence have a slower electrophoretic mobility which is similar to that of γ-globulin (Weigle, 1958b). What, if any, effect the net charge of the complexes would have on their *in vivo* behavior is not known at the present.

Soluble complexes formed with nonprecipitating antibody (Heidelberger and Kendall, 1935) and an equivalent amount of antigen are rapidly removed from the circulation following their injection into rabbits (Weigle, 1958b). However, if soluble complexes are prepared with nonprecipitating antibody and a large excess of antigen, they persist in the circulation in the same manner as complexes prepared with precipitating antibody and excess antigen. The complexes formed at equivalence with nonprecipitating antibody are probably aggregates of complexes and their failure to precipitate is a result of something other than an 'univalence' of the antibody (Weigle and Maurer, 1957a).

The ability of complexes, prepared *in vitro* with a large amount of excess antigen and those formed *in vivo* early during an immune response, to persist in the circulation indicates that immune elimination of serum protein antigens depends on more than the mere reaction of circulating antigen with newly synthesized antibody. It appears that the first antibody synthesized combines with the circulating antigen in great antigen excess and that the resulting complexes are small and remain in the circulation. As additional antibody is synthesized, it probably combines with both the free antigen and the antigen present in the complexes. A continuation of antibody synthesis would result in the formation of larger complexes. When the complexes reach a certain size, they are probably rapidly removed from the circulation by the reticulo-endothelial cells of the liver, lung, and spleen, and possibly by circulating phagocytic cells. If this is the case, the rate of immune elimination of antigen would then be determined by the amount of antigen injected, the rate of antibody synthesis, and, perhaps, the quality of antibody produced.

The immune elimination of antigen is not influenced by cellular

sensitivity. This is evidenced by the results of Dixon and Maurer (1953) who demonstrated that rabbits either actively or passively sensitized eliminated antigen from the circulation at the same rate, provided the level of circulating antibody was comparable. Also, guinea pigs injected with bovine γ-globulin (BGG)–anti-BGG precipitates in adjuvant possessed delayed sensitivity to BGG several days before immune elimination of BGG occurred (Sell and Weigle, 1959).

III. Biological Activity of Antigen–Antibody Complexes

A. Serum Sickness

Serum sickness is one of the experimental diseases of hypersensitivity in which a pathogenic role of antigen–antibody complexes has been most seriously implicated (Dixon *et al.*, 1958). Past investigations have shown beyond much doubt that the morphologic lesions of serum sickness are the result of antigen–antibody reactions (Longcope and Rackemann, 1918; MacKenzie and Leake, 1921; Hopps and Wissler, 1946; Hawn and Janeway, 1947; Ehrich *et al.*, 1949; Germuth, 1953; Germuth *et al.*, 1955). The lesions of serum sickness are produced as antibody production begins and while antigen is still persisting in the blood and tissues. With the continued production of antibody, antigen is eliminated, free antibody appears in the serum, and the lesions of serum sickness disappear. It has recently been possible to demonstrate the persistence of antigen–antibody complexes in the circulation at the time of serum sickness development (Weigle and Dixon, 1958; Dixon *et al.*, 1958). A relative measurement of these complexes was permitted, as already described, by the differential solubility of BSA and the globulin antibody in 50% saturation with ammonium sulfate. Rabbits were injected with 250 mg. per kilogram body weight and bled periodically. The sera were analyzed for both total I*BSA and globulin-bound I*BSA. The formation of circulating I*BSA–anti-BSA complexes and their elimination from the blood can be seen in Fig. 4. The dot-dash line indicates the per cent of the totally injected I*BSA bound to globulin (antibody) in the serum of a typical rabbit. After the seventh day following injection, the amount of I*BSA in complex form increased sharply to a maximum value on the eleventh day and then decreased, paralleling the fall in total circulating I*BSA. During the rapid immune elimination of I*BSA. as much as from 50 to 75% of the total circulating I*BSA was bound to the host's globulin. Also shown in Fig. 4 is the percentage of animals killed at various intervals which had the glomerular and the arterial lesions of serum sickness. It is apparent that the lesions of serum sick-

ness develop in rabbits at the time when there are high levels of circulating antigen–antibody complexes. In addition, glomerulonephritis has been observed in rabbits which showed no immune elimination of

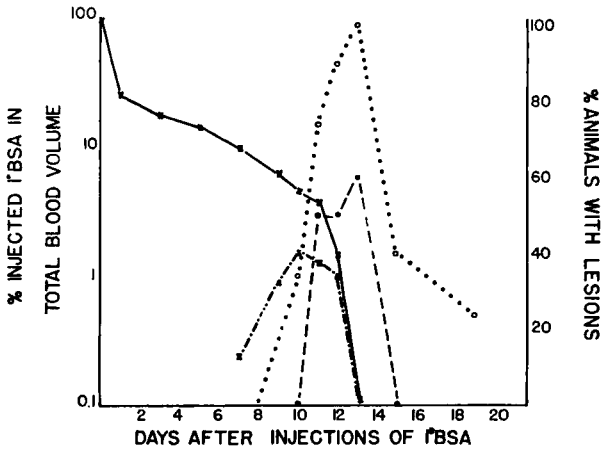

FIG. 4. I*BSA induced serum sickness in the rabbit. ———, Total circulating I*BSA; ——·——·——, globulin-bound I*BSA; · · · · ·, glomerulonephritis; – – – –, arteritis. Reprinted from *Arch. Pathol.* **65**, 18 (1958).

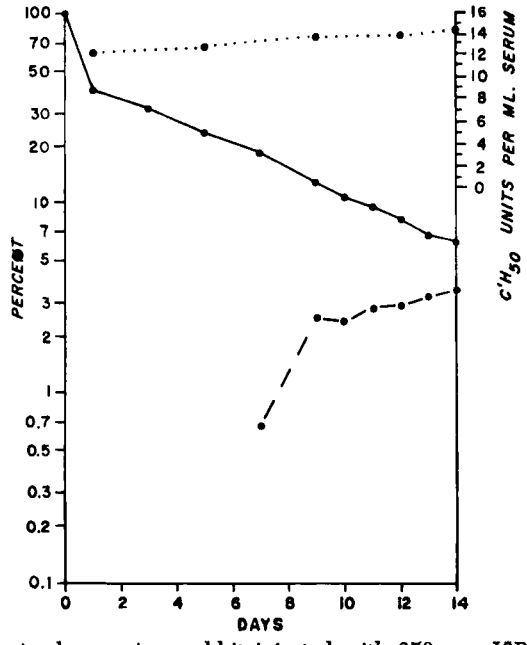

FIG. 5. Serologic changes in a rabbit injected with 250 mg. I*BSA per kilogram body weight. Reprinted from *Proc. Soc. Exptl. Biol. Med.* **99**, 226 (1958).

antigen, but which did contain high levels of circulating complexes prior to autopsy. Data obtained from such a rabbit are shown in Fig. 5. This rabbit had a moderate glomerulonephritis when autopsied 14 days after injection of 250 mg. I^*BSA per kilogram body weight. Such results were observed in 3 of the 26 rabbits injected. That antigen–antibody complexes initiate the lesions of serum sickness is further indicated by the localization of antigen and, presumably, antibody in tissue during the development of lesions, but not prior to their development (Dixon *et al.*, 1958).

While antigen–antibody complexes are probably involved in the pathogenesis of serum sickness, they do not themselves always induce typical lesions of this disease. Almost all rabbits respond to injections of large amounts of BSA by making antibody capable of forming complexes with the circulating antigen, but not all animals develop lesions (Weigle and Dixon, 1958). Similarly, infusion of rabbits with soluble antigen–antibody complexes has not produced lesions of serum sickness with the same distribution, consistency, and severity as those produced during a primary immune response to injected antigens. Germuth and McKinnon (1957) showed only slight glomerulonephritis and necrotizing pulmonary arteritis in rabbits sacrificed 36 hours after infusion for 12 to 36 hours with large amounts of soluble BSA–rabbit anti-BSA complexes prepared in a moderate excess of antigen. They observed a slightly more severe glomerulonephritis in rabbits either infused or intermittently injected with anti-BSA 24 hours following an injection of 500 mg. of BSA (Germuth and Pollack, 1958). It was suggested that the lesions might have been provoked by antigen–antibody complexes formed in the circulation rather than by the actual interaction of antigen and antibody in the tissues. Less than 50% of the infused rabbits developed glomerulonephritis, but almost 100% of the rabbits developed glomerulonephritis during a primary immune response to an injection of a large amount of BSA alone (Fig. 4). Also, necrotizing arteritis was observed in the lungs of some of the rabbits infused with antibody but only very rarely in the coronary arteries where it is most commonly found during active serum sickness resulting from a primary antibody response. The failure of infusion of either antibody into rabbits previously injected with antigen, or of antigen–antibody complexes into normal rabbits, to produce severe and typical lesions may be the result of the quality of the antibody employed. The antibody used by Germuth and co-workers was obtained from hyperimmune rabbits, whereas the antibody involved in active serum sickness results from a primary antibody response. That the antibody formed early during a primary anti-

body response is of a different quality than the antibody formed during a secondary response has been shown by Farr (1958).

The distribution and severity of the lesions in rabbits injected with heterologous serum proteins may be dependent on several factors. The injection of large doses of BGG, which is cleared from the circulation more rapidly than BSA, produced only mild glomerular lesions and no arteritis (Heptinstall and Germuth, 1957). West *et al.* (1960) increased the severity of glomerulonephritis in rabbits by sensitizing the animals with BGG in Freund's adjuvant 2–6 days prior to intravenous injection of BGG; arterial lesions, however, were still absent. Germuth and Heptinstall (1957) suggested that the arterial lesions of serum sickness are dependent on the duration of antigen exposure, since following multiple injections of BGG, prominent valvular and arterial alterations appeared while glomerulonephritis was less frequent. However, Dixon *et al.* (1960) and Germuth (personal communication, 1960) produced chronic glomerulonephritis but no arteritis in rabbits injected daily for several months with small doses of BSA. Arterial lesions were absent, although antigen–antibody complexes were almost constantly present in the circulation. Both the duration of interaction between antigen and antibody and the physical nature of the complexes formed are probably responsible for the distribution of lesions in rabbits injected with heterologous serum proteins.

Contrary to the results obtained in rabbits, McCluskey and Benacerraf (1959) consistently observed glomerulonephritis, arteritis, and endocarditis in mice receiving multiple injections of either BSA–rabbit anti-BSA or egg albumin (Ea)–rabbit anti-Ea complexes prepared in moderate to extreme excess antigen. The glomerular lesions were characterized by a more intense exudative reaction than was observed in the lesions usually described in serum sickness resulting from a primary antibody response in rabbits. The total amount of antibody injected into the mice ranged from 44 to 73 mg. per kilogram body weight, which was similar to the amount of antibody infused into rabbits previously injected with antigen (Germuth and Pollack, 1958). Why soluble antigen–antibody complexes are more effective in the production of serum sickness lesions in mice than in rabbits is not known.

Circulating antigen–antibody complexes also are probably involved in the production of the experimental chronic glomerulonephritis in rabbits reported by Dixon *et al.* (1960). Rabbits were injected daily for several months with a given dose of BSA ranging from 5 to 100 mg. Some of the animals achieved antibody levels at 2–3 mg. antibody N per milliliter of sera within 4 to 6 weeks; others made moderate

amounts, enough to maintain an antibody excess in the circulation; some made just enough antibody to combine with all of the injected antigen; and some made no detectable antibody. Some of the high antibody producers developed arteritis and glomerulonephritis of the serum sickness type during the second and third weeks of injection, but thereafter these high producers showed only occasional anaphylactic or embolic phenomena, a slight thickening of glomerular capillaries, and inconsistent minimal proteinuria. The rabbits which produced either moderate levels of antibody or no detectable antibody showed no consequences of the antigen injections. However, the rabbits which made barely enough antibody to combine with all of the antigen and therefore contained high levels of circulating antigen–antibody complexes, developed a chronic, progressive glomerulonephritis, either proliferative or membranous, associated with edema, uremia, proteinuria, and hypercholesterolemia.

B. Anaphylaxis

The initial observations demonstrating the anaphylactogenic property of preformed antigen–antibody complexes were made by Frieberger (1909) and Friedemann (1909). They showed that when serum from a sensitized animal was mixed with the specific antigen and the mixture injected into a normal guinea pig, toxic reactions resembling anaphylaxis appeared. The toxicity was believed to have resulted from an *in vitro* production of a toxic substance (anaphylatoxin) during the reaction of antigen with antibody. Although it has been clearly demonstrated that anaphylatoxin is released *in vitro* by antigen–antibody mixtures (Osler *et al.*, 1959), recent studies indicate that soluble antigen–antibody complexes per se will produce anaphylactic reactions when injected into guinea pigs (Germuth and McKinnon, 1957; Weigle *et al.*, 1960), mice (Tokuda and Weiser, 1958a; Treadwell *et al.*, 1960) and rabbits (Trapani *et al.*, 1958; Weigle *et al.*, 1960). A stimulating effect of antigen–antibody complexes on isolated guinea pig smooth muscle has also been demonstrated (Kulka, 1942; Trapani *et al.*, 1958). Kulka (1942) showed that the ability of antigen–antibody mixtures to induce contraction of isolated smooth muscle was governed by the quantitative relationship between the antigen and antibody. The nature of the complexes responsible for anaphylactic reactions was further explored by Germuth and McKinnon (1957), who used preformed BSA–rabbit anti-BSA complexes and normal guinea pigs. They observed that the soluble complexes prepared in moderate excess antigen ranging from 2 to 8 times equivalence were effective, while the supernatants

of the complexes prepared either at equivalence or in excess antibody were ineffective. Furthermore, supernatants of soluble complexes prepared with excess antigen and then afterwards precipitated by the addition of antibody were unable to induce anaphylaxis when injected into guinea pigs. Soluble complexes, prepared either by adding excess antigen directly to antisera or by dissolving washed antigen–antibody precipitates formed at equivalence with excess antigen, were effective in producing anaphylactic reactions. Similar observations were made by Trapani *et al.* (1958), who showed that complexes prepared in slight to moderate excess antigen were most effective in producing anaphylactic contraction of normal guinea pig ileum, whereas the supernatants of precipitates prepared at equivalence were ineffective. In greater than moderate antigen excess, the ability of antigen–antibody complexes to induce either contraction of guinea pig smooth muscle (Trapani *et al.*, 1958) or acute anaphylaxis in the mouse (Tokuda and Weiser, 1958b; Treadwell *et al.*, 1960) and guinea pig (Weigle *et al.*, 1960) decreased with an increase in the amount of antigen added. Thus, there appears to be a correlation between the anaphylactic properties and the size of antigen–antibody complexes, since it has been repeatedly shown that the average size of antigen–antibody complexes decreases with an increase in the amount of excess antigen present.

The acute anaphylactic reaction produced in normal guinea pigs by soluble complexes is similar in many respects to the reaction seen in actively and passively sensitized guinea pigs challenged with an intravenous injection of antigen. The symptoms following the injection of complexes are the same as those observed in both the active and passive reactions (Germuth and McKinnon, 1957). Also, in fatal reactions, the degree of emphysema observed in the lungs of normal guinea pigs injected with complexes is identical to that observed in sensitized guinea pigs injected with antigen (Germuth and McKinnon, 1957). In addition, the complex induced reactions can be inhibited with a quantity of antihistamine similar to the amount needed to inhibit classic passive reactions (Weigle *et al.*, 1960). The preferential localization of I*trace-labeled anti-BSA in the lungs of guinea pigs injected with soluble BSA-I*anti-BSA complexes is similar to that observed in guinea pigs passively sensitized with I*anti-BSA and injected with BSA after a 48-hr. latent period. These latter results are in agreement with those of Dixon and Warren (1950), who demonstrated a preferential localization of I*BGG in the lungs during anaphylaxis in guinea pigs actively sensitized with BGG. The similarities between the classic anaphylactic reactions and the complex-induced reactions suggest that

there may be a similarity in the mechanisms responsible for these reactions.

In addition to the acute anaphylactic reaction produced in guinea pigs injected with soluble antigen–antibody complexes, delayed reactions are commonly observed. Germuth and McKinnon (1957) reported in some animals injected with preformed complexes a delayed reaction which occasionally led to death within several to 24 hours instead of the immediate acute reaction. Other studies have shown that soluble complexes commonly induce both acute and delayed symptoms (the latter leading to death) in the same guinea pig (Weigle *et al.*, 1960).

Typical anaphylactic reactions were produced in rabbits by injecting large amounts of soluble BSA–anti-BSA complexes prepared with either precipitating antibody and excess antigen or with non-precipitating antibody[2] and an equivalent amount of antigen (Table I).

TABLE I[a]

ANAPHYLACTIC REACTIONS PRODUCED BY INJECTION OF SOLUBLE BSA–ANTI-BSA COMPLEXES INTO RABBITS

Antibody N (mg.)	Times equivalence	Number of rabbits	Anaphylactic symptoms		
			Neg.	Nonfatal symptoms	Death
Precipitating antibody					
60	3	6	0	1	5[b]
30	3	10	0	9	1[c]
60	10	5	0	5	0
Nonprecipitating antibody					
5	1	3	0	2	1[d]

[a] Reprinted from Weigle *et al. J. Immunol.* **85**, 469. (1960).
[b] Two died within 10 minutes following injection and 3 died 6–12 hours later.
[c] Died within 3 hours following injection.
[d] Died within 10 minutes following injection.

These anaphylactic reactions occur in the absence of an obvious embolization of the pulmonary circulation. Trapani *et al.* (1958) also were able to produce anaphylactic reactions in rabbits with soluble antigen–antibody complexes prepared with precipitating antibody and excess antigen. On the other hand, McKinnon *et al.* (1957) failed to produce anaphylaxis in rabbits with large doses of soluble complexes, but they could obtain anaphylaxis by injecting suspensions of antigen–antibody precipitates prepared at equivalence. McKinnon and asso-

[2] Prepared by serial absorption with small additions of antigen as described by Heidelberger and Kendall (1935).

ciates believed that anaphylaxis in the rabbit was caused by vascular obstruction resulting from embolization of the pulmonary circulation by antigen–antibody precipitates. That pulmonary embolization plays some role in anaphylaxis in the rabbit was previously suggested by Dixon (1954). On the basis of both the present and past observations, it appears that anaphylaxis in the rabbit, while perhaps enhanced by embolization of small pulmonary vessels by antigen–antibody precipitates, is not dependent on this event. Rather, other mechanisms, such as vasoconstriction of pulmonary arteries by a humoral factor which is released secondary to antigen–antibody interaction, are probably equally or more important.

C. Cutaneous Reactions

Antigen–antibody mixtures were first shown to have skin-irritating properties by Opie (1924), who was able to elicit inflammatory reactions in the skin of rabbits by intradermal injection of mixtures of horse serum and rabbit anti-horse serum. Later, a number of investigators demonstrated that mixtures of serum from hay fever patients and the antigen to which they were sensitized produced immediate reactions when injected into the skin of normal patients (Chant and Gay, 1927; Foran and Lichtenstein, 1931; Cooke *et al.*, 1935; Loveless, 1940). More recently Humphrey and Jaques (1955) showed that mixtures of rabbit antibody and soluble antigen resulted in increased capillary permeability when injected into the skin of rabbits. The nature of the soluble BSA–anti-BSA complexes responsible for producing increased capillary permeability in the skin of guinea pigs has been studied extensively by Ishizaka and associates (1959a), who demonstrated that soluble BSA–anti-BSA complexes prepared in moderate excess antigen were most effective. As in anaphylaxis, the effectiveness decreased with an increase in the amount of antigen employed. Experiments employing different electrophoretic fractions of preparations of soluble BSA(Ag)–anti-BSA(Ab) complexes suggest that complexes of the Ag_3–Ab_2 molecular species and more complicated complexes were capable of producing increased capillary permeability, whereas the simple Ag_2–Ab molecular species were ineffective. Also levorotation increased in the formation of the skin-irritating complexes, but not in the formation of the Ag_2–Ab complexes (Ishizaka and Campbell, 1959). The composition of these complexes was calculated on both the basis of electrophoretic analysis and the assumption that the valence of precipitating rabbit anti-BSA is 2. The ability of soluble antigen–antibody complexes to produce increased capillary permeability appears to de-

pend on the properties of the antibody rather than on the antigen (Ishizaka *et al.*, 1959b). With a few exceptions, complexes composed of rabbit antibody and homologous antigen produced increased capillary permeability irrespective of the nature of the antigen, but the complexes composed of either horse or chicken antibody and the same antigens were not active in the skin.

An inflammatory reaction was observed by Cochrane and Weigle (1958) in the skin of rabbits injected intradermally with soluble BSA–

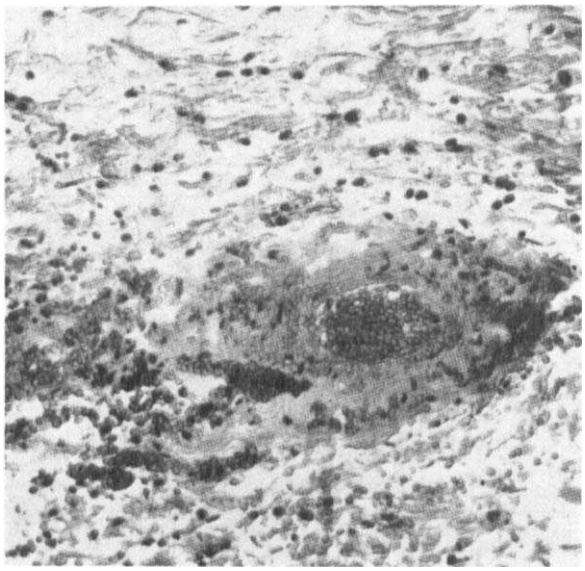

FIG. 6. Necrotic vessel seen in a cutaneous lesion produced by soluble complexes. Hematoxylin and eosin stain. Magnification: × 240. Reprinted from *J. Exptl. Med.* **108**, 591 (1958).

anti-BSA complexes prepared with excess BSA at 3 times equivalence. This complex-induced reaction reached a maximum in 20 hours and was compared with the local passive Arthus, the reversed passive Arthus, and the active Arthus reactions, with respect to the amounts of antibody and time needed to produce a lesion, the morphologic alterations, and the distribution of antigen and antibody within the lesion. The complex-induced reaction and the local passive Arthus reaction, in which antibody is injected locally followed in 30 minutes by a local injection of antigen, were similar in all respects. The amount of antibody needed to produce a given lesion was the same in both reactions, as was also the sequence of morphologic changes and the

distribution of antigen and antibody in the tissues. These two reactions, in which both the antigen and the antibody are given locally in the same site, were characterized by a diffuse acute inflammatory infiltration with little vascular damages at 8 hours, a persistence of mono-

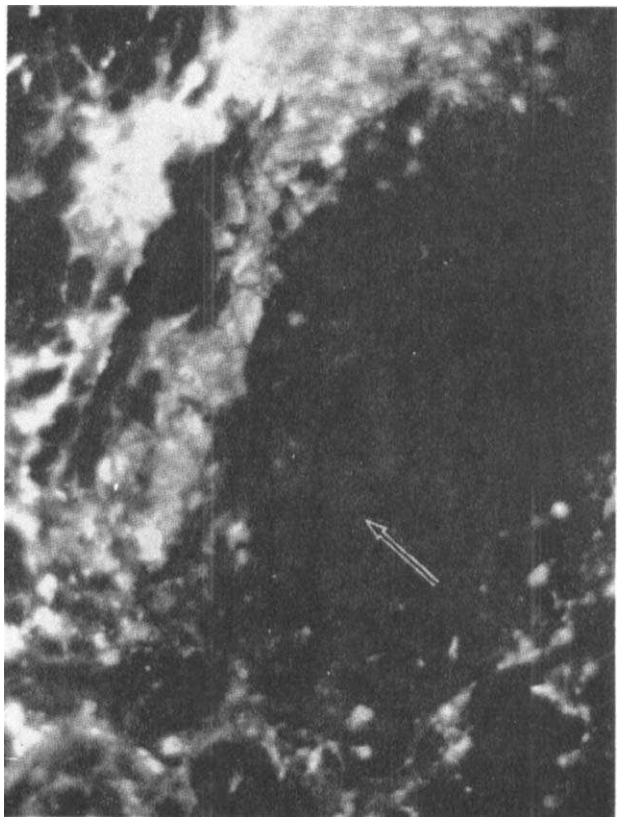

FIG. 7. A section of a complex-induced reaction stained for BSA with fluorescent anti-BSA. Granular white areas mark the presence of BSA. Arrow marks outer portion of vessel wall. The wall contains no detectable BSA, while an abundance of the antigen is seen in surrounding areas. Magnification: × 440. Reprinted from *J. Exptl. Med.* **108**, 591 (1958).

nuclear cells and occasional degenerative changes in the blood vessels without particular involvement by leucocytes at 24 hours (Fig. 6), and progressive necrosis of blood vessels and hemorrhage in the more severe lesions during the second day. Throughout the course of the reaction, the antigen and the antibody were present in diffusely scattered granular aggregates with little or none present in the blood vessel

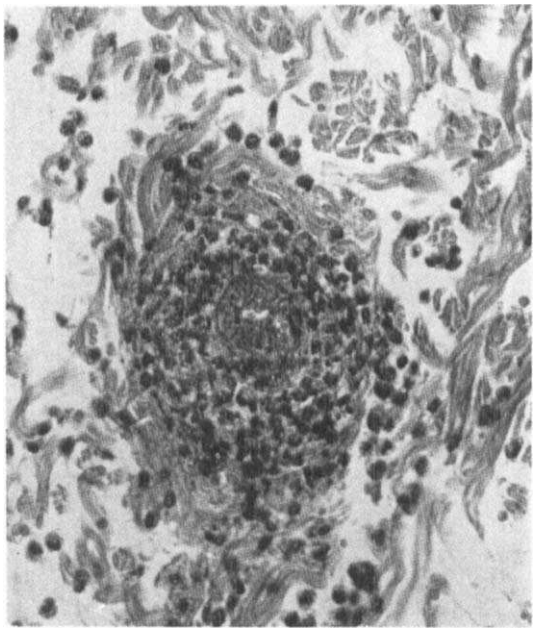

FIG. 8. Vessel in an active or classic Arthus reaction showing inflammation of the vessel wall. Hematoxylin and eosin stain. Magnification: × 280. Reprinted from *J. Exptl. Med.* **108**, 591 (1958).

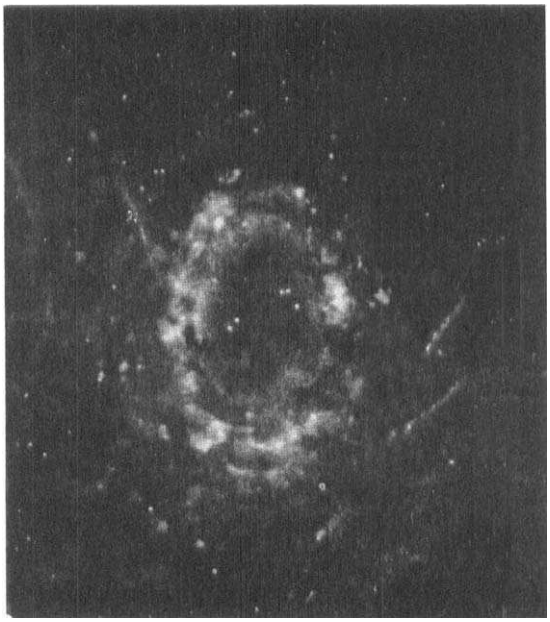

FIG. 9. A section of an active Arthus reaction stained for BSA with fluorescent anti-BSA. The BSA is found concentrated in the area of vascular inflammation. Magnification: × 240. Reprinted from *J. Exptl. Med.* **108**, 591 (1958).

walls. Necrotic vessels were conspicuously devoid of the antigen (Fig. 7). On the other hand, the reverse passive and active Arthus reactions required less antibody to produce a given lesion and developed more rapidly than the complex-induced or local passive Arthus reactions. In these situations the reaction seemed to be concentrated in the blood vessel walls. The vessels were heavily infiltrated by leucocytes and showed signs of fibroid necrosis within a few hours (Fig. 8). After 4 to 5 hours, the blood vessels were severely damaged with resultant thrombosis and hemorrhage. In these lesions, in which one of the immunologic reactants was in the circulation and the other injected locally, there was a massive concentration of antigen and antibody in the walls of the small blood vessels corresponding to the sites of vascular necrosis (Fig. 9). The foregoing observations suggest that, in the complex-induced reaction, the complexes might be exerting their influence throughout the injection site and that physiologically active products released by these complexes are secondarily causing damage of neighboring blood vessels.

D. Effect on Blood Cells

Antigen–antibody complexes have been shown to cause changes in blood cells both *in vivo* and *in vitro*. Miescher and associates (Miescher and Straessle, 1956; Straessle and Miescher, 1956; Miescher, 1959) studied the pathological effect of antigen–antibody complexes on leucocytes and thrombocytes. They showed that intravenous injection of soluble antigen–rabbit antibody complexes into rabbits produced various effects on the leucocytes and thrombocytes. Also, such complexes became fixed to and caused agglutination of these cells *in vitro*. The effect of antigen–antibody complexes on the cells depended on the nature of the antigen, the nature of the antibody, and the ratio of antigen and antibody. Miescher concluded from these data that antigen–antibody complexes may be of pathological importance in blood disorders such as leucopenia, thrombopenia, and anemia of allergic etiology (Miescher, 1959). Boyden and Anderson (1955) reported that mixtures of tuberculoproteins and certain rabbit antisera produced by immunization with tubercle bacilli caused agglutination of normal sheep erythrocytes. This reaction depended upon the affinity of the erythrocytes for tuberculoproteins. More recently, Robbins and Stetson (1959) have shown that the addition of mixtures of antigen and antibody to normal rabbit blood accelerated coagulation. They suggested that this phenomenon may be mediated by an effect of the complexes on the platelets.

IV. Effect of Complement on the Behavior of Antigen–Antibody Complexes

A. *In Vitro* Interaction between Antigen–Antibody Complexes and Complement

The *in vitro* reaction of complement with antigen–antibody complexes results in a number of secondary phenomena such as lysis of sensitized erythrocytes, activation of proenzymes, production of anaphylatoxin, and precipitation of soluble antigen–antibody complexes. As evidenced by the reaction between complement and sensitized erythrocytes (Mayer *et al.*, 1954), the interaction between complement and antigen–antibody complexes is not a simple one but consists of a series of known and probably unknown events. Thus, the exact reactions involved in most of the secondary phenomena produced by the interaction between complement and antigen–antibody complexes are not as yet known. The action of complement is discussed by Osler in this volume.

In the past 70 years an enzymatic nature of complement has been suggested by a number of workers who associated complement with several enzymes, including proteases, peptidases, and lipases. Recently Lepow and co-workers (1956) demonstrated that the first component of complement C'1 was converted to an esterase as a result of its reaction with antigen–antibody complexes. The esterase could then inactivate the fourth (C'4) and to lesser extent the second (C'2) component of complement. As yet there is no known substrate in the animal body which this esterase can attack to produce changes which result in biological reactions. On the other hand, the addition of sera containing complement activity to antigen–antibody complexes produces a toxic substance (anaphylatoxin) which induces an anaphylacticlike reaction when injected into guinea pigs (Osler *et al.*, 1959). The third component (C'3) is probably involved in this reaction. Although complexes formed both at equivalence and in the region of excess antigen are effective in the production of anaphylatoxin, the complexes become less effective as the amount of antigen added is increased.

Certain soluble antigen–antibody complexes prepared with excess protein antigens and rabbit antisera are precipitated in the presence of complement (Weigle and Maurer, 1957a), probably as a result of the fixation of the C'1 component (Weigle and Maurer, 1957c). As in the *in vivo* production of biological activity and in the *in vitro* production of anaphylatoxin, the ability of complement to fix to and precipitate these soluble complexes is directly proportional to the size of the complexes (Weigle and Maurer, 1957b). The smaller complexes prepared

in extreme excess antigen not only remove less hemolytic activity from fresh sera than do the larger complexes prepared in slight excess antigen, but also are precipitated to a lesser extent. Complexes with an antigen/antibody molecular ratio of 1.5 or greater fail either to be precipitated by rabbit complement or to remove hemolytic activity from fresh rabbit sera. If a valence of 2 is accepted for rabbit antibody, these data would suggest that the molecular species Ag_2–Ab and probably Ag_3–Ab_2 are incapable of fixing rabbit complement. Certain antigen–antibody complexes which are formed *in vivo* during a primary antibody response to BSA are incapable of reducing the level of circulating complement (Weigle and Dixon, 1958). In a large number of rabbits injected with I*BSA, a drop in complement never preceded a detectable immune elimination of antigen even when large amounts of circulating complexes were present. These data suggest that only the smaller non-complement-fixing complexes of the Ag_2–Ab and possibly the Ag_3–Ab_2 molecular species are capable of persisting in the circulation of rabbits.

Soluble complexes prepared with nonprecipitating antibody also are capable of reacting *in vitro* with complement. Antigen–antibody complexes prepared at equivalence with BSA and either precipitating or nonprecipitating rabbit anti-BSA fixed comparable amounts of guinea pig, human, and rabbit complement (Weigle and Maurer, 1957a). Of the complexes prepared with nonprecipitating antibody, 80–90 % were precipitated in the presence of normal guinea pig and rabbit sera. Whether the *in vivo* aggregation of these complexes by complement is in part responsible for their rapid immune elimination from the circulation of rabbits is not known. As mentioned previously, complexes prepared with nonprecipitating anti-BSA produce anaphylactic-like symptoms when injected into rabbits.

B. Possible Role of Complement in the Biological Reactions Mediated by Soluble Antigen–Antibody Complexes

Over the years complement has been implicated in most of the experimental diseases of hypersensitivity. Furthermore, recent data suggest that complement plays a role in the biological activity mediated by preformed soluble antigen–antibody complexes. However, no data are available which conclusively implicate complement in either the diseases of hypersensitivity or in the biological reactions produced by soluble complexes. At this time it should be mentioned that the mechanisms involved in the various biological reactions produced by preformed complexes may be different. Therefore, it is possible that complement plays a role in some of the reactions but not in others.

1. Serum Sickness

It has not been demonstrated that factors other than antigen–antibody complexes are involved in the development of lesions of serum sickness. Complement would, of course, be one of the first factors considered, since it is capable of reacting with certain soluble antigen–antibody complexes. Also, the reduction of circulating complement has been associated with both the immune elimination of antigen and the occurrence of glomerulonephritis in rabbits injected with large doses of serum protein antigens (Schwab *et al.*, 1950; Moll and Hawn, 1952). On the other hand, a mild glomerulonephritis has been observed in rabbits in the absence of either an immune elimination of antigen or a reduction in serum complement (Weigle and Dixon, 1958). The levels of circulating I*BSA–antibody complexes in the serum of a rabbit in which both glomerulonephritis and endocarditis were observed is shown in Fig. 5. This rabbit showed neither an immune elimination of antigen nor a reduction in serum complement, but at one time had over 50% of the circulating I*BSA bound to antibody. It appears that circulating antigen–antibody complexes, in the absence of either immune elimination of antigen or detectable reduction in serum complement, can initiate lesions of serum sickness. But this does not mean that complement plays no role in the production of lesions. Certainly more numerous and more severe lesions are usually found in rabbits that show an early and rapid immune elimination of antigen and have a large reduction in serum complement than in rabbits that contain circulating complexes in the absence of either immune elimination or reduction in serum complement. Also, in the afore-mentioned experiments, only the total hemolytic activity of the sera was determined and not the activity of the individual components of complement. If it is possible to compare the noncomplement-fixing complexes which circulate *in vivo* with the noncomplement-fixing complexes prepared *in vitro,* it would appear that small complexes of the Ag_2–Ab and/or Ag_3–Ab_2 molecular species were capable of inducing morphologic lesions of serum sickness in rabbits.

2. Anaphylaxis

There is evidence available both for and against the role of complement in the acute anaphylactic reactions mediated by either the injection of soluble antigen–antibody complexes into animals or the addition of these complexes to isolated tissues. Although the presence of complement during the preparation of soluble antigen–antibody complexes is not important for the activity of the complexes, there is no way

of preventing the interaction of the host's complement with the complexes after injection. Humphrey and Jaques (1955) and Kulka (1942) demonstrated that complement-like substances increase the *in vitro* anaphylactogenic activity of complement deficient antigen–antibody complexes. Humphrey and Jaques (1955) showed that the release of histamine by addition of antibody and antigen to normal rabbit platelets required the presence of both unheated plasma and Ca^{++}. Also, Kulka (1942) noted that fresh normal sera enhanced the contraction of smooth muscle tissue resulting from exposure of such tissues to antigen–antibody complexes. Additional support for the active role of complement in anaphylaxis produced by soluble antigen–antibody complexes is found in the correlation between the ability of soluble complexes formed with excess antigen to fix complement and to produce anaphylactic reactions. As mentioned previously, the anaphylactic properties of soluble antigen–antibody complexes decrease with an increase in excess antigen (Trapani *et al.*, 1958; Tokuda and Weiser, 1958b; Weigle *et al.*, 1960) as do their ability to fix complement *in vitro* (Weigle and Maurer, 1957a). Contrary to these observations, other data show little correlation between the ability of a substance to fix complement and its ability to produce toxic reactions when injected into animals. Preformed protein–rabbit anti-protein complexes prepared at equivalence fix larger amounts of complement *in vitro* than do the soluble complexes formed in excess antigen, but the former produce less anaphylactogenic activity when injected into guinea pigs than is produced by the latter (Germuth and McKinnon, 1957). Furthermore, the amount of complement fixed *in vivo* does not determine the degree of anaphylaxis produced. In guinea pigs injected with complexes prepared at 3 to 4 times equivalence with 1.65 mg. anti-BSA N and bled 5 minutes later, over 90% of the blood complement was removed. But only from 11 to 24% of the blood complement was removed from guinea pigs passively sensitized with 0.10 mg. anti-BSA N and challenged 48 hours later with BSA (Table II). The latter group of animals was bled after collapse and prior to cardiac failure (4 to 5 minutes after injection). Although considerably more complement was fixed following the injection of soluble complexes into normal guinea pigs than following the injection of antigen into passively sensitized animals, the degree of anaphylaxis in the latter group was more severe than the former. Similarly, the injection of 5 mg. of aggregated human γ-globulin N into normal guinea pigs almost completely depleted the animals of circulating complement, but produced only occasionally mild anaphylactic reactions (Christian, 1960).

If anaphylactic reactions produced by soluble antigen–antibody

complexes do depend on complement, it may be that the rate at which complement is fixed is more important than the total amount fixed, since these acute reactions are completed in a few minutes or less. Although there are no data available that compare the rate of fixation of complement during the reaction between antigen and antibody and the rate of fixation by preformed soluble complexes, Johns (1953) showed that the rate is greater when antibody and antigen are added

TABLE II[a]
In Vivo FIXATION OF COMPLEMENT DURING ANAPHYLAXIS

Guinea pig No.	% Circulating complement[b] ($C'H_{50}$ units) removed	Degree of anaphylaxis
	Passively sensitized with anti-BSA[c]	
1	12	Fatal
2	22	Fatal
3	14	Fatal
4	11	Fatal
5	17	Fatal
6	24	Fatal
	Injected with BSA–anti-BSA complexes[d]	
1	97	Mild
2	99	Negative
3	97	Moderate
4	97	Fatal
5	95	Fatal
6	97	Mild

[a] Modified from Weigle *et al. J. Immunol.* **85**, 469 (1960).

[b] Guinea pigs undergoing fatal anaphylaxis were bled after they had collapsed and prior to cardiac failure. All others were bled 5 minutes after the challenging injection.

[c] Passively sensitized with 100 μg. anti-BSA N, 48 hours prior to a challenging injection of 1.0 mg. BSA N.

[d] Injected with 1.0 ml. of complexes prepared at 3 times equivalence with 1.65 mg. anti-BSA N per milliliter.

separately to complement than when they are added as preformed precipitates. In addition, the site of fixation of complement may be important, e.g., complement fixed in extravascular spaces or at the site of target tissues may be more significant than complement fixed in the circulation. Another possibility is that the preformed complexes do not fix very well the component or components which are most important in the anaphylactic reaction, e.g., C'3 (Osler *et al.*, 1959).

3. *Cutaneous Reactions*

Considerable data exist which implicate complement in the production of increased capillary permeability in the skin by preformed antigen–antibody complexes. Osler *et al.* (1957) showed that the immediately increased capillary permeability which results from an intradermal injection of antibody in rats, followed, after a latent period, by an intravenous injection of antigen, is dependent on the presence of a circulating complement-like substance. Also, there is a relationship between the ability of antigen–antibody systems to fix complement and their ability to produce increased capillary permeability. In most instances, soluble complexes prepared with rabbit antibody both fix complement and produce increased capillary permeability, while soluble antigen–antibody complexes prepared with either horse or chicken antibody do neither (Ishizaka *et al.*, 1959b). Similarly, the skin-irritating activity of complexes prepared with a given antiserum and various amounts of excess antigen parallels the ability of these complexes to fix complement. With a given antigen–antibody system, the ability of complexes to both fix complement and produce increased capillary permeability in the skin decreases with an increase in the amount of excess antigen present. However, when comparing one antigen–antibody system with another, the complement fixability of the soluble complexes does not always parallel their skin reactivity. Contrary to the situation in acute anaphylaxis, the data on complement fixation and production of increased capillary permeability by aggregated human γ-globulin further implicate complement in this cutaneous reaction of the immediate type. These complement-fixing aggregates do produce increased capillary permeability in the skin of guinea pigs, while aggregated BGG, which does not fix complement, also does not produce cutaneous reactions (T. and K. Ishizaka, 1959). The inability of aggregated BGG to produce skin reactions is in accord with the observation that bovine antibody is incapable of sensitizing guinea pigs to subsequent anaphylaxis (Kabat and Mayer, 1948). As in anaphylaxis, the *in vitro* addition of complement to soluble antigen–antibody complexes has no effect on their ability to produce skin reactions (Ishizaka *et al.*, 1959a).

4. *Pathological Changes in Blood Cells*

Whether complement is involved in the pathological changes produced in blood cells by soluble complexes has not been extensively investigated. However, normal sheep cells which agglutinate in the presence of tuberculoprotein and specific antisera will also lyse if com-

plement is added to the system (Boyden and Anderson, 1955). Also, a complement-like substance apparently is required for the *in vitro* release of histamine from platelets by antigen–antibody mixtures (Humphrey and Jaques, 1955).

V. Possible Mechanisms Responsible for the Biological Activity of Soluble Antigen–Antibody Complexes

It would appear from the data so far accumulated that the biological reactions produced by either antigen–antibody complexes or the injection of antigen into sensitized animals are mediated by a series of biochemical events. These events may involve complement, enzymes, anaphylatoxin-like substances, etc., and probably are ultimately set off by pharmacologically active substances. The manner in which soluble antigen–antibody complexes initiate these events has not been conclusively established. The data available at the present suggest that the ability of soluble antigen–antibody complexes to produce biological reactions could result from either the action of the complexes as a stable unit or the process of dissociation and reassociation.

A. The Role of the Antigen–Antibody Complexes as a Stable Unit

A number of reports support the contention that soluble antigen–antibody complexes as a stable unit can produce certain pathological changes in tissue. It has been suggested that the ability of complexes to produce increased capillary permeability in the skin is a result of a molecular change in the antibody portion of the complexes (Ishizaka *et al.*, 1959a). That a molecular change takes place in the antibody as a result of its reaction with antigen is evidenced by the change in optical rotation of the antibody once combined with antigen (Ishizaka and Campbell, 1959). Levorotation of antibody increases in the formation of complexes which are capable of producing increased capillary permeability in the skin of guinea pigs, but the levorotation of antibody does not increase in the formation of complexes which are not capable of producing increased capillary permeability in the skin.

That complexes can, as a stable unit, produce biological reactions is further indicated by the ability of soluble heat-aggregated human γ-globulin both to fix complement and to produce increased capillary permeability in the skin of guinea pigs (T. and K. Ishizaka, 1959). Aggregated human γ-globulin is comparable on a weight basis with soluble antigen–antibody complexes in respect to its abilty to produce

skin reactions. The function of antigen in antigen–antibody complexes may be to convert the antibody to aggregates of γ-globulin; presumably enabling the antibody to fix complement and to initiate the events leading to tissue damage. It has been suggested that the ability of both aggregated γ-globulin and soluble antigen–antibody complexes to produce skin reactivity depends on the ability of the aggregated γ-globulin and the antibody portion of the complexes to fix to tissue (T. and K. Ishizaka, 1959). Recently, Biozzi *et al.* (1959) have shown that the ability of antibody to produce increased capillary permeability in guinea pigs injected intravenously with antigen also depends on the ability of the antibody to fix to tissue. This latter reaction could be blocked by a prior injection of normal γ-globulin into the skin.

The similarity of the amounts of antibody in complexes producing allergic type reactions and the amounts needed to produce passive reactions in which no latent period is involved also has been used as evidence that the complexes act as a stable unit and do not depend on a process of dissociation and reassociation. This evidence is of little significance, however, since a quantitative comparison cannot be made between the antibody involved in the complex reactions and the antibody involved in the immediate passive reactions. The complexes formed *in vivo* as a result of injecting antigen into sensitized animals are with little doubt of different composition and behavior than the complexes prepared *in vitro* where an equilibration takes place prior to injection. This difference is made more evident by the data of McKinnon *et al.* (1957), who showed that an injection into rabbits of washed antigen–antibody precipitates, prepared at equivalence, produced anaphylactic reactions, while an injection of antibody followed immediately by an equivalent amount of antigen produced no symptoms.

The rapidity with which acute anaphylactic reactions takes place following the injection of soluble antigen–antibody complexes and the low dissociation constants ascribed to complexes prepared with hyperimmune antibody, further suggest that the biological reactions produced by complexes are a result of changes produced by stable antigen–antibody units and do not depend on the process of dissociation and reassociation. Only a small amount of dissociation may be necessary, however, since the amounts of antibody involved in the complex reactions are much greater than the amounts needed to produce active or passive anaphylactic reactions. Also the rate of dissociation of *in vitro* prepared complexes may be greatly increased following their injection into animals or their addition to isolated tissue as a result of dilution.

B. The Role of the Process of Dissociation and Reassociation of Antigen–Antibody Complexes

There is also evidence which indicates that the biological activity of soluble antigen–antibody complexes is influenced and, in some instances, dependent on a process of dissociation and reassociation. It is possible that the events leading to the pathological changes produced by complexes are largely initiated during the reaction between antigen and antibody and not by the stable complexes. If this is the case, then the biological reactions produced by the injection of preformed complexes would require a dissociation and reassociation of the complexes involving an *in vivo* reaction between antigen and antibody.

It is evident from an accumulation of data that something besides the mere presence of soluble antigen–antibody complexes in the circulation or tissue baths plays an important role in the pathological changes produced by antigen–antibody complexes. Large amounts of anti-BSA (3–5 mg. antibody N), complexed with antigen, frequently produce little or no anaphylactic reactions when injected intravenously into guinea pigs (Weigle *et al.*, 1960), while an injection of as little as 0.03 mg. of anti-BSA N produces 100% fatal reactions in guinea pigs challenged with BSA 48 hours later. Similar evidence was given by Benacerraf and Kabat (1949), who demonstrated that large amounts of antibody were required to produce passive anaphylaxis in the guinea pig when the antigen was injected immediately after injection of the antibody. Also, the amount of antibody needed to produce increased capillary permeability in the skin of guinea pigs is considerably less if the antibody is given intradermally and followed 3–18 hours later by an intravenous injection of antigen than if the antibody is injected in the form of antigen–antibody complexes. Furthermore, lesions of the serum sickness type produced by infusion of rabbits with large amounts of soluble antigen–antibody complexes are both less severe and less consistently produced than similar lesions in active serum sickness. However, the difference in the quality of antibody used to prepare the soluble complexes and the antibody formed during active serum sickness in a primary response might account in part for the difference in the lesions produced. Nielsen *et al.* (1959) observed that the addition of only 0.002 mg. of antibody N to strips of guinea pig ileum was sufficient to render them sensitive to a subsequent addition of antigen. On the other hand, Trapani *et al.* (1958) showed that anaphylactic contraction of guinea pig ileum was achieved (at 63% maximum) with the addition of antigen–antibody complexes containing from 0.8 to 0.9

mg. antibody N. They noted that there was a lag of 30 to 90 seconds between the addition of the complexes and the contraction of the ileum. Since the amount of antibody employed by Trapani and associates was approximately 400 times the amount used by Nielsen and coworkers and since there was a lag period between the addition of complexes and contraction, it is not unreasonable to suggest that the process of dissociation and reassociation could have been responsible for the activity of the complexes.

The effect of a prior injection of antigen on the ability of soluble antigen–antibody complexes to produces acute anaphylaxis in the guinea pig suggests that the process of dissociation and reassociation plays a role in the biological reactions induced by these complexes (Weigle *et al.*, 1960). Guinea pigs given from 0.25 to 15 mg. BSA N, 4 to 5 minutes prior to the injection of soluble BSA–anti-BSA complexes, exhibited considerably more severe symptoms than guinea pigs injected with only the complexes (Table III). Similarly, Rosenberg *et al.* (1958) demonstrated that exceedingly small amounts of antigen–antibody complexes prepared with excess antigen could not themselves produce increased capillary permeability but could sensitize the skin so that an intravenous injection of antigen given 3 to 18 hr. later resulted in a marked reaction. On the other hand, Germuth and McKinnon (1957) and Tokuda and Weiser (1958b) failed to observe an enhancing effect of the injection of large amounts of BSA prior to the injection of soluble BSA–anti-BSA complexes on subsequent anaphylaxis. Also, Ishizaka *et al.* (1959a) failed to alter the ability of soluble complexes to produce increased capillary permeability in guinea pigs by a prior injection of antigen. While reasons for these differences in observations are not apparent, results demonstrating an enhancing effect are more significant than the negative results and might be explained on the basis of *in vivo* dissociation of the complexes followed by a reassociation of the antigen and antibody.

The ability of stable aggregates of human γ-globulin to produce increased capillary permeability but not acute anaphylaxis (Christian, 1960) also indicates that the process of dissociation and reassociation plays a role in acute anaphylaxis in the guinea pig. Complex-induced anaphylaxis in the guinea pig may depend on a dissociation of the antigen–antibody complexes followed by a diffusion of the free antigen and antibody into extravascular spaces. The reassociation of the antigen and antibody in the extravascular spaces may then initiate the events leading to anaphylaxis. The foregoing suggestion may explain; (1) why stable aggregates of γ-globulin do not produce acute anaphylaxis in the

guinea pig; (2) why much larger amounts of antibody in complex form are needed to produce anaphylaxis than amounts of free antibody injected 48 hours prior to an injection of antigen; (3) why a latent period is required in classic passive anaphylaxis; and (4) why the in-

TABLE III[a]

EFFECT OF A PRIOR INJECTION OF BSA ON THE ANAPHYLACTIC PROPERTIES OF SOLUBLE BSA–ANTI-BSA COMPLEXES

Expt. No.	Prior BSA N injection[b]	Antibody N in complexes[c]	Acute Anaphylaxis					Anaphylactic index[d]
			Neg.	Mild	Mod.	Severe	Fatal	
380	0	1.12	0	5	1	0	0	1.2
	2.5	1.12	0	2	0	0	3	2.8
383	0	0.94	4	0	0	0	0	0
	0.25	0.94	3	4	0	1	1	1.2
	2.50	0.94	3	2	2	0	1	1.2
	15.00	0.94	0	4	2	0	2	2.0
	0	1.87	6	2	0	0	0	0.2
	0.25	1.87	2	5	0	0	1	1.1
	2.50	1.87	2	3	1	0	2	1.6
	15.00	1.87	2	5	1	0	0	0.9
400	0	0.94	9	5	4	0	1	0.9
	2.50	0.94	2	8	3	0	4	1.8

[a] Reprinted from Weigle *et al. J. Immunol.* **85**, 469 (1960).

[b] Prior injection of BSA given intravenously 4–5 minutes before injection of complexes.

[c] Prepared at 3 times equivalence. Complexes in experiments 383 and 400 were incubated at 0–3°C. for 72 hours prior to injection, and complexes in experiment 380 were incubated for 36 hours

[d] $$\frac{(a \times 4) + (b \times 3) + (c \times 2) + (d \times 1) + (e \times 0)}{\text{total number of animals}} = \text{anaphylactic index}$$

where a = No. of animals with fatal symptoms; b = No. of animals with severe symptoms; c = No. of animals with moderate symptoms; d = No. of animals with mild symptoms; and e = No. of animals with no symptoms.

jection of antigen–antibody complexes can remove a large amount of complement from the intravascular fluid spaces of guinea pigs and yet produce mild symptoms or none at all.

C. CONCLUSION

It is obvious from the data discussed in the foregoing that it is impossible to evaluate the roles of either the complexes as a stable unit or the process of dissociation and reassociation in the initiation of the biological reactions produced by soluble antigen–antibody complexes. Since the target tissues and possibly the events involved vary from one reaction to another, it is possible that the manner in which

the complexes initiate these events also varies. The process of dissociation and reassociation may play a major role in some reactions and minor role or none at all in other reactions. Certainly the process of dissociation and reassociation could well play a role in complex-induced acute anaphylaxis in the guinea pig and in the more delayed reactions produced by complexes, i.e., lesions of serum sickness, cutaneous reactions of the Arthus type, delayed anaphylactic reactions in the guinea pig, and possibly anaphylactic reactions in mice. However, even in these reactions the process of dissociation and reassociation may be important only in allowing the antigen and antibody to diffuse as separate entities to the target sites; the pathological changes may be initiated by the stable complexes that result from reassociation in these sites.

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Delayed Hypersensitivity to Simple Protein Antigens

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I. Introduction

Before describing advances made in recent years in the analysis of "delayed hypersensitivity," it is necessary to define with some care exactly what we are talking about. We shall therefore use as a working definition of a pure "delayed reaction" *an immunologically specific inflammatory reaction, which takes some hours to reach a maximum, occurring in the absence of demonstrable antibody of the conventional type.* Other usual but not necessary characteristics of this state are: (a) that the reaction is erythematous and/or indurated (but it is pos-

sible that a reaction may be invisible macroscopically but still demonstrable histologically), (b) that it is transferable by cells and not transferable by serum (though Cole and Favour, 1955, claim to have transferred a form of delayed reactivity with a chemically separated fraction of serum), and (c) that it is reduced by short-term treatment with corticosteroids but unaffected by antihistamines. The phrase "some hours" is vague and so indeed is the phrase "reach a maximum"; in rabbits and guinea pigs, a fairly strong reaction without necrosis (which would lead to secondary inflammation) is at its most intense somewhere between 10 and 18 hours—exact data on this are apt to be inhibited by the normal limitations of the working day—and is appreciably less at 36 hours; in larger animals (man, bovines) the time scale is rather more extended.

The definition is designed to exclude (a) reactions which, though delayed, are nonspecific, such as the skin irritation produced by high concentrations of dinitrofluorobenzene, (b) reactions such as passive cutaneous anaphylaxis (PCA), which are inflammatory and in which no free antibody is demonstrable, being virtually all bound at the skin site, but which come up within 20 minutes at most, (c) Arthus reactions which are inflammatory and take some hours to reach a maximum macroscopically but in which antibodies are demonstrable in the circulation or in the serum used to sensitize in passive reactions, and (d) the Shwartzman reaction which is usually considered nonspecific, though Stetson (1955) has argued that delayed hypersensitivity may be involved. Finally, one must differentiate a pure delayed reaction from a so-called "combined" reaction occurring in animals in which the state of delayed reactivity coexists with that of active antibody production; the recognition of this condition and its certain differentiation from an Arthus reaction is never entirely satisfactory. It may, however, be imitated experimentally (R. T. McCluskey, unpublished observations): if a group of guinea pigs with active "pure" delayed hypersensitivity to bovine seralbumin (BSA) are given a dose of anti-BSA equivalent to about 1 mg. antibody nitrogen per kg., the 18-hour reaction to 20 μg. BSA is rather more intense than the moderate passive Arthus reaction resulting in normal pigs from this dose of antibody alone. It takes at this time the general appearance of a delayed reaction macroscopically but, if observed during the first 4 hours of its development, it shows then the increasing edema characteristic of an antibody-dependent reaction.

The 18-hour macroscopic appearance of a typical pure Arthus reaction and of a typical pure delayed reaction are indeed very different; the Arthus reaction is edematous and frequently hemorrhagic in the center with ill-defined margins, whereas the delayed reaction is indurated

and erythematous with well-defined margins. However, these criteria, as well as being somewhat subjective, are not constant, the appearance of the delayed reaction varying considerably with the antigen used to elicit it.

It should be clear from the foregoing discussion that, whatever hypersensitivity is, it is not simply the result of the presence of *very small* amounts of antibody, insufficient to provoke an Arthus reaction (cf. Benacerraf and Kabat, 1950), since passive administration of less and less antibody leads to progressively milder Arthus reactions, taking less and less time to reach a maximum, until eventually no reaction at all can be observed.

II. Early Studies on Delayed Hypersensitivity

A. Bacterial Hypersensitivity

It has long been established (Rich, 1944) that the proteins of the tubercle bacillus, whether purified (Seibert *et al.*, 1934) or obtained crude from the medium in which they have grown, "tuberculin," are able to elicit delayed sensitivity reactions in tuberculous animals or in animals immunized with whole killed mycobacteria. It should be kept in mind in relation to what is known today of delayed hypersensitivity to native proteins and denatured proteins, to be discussed in another section, that the methods of preparation and extraction of tuberculin and purified protein derivative (P.P.D.) are known to denature proteins extensively. Therefore, all tuberculin tests are performed with denatured antigens, which may be different from those existing in the native state in the mycobacterium and in the infected animal. Attempts to provoke delayed tuberculin sensitivity by immunization with these tuberculoproteins alone had consistently failed; serum antibodies and immediate anaphylactic sensitivity resulted from such treatment. This problem was investigated again recently by Boyden (1957) in a comparative study of the immune response to tuberculoprotein or to live or killed tubercle bacilli. He observed that delayed reaction to tuberculoproteins occurred only when the whole bacillus was used for immunization. It should be stressed that, in addition to delayed sensitivity, the animals also showed a small titer of antituberculoprotein antibodies in the serum, detected by the sensitive tanned cell agglutination technique. If tuberculoprotein was used for immunization, either in saline or oil emulsion, only immediate wheal-like reactions could be elicited. It should be noted that the animals were tested on the forty-second day postimmunization, which is very late if the state of delayed sensitivity was an early and transient

one, as would be expected from other data on immunization with pure protein (to be discussed in Section III, C).

For some time, delayed sensitivity was a mode of reactivity thought to be characteristic of the proteins of the tubercle bacillus, but it became clear that many infections other than tuberculosis give rise to delayed hypersensitivity to protein components (Zinsser, 1928; Chase, 1952), among them brucellosis, typhoid, syphilis (Rich *et al.*, 1933), streptococcal infections (Swift and Derick, 1929), virus infections, and the various mycoses.

The relationship between bacterial hypersensitivity and the reactions to the lipopolysaccharide of Gram-negative bacteria is still not well understood. Stetson (1959) has pointed out many points of resemblance between endotoxin reactions and delayed hypersensitivity. The skin reaction to an intradermal injection of endotoxin is like a tuberculin reaction in timing and in gross and histologic appearance. The systemic reactions of animals hypersensitive to tuberculin appear to be identical with endotoxin shock, except for the difference in dosage. Animals with delayed sensitivity to protein antigens show fever reactions upon intravenous injection with the specific antigen, similar though not identical to what is observed with small doses of endotoxin (Uhr and Brandriss, 1958). Acquired endotoxin tolerance protects tuberculin-sensitive animals against tuberculin shock (Stetson *et al.*, 1958). Just as skin sites injected with endotoxin are prepared for the Shwartzman reaction, sites undergoing a delayed hypersensitivity reaction whether to tuberculin, to protein antigens, or to contact with simple chemicals (Gell and Benacerraf, unpublished observations) also undergo hemorrhagic necrosis upon intravenous injection of endotoxin.

In an evaluation of the meaning of these similarities, two facts should be considered: (a) preparations of old tuberculin have been shown to possess biological activities of endotoxin (Stetson *et al.*, 1958) and (b) animals infected with tubercule bacilli (Bordet, 1936; Suter *et al.*, 1958; Halpern *et al.*, 1958) or having developed visceral inflammatory granulomatous lesions due to zymosan (Benacerraf *et al.*, 1959) or to talcum show a highly enhanced sensitivity to endotoxins for as yet unexplained reasons.

B. Hypersensitivity to Proteins and the Effect of Adjuvants

The observations of Dienes (1930, 1936) showed that delayed hypersensitivity reactions were not an immune response limited to bacterial protein antigens or infectious processes, but could be observed in the course of immunization with protein antigens in animal species which

are best capable of exhibiting this type of immune reactivity, guinea pig and man. Guinea pigs injected with a few milligrams of egg white or horse serum showed, on the fourth and fifth days, skin reactions to these antigens similar in appearance and timing to slight tuberculin reactions, before antibody could be detected; later, antibody-dependent reactions, anaphylaxis and Arthus reactions could be observed. Dienes stressed that delayed sensitivity reactions preceded other immune responses. The reaction was far stronger in tuberculous guinea pigs or if the antigen was injected in tuberculous lesions. These observations were confirmed and extended by Freund and MacDermott (1942) and by Raffel (1948) who observed delayed sensitivity to proteins in guinea pigs if "complete" adjuvants or wax from the tubercle bacillus were injected with the antigen.

While these early observations establish that delayed hypersensitivity can be an immune response to protein antigens, the fact that antibodies were soon produced also in large amounts made the study of these reactions and their differentiation from Arthus reactions difficult. In the last few years, techniques have been developed to dissociate in protein antigens the capacities to induce delayed sensitivity and serum antibody production. These techniques which allowed these two properties of protein antigens to be studied independently are discussed in Section III.

C. Contact Sensitivity

Application to the skin or injection with a few micrograms of any of a large number of low-molecular-weight chemical substances capable of reacting stably with the free amino groups or the sulfhydryl group of proteins sensitize guinea pigs in about a week, so that the application to the skin of the same substance produces an inflammatory response with all the characteristic features of delayed sensitivity, both in morphological and in time sequence. Such "contact reactions" to simple chemicals as picryl chloride or 2,4-dinitrofluorobenzene are indistinguishable from tuberculin tests. Extensive studies have been made by Eisen (1959) of the reaction of these chemicals with skin proteins. Using dinitrochlorobenzene labeled with C^{14}, the fate of the chemical applied to the skin was investigated: 20% is excreted with urine, 70–80% is disseminated in noncutaneous structures, 5% remains in the skin. The amount remaining in the skin was located almost exclusively in the epidermis, half of it being accounted for as *N* 2,4-dinitrophenol (DNP) lysine. Besides contact sensitivity, animals treated with sufficient doses of these chemical reactants often develop antibodies reacting with the same hapten conjugated with a carrier protein. Such antibodies are irrelevant to the

contact reaction. They cannot transfer contact sensitivity and do not seem to affect it. The relationships of contact sensitivity and of delayed sensitivity to antibody production are further discussed in Section III.

III. Methods of Producing Pure Delayed Hypersensitivity to Simple Proteins in Guinea Pigs

Recent workers have concentrated on producing a state of pure delayed hypersensitivity demonstrable at a time before antibodies appear, or at least before they can be shown by the most sensitive techniques available. For work of this sort, the guinea pig is far the most suitable animal for two reasons: first because in this animal the dissociation between the two modes of response can be readily achieved, and second because it is easy to show in it the presence of very small amounts of antibody by tests for systemic or local anaphylaxis. Hence, in the discussion which follows, this is the experimental animal referred to throughout unless the contrary is stated.

A. Antigen–Antibody Complexes

The first reliable method for the demonstration of pure delayed hypersensitivity to protein antigens in guinea pigs was published by Uhr, Salvin, and Pappenheimer (1957). They showed that if small amounts of antigen–antibody complexes precipitated in antibody excess and incorporated in adjuvants were injected into guinea pigs, the animals showed typical delayed reactivity, between 5 and about 12 days, to the antigen component: rigid tests for the presence of antibody were negative. Complexes precipitated at equivalence or in antigen excess did not produce this effect. This method tends to be unreliable if nonavid antibodies are used to make the complexes. Scharff, Uhr, and Pappenheimer (1957) and Sell and Weigle (1959) have shown that animals sensitized by this technique will show immediate "immune elimination" of intravenously injected antigen and are, therefore, highly primed for antibody production.

Uhr *et al.* tended at first to attribute to these results a basic significance in the immune process, suggesting that the essential way in which delayed hypersensitivity is produced is via the complexing of traces of antigen with very small amounts of antibody as it is formed. It is not legitimate to argue against this concept that delayed hypersensitivity is demonstrable before the antibody is, since this is a function of the sensitivity of the respective tests. An alternative explanation, that the antibody merely blocks or interferes with the salient antibody-stimulating

and combining sites so that they are unavailable at the crucial stage for "recognition" within the antibody-producing cell, is perhaps more consistent with the work of Benacerraf and Gell (1959a) quoted in the following section. They found that the substitution of chemical haptenic dominant sites, themselves potently antibody-stimulating, was another good method for producing a pure delayed hypersensitivity to the carrier protein.

Raffel and Newell (1958) have argued that the results of Uhr *et al.* represent a type of biological reactivity different from classic delayed or tuberculin type hypersensitivity, chiefly on the ground of "its fleeting appearance limited to the few days following the antigenic stimulus." It is impossible to compare the data given in their paper directly with those of Uhr *et al.* since the large test dose antigen used throughout (0.5–1.0 mg. ovalbumin) would be expected to desensitize most animals sensitized to ovalbumin with complexes, judging from the data given by Uhr and Pappenheimer (1958); it is difficult not to conclude that at least some of the reactivity exhibited was to contaminating proteins present in a small amount. Nevertheless, their arguments must be seriously considered, especially their proposal to separate hypersensitivity of the sort demonstrated by Uhr *et al.* from "classic" delayed hypersensitivity (by which is meant tuberculin and presumably other bacterial and parasitic delayed reactivities) under the title of "Jones-Mote sensitivity" (Jones and Mote, 1934).

Here it might be as well, in dealing with objections based on gross morphological appearance, to re-emphasize that the exact macroscopic characteristics of the delayed reaction, and still less the histological appearance, do not constitute a satisfactory criterion for defining a type of reactivity, since in the experience of the writers this is largely dependent on the antigen used. Thus, delayed hypersensitivity reactions vary from small but intensely indurated and often necrotic reactions given to salmine, through larger, well-defined, and indurated reactions given by most hapten conjugates and by γ-globulins, to widespread, not appreciably indurated, and not even very erythematous reactions given by some animals to ovalbumins and some seralbumins. It is reasonable to suppose that these differences depend on the holding power of the tissues for the particular proteins used, though we have no direct evidence for this. The other point on which, as has been said, Raffel and Newell placed the main emphasis, is the transient nature of the reactivity as compared with tuberculin sensitivity. The transience does not, however, appear to be so evident in the data presented by Uhr *et al.* in their various publications, and still less so in the observations concerning delayed hypersen-

sitivity to the protein component of conjugates (see Section III, B). In the latter, after efficient initial sensitization, delayed hypersensitivity lasts at least for 6 to 8 weeks at high level and probably much longer. Moreover, there is no evidence that very small doses of bacterial sensitizers also would not produce an equally transient reactivity, particularly if the source of antigen were completely eliminated. Thus we do not feel that hypersensitivity to simple proteins need be separated from classic delayed hypersensitivity but, on the contrary, that it constitutes a very much cleaner system by means of which the mechanism of delayed hypersensitivity may be elucidated.

B. The Use of Conjugates with Chemical Haptens and Altered Proteins

Benacerraf and Gell (1959a) showed that if conjugates of proteins with simple chemical groups were used as antigens in small amounts in complete adjuvant (e.g., 0.1–1.0 μg. of picrylated proteins) the animals rapidly developed antibody reacting with the haptenic group, but if tested with the "carrier" protein, alone or carrying another noncross-reacting hapten, they showed only delayed hypersensitivity without any evidence of antibody production, as judged by tests for active anaphylaxis or PCA. These tests were done from 5 to 16 days or more after the primary immunization. With some antigens the state of pure delayed hypersensitivity could be shown to persist for 6 to 8 weeks; with strongly antigenic carriers such as ovalbumin, some animals of a group tended to produce antibody to the protein by the third week, whereas with very weak antigens, such as gelatin, pure delayed reactivity was shown to persist at high level for 17 weeks in spite of intermediate testing. With the use of a suitable protein carrier [such as bovine γ-globulin (BGG)] and a sufficient coverage with hapten (about 40 groups in the picryl-BGG conjugate used in this work; see Benacerraf and Gell, 1959b), a satisfactory method for the durable dissociation of delayed hypersensitivity from antibody production could be demonstrated.

Why should the presence on the antigen molecule of a number of groups of a highly foreign and strongly antibody-stimulating hapten suppress to so great an extent the antibody-stimulating potency of a protein, while leaving its power to stimulate delayed hypersensitivity unimpaired? Presumably most of the potential original combining sites on the antigen contain a chemical haptenic group, leaving insufficient sites uncovered to supply an adequate stimulus for antibody production. There is reason to suppose that, in rabbits at least, the mechanisms of both delayed hypersensitivity and of antibody production are simul-

taneously activated during normal immunization (Gell and Hinde, 1954; Tremaine and Jeter, 1955). In the case of chemical conjugates, the former mechanism seems not to be interfered with by the presence of the hapten, possibly because in this case a much larger area of the molecule is involved in the specificity. This point is further discussed in the following paragraph.

The same authors have found that denatured proteins and, in large (100 μg.) doses, protein fragments, such as gelatin (Gell and Benacerraf, 1959) and even low-molecular-weight polypeptides such as salmine (unpublished observations), also produce pure delayed reactivity. Very weak antigens, such as γ-globulin allotypes in adjuvants, can induce in the guinea pig typical delayed sensitivity in the absence of detectable serum antibodies (Benacerraf and Gell, 1961). Delayed hypersensitivity can also be used to demonstrate immunological cross reactivity. Human and bovine serum albumins, which are known to cross-react when tested with hyperimmune rabbit antisera, each elicit delayed reactivity only when tested in animals immunized with the other antigen (Gell and Benacerraf, 1959).

Salvin and Smith (1960) have made an extensive study of cross reactivity, using avian albumins and a number of picryl and DNP conjugates, with results which are essentially consonant with the foregoing. The significance of these studies of cross-reacting antigens is discussed in Sections IV, A, B and VII.

C. The Use of Small Doses and Early Testing

Salvin (1958) showed that the use of very small doses of antigen and/or early testing also produced a situation where delayed hypersensitivity to protein antigens was demonstrable in the absence of antibody. The pure delayed state persisted even here for 2 or 3 days at least and there is no reason to suppose that the delayed reactions demonstrated by Salvin were any different from those produced by other techniques.

D. Hypersensitivity to Simple Chemicals and Pure Delayed Hypersensitivity to Simple Haptenic Groups

Contact sensitivity to simple chemicals does not fall strictly within the terms of reference of this discussion. Nevertheless, so much light has been thrown upon the mechanism of delayed hypersensitivity to proteins by work along these lines, that it is desirable to include some discussion of the relationship between hypersensitivity to simple chemicals and specific delayed hypersensitivity to the haptenic group of conjugates.

Eisen (1959) has, in a recent review, given a most lucid discussion of

his own and other work in this field, pointing out the apparent contradiction between the facts that contact sensitivity to simple chemicals shows a very great operational similarity to delayed hypersensitivity, but that nevertheless it has not been possible, except in a few instances, to demonstrate contact sensitivity in animals immunized by any method with protein conjugates carefully freed of unreacted chemical sensitizer; this is despite the fact that these conjugates react readily with the antibodies which often appear in the circulation of animals sensitized to contact by means of the simple chemical. The main exception to this is the work of Landsteiner and Chase (1941) who showed that picrylated red-cell stromata and, in rare cases, in low degree, other picrylated proteins could stimulate contact sensitivity. Chase (1959), however, in a review in the same volume containing much new material, agrees that picryl chicken albumin, at least, is unable to provoke contact sensitivity to picryl chloride, though it readily produces picryl-specific antibodies.

The work of Benacerraf and Gell (1959b and Gell and Benacerraf, 1961) serves in some measure to illuminate this problem. They found, in agreement with previous workers, that picrylated foreign proteins, such as picryl BGG, were very inefficient in producing the state either of contact sensitivity or of pure picryl-specific intradermal delayed hypersensitivity, though the latter could be detected around the sixth day if large doses were used both for immunization and testing. Animals sensitized with picryl chloride also showed regular but weak delayed hypersensitivity to the injection of 200 μg. (a large dose) of picryl BGG. However sensitization of guinea pigs with conjugates in which the carrier protein is homologous and of very low antigenicity—in particular with conjugates of homologous albumin (in 10-μg. doses or less)—has been found to give quite strong and reliable contact reactivity to the hapten together with strong delayed reactivity to the homologous albumin conjugate which was used to sensitize the animals.

In all these studies, however, the animals sensitized with the simple chemical always showed a relatively greater degree of contact sensitivity than of delayed anticonjugate hypersensitivity even to picryl guinea pig albumin, while the reverse was true of animals sensitized with the homologous albumin conjugate. The authors interpreted these results as indicating that the simple chemical combines *in vivo* with a number of homologous proteins, only one of which is seralbumin; the reactivity to intradermal injection of conjugate produced under these circumstances has a high degree of "carrier specificity" (see Section IV, C). Hence, since only one of the available kinds of cellular antibodies is activated by picryl guinea pig albumin, the response is comparatively weak. This

concept is essentially similar to that suggested by Eisen (1959). It was confirmed by experiments on desensitization, in which animals sensitized by means of picryl guinea pig albumin conjugates can be desensitized to picryl chloride contact by intravenous injection of the conjugates, while those sensitized by means of the simple chemical cannot. This experiment, together with quantitative measures of the relative amounts of hapten combined with protein and of the simple compound itself needed for minimal contact sensitization (which are found to be approximately equivalent in amount), render untenable for the picryl system the suggestion that traces of unreacted chemical may be responsible for the contact sensitivity found in guinea pigs sensitized with homologous conjugates.

These results can be duplicated in systems using chlorobenzoyl chlorides as sensitizers. But, in agreement with Eisen, they cannot be duplicated in systems using fluoro- and chlorodinitrobenzene; with these systems, only the simple chemicals produce contact sensitivity. This abnormal behavior is difficult to explain, but possibly it may be due to a special type of chemical reactivity of such compounds or to the fact that these chemicals are very irritant and can only be applied in low concentration to the skin.

These results are relevant to the question of delayed hypersensitivity to simple proteins, as they emphasize the importance of "carrier specificity" in delayed sensitivity. They also indicate that the immune mechanism cannot readily be induced to exhibit delayed reactivity to a small antigenic determinant only the size of one picryl (trinitrophenyl) group. The fact that large doses of foreign conjugates were needed to demonstrate such delayed reactivity (about 200 μg.) suggests that there may have been in those cases only a few sites on the proteins where the amino acid pattern was sufficiently similar to that on a homologous protein for a cross reaction to occur when that site carried a haptenic group.

IV. Nature of the Antigenic Specificity

A. Cross Reactivity

There is a fair amount of data in the literature on the cross reactivity, as far as antibody production in rabbits is concerned, between related proteins in different species, in particular the seralbumins and ovalbumins. This cross reactivity is observed in the field of delayed hypersensitivity also. Gell and Benacerraf (1959) showed that horse and bovine serum albumins could cross-sensitize to one another; with an immunizing dose of 10 μg., guinea pigs tested on the ninth day showed

delayed reactivity only to the cross-reacting antigen, but were anaphylactic if tested with the homologous antigen. In more extensive studies, Salvin and Smith (1960) used avian egg albumins in 5-μg. doses and also found cross reactions among hen, duck, and goose proteins. The persistence of pure delayed reactivity to the cross-reacting antigen is not as striking in their studies, possibly because hen ovalbumin is an extremely powerful antigen, whereas BSA is rather poorly antigenic in the guinea pig. As little as 0.1 μg. of ovalbumin in complete adjuvant is sufficient to elicit antibodies by the twelfth day, while 0.004 μg. can provoke delayed reactivity (Gell and Benacerraf 1959).

B. The Effect of Denaturation

Since the time when tuberculin supplied the *locus classicus* of a delayed sensitivity antigen, it has been evident that a somewhat degraded protein fragment can function at least to elicit a delayed hypersensitivity reaction, though it was then considered that it could not induce the delayed reactivity state. More recent work has shown that heat-denatured proteins, protein fragments such as gelatin, and even quite low-molecular-weight polypeptides such as salmine (Benacerraf and Gell, 1959a and unpublished observations) can readily provoke delayed reactivity in guinea pigs. In most of these cases, sufficient antibody to cause the anaphylactic state eventually developed, but there is no doubt that the ability of these denatured antigens to give rise to antibodies reacting with the native protein is extremely poor. Nevertheless, in the case of heat-denatured ovalbumin at least, the ability to provoke delayed reactivity was as great quantitatively as that of the native protein, and, whichever antigen was used to sensitize the animal, the delayed reactions were rather better with the native than with the denatured protein. Thus, it would appear that disorganization of the protein tertiary structure, which impairs or alters its ability to provoke antibodies, does not affect the ability to provoke delayed hypersensitivity. It could be argued from this that quite different groupings on the molecule are responsible for provoking the two kinds of immune response. Against this theory, which implies a dichotomy between the two modes of response from the beginning of the immune response, is the evidence of the close correlation between the intensity of delayed hypersensitivity and the subsequent production of antibodies after restimulation and other evidence on the secondary response (Section VII). These differences may, therefore, merely illustrate the greater sensitivity in the guinea pig of delayed hypersensitivity over the antibody response. Moreover, even gelatin has some ability to produce antibodies, while foreign haptenic groups, such as the picryl group,

even on fully denatured proteins can be potent antibody producers. It is, therefore, not a *sine qua non* condition of antibody production that the antigen should retain its folded structure.

C. Carrier Specificity

A rather more subtle distinction has, however, recently been tentatively demonstrated between the kind of antigenic structure capable of provoking antibody production and that capable of provoking delayed hypersensitivity, arising out of work with conjugates with simple proteins. It is clear from recent work that a conjugate of, for example, ovalbumin with picryl chloride functions antigenically in two independent ways; the picryl component tends to provoke picryl-specific antibodies with only a very transient phase, if any, of delayed reactivity specific to picryl and the protein carrier produces long lasting delayed reactivity, as described in Section III, B. Benacerraf and Gell (1959b) showed further that reducing the antigenicity of the protein carrier tended to prolong the phase of hapten-specific-delayed hypersensitivity. [To demonstrate delayed sensitivity to the hapten, it is necessary to test with a conjugate with a different noncross-reacting protein carrier, in large doses (200 μg.)]

More recent work (Gell and Benacerraf, 1961) has extended these observations. If homologous serum albumin is coupled with a chemical hapten of moderate molecular size, such as the picryl or *p*-chlorobenzoyl group, the tendency to produce antibodies against the hapten is much reduced. The animals, however, exhibit clear-cut pure delayed reactivity against small doses of sensitizing antigen, a lesser reactivity against a conjugate made with a related cross-reacting protein, such as bovine seralbumin, but none against comparable doses of a conjugate made with a noncross-reacting protein, such as ovalbumin; nor, as one would expect, do the animals react to homologous albumin, either directly or when it is conjugated with a totally different chemical hapten (a rather pure albumin needs to be used to avoid the possibility of allotypic sensitization to homologous globulins). In spite of this marked "carrier specificity" of the delayed reaction, when antibodies are eventually produced their cross-reactivity is very much greater, the antibodies reacting with any protein made with the appropriate conjugate—a point also emphasized by Salvin and Smith (1960).

No strictly quantitative work in this area has yet been published; but one may perhaps be permitted to theorize from rather preliminary data of this sort. It would appear that for a delayed reaction to be demonstrable in this type of situation, the environment of the hapten group

(attached to free terminal —NH_2, the ε-NH_2 groups of lysine, and possibly to any available —SH groups), extending probably over several amino acid residues in the chain, must be identical with that of the sensitizer; while an antibody may react just with the haptenic group, or at most with the hapten —NH_2 lysine complex, though, as Landsteiner (1945) showed by using inhibition methods, the binding may be influenced to some extent by the nearer amino acids in the chain. The high carrier specificity of the delayed reaction may, therefore, depend on a larger "area of recognition" on the antigen molecule, while the area of recognition of the antibody is focused onto a smaller area, presumably because greater binding forces are involved. The change-over from a state of delayed hypersensitivity to that of antibody production can then be conceived as part of a single process, but has to be correlated with an increase and focusing of the binding forces between antigen and a hypothetical intracellular antibody on the one hand and the extracellular antibody on the other.

This concept, suggested by Benacerraf and Gell (1959a) has been elaborated on the basis of extensive further data by Salvin and Smith (1960). The relevance of this approach, together with that of results on anamnestic or secondary responses to the components of conjugates presented by the latter authors, to the question of the biological interrelationships of delayed and antibody-dependent hypersensitivities are discussed in more detail in Section VII.

V. Characteristics of the State of Delayed Hypersensitivity

A. Histology and Physiopathology

A study of the histopathology of the delayed reaction has been recently made by Gell (1958). The most prominent appearance is a perivascular (and perineural) infiltration with mononuclear cells, including hypertrophied adventitial cells; there are very few if any polymorphonuclear cells. These islands of mononuclear cells occur especially in the loose areolar tissue, though very characteristic and clear-cut islands may surround the rather less frequent bundles of vessels in the dense dermis. Within the dilated vessels there are sometimes large numbers of polymorphonuclear cells. The nature of the infiltrate depends on the intensity of the reaction, fairly well-marked polymorphonuclear infiltration often occurring in the center of intense lesions (presumably owing to incipient necrosis), although at the periphery of the lesion mononuclears predominate.

Recent experiments by Voisin (1960) demonstrated that changes in

capillary permeability to serum proteins take place during the delayed hypersensitivity reaction. The site of a very mild tuberculin reaction shows accumulation of Evans blue dye beginning 10 to 12 hours after testing. These permeability changes are different from those observed during PCA. The intensity of accumulation of the dye is not as marked; it takes place over a longer period of time and it is not due to the release of histamine, as it is completely unaffected by antihistaminic drugs. Using the techniques of immunization described in Section III, identical changes were observed in delayed reactions caused by other antigens besides tuberculin, such as ovalbumin and bovine serum albumin. These experiments further show that the delayed sensitivity reaction has a different pathogenesis from the antibody-dependent local anaphylactic reaction.

B. Passive Transfer

It is a characteristic feature of delayed hypersensitivity that it cannot be transferred by serum but only by cells of the sensitized animal. Landsteiner and Chase (1942) were successful in transferring contact sensitivity in guinea pigs with peritoneal exudate cells. Three years later, Chase (1945) was able to transfer tuberculin sensitivity in guinea pigs also with peritoneal exudate cells. Since these early experiments, numerous studies were made in the guinea pig. Metaxas and Metaxas-Buehler (1955) showed that delayed sensitivity to tuberculin is transferred by the sensitized cells injected intravenously without a latent period and that the severity of the reactions decreases with the number of skin sites tested. These observations suggest that it is the transferred cells that participate in the reaction in this species. No definite proof, however, has been obtained. Delayed sensitivity to protein antigens induced by antigen–antibody complexes or by small amounts of antigen have also been transferred passively in the guinea pig. Lymph nodes cells could be used in these transfers.

The most important advance in the transfer of delayed sensitivity in man stems from the work of Lawrence. This investigator was able to transfer passively in man delayed hypersensitivity to tuberculin (1949), to streptococcal proteins (1952), and also to protein antigens (such as diphtheria toxoid (Lawrence and Pappenheimer, 1956) by blood leucocytes from sensitized human subjects. Transfer of delayed sensitivity to viral antigens (Warwick *et al.* 1956) and to contact chemicals (Epstein and Kligman, 1957) was also achieved in man by these techniques. In the initial studies, care was taken in the preservation of the integrity and viability of the leucocytes used for transfer, because it was known that in guinea pigs freezing and thawing abolished the capacity to transfer sensitivity.

In an attempt to identify the factors ("transfer factor") in sensitive human leucocytes responsible for the transfer of delayed sensitivity, Lawrence performed experiments with leucocyte extracts, which are described in two review articles (1956, 1959). Various treatments of the leucocytes (lysing by distilled water, freezing and thawing) [treatment of the extracts with deoxyribonuclease (DNase) or ribonuclease (RNase) or trypsin] were attempted and all failed to abolish or diminish their capacity to transfer sensitivity. The result of the treatment with DNase was verified by the Feulgen stain. Further evidence of the existence of a transfer factor in man relates to the fact that many recipients have remained sensitive for 1 or 2 years after the transfer. It is possible that the difference between man and the guinea pig who requires live cells for transfer results from the fact that man is the species which shows the highest degree of delayed sensitivity, as the dose of leucocytes required to sensitize man passively is much smaller than for the guinea pig or the rabbit. Lawrence (1959) observed also with the tuberculin system in man that exposure of sensitized leucocytes to P.P.D. *in vitro* allowed the "transfer factor" to pass in the cell-free supernatant, which then could transfer tuberculin sensitivity. The cells themselves lost the ability to transfer sensitivity, but did not show morphological evidence of any significant damage. Sensitivity to coccidioidin was also transferred in man with DNase-treated leucocyte extracts (Rapaport *et al.*, 1960). This system was particularly important since the sensitivity to coccidioidin was transferred by leucocytes from residents of an area endemic for *Coccidioides immitis* to individuals who were lifelong residents of an area free of the organism and therefore had no possible previous contact with the antigen.

Among the forms of delayed hypersensitivity transferred in man by DNase-treated leucocyte extracts, the transfer of skin homograft sensitivity should also be mentioned. A dosage of 4 successive grafts is required to sensitize donor leucocytes to ensure a systemic transfer of delayed hypersensitivity (Lawrence *et al.*, 1960).

The chemical nature of the human transfer factor is a matter for speculation. It is certainly not an antibody of the conventional type.

C. Effects of Irradiation

Uhr and Scharff (1959, 1960) and Salvin and Smith (1959) have studied the differential effect of X-irradiation on delayed hypersensitivity and antibody production to protein antigens. Both groups have shown that a dose of irradiation, which will very much delay or suppress antibody production, will leave the capacity to develop delayed reactivity

unimpaired. Salvin and Smith found that, in guinea pigs sensitized with 1 Lf of diphtheria toxoid in adjuvant without mycobacteria, 300 r whole-body radiation before sensitization caused the period of delayed hypersensitivity (normally 12–14 days with this dose) to be extended to 19–21 days, when antibody began to appear. When the same dose of irradiation was administered 18 hours after sensitization, both delayed hypersensitivity and antibody production appeared at much the same time as in controls. They also noted that a considerable decrease in circulating lymphocytes could occur in X-rayed animals without markedly influencing the delayed reaction; when the number fell to 100–200 leucocytes per cubic millimeter of blood, no skin response of any kind was elicitable. This interesting observation is clearly relevant to the question of the cell responsible for delayed hypersensitivity. Uhr and Scharff, though in the main their results are similar, found that doses of 200–300 r did not completely suppress antibody formation in the early stages, as judged by the very sensitive active cutaneous anaphylaxis test, and had a slightly suppressive effect on delayed reactivity; but they consider that these differences are mainly owing to variations of the experimental setup. The essential contrast between the radioresistance of delayed hypersensitivity and the radiosensitivity of the primary antibody response certainly holds.

D. The Systemic Reaction

Guinea pigs infected with tubercle bacillus, whether virulent or BCG, show a typical shock syndrome when injected intravenously with old tuberculin or tuberculoprotein. This condition, indistinguishable from endotoxin shock, has been known as tuberculin shock and was accepted as being the systemic manifestation of delayed sensitivity corresponding to the local tuberculin reaction. However, guinea pigs with strong delayed hypersensitivity to simple proteins do not have such a shock reaction but, as Uhr and Brandriss (1958) have demonstrated, the intravenous injection of antigen is followed by a fever reaction of 2° to 3° F. which is specific for the antigen to which the animals are sensitized. This hyperthermia is markedly decreased by specific desensitization, but delayed in appearance by endotoxin tolerance. If small amounts of specific antibody are present (5 μg. or more of antibody nitrogen), the animals show hypothermia rather than fever when injected with the antigen.

The question arises whether the systemic tuberculin shock and the fever reaction to protein antigens, to which hypersensitivity has been induced, are two stages of the same process of different severity. This would mean that simple protein-delayed sensitivity induced by the methods described in the foregoing never reaches the intensity observed

in tuberculin sensitivity which causes lethal shock. In favor of this interpretation, we may cite the observation that early in the course of delayed hypersensitivity in the guinea pig, when skin reactivity is already present, tuberculin shock could not be elicited, and also the observations of Johanovský (1959a) who reported that incubation *in vitro* of washed leucocytes, spleen cells, and lymphocytes from hypersensitive rabbits with diluted tuberculin led to the appearance of substances with a pyrogenic effect on normal rabbits. Serum collected from rabbits during systemic tuberculin reactions also had a pyrogenic effect on normal nonsensitized rabbits (Johanovský, 1959b).

The other possibility remains that the fever reaction is the only specific manifestation of systemic-delayed hypersensitivity and that the lethal shock observed with tuberculoprotein in tuberculin-sensitive animals is, in fact, endotoxin shock, resulting from the hypersensitivity of such animals to endotoxin and the small amount of endotoxin activity present in tuberculoprotein.

The data available do not allow, as yet, a decision between these two interpretations, which depend also, to some extent, on the problem discussed in the foregoing paragraphs.

E. Desensitization

Desensitization of delayed sensitive animals has been extensively studied in relation to tuberculin hypersensitivity in order to ascertain whether hypersensitivity to tuberculoprotein was in any way associated with immunity to tuberculous infection. The weight of evidence, as reported by Rich (1944), showed that immunized hypersensitive animals can by appropriate treatment with tuberculin be desensitized, so that they no longer react to tuberculin locally or systemically. Such animals, when infected in the anterior chamber of the eye with virulent tubercle bacilli, remained as resistant to the proliferation and invasion of the mycobacteria as were the immunized hypersensitive ones.

A detailed study of specific desensitization of guinea pigs sensitized to protein antigens was carried out by Uhr and Pappenheimer (1958). These investigators showed that animals with delayed sensitivity to protein antigens could be desensitized by a single injection of the specific antigen in proper amount (about 1 mg.). The reappearance of skin reactivity required several days. If, however, 5 hours had elapsed after the skin injection, before the desensitizing injection, complete desensitization was not achieved. If animals were sensitized to two antigens, specific desensitization to one decreased but did not abolish the sensitivity to the other. This nonspecific desensitization was found to last

only 24 hours. The mechanism of desensitization is not clearly understood, although it is evident that it must involve an effect on the sensitized cells themselves, either by destroying them or by combining with specific sites on these cells and thus neutralizing them.

Inhibition of contact sensitivity can be obtained in the guinea pig, as demonstrated by Chase (1946), by prior feeding of the chemical sensitizer.

F. Agammaglobulinemia, Hodgkin's Disease

Extensive studies by Good and co-workers (1959) on human agammaglobulinemic patients (Good and Zak, 1956) have shown that, while these patients are unable to form significant amounts of γ-globulins, delayed sensitivity to protein antigens and to contact with chemicals can be induced. Such delayed sensitivity can also be passively transferred to agammaglobulinemic patients.

The loss of delayed sensitivity to tuberculin has been reported during measles and also in terminal infections. Recently, however, it was also found that patients with Hodgkin's disease showed a high incidence of energy to bacterial antigens to which the vast majority of the population shows delayed sensitivity. Delayed hypersensitivity to simple proteins cannot be induced in patients with Hodgkin's disease, while their ability to form serum antibodies does not seem to be affected. Furthermore, delayed hypersensitivity cannot be passively transferred to patients with Hodgkin's disease contrary to what is observed in the case of control patients (Kelly *et al.*, 1960).

G. Status of the Homograft Reaction

Apart from the fact that excellent recent reviews are available on the immune status of the homograft reaction (Brent, Brown, and Medawar, 1959), its relevance to the present discussion is equivocal for two reasons; first because there is still some controversy as to the part played in it by antibody, if any, and second because the antigens responsible have not been separated and demonstrated to be proteins. Brent *et al.* (1959) showed that animals sensitized for homograft rejection would give characteristic delayed reactions to the intradermal injection of splenic extracts from the donor and that cells from the local lymph nodes of these sensitized animals would give a specific inflammatory lesion on injection into the donor intradermally, whereas passive transfer of serum was ineffective. Although the macroscopic and microscopic appearances gave good reason to assume that these were all pure delayed reactions, the mediation of antibody, however unlikely, was not entirely excluded.

The extracts were unavoidably crude and it is likely that the active antigen comprised only a very small proportion of the total material present; Medawar (1959) gives evidence to suggest that it is a polysaccharide-amino acid complex.

VI. Nature of the Sensitized Cell

The problem of the nature of the hypothetical cellular antibody in delayed hypersensitivity to simple proteins is part of that of delayed hypersensitivity in general and cannot be discussed in detail here. As far as the available data go, the results of the investigations on sensitized cells appear to be equally applicable to either system; that is to say, cell transfer of simple protein hypersensitivity is feasible (see Section V, A), the reactions to demonstrate the "transfer factor" in humans are successful in this situation (Lawrence and Pappenheimer, 1956), the reactivity persists in hypogammaglobulinemic patients (Good *et al.*, 1959), and the histological characteristics of the lesions are similar (Gell, 1958). No data are yet available on *in vitro* cytotoxicity studies along the lines of those of Waksman and Matoltsy (1958) on the tuberculin system. One apparent difference is the fact that animals with delayed sensitivity to complexes are strongly primed for antibody production (see Section III, A), whereas with the tuberculin system antibody production is the exception. This may be the result of either a low intrinsic antigenicity of the bacterial antigens, analogous to that of gelatin, or the precision with which antibody priming can be measured when a well-defined system is used, or, of course, it may indicate a real difference between the two kinds of immune reactivity.

VII. The Relationship between Delayed Hypersensitivity and Antibody Production

It has been suggested by many workers in the field, from the times of Dienes (1936) onward, that delayed hypersensitivity was a stage in the production of antibodies. This was mainly on the basis of the numerous experiments in which such a stage was shown to precede in time the appearance of demonstrable antibody production and often (as in the experiments of Leskowitz and Waksman, 1960, on rabbits) to be correlated in some degree with its intensity. However, there is considerable evidence against the assumption that delayed hypersensitivity is completely superseded by antibody production, since in the analysis of "combined reactions," especially by cell-transfer experiments (Chase, 1959; Gell and Hinde, 1954; Tremaine and Jeter, 1955), the two types

can usually be shown to be coexistent, the Arthus component sometimes masking the delayed component in the active skin reaction. Moreover, it is clear from the foregoing discussion that animals, given the proper antigenic stimulus, can persist for long periods at the stage of delayed hypersensitivity without producing antibody.

Chase (1959) has argued against the linkage of the two modes of response, for example, on the basis of transfer experiments of cells from animals just about to produce antibody against picryl ovalbumin; the recipients at no time showed evidence of contact sensitivity to picryl chloride. Here, however, the demonstration of the carrier specificity of the contact reaction, discussed in Section III, D, may make the argument invalid.

Perhaps the most interesting evidence in favor of the linkage theory is that produced by Salvin and Smith (1960) on anamnestic responses to picryl and DNP haptens on different carriers (these workers use the phrase "anamnestic response" as equivalent to secondary or "booster" response, i.e., the response of an animal to a second injection of antigen). They showed that in animals minimally sensitized to a conjugate, such as 1 μg. picryl BGG in saline, an accelerated antibody response to picryl was not produced by injection of picryl ovalbumin (15 μg. in adjuvant) although it was produced when picryl BGG was reinjected. Thus, the occurrence of a secondary type of response appeared to depend on a pre-existing delayed reactivity to the carrier protein, even though the antipicryl antibodies, when they appeared, did not have this limitation on their specificity. Using these protein carriers, they were able only to consider hapten-specific antibody production and did not demonstrate any hapten-specific delayed reactivity. Thus, testing with the actual conjugate used for sensitizing would combine the effects of carrier-specific and hapten-specific (if any) delayed reactivity, whereas testing with hapten on another protein would be negative in their dose range owing to carrier specificity. Therefore, the contribution of the hapten in this case to the *initial* level of the delayed reactivity (when the first immunizing dose was this small and without adjuvant) remains in doubt.

Gell and Benacerraf (1960) have reported an experiment using the triple cross-reacting system, picryl ovalbumin/ovalbumin/denatured ovalbumin. Guinea pigs were sensitized with picryl ovalbumin and were divided into three groups, some skin tested with ovalbumin, some with denatured ovalbumin, and some untested controls; as expected, strong and equipotent delayed reactions were obtained in all tested animals. The booster effect of this reaction was then evaluated by testing all three

groups for anaphylaxis to intravenous ovalbumin 14 days after the skin test. The animals which had been boosted with ovalbumin were found to be uniformly positive, while among those tested with denatured ovalbumin, there were no more positives than in the unboosted group. Since denatured ovalbumin failed to boost antibody production to ovalbumin, although the delayed reactivity to each appeared to be identical, it might be suggested that the delayed hypersensitivity and sensitization to antibody production were independent effects of the primary injection. It is unfortunate that the denatured-ovalbumin-boosted animals were not tested for anaphylaxis also with denatured ovalbumin, since this is known to have an antigenic specificity of its own. Had they been positive to this, the results could be used as further evidence for Salvin and Smith's contention that stimulation of a pre-existing delayed hypersensitivity controls to a certain extent the specificity of the antibodies produced as a result of this restimulation, allowing for the narrowing of specificity referred to in Section V, C.

Many of the arguments against the linkage theory of the two immune responses on qualitative and quantitative grounds, including some of our own, can be negated if the necessary attention is paid to the facts of carrier specificity and if the hypothesis of the narrowing of specificity between the two stages, which has been outlined in the foregoing, is given due weight.

It is not yet possible to conclude, however, that antibody production must always be preceded by a stage, however transient, of delayed hypersensitivity; and a single example of antibody production during the course of which a stage of delayed reactivity could incontrovertibly be excluded, would throw grave doubt on the linkage hypothesis. A particularly interesting field of research is offered by anticarbohydrate immunization, which has never been shown to be associated with delayed reactivity. It is, of course, possible that this is an extreme example of carrier specificity, a conjugate or complex of the carbohydrate with protein being the actual antigen and thus the whole complex being needed to demonstrate the delayed hypersensitivity stage.

If the two modes of response are indeed linked, a wide field of cytological investigation is opened up. There is, in fact, a yawning gap between our knowledge of the cytology of antibody production, which is considerable, and our knowledge of the cells which take up antigen and of those which develop the ability to respond, by approaching the site of antigen deposition in the tissues, in the delayed reaction. Indeed, all speculations on antibody production, especially those hypothesizing definite biochemical mechanisms, will have to take this stage into account.

Particularly interesting in this connection are those theories which postulate a change of the surfaces of cells analogous to the production of permeases by bacteria, which could account for many of the "steady-state" characteristics of immunity without invoking the existence of preformed clones of cells capable of collectively reacting to any possible antigen: theories along these lines have been suggested by Pappenheimer, Scharff, and Uhr (1959) specifically in connection with delayed hypersensitivity and by Sorkin (1960) who suggests the name "antigen accumulases," although he was considering cells fully sensitized for antibody production. Similarly, the biochemical nature of Lawrence's "transfer factor" is clearly relevant to the analysis of the cytological transition from the state of delayed hypersensitivity to that of antibody production.

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The Antigenic Structure of Tumors

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I. Introduction

It was originally intended that this article should be entitled "Immunology and Cancer Research" and it was first written with this in view. However, on revision it became clear that the contents did not justify the title. The author could be honorably excused for not considering the relationships between the human ABO blood groups and cancer since this is really the province of statisticians rather than immunologists. Unfortunately the same cannot be said of virology and cancer. An article that deals adequately with immunology and cancer research must deal adequately with viruses. This is not only beyond my powers but would occupy far too much space. However I do not think virology can be ignored altogether even with the more limited title now chosen. It seems that viruses may "borrow" antigenic material from their hosts and thus their antigenic constitution may be altered by that of the host in which they have proliferated and it is possible that they may also alter the antigenic constitution of the host cells.

Even with these limitations, the task is no small one since one has to touch upon so many related fields. Immunogenetics of necessity occupies a large part of this article, but in no sense can it be said to be a comprehensive review since this would involve a review of the homograft problem. I have, therefore, concentrated on certain aspects that seem of special relevance to the subject in hand. No two authors would have done this in exactly the same way. The human macroglobulins are mentioned but no attempt has been made to review this important field.

Immunological techniques may be used in the differentiation of macromolecules. In its original sense "immunology" meant the study of

the resistance of the organism to noxious agents. In the present context we use immunology to define the differences between normal and malignant cells and to study the variability of malignant cells among themselves. Some of these differences are best observed by the study of intraspecific antigenic differences. It is very likely that some of the most important modifications involve changes that are not recognizable as antigenically different by the homologous species. This would apply to deletion of any antigens that do not act as histocompatibility antigens and probably applies to all organ-specific antigens. The ability of the host to react against an autochthonous tumor in any immunological sense implies that the growth must have an antigen that the host lacks. For technical reasons, it may be impossible to show that such an antigen is functional as an autoantigen, but it should be shown to be antigenic for an individual genetically identical with the primary host before it can be claimed to have potentialities in the initiation of an autoimmune response. Antigenic differences detected in foreign species need not be functional in this respect.

It is unfortunately necessary to add a short note on terminology. I recently referred to the jargon used in the study of tissue transplantation as "Transplantese." Three adjectives concern us: "autologous," "isologous," and "homologous." The first is a recent addition to the vocabulary. By derivation it does not appear to mean anything. It is usually (but not always) used in place of "autochthonous," the meaning and derivation of which may be found in any dictionary. "Isologous" by derivation would have almost exactly the same meaning as "homologous," but it is not really suitable to denote genetic identity. "Isogenic" has been used for this purpose and will be so used here. While neither of the former terms are suitable for the purpose for which they were introduced, they have no popular meaning to contradict. "Homologous" is very widely used especially by immunologists. It should always be employed to denote some kind of agreement or correspondence. Its continued use to mean "genetically different" is indefensible. I have suggested the term "allogeneic" for genetically different. "Homologous" is used, therefore, in its ordinary sense (Gorer 1960a).

There have been a number of reviews on the immunology of cancer. That of Hauschka (1952) is outstanding, not only because of its intrinsic merits but because it was the first to show the impact of genetics upon the immunology of cancer. All the themes to be found in the present article were dealt with in this review, although much detail has been added since. Snell (1953, 1957a, b) has also written reviews on various aspects of the subject, the first of which is the most comprehensive, and

the present writer reviewed some aspects in 1956 (Gorer, 1956). A very condensed review is that of Southam (1960). The Russian contributions have been reviewed by Zilber (1958, 1959). Reviews on special subjects will be found in appropriate sections.

II. Some Comments on Viral Antigens

The immunology of oncogenic viruses can hardly be divorced from a general discussion of such agents. Among the more recent reviews are those of Oberling and Guerin (1954) and of Harris (1960). Discussions by numerous authors may be found in Monograph No. 4 of the National Cancer Institute and *Cancer Research* for June 1960. Here I shall try to draw attention to a few points that have been little stressed elsewhere. One could hardly fail to mention the remarkable oncogenic viruses that have recently come to light in mice although much has been written by better informed authors. Lastly, it is necessary to draw attention to some possible ways in which immunological tolerance may influence viral carcinogenesis and possibly the antigenic structure of tumors.

Within recent years attention has been concentrated on the oncogenic viruses of mice. The first of these to be described was the mammary tumor agent (MTA) of Bittner (1936). It may seem surprising how little is known about the serology of this agent, but it is an extremely difficult virus with which to work. The only lesions it is known to produce are mammary carcinomata and these may not appear for several months, thus the titration of a viral preparation would take little less than a year. So far as the author is aware there are no experiments on virus neutralization or on passive immunity in which the number of tumor-producing doses is known.

There are no acceptable demonstrations of antibody formation against the agent by the homologous species. As will be seen, the failure to demonstrate neutralization may merely be a reflection of inadequate technique. The same applies to flocculation or complement fixation, since mouse sera are notoriously inefficient in these respects.

Andervont and Bryan (1944) reported some experiments demonstrating antiviral bodies. The sera were obtained from rabbits immunized with a preparation of the MTA, the Brown-Pearce rabbit carcinoma, and an untreated rabbit. In one experiment antigen and antibody were allowed to react *in vitro* prior to injection (neutralization) while in another the serum was injected prior to the agent (passive immunity). R. G. Green *et al.* (1946) immunized rabbits and rats with preparations of the MTA and with a mixture of agent-free organs. Neutralization was

obtained with the sera which were produced against the agent but not with those produced against the normal tissues. The work of Bittner and Imagawa (1955) will be dealt with again later but their controls with both neutralization and passive immunity were particularly good. Antiserum was obtained against mammary glands of $(AZ)F_1$ mice possessing the MTA and against the glands of $(AxZb)F_1$ mice whose parents had been freed of the agent by foster nursing. The former serum was active against the MTA from an $(AZ)F_1$ tumor while the latter was not. Admittedly only two pertinent experiments were reported, but, in all, 83 mice were tested with anti-$(AZ)F_1$ and 76 with anti-$(AxZb)F_1$ in addition to which there were controls with no serum and with normal guinea pig serum.

Tests *in vitro* are less extensive than those *in vivo*. Imagawa *et al.* (1948) performed qualitative precipitin tests with sera produced in rabbits against tissues with and without the MTA. Their absorption test was not quite conclusive since absorption with agent-free material did not quite exhaust the serum against the homologous antigen; however, reactions with MTA preparations were markedly stronger. Heidelberger *et al.* (1952a) performed quantitative precipitin tests with a concentrated preparation of the MTA produced from mouse milk according to the method of Graff *et al.* (1949). After removal of fat, the milk was spun and the casein and the bulk of the agent left in the deposit (fraction A). The casein was removed by treatment with chymotrypsin (trypsin destroyed the agent). The supernatant was called fraction B by Heidelberger *et al.* Fraction A from the high cancer RIII strain was strongly antigenic for rabbits, whereas that from the agent-free C57BL was but feebly so. The strongest cross reactions with anti-RIII fraction A occurred with C57BL fraction B. However, repeated absorptions with the latter sometimes failed to influence the amount of nitrogen precipitated with the homologous antigen and never reduced it by more than 50%. A brief account of experiments with complement fixation by Heidelberger *et al.* (1952b) throws no light on the question of specificity. Perhaps none of the foregoing experiments is conclusive by itself but if we consider them as a whole it would appear that Dmochowski (1953) was being overconservative when he said there was no convincing proof of antigenic differences between the MTA and the tissues from which it originated. As Heidelberger *et al.* (1952a, b) pointed out, their results were very similar to those obtained with other viruses.

Bittner and Imagawa (1955) tested a preparation of the MTA from an A strain mammary tumor with sera prepared against A strain mammary tumor and against normal mammary glands of the $(AZ)F_1$ and the

(AxZb)F_1 (agent free). Neutralization was only obtained with the homologous antibody, those directed against either F_1 failed to protect. The second experiment involved an A strain agent from normal mammary gland, and passive immunity was used instead of neutralization. The result was precisely the same as in the previous experiment. For the reciprocal experiments employing MTA from transplanted AZ tumor, guinea pigs instead of rabbits were used for making antiserum. As already mentioned, sera produced against agent-free mammary glands gave no protection while those against AZ glands did so. The sera against the A strain MTA gave somewhat ambiguous results. There was an indication of protection against the AZ agent at the twelfth transfer of the homologous tumor but none from the agent obtained from the thirty-first. Later Bittner *et al.* (1959) reported results obtained with sera prepared in guinea pigs from an A strain mammary tumor carried in As and in the agent-free (AxZb)F_1. Some of the groups of mice are rather small, but in two experiments an antiserum against the (A in F_1)MTA was ineffective against the (A in A)MTA, whereas the homologous serum was effective. In the case of an MTA from the (A in F_1) tumor, either antiserum might protect. Blair (1960) tested samples of the MTA from four different sources with rabbit sera and observed no antigenic differences between them. No absorption experiments were reported.

Experiments such as those of Blair (1960) show that all the agents studied have certain antigens in common. In the absence of absorption, differences can only be shown if an antibody acting upon a differential antigen is of considerably higher titer than those acting upon common antigens. This may well be the case in the experiments of Bittner and Imagawa (1955), in which it would appear that, in the anti-AZ serum, "anti-Z" was at effective titer, whereas "anti-A" was not. There is nothing very surprising in the finding that there are different antigenic types of MTA or, indeed, that such properties are influenced by the type of cells in which the agent proliferates. However, the later experiments of Bittner *et al.* (1959) are surprising. In this case it would seem likely that the agent was modified by the environment of the cell. This does not mean that the observation is incorrect but does suggest the need for supporting evidence. In no case has a serum been tested against titrated MTA, and an apparent antigenic difference might result from the fact that preparation A contained ten times as much antigenic material as preparation B. Tumor incidence is not a completely safe criterion since Bittner (1948) showed that preparations of MTA may show zoning, that is to say, that low concentrations of agent may show a higher tumor incidence than do high concentrations. Absorption experiments would undoubtedly be of value.

It is always notoriously unsafe to draw conclusions from negative results. The fact that Dmochowski *et al.* (1952) failed to find complement-fixing antibodies in mice against the MTA is unfortunate, but virus-neutralizing antibodies may not fix complement. The failure of Gorer and Law (1949) to show virus neutralization with mouse sera may be due to antigen excess or other technical reasons pointed out by them. Lastly it is possible that immunity to the MTA depends wholly or in part on specifically sensitized cells. The writer is fully aware of the technical difficulties involved, but the most pressing need would seem to be for a more quantitative approach to some of these problems.

The extraordinary polyoma virus of S. E. Stewart and her co-workers must be mentioned. Serological tests for signs of infection are now well established. The virus seems to be widely distributed in laboratory stocks (see Harris, 1960; Law *et al.*, 1960). It is unlikely that it has played any major role in spontaneous tumors of mice or, indeed, under any circumstances other than injection of the virus itself.

Three groups of workers have described leukemogenic agents derived from long transplanted tumors. Graffi (1957) described the induction of myelogenous leucoses with extracts from five different transplanted tumors in newborn mice from three strains. Later Graffi and Gimmy (1958) were able to induce thymomas in newborn rats and to transmit the agent back to mice (Gimmy and Graffi, 1959). According to Graffi and Krischke (1957) and Graffi *et al.* (1958) the agent is feebly antigenic for mice, but satisfactory antisera can be obtained from rabbits.

Friend (1957) obtained leukemia in adult Swiss and DBA/2 mice with an agent derived from the Ehrlich carcinoma. Some people threw doubt on the leukemic nature of the disease because subcutaneous tumors did not follow subcutaneous inoculation of cells. This, in fact, is a very common finding in the early transfers of "respectable" leukemias. Although the virus is strain specific, it does not appear to have set any difficult immunological problems, and antibodies have been produced against it in the homologous species and in rabbits (Friend, 1959).

Moloney (1960) has reported extensive studies on an agent derived from Sarcoma 37. It is effective in adult mice of eight different strains and in newborn Sprague-Dawley rats. Its immunological behavior is now being studied.

The late Dr. F. Duran-Reynals once referred to certain viruses as "orphans" since they were first isolated in tissue culture but were in search of a disease. One suspects that polyoma and the foregoing agents were also originally orphans. The first was obtained from culture *in vitro*, while in the case of the other three, the tumors supplied the culture

medium. The conditions necessary for the exhibition of their oncogenic properties vary. The Graffi agents apparently need newborn hosts but do not seem to show much dependence on the host's genotype. The Friend agent will affect adult mice but does show strain specificity. The Moloney agent is the least exacting of all.

One cannot help wondering how many cultured cell lines owe their existence to infection by some convenient potentially oncogenic virus. Unfortunately some statements about them may result from errors in identification. Thus alleged loss of species-specific antigens cannot be accepted without reserve in view of the finding by Defendi *et al.* (1960) that three "human" and one "rabbit" cell line were all really murine (probably L cells). However, if De Somer and Prinzie (1961) are correct that all lines of diverse origin have common new antigenic components, the possibility exists that these components may be viral.

It is not clear where the Schwartz agent fits into the viral hierarchy. The original agent was derived from the brain of a leukemic Swiss mouse and produced the disease in adult Swiss mice after a latent period of 1–3 weeks (Schoolman *et al.*, 1957). Later a very similar agent was found in the brain of a leukemic C3HeB mouse. It could be transmitted to adult mice of several C3H sublines with a latency of under 7 weeks (Schwartz *et al.*, 1959).

The leukemogenic agent originally described by Gross (1951) appears, like the MTA, to be associated with the spontaneous disease. Very young animals have to be used, and the virus is strongly strain specific (see Gross, 1960). The agent most commonly used by Gross was of AK origin although the C58 strain has been used as well. Gross (1957) enhanced the virulence of the agent by repeated passage through C3H/Gs mice. This agent has been called "Passage A" virus. It will affect older mice but less efficiently than young mice. Miller (1960) finds it more effective in Gross's C3H than in the Pollards Wood substrain, although both were originally C3H "Bittner."

Gross (1958) and Lieberman and Kaplan (1959) showed that a leukemogenic agent could be demonstrated in radiation-induced leucoses from C3H and C57BL, respectively. Kaplan's agent seems specific for isogenic mice or F_1 hybrids (Kaplan 1959, 1960). Both Gross (1959) and Lieberman and Kaplan (1959) were able to enhance the virulence of the agent by repeated passage. Immunological studies have shown that rabbit antisera will neutralize Passage A and Passage X agents. The latter is the agent derived from an irradiated C3H/Gs mouse.

If we equate immunology strictly with serology, it would appear that most of the leukemogenic agents, like the MTA, are of doubtful or low

antigenicity for the homologous species, but genetic studies give rather a different picture. The genetics of spontaneous leukemias have been admirably reviewed by Law (1954). As a rule leukemia shows the phenomenon of maternal inheritance which was first reported by MacDowell and Richter (1935). The results are not so clear-cut as with mammary cancer. Since the time of the original work of Bittner (1936), it has been known that foster nursing may cause a permanent reduction in the incidence of mammary cancer. Furth *et al.* (1942) showed that a temporary reduction of the incidence of leukemia might be obtained by foster nursing. Since this was temporary, it could not be due to removal of a virus as in mammary cancer. This type of resistance has been called the "maternal resistance factor" by Law.

MacDowell and Taylor (1948) found that STOLI mothers about 16 weeks of age when crossed with C58 males showed no such resistance and the incidence in their progeny was the same as in the C58 strain. However, mothers of 6 months of age or more had young with a considerably reduced incidence. Later MacDowell *et al.* (1951) showed that this resistance could be transmitted by older mothers either *in utero* or through the milk. Law (1954) showed that the maternal resistance factor was common but not universal in low leukemia strains. In most of these the resistance is manifest at the beginning of the reproductive age, but the nature of the experiment makes it impossible to know at which age prior to puberty it develops. The nature of the resistance is unknown but the phenomena do suggest some type of antibody response.

Bittner (1958) has given an exhaustive review of the genetics of mammary cancer in mice. We have little evidence of anything akin to the maternal resistance factor but hormonal factors are far more prominent than is the case with leukemias. Bittner points out that genes may affect the hormonal pattern and the ability of the agent to proliferate. It would appear that the latter class of genes in both spontaneous mammary cancer and in leukemia are active when heterozygous. This could suggest one of two things. It is possible that "resistance" in its strict sense is not involved. The virus fails to proliferate in strains of low incidence because they lack certain necessities supplied by high incidence strains. The evidence available is not altogether favorable to such a view. An alternative is suggested by analogy with tissue transplantation. Here susceptibility is "dominant" because the F_1 is naturally tolerant to antigens from either parent strain. It appears that some viruses may coat themselves with the cell membrane of the host (see Bernhard, 1960, for references). It is possible that this may account for the genetic and immunological behavior of the MTA and other agents.

Failure to obtain antibodies against such agents could be due to the fact that the viruses must first be "skinned" by a homograft response. It is also possible that histocompatibility antigens influence the ability of the agent to enter cells.

It will, perhaps, be easier to obtain evidence on the former point than on the latter. It is certain that no *simple* relationship exists between histocompatibility and susceptibility. If this were so we would expect only a small number of genes to be concerned. The most obvious candidate for consideration is the H-2 system. There is no clear association between H-2 type and the incidence of mammary cancer. Of the best known "high cancer" strains, A is H-2a, DBA/1 is H-2q, DBA/2 is H-2d, C3H is H-2k, and RIII is H-2r. So far as is known no H-2b strain is susceptible, but the low cancer C57BR (H-2k) is resistant. The data for leukemic strains are less extensive. Both C58 and AK are H-2k but, as will be seen later, this is a common H-2 type. The F strain of Strong which is rather less susceptible than the two preceding (Kirschbaum and Strong, 1942) is of an entirely different H-2 type (H-2n, see Amos, 1959).

Unfortunately our ability to type strains by serological means is confined to H-2, and it may be that some other H-systems show a good correlation with susceptibility to one or other type of agent. However this may not be so. It is possible that antigenic structure simply influences the ability of the agent to enter a cell and once there this becomes indifferent. It should be easy to design a genetic experiment involving a cross such as $H\text{-}2^a \times H\text{-}2^b$ and to see if a majority of animals developing mammary cancer are $H\text{-}2^a$.

The possible effects of the MTA on the antigenic constitution of tissues has already been reviewed by the author (Gorer, 1956). If such modifications exist they have not been conclusively demonstrated to effect the result of transplantation experiments. However, Imagawa *et al.* (1954a) found evidence to suggest that guinea pigs may be able to differentiate between infected and uninfected cells. Serum prepared against agent-free material appeared less effective in inhibiting the growth of a transplanted mammary tumor than sera prepared against material with the agent. Unfortunately the only absorption experiments were with agent-free material. Absorption with infected mammary glands and mammary tumor was essential to determine the amount of antigen necessary for absorption. In a later experiment Imagawa *et al.* (1954b) studied the cytotoxic effect of such sera on tissue culture. They used a mammary tumor and an embryonic agent-free intestine as a control. The latter is not suitable since a number of antigens such as H-2 antigens might not

have developed in the embryonic material. It is surprising that this type of work has not been followed since it suggests the possibility of a more rapid test for the MTA.

It is not possible to consider the antigenic structure of fowl viruses in the present article. Reference may be made to the reviews quoted earlier or to those of Darcel (1960) or of Harris (1958). The close association between such agents and normal tissue constituents has been known for many years and various explanations of this association have been offered. Recently Harris and co-workers have conducted a number of experiments on the ability of fowl blood to confer tolerance to the Rous agent in young turkeys. Together with Simonsen, they showed that turkeys inoculated *in ovo* with fowl blood produced tumors if inoculated with the Rous agent at 8 weeks of age (Simonsen, 1955). The mortality in embryos so treated is high, and Harris (1956) found that 1-day-old turkey chicks could be used. One of the tumors so induced was transplanted to normal turkeys. Virus recovered from the transplanted tumors was less infective for fowls than that maintained in the homologous species. In contrast with what has been found with skin transplants, living cells are not necessary since lyophilized blood was effective. Harris (1956) in discussing these results pointed out that resistance to the Rous agent that develops with aging in chickens is associated with the formation of antibodies against the viral particles but that this is not so in turkeys, and he has suggested that in this case resistance was against fowl antigens. Subsequent work showed that the situation is not so simple. Harris and Simons (1958) showed that tolerance could be transferred by substances having the antigenic constitution of the human group A substance. This type of antigen has not been found to be associated with the viral particle.

Svoboda (1960a, b) has conducted some small-scale experiments on tolerance to the Rous agent in ducks. One very surprising result was that a tumor induced in a tolerant duck would grow in chickens but not in ducks. She confirmed that human group A red cells would confer tolerance and found that deoxyribonucleoprotein from fowl erythrocytes would also do so. It may well be that the situation in ducks is different from that in turkeys but one would like to see the experiments done on a larger scale before drawing conclusions. The failure of a tumor induced in ducks to grow in the homologous species is not surprising but the alleged ability of the same tumor to grow in chickens is without precedent. Possibly some error was made or the fowl tumors were the result of viral infection rather than cellular transplantation.

It is not possible to say to what extent our views on the immunology of cancer will be modified by the concept of immunological tolerance.

The necessity to use very young animals with some viruses does not necessarily involve the induction of tolerance. As will be seen later some mouse tumors are potentially autoantigenic while others are not. It is possible that viruses infecting newborn mice may render these tolerant to some viral product which forms a very small component of normal cells but which will form a major component of malignant cells.

III. Histocompatibility Systems

Nobody would suggest that the study of the immunology of cancer should be confined to inbred strains since to do so would limit our activities to mice, rats, and guinea pigs. There are several strains of inbred rats and it is to be hoped that they will be used more in future. Their only disadvantage compared to mice is their larger size. Unfortunately there are not many inbred strains of guinea pigs (see Billingham and Silvers, 1959, for details on rats and guinea pigs). However, if mouse or rat tumors are being studied from this point of view, inbred strains and tumors derived from them should always be used. Tumors such as Sarcoma 180 or the Walker rat carcinoma may be admirable research tools in some fields of study but not for the investigation of the antigenic structure of tumors, yet they are constantly being so used.

Although a start has been made on histocompatibility systems in rats (Bogden and Aptekmann, 1960), our knowledge is virtually confined to mice. The H-2 system is by far the most intensively studied of any. The most recent publications on its antigenic components are those of Hoecker *et al.* (1959), Amos (1959), Gorer (1959), and Gorer and Mikulska (1959). Nobody would care to guess how many H-2 combinations actually exist but twenty are known in varying detail. It appears clear that we are dealing with a system of pseudoalleles and crossing over has been clearly demonstrated. Crossing over between the D and K regions of the chromosome occurs with a frequency of rather more than 1% (references in Gorer and Mikulska, 1959) but the actual frequency may vary in different crosses. Although the number of possible H-2 types is obviously very large, it would appear that certain types have a distinct selective advantage under laboratory conditions. In view of the great potential polymorphism of the system, the probability of finding two *unrelated* strains of the same type is very small. The fact that strains CBA, C3H, and CHI are H-2k is of no significance if considered alone since all derive from a cross between a mouse of Bagg's albino strain and the DBA made by Strong in 1920. Similarly strains C57BL, C57BR, C57L, and C58 derive from a mating between litter mates by Little and Bagg

(1924). Most C57BL substrains are H-2b as is C57L. Some C57BL are H-2d and it is not certain how this combination came into these substrains; it could have been ancestral. The C57BR strains and C58 are H-2k. There is no known ancestral connection between the C57,58 family and Strong's famous family of strains, and we can thus count these as two independent occurrences of H-2k. Seven independent occurrences of H-2k are now known. Amongst them are AKR, 101, STB (formerly called ST), and CE (recently typed by Rubin, 1960). There appear to be five independent occurrences of H-2b. The best known of the H-2b strains is C57BL but its occurrence in the STA strain is of interest. Snell (1953) had classified the Danish ST strain as H-2k. Simonsen sent us examples of his mouse strains from Copenhagen for serological typing. We were very surprised to find that his ST were H-2b. We then learnt that he had sent us his STA strain while that sent to America was apparently his STB. Subsequent typing of STB showed it to be H-2k. We may mention here that it is desirable to get H-2 types confirmed where possible. Usually strains conform to the published pattern, but, in addition to STA, we found that the Rijwsijk CBA was not H-2k as expected but some unidentified H-2 type.

H-2d is the third common type of which seven occurrences are known. The best known strains are BALB/c and DBA/2. Strain DBA/1 is H-2q. The only other example of H-2q is C/St which is descended from DBA (Strong, 1935, 1936, 1942; Amos, 1959). Indeed the only other H-2 combination to occur more than once is H-2a. It occurs in the A strain and in the AK.H-2a (also called AK.K.). In the latter case it was introduced onto an AKR genetic background by Snell by crossing with the heterogeneous Ki stock. It is most probable that H-2a resulted from a crossover between H-2d and H-2k (see Gorer and Mikulska, 1959). We do not know in what way these three common combinations confer an advantage. It is of interest to note that Snell (1958b) has found all three in heterogeneous stocks. The Swiss has H-2b and H-2d in addition to H-2s.

The antigenic components of the most commonly used H-2 combinations are shown in Table I. It will be seen that H-2a has 11 named antigens, H-2d has 8, H-2k has 7, and H-2b has 6. I should be inclined to regard these as underestimates of the real number, particularly with regard to the last three. In a recent discussion, Owen (1959) took the opposite view basing his arguments upon studies with synthetic antigens. In a simplified form his reasoning is as follows. Suppose we make an antibody against *m*-aminobenzenesulfonic acid (*m*-ABS), we shall probably find it will give strong reactions with *o*-aminobenzenesulfonic acid

TABLE I
SOME WIDELY USED H-2 COMBINATIONS[a]

H-2 symbols	Antigen																
all	A	C	D	E	F	G	H	I	J	K	M	N	Q	S	V	Y	Some typed strains
a	A	C	D	E	F	—	H	—	J	K	M	N	—	—	—	Y	A,AKR.K
b	—	—	D^b	E	F	—	—	—	—	K^b	—	N	—	—	V	—	C57BL,129, LP,STA, etc.
d	—	C	D	E^d	F	—	H	—	J	—	M	N	—	—	—	—	BALB/c, DBA/2, etc.[b]
f	nt[c]	nt	—	nt	?[d]	G	H	I	nt	—	nt	nt	—	—	nt	—	A.CA
k	A	C	D^k	E	—	—	H	—	—	K	—	—	—	—	—	Y	CBA,C3H,C57BR, C58,AKR, 101,STB
q	—	C	—	E	F	—	—	—	—	—	M	—	Q	—	—	nt	DBA/1
r	nt	?	—	E	nt	—	—	—	nt	K	nt	nt	—	—	nt	Y	RIII
s	nt	C	—	E	F	G	—	—	nt	—	nt	nt	—	S	nt	—	A.SW

[a] From Amos (1959), Hoecker *et al.* (1959), Gorer and Mikulska (1959).
[b] C57BL/Ks and some other C57BL substrains are H-2d.
[c] nt = no test reported.
[d] ? = result of test uncertain.

(*o*-ABS) as well. Absorption with the former will remove all antibodies, whereas the latter will leave antibodies specific for *m*-ABS acid. Without prior knowledge, one might deduce that *m*-ABS consisted of two antigenic components, one of which was shared with *o*-ABS. Therefore one might get the appearance that H-2a contains antigens D and K when, in fact, there is only a single antigen A that cross-reacts with D and K. I doubt very much if the complexity of the H-2 system is exaggerated much in this way. If one has two antigens showing strong cross reactions such as *m*-ABS and *o*-ABS, one will usually find that absorption with the latter greatly lowers the titer for the former. In the absence of prior knowledge, one would be inclined to invoke a quantitative difference rather than a qualitative one. Where cross reactions are weak as with *m*-aminobenzenearsenic acid, the possibility of error is greater but is probably not of much practical importance. In the specific case of D and K, the analogy breaks down at several points. We can easily produce anti-D and anti-K by immunizing H-2b mice against H-2a strains. Several sera of this type have been tested and the titer of the two antibodies is approximately the same when tested on H-2d and H-2k, respectively. This would indicate strong cross reactions, but adequate absorption experiments show that absorption with H-2d does not cause any appreciable fall in the titer of anti-K and vice versa. We can, of course, produce anti-K in H-2d mice and anti-D in H-2k mice. If we cross H-2d and H-2k strains, the F_1 will form no antibodies against H-2a. Lastly D and K can be separated from one another by genetic crossing over. None of these phenomena can be paralleled with synthetic antigens.

These facts do not mean that Owen's arguments should be completely ignored. Cross reactions may be seen between H-2 components particularly where immunization has been especially intense. We have taken strong cross reactions to indicate antigenic similarity and hence allelomorphism. The best documented example is K^b of H-2b. The first suggestion of an allelomorph of K in H-2b came from occasional strong cross reactions with anti-K. Crossover data showed the location of the supposed allelomorph to be as expected. An example of anti-M was sent to us by Dr. Stimpfling from Bar Harbor, Maine, and it showed strong reactions with H-2b, which is classified as M-. This probably indicates an allelomorph of M but lacks genetic confirmation.

Most of the antigens of the H-2 system are shared by red cells and nucleated cells but some are absent from or very poorly represented on red cells. If we ignore this complication, it can be shown that the number of components that can be distinguished depends on the number of combinations we have available for study. If we have four such com-

binations it is obvious that a component shared by all would not be recognizable. We can, in effect, only recognize three categories, those components shared by two combinations, by three combinations, or unique to a single combination. The totals for any number of combinations may be calculated, and the number is 14 for four H-2 types and 126 for seven H-2 types (see Gorer and Mikulska, 1959). Suppose we consider the commonest types, H-2a, H-2b, H-2d, and H-2k. Reference to Table I will show that antigen A is shared by H-2a and H-2k but that it is absent from the other two. Under these conditions A and K are indistinguishable. Their distinction depends on the study of a number of further strains of different H-2 types as was done by Amos (1959) in the detection of A. Ideally one should have both A+K— and A—K+ but either will show that "anti-K" contains at least two antibodies.

If one does not have new combinations "ready made" one may sometimes obtain new ones by crossing over. The manner in which crossover combinations may reveal new components may be seen from Table II in which three new H-2b components were dissociated. The table only illustrates antigens pertinent to the present discussion. The two new combinations obtained from the cross ($H\text{-}2^a \times H\text{-}2^b$) are the most pertinent. The component V was detected by hemagglutination. It could not have been detected in the absence of new combinations since it is absent from all three of our other combinations. However the new H-2g is D^b+V+ and H-2i is D^b—V+ and this makes differentiation possible. The situation with regard to K^b is more complex. This antigen is not detectable on red cells, and the antigen was detected by the cytotoxic test of Gorer and O'Gorman (1956). Antibody can be formed by the new H-2g but here the picture is complicated by the presence of anti-E. Antibody is very readily formed by H-2h. It is present in H-2b and H-2i only of our combinations. Amos (1959) finds that V is present in other combinations not listed here.

The majority of H-2 components have been detected by the hemagglutination technique of Gorer and Mikulska (1954). The leucocytic agglutination system of Amos (1953) has not been used for this system but has proved useful in others. The cytotoxic technique of Gorer and O'Gorman (1956) has already been mentioned in connection with K^b. As an antibody detector it is much less sensitive than hemagglutination. With the latter technique, titers of over a thousand are common and they may be considerably higher. With the cytotoxic technique, a titer of 128 must be considered good. It is, therefore, impossible to be certain whether all antibody molecules are capable of both activities; it may well be that they are not. The demonstration that H-2 antibodies are

TABLE II

A PARTIAL GENETIC MAP OF SOME H-2 COMBINATIONS[a]

Parental combinations[b]						Crossover combinations					
H-2 symbols	Loci					H-2 symbols	Loci				
H-2^d	D	C	v	'E^d'	'k'	H-2^g	D^b	c	V	'E^d'	k
H-2^b	D^b	c	V	E	K^b						
H-2^a	D	C	v	K		H-2^h	D^b	C	v	K	
H-2^b	D^b	c	V	K^b							
H-2^a	D	C	v	K		H-2^i	D	C	V	K^b	
H-2^b	D^b	c	V	K^b							

[a] From Gorer and Mikulska (1959).

[b] Antigen F was not segregating in any parental combination. Antigen E was not segregating in H-2^a/H-2^b. The vertical lines denote the point of crossing over.

responsible for cytotoxic activity involved genetic experiments. These were first done with known components such as D and K (Gorer and O'Gorman, 1956). Later new components were studied such as K^b already mentioned and D^k of H-2k (Gorer and Mikulska, 1959).

So far, pronounced cytotoxic activity has only been shown to be associated with H-2 antibodies. It is impossible to assess the incompletely analyzed results of Sachs and Feldman (1958). Briefly they found that some tumors might elicit hemagglutinating antibodies that showed no cytotoxic activity toward the leukemia 6C3HED. This could be due to the fact that the leukemic cells which they used lacked certain antigens present in other tumors arising in the same inbred strain. It could result from the fact that some of their sera were anticomplementary. It is not known how many of their antibodies were directed against the H-2 system or what components were involved. It is known that with human red cells anti-A and anti-B are strongly hemolytic *in vitro,* while Rh antibodies are not. The same may be true of different H- systems in mice. One should not draw any conclusions concerning the nature of H-2 antigens from data such as these and their suggestion that toxinogens and agglutinogens are part of the same antigenic molecule is an unnecessary complication.

Although it has been emphasized that the allocation of any antigen to the H-2 system must be confirmed by a genetic test, it is an empirical fact that, if two strains of mice have different H-2 systems and any antibodies are formed, H-2 antibodies will certainly be present and often they are the only antibodies detectable. Since serological work has been begun, hemagglutinating antibodies associated with other unidentified systems have occurred from time to time. For simplicity we shall assume we are concerned with a single antigen "D." It will probably be tested in a backcross Dd×dd which should give 50% D+ and 50% D—. If a given antiserum were to give a significant excess over this expectation, we should know that we were dealing with more than one genetic system. Occasionally one may find sera that will give results suggestive of more than two genetically independent systems. Such sera are of somewhat irregular occurrence and the antigens with which they react have not been investigated.

If one wishes to make a systematic study of systems other than H-2, one must use inbred strains of the same H-2 type. Amos (1958) made a serological study of substrains of C3H. All of these appeared to be H-2k, but Amos did not ignore the possibility that mutations might have occurred within the system and he, therefore, performed genetic tests to make sure that the antigenic differences that he observed were really

not of the H-2 system. He described three antigens which he called alpha, beta, and gamma. Of these only alpha need detain us. This antigen was found in the leukemia 6C3HED and on the red cells of certain C3H substrains but on no other types of nucleated cells. This type of complication did not occur to Sachs and Feldman (1958). Unfortunately the antibodies concerned are unstable and further genetic work has not been done on these interesting antigens. Similarly Amos (1959) has shown that strain 129 can form strong hemagglutinins against C57BL, and we have shown the same with STA anti-C57BL (unpublished). All three strains are apparently H-2b, but we have not performed genetic experiments to show whether the H-2 systems in all three strains are really exactly the same.

Snell (1948) conceived the idea of building strains of mice which were genetically identical except for a single histocompatibility locus, such strains being called "isogenic resistant" or IR strains. Today it would be a simple matter to put H-2b or some other H-2 type onto an A strain background. One would cross A and C57BL and then backcross the F_1 to the A strain. This cross would be typed and the H-2b mice again crossed to the A strain. The proportion of C57BL genes is, of course, 50% in the F_1 and is halved at each backcross. After six backcrosses, under 1% of C57BL genes should remain and after nine, less than one in a thousand. At any desired point the H-2b mice can be bred inter se and the homozygotes found by typing. This could not have been done with many H-2 types when Snell began building his strains and cannot be done even now for the H-1 and H-3 types which have been discovered more recently. Snell had to use rejection of a tumor graft as a criterion of antigenic difference. He could not do this by continual backcrossing since members of the backcross to the susceptible strain are all susceptible. He, therefore, had to mate every backcross inter se and challenge them with a tumor. This doubles the number of generations that has to be raised to obtain the desired degree of homozygosity. Probably his most widely used IR strains are those with an A strain background or C57BL background.

Before H-2 types were properly defined any attempt to establish a resistant partner would almost inevitably result in antagonism at H-2. Later Snell was able to start his IR lines with parents of the same H-2 type. Thus the C57BL.LP has both parents of H-2b. In this way he has defined the H-1 locus that is linked with albinism and the H-3 locus that is linked with the agouti locus (Snell 1958a, b). Compared with H-2, these loci determine relatively weak antigens, and in order to select his lines Snell had to give preliminary immunization with normal tissues

(usually thymus) from the tumor donor. With his usual ingenuity Snell has exploited this apparent drawback to show that H-3 is probably a complex of antigens like H-2 (Snell *et al.*, 1957). Neither H-1 nor H-3 induce a good antibody response. Amos (1959) obtained feeble leucocytic agglutination.

With IR pairs one would expect both normal and malignant tissue transplants to give the results expected from a single gene difference. Berrian and McKhann (1960) studied crosses between C57BL/10(H-2b, H-3a) and B.10.D2(H-2d,H-3a) and found the results consistent with a single gene difference, in this case H-2b vs. H-2d. They also studied C57BL/10 and B.10.LP. Both are H-2b but the latter is H-3b. Again the results were consistent with a single gene difference (H-3a vs. H-3b). On the other hand, Linder and Klein (1960) studied A/Sn, A.SW, A.CA, and A.BY which should only differ at H-2. Tumors behaved according to expectation even when prospective hosts were immunized with normal tissues. Skin grafts exchanged within the A strain survived well but grafts performed within the A.SW, A.CA, and A.BY did not. Under these circumstances it could not be expected that skin grafts between the strains should agree with a single gene difference. Indeed, one cannot give any assessment of the number of differences between the strains, since A skin might differ from A.SW No. 1 by five factors and from No. 2 by seven, etc. This is an unexpected result; one would not be surprised if A and A.SW differed at two or three loci, but it is difficult to understand why strain A should apparently be uniform and A.SW extremely heterogeneous. Further consideration is better left until later when we come to consider the question of interactions between different systems.

The last system to be considered is the Y chromosome system of Eichwald and Silmser (1955). They found that, in certain pure strains, female mice reject male skin. Amos found that leucocytic agglutinins may be formed following such rejection and also that there is questionable evidence of cytotoxic activity (see Hauschka *et al.*, 1960).

Humoral antibodies are very readily formed by mice where H-2 antagonisms occur. They are also readily formed with other histocompatibility systems that have not been studied genetically. The remaining systems, H-1, H-3, and the Eichwald-Silmser systems are not associated with high titers of antibodies. This raises a complication for those who wish to formulate a theory of homograft rejection. A scheme which might work well for the H-2 system may not apply to H-1 or H-3.

We still know very little concerning the chemistry of the antigens involved in graft incompatibility. Billingham *et al.* (1956a) gave a method for preparing cell-free antigens that would give a short-lived

immunity to skin grafts which was followed by an improved method of extraction 2 years later (Billingham *et al.*, 1958). These preparations were called "T-antigens" in contrast to "H-antigens" which usually, but not invariably, result in immunological enhancement (for references see Gorer, 1956; Medawar, 1959). It does not appear likely that the genes of the H-2 system elaborate two distinct groups of antigens. The writer would much prefer "T-preparations" and "H-preparations" to "T-antigens" and "H-antigens." The determinant groups in both are probably the same (Berrian, 1959; Medawar, 1959) and a T-preparation may differ from an H-preparation by subsidiary molecular attachments. Hildemann and Medawar (1959) did not detect hemagglutinins following injection of T-preparations. This is not conclusive evidence that no humoral antibody is formed. Gorer *et al.* (1959) found that some H-preparations gave negative results with the ordinary hemagglutinating tests. They gave a positive result with the synergic test of Gorer *et al.* (1959) and also gave enhancement of Sarcoma 1 *in vivo*. As will be seen later, grafts of different types differ in their response to humoral antibodies *in vivo*. It seems certain that specifically sensitized cells are essential for the destruction of certain types of graft including skin grafts. It would appear that T-preparations are much more efficient at eliciting a cellular response than H-preparations. Some people seem to have the idea that T-preparations elicit a special type of humoral antibody that is different from the hemagglutinins. This is most unlikely. The only tumor used for the extractions of T-preparations is a leukemia (Manson *et al.*, 1960). This is not a very good choice as a test object since such neoplasms are very susceptible to humoral antibodies. It now remains to consider some of the ways in which sensitized cells and humoral antibodies interact.

IV. Enhancement and Destruction of Grafts

The nature of the homograft reaction is a subject of lively investigation and debate which has been the subject of several lengthy reviews (Gorer, 1956; Snell, 1957a, b; Medawar, 1958; Brent, 1958). A judicial discussion of the problem is obviously impossible in the present article and all I can hope to do is to present certain ideas that are widely accepted, accentuating those that are of special pertinence to cancer research. Five years ago all of us argued about "the homograft problem" as if there were only one possible method of graft destruction. Today no well-informed person takes this view. We all agree that specifically sensitized cells play a major role and that humoral antibodies may be found at high titer. In those cases where both types of immune response are

known to occur we are very interested in finding out the relative importance of each and the ways in which the sensitized cells and humoral antibody may interact. The cellular response is not the same for all types of tumor homograft. In the case of ascites sarcomata, histiocytes are clearly the dominant cells (Gorer, 1956, 1958a, b). With other solid tumors, lymphocytes have long been accepted as playing a leading role (da Fano, 1912). The same has generally been accepted for skin grafts but Waksman (1960) states that histiocytes predominate in skin graft rejection in guinea pigs.

Leukemic grafts were the first type of graft in which humoral antibodies were shown to play a major role (Gorer, 1942) and this has been amply confirmed since. In the case of tumors such as the A strain Sarcoma 1 the role of antibodies is very different, while the C3H ascites sarcoma B.P.8 differs from both of these.

Mouse sera very seldom show complementary activity *in vitro* and this property has probably delayed our understanding of the role of antibodies in various types of homograft response. Gorer and O'Gorman (1956) showed that mouse isoantibodies were cytotoxic to normal splenic cells and to leukemic cells in the presence of guinea pig or rat complement. The A strain Sarcoma 1 appeared to be completely resistant while B.P.8 seemed to consist of a mixed population of cells. Gorer and Boyse (1959) showed that bone marrow contained both resistant and susceptible cells. They found that thymocytes were very susceptible to cytolysis by most samples of normal guinea pig serum. They were less susceptible to rat serum and, in the presence of rat complement or suitably absorbed guinea pig sera, most samples of thymocytes are resistant to the action of antibodies. A similar finding was made independently by H. J. Winn (quoted by Snell *et al.*, 1960). This is of some interest since lymph node cells are not particularly susceptible to any cytotoxic activity of guinea pig serum alone but are highly susceptible in the presence of antibodies. Hellström (1959) examined a spectrum of ascites tumors and of solid tumors dissociated by mechanical means. The study of the latter is handicapped by the high proportion of dead cells that is always present following mechanical disintegration of solid tumors. He found leukemias to be susceptible and carcinomata to contain susceptible cells. He considered sarcomata to be resistant. This finding must be treated with some reserve. One of his "carcinomata," the A strain 15091a, is almost certainly a sarcoma which is susceptible like B.P.8. Boyse (1960) obtained suspensions from solid tumors containing a small percentage of dead cells following treatment with trypsin and deoxyribonuclease. So far as we have been able to ascertain such treatment does not affect the reactivity

of leukemias or B.P.8 but it is impossible to be sure that this is true of cells obtained from solid tumors. Boyse *et al.* (1961) found that suspensions prepared from mammary tumors consisted of mixed populations. The proportion of susceptible cells varies with the tumor from about 50% to about 90%. A tumor that had never been transplanted showed one of the lowest proportions of susceptible cells while a long transplanted tumor contained about 90% susceptible cells. Sachs and Feldman (1958) confirmed that tumors vary in their susceptibility as tested *in vitro,* as has also been shown by Amos (Amos and Wakefield, 1959; Amos, 1960).

The effects of antibody *in vivo* may be studied by allowing antibodies and target cells to meet first *in vitro* (neutralization) or *in vivo* (passive immunity). Gorer (1956) stated that the two might give somewhat different results. This is probably wrong except that neutralization may be rather more delicate. Either technique may inhibit the growth of leukemic cells in foreign strains or in the strain of origin (Gorer, 1942; Gorer and Amos, 1956; Amos and Day, 1957; Winn, 1960). Siskind and Thomas (1959) showed that antibody could inhibit the development of runt disease in infant mice, and Gorer and Boyse (1959) showed that it could abolish the protective effect of allogeneic bone marrow in irradiated mice. S. Harris *et al.* (1958) showed that antibodies could destroy immunologically competent cells in rabbits.

Although these experiments show that antibodies are of importance in the destruction of leukemic cells, bone marrow cells, etc., *in vivo,* they do not show that this results from a direct cytotoxic effect. Amos and Wakefield (1958, 1959) conducted a long series of experiments with a number of ascites tumors in a special type of diffusion chamber in mice that had not been actively immunized. They showed that both cytotoxic and cytostatic effects occurred. A limiting factor appears to be the concentration of complement. In contrast to what happens *in vitro,* fresh normal mouse serum can reinforce complementary activity *in vivo.* Using the neutralization technique, Winn (1960) found that the amount of complement had a great effect on neutralization of 6C3HED. Gorer and Boyse (1959) and Algire (1959) confirmed the essential correctness of the observations of Amos and Wakefield on leukemias in diffusion chambers in actively immunized mice. Sachs and Feldman (1958) found that cells of 6C3HED and MCIM taken from the peritoneal exudate in Swiss mice were apparently undamaged unless placed in guinea pig serum. They deduced that cytotoxic antibodies were not formed in "the homograft reaction." Their work was published before that on diffusion chambers but one would have thought the possible deficiency of com-

plement would have been considered. A brief note by Weaver (1958) had, in fact, described direct cytotoxic activity in peritoneal exudates with another leukemia. Even more surprising is a reiteration by Feldman and Globerson (1960) that cytotoxic antibodies are not formed. Samuel Johnson once gave the advice, "Never argue, repeat your assertion." It has not yet been shown that all types of normal reticulo-endothelial cells are destroyed by direct cytotoxic action; some, such as the resistant cells of bone marrow, may be taken up by normal histiocytes after contact with antibodies. Although humoral antibodies undoubtedly play a part in the rejection of the types of graft just mentioned, there is no doubt that cellular sensitization is also important. Any of the foregoing cell types can induce active immunity against other types of graft. Sensitization of the hosts' cells must play a part in the extensive exudation that can be seen in subcutaneous grafts of leukemias in allogeneic hosts (Gorer, 1958a, b, 1960b). In the case of intraperitoneal grafts, sensitized histiocytes may play the leading role early in graft destruction (Weaver, 1958; Amos, 1960).

While much remains to be learned concerning grafts of cells such as those just described, their general behavior shows a recognizable kinship with the reactions to infection. With all other types of graft the reactions are far more complex. Students of transplantation immunity have naturally drawn upon knowledge common to immunology in general. The debt has been repaid by the discovery of immunological tolerance and immunological enhancement. The general importance of the former has won universal acceptance and official recognition. It is not claimed that the latter is of such theoretical interest but its applications may be wider than generally realized (see Gorer, 1960b). The subject has been admirably reviewed by Kaliss (1958) who is responsible for the discovery that humoral antibody can enhance the survival of grafts that would normally be rejected. It is not an "all-or-none" phenomenon, and we may see nothing except a slight delay in regression, or a permanent, very indolent growth that kills indirectly by becoming infected, or a graft that grows more rapidly than one in the strain of origin, or numerous other variations. It is of great theoretical interest and possible practical importance that immunological enhancement has been demonstrated with normal tissues (Parkes, 1956; Billingham *et al.*, 1956b; Medawar, 1959).

It is not difficult to construct a theory of enhancement provided one concentrates on a single tumor–host combination. Kaliss (1958) considers that a direct action of antibody on the tumor is responsible for enhancement although he does not speculate upon the nature of the

effect. Feldman and Globerson (1960) take much the same view but are more specific. They consider that antibody stimulates the production of excess antigen in cells that are exposed. It may well be that sublethal doses of antibody or antibody acting upon resistant cells modifies these cells. It has been known for some years that antibody can stimulate mitosis (Gorer, 1942), and Kaliss advances other evidence in favor of some direct effect. It is by no means unreasonable to suppose that antigen excess may influence the outcome of an immunological reaction. None of these can explain enhanced survival of grafts of normal tissues. Kaliss (see Gorer and Kaliss, 1959), while not abandoning his ideas concerning direct modification of a tumor by antibody, agrees that some effect upon the response of the host must be considered.

Possible effects upon the cellular responses have been discussed by Gorer (1956, 1958a, b), Snell (1956), and Billingham *et al.* (1956b). All of these authors have used different words to express the idea that antibody interferes with the cellular responses of the host. Gorer (1958b) put forward the concept of "anergy," Snell (1956) preferred the verbal formula of "walling off." These terms sound very imposing but it may be a good idea to examine the stages at which humoral antibody might interfere with the cellular responses. If we have understood Snell *et al.* (1960) correctly, "walling off" amounts to a reduction of the amount of antigen reaching the regional lymph nodes thus reducing the magnitude of the cellular response. This may have occurred in the particular circumstances covered by them but it will not account for all cases. The amount of antibody that can result in enhancement is very small. With hyperimmune sera, it is certainly less than 0.001 ml. (Batchelor *et al.*, unpublished). On the basis of data supplied by Amos (1955), this amounts to a dilution of 1 in 2000–4000 which should be detectable at a titer of 2:8 by hemagglutination immediately after injection. If a half-life of 4–5 days is correct, little antibody will be left by the fourth day. With a simple "walling off" theory, it is difficult to see why there should be anything other than a slight delay in regression, but, in fact, these very small doses may result in fatal tumors. Another possibility is that the type of response given by an unsensitized host is changed if it first encounters antigen in combination with antibody. Data from other branches of immunology show that this suggestion is not unreasonable although no exact parallel suggests itself. In the present case we should expect the result to be the production of a humoral response at the expense of a cellular response.

While it seems very likely that enhancement may depend on effects very early in the homograft response, this does not abolish the possibility

that antibody *excess* may not also interfere with the action of sensitized cells, and there is a great deal of evidence that this is so. If one studies the morbid anatomy of enhancement in more than one tumor–host combination, one is bewildered by the variety of phenomena that may be seen. One may see a dramatic acceleration of growth and the animal may die without any cellular invasion of the tumor. One may also see growth of the tumor retarded for several days, but it ultimately grows and kills the host. On the other hand, there may be no visible difference between experimental and control groups for about the first 10 days. The regional lymph nodes do not differ in macroscopic or microscopic appearance. More important is the observation that the local cellular response may be obviously functional in both groups but that after a variable time the local reaction fails in the serum-treated group. This is consistent with the observations of Kaliss with Sarcoma 1 that serum administered as long as 10 days after grafting may inhibit the homograft response.

Both of these indicate that excess antibody can interfere with cells that have previously become sensitized. If immunity to grafts is transferred by cells from regional lymph nodes or spleen, this is usually referred to as "adoptive transfer." In the earlier experiments the immune cells and graft were inoculated remotely from one another. Winn (1960) and Klein and Sjögren (1960a, b) have recently revived a technique apparently first used by Kidd and Toolan (1950) in which immune cells and tumor were mixed prior to inoculation. We have used this technique together with intravenous inoculation of the immune cells to try to elucidate the interactions between immune cells and humoral antibodies. Some of the results have already been published (Batchelor *et al.*, 1960). In the case of B.P.8 inoculated subcutaneously, we have strong evidence that cells and antibody act synergically regardless of the route of inoculation of the sensitized host cells. Using the Kidd-Winn technique, we have found that synergy may be converted into antagonism by injecting B.P.8 and antibody into the peritoneum. We do not know why this reversal should happen but the fact that it does happen shows that antibody excess can interfere with the action of sensitized cells. This particular tumor grows much better in the peritoneum than in the subcutaneous tissues and must, therefore, produce antigen more rapidly, which again shows that reduction of the antigenic dose cannot be the only factor in enhancement. Using the same tumor, Gorer and Kaliss (1959) had pointed out that the intramuscular route gave both faster growth and a more rapid evolution of the homograft response than the subcutaneous route, and yet enhancement was more easily obtained following intramuscular injection.

It would seem that antibody excess may interfere with the immune responses in more than one way. The fact that such very small doses of antibody can be effective makes it seem rather unlikely that a simple reduction of antigenic stimulus can be of general importance and suggests that some qualitative change may be brought about by confronting unsensitized hosts with antigen combined with antibody. Thereafter antibody excess can interfere with the cellular responses for reasons that remain to be determined.

Although enhancement is easy to demonstrate with many tumor–host combinations, certain variables may have a profound effect upon the results obtained. It has long been known that sex plays an important role, although the reasons for this remain to be determined (see Kaliss, 1958). The importance of the genetic constitution of the host has also been recognized and the manner in which these variables complicate the results may be demonstrated by some experiments of Gorer and Kaliss (1959) using B.P.8. In the A strain, doses of serum of 0.2 ml. or greater inhibited growth of the tumor especially in females. Smaller doses might cause a delay in regression but all the animals survived. In the BALB/c strain inhibitory effects were less pronounced than in the A strain, particularly in males. Smaller doses of serum resulted in fatal tumors. If C57BL mice were tested with C57BL anti-B.P.8, no serum dosage effect was observed in either sex but all tumors regressed after reaching a large size. When A anti-B.P.8 was used at a single dose (0.2 ml.), there was no enhancement in males and there was inhibition in females. This suggests some qualitative difference between antibodies that may be genetically determined. In the case of Sarcoma 1 no inhibitory effects were found, but, of course, the A strain could not be used. Boyse *et al.* (1961) in a pilot survey of six carcinomata found a general tendency resembling the behavior of B.P.8. Unfortunately some of these tumors grew so badly in allogeneic hosts that it was impossible to tell if any inhibition occurred. With many tumors, including B.P.8, initial inhibition may be followed by enhancement.

Gorer (1958b) made a histological study of the behavior of arrested tumors. The serum does not appear to be cytocidal. For the first 3 days there is very little difference between treated and untreated tumors. In those cases (e.g., in A strain females) in which inhibition is permanent, the serum caused an almost complete inhibition of growth and there may be numerous mitotic abnormalities. There may be a precocious invasion by host histiocytes which begin to attack the tumor by about the sixth day. The process is quite unlike that seen in leukemias where the cells are very rapidly destroyed without any active intervention by the

hosts which are left fully susceptible to further challenge. Destruction of B.P.8 does require the active cooperation of the hosts and if this is successful they are immune to further challenge. The histological appearance of arrested tumors is very like that seen in some actively immunized hosts. They may show complete inhibition of growth, or some growth with numerous arrested mitoses. In the fully sensitized hosts, these tumors do not become vascularized. They may be invaded by host cells or the grafts may become infarcted with or without such invasion. It would seem that the cytostatic effects of antibody are important in graft destruction.

It is most unlikely that the effects of antibody dosage that may be observed result from the presence of "enhancing" and "inhibitory" antibodies. The minimum enhancing dose may be less than one-hundredth that of the inhibitory dose, and the enhancing effect should always predominate if two antagonistic antibodies are present. It would appear rather that the cytostatic effects of high doses of antibody may counterbalance any desensitizing effects of antibody. Quite often the cytostatic effects may wear off and enhancement ensue. It is very difficult to interpret synergy or antagonism between immune cells and serum when passively administered. We have not yet produced a cell suspension that consists entirely of cells with "cell bound antibody," all contain cells which can produce humoral antibody as well. When we get synergy, the cell suspension may be producing insufficient humoral antibody to give a full cytostatic effect. We have already referred to antagonism but a third effect may be seen that falls between the two. In some cases immune cells merely abolish the enhancing effect of serum. We cannot say that this shows antibody to be indifferent. It may be that the cells are producing an effective dose and we have not succeeded in bringing about antibody excess. It is quite possible that in some graft–host combinations the reactions are entirely cellular and that humoral antibodies are either indifferent or protective to the graft. At present all we can say is that antibody *excess* may favor graft survival.

The foregoing arguments apply pre-eminently to mice of different H-2 types, and with a single tumor we might find differences in behavior in different H-systems. The antibody response in the case of H-1 and H-3 is generally poor, and destruction may be more purely "cellular" than with H-2. As Kaliss (1958) notes in his review, enhancement has been shown in rats and rabbits and its possible occurrence should be borne in mind particularly since serum treatment of human cancer has reappeared in the literature (Murray, 1958; see also Southam, 1960). We know very little about the sensitivity of different tumor types to antibody

in vivo in species other than mice. Sekla and Barvic (1956) were able to produce passive immunity to the Walker rat carcinoma. The old observations of Lumsden *et al.* (1934) indicated that the Jensen rat sarcoma was sensitive to isoantibodies. Enhancement in mice is usually produced by isoimmune sera but many years ago Kaliss found that rabbit anti-mouse sera might do so. We do not know which antibodies are responsible, but the original antigen II from which H-2 was named was detected with immune rabbit serum (Gorer, 1936). Stimulation of growth is a risk that should not be neglected in man.

Southam and Moore (1958) studied grafts of certain human cultured cell lines in healthy volunteers and cancer patients. In the former the cellular response was predominantly polymorphonuclear. So far as I know this type of response has not been seen in homografts of other types in man or in any other species. In cancer patients regression might be delayed and the cellular response mononuclear. Grace and Kondo (1958) exchanged skin grafts and tumors between patients. As a rule regression of both occurred at a normal rate but delays sometimes occurred. It would appear that some depression of resistance may occur as a terminal event in some human cancers but it does not seem to be a general property of malignant disease. Kelly *et al.* (1960) showed that Hodgkin's disease is a special case since sufferers from it are generally anergic with respect to a large number of reactions of the delayed type. They may retain skin homografts for considerable periods. The results are not to be explained by cachexia since many of the patients were in good condition and patients with other types of malignancy do not show this pattern of reactions. Cultured human tumors are not ideal for studying the antibody response to homografts. If they are antibody resistant, direct cytotoxic effects cannot be observed, and there are no normal tissues of similar genetic constitution to enable one to perform hemagglutination tests, etc. However Aizawa and Southam (1960) coated tanned sheep red cells with extracts from four such tumors. Antibodies were detected but their significance cannot be assessed.

In summary we see that the interactions between immune cells and humoral antibodies are far from simple. Antibodies have been shown to be cytotoxic and cytostatic toward certain types of target cell. They may sometimes show a cytotrophic action on the hosts' histiocytes. However, if all grafted cells are not killed in nonimmune animals, immunological enhancement may occur. We do not know why cells and serum may sometimes act synergically and at other times antagonistically. No explanation or verbal formula put forward to date can be felt to be entirely satisfactory. A simple reduction of antigenic dose reaching the draining

nodes can hardly be sufficient since the doses of serum capable of producing enhancement are so small and since enhancement with a given serum dose occurs more easily where the tumor grows particularly well. It would appear that antibody may sometimes interfere with the sensitized cells but we do not know how. The possibility that serum may have a qualitative effect on the type of immune response should be kept in mind.

V. Genetic and Antigenic Analysis

Probably most people are now aware of the genetic behavior of transplanted tissues. The skins of mice from unrelated pure strains appear to differ from one another by about fifteen genetically independent antigenic systems (Prehn and Main, 1958; Barnes and Krohn, 1957). Nobody would claim that this figure is more than an approximation and Prehn and Main (1958) pointed out that a possible error might arise from additive effects between weak systems. Some recent work makes it appear probable that there may be synergic effects between strong and weak systems. Berrian and McKhann (1960) used three IR strains. Of these B10 was H-2b:H-3a, the second B10.LP was H-2b:H-3b, and the third B10.D2 was H-2d:H-3a. It will be noticed that the first two differ only at H-3, whereas the second and third differ at H-2 and H-3. If the difference was confined to H-3, it was found that H-3a splenic cells were very inefficient at immunizing H-3b hosts against a subsequent skin graft. However, if splenic cells of the constitution H-2d:H-3a were used, immunity was induced to a skin graft that only differed at H-3. It should be pointed out that this effect was found with spleen and not with skin as the immunizing agent.

Some further evidence comes from our experience with crossover stocks. The crossover that ultimately gave rise to the H-2g stock was found in a backcross (BALB/c $\times$ C57BL) $\times$ BALB/c. The original animal was again crossed with BALB/c and the progeny of the required H-2 type bred inter se to obtain homozygotes. This means that the H-2g type has about 87.5% BALB/c genes. The history of the second type (H-2h) is rather more complex but it has about 43.75% C57BL genes, 6.25% A genes, and 50% BALB/c genes. The last H-2i has about 87.5% C57BL genes since it was formed following two backcrosses of (A $\times$ C57BL) to C57BL. Both the former two give a good antibody response to C57BL leukemias and seem as resistant as any unrelated strain. Not only are H-2 antibodies formed readily but anti-X antibodies as well (see Section VI). However the H-2i stock is extremely susceptible, and the antibody response is very poor and they seem unable to form anti-X's. Reference to Table II shows that this type can only respond to the

"D-region" of H-2b and perhaps this is less antigenic than the "K-region." This argument breaks down because strains STA and 129 which are both H-2b respond very much better than H-2i. As has been pointed out previously, H-2d and H-2k appear to share between them all the antigens of H-2a, and resistance and antibody production between strains A, C3H, and BALB/c are perfectly efficient. It, therefore, appears that the general genetic background must play some part. If one wished to make an IR strain with H-2i on a C57BL background, using tumor resistance as an indicator of antigenic difference, one would need to immunize with normal tissue prior to challenge. It would seem desirable to test other H-2 combinations that are introduced into a new strain by serological typing of backcrosses. This offers no great technical difficulties, and, if only two backcrosses were needed to unmask the synergic effects of minor antigens, the experiments need not take very long.

In several previous publications we have pointed out that the genetic behavior of tumors is not a completely reliable index of antigenic constitution (Gorer, 1938, 1956; Gorer *et al.*, 1948). There can be no doubt that tumors may grow and kill in the presence of potentially useful antigenic differences and this is most easily shown by preimmunizing prospective hosts with normal tissues from the same strain as the tumor. Using this approach, Amos *et al.* (1955) found that the gene requirements of the C57BL leucosis E.L.4 might be changed from less than one to about eight in a cross with BALB/c. We expressed doubts whether E.L.4 had lost any isoantigens, but because of the results obtained with skin grafts this view seems untenable and some antigens must have been either lost or so diminished as to be ineffective. This seems to be reinforced by the results obtained in a cross between C57BL and A, where in immunized hosts the number of independent systems appeared to be about five. In a small experiment with an A strain sarcoma, preimmunization gave an increase in "takes" but the number of factors was again much less than would be expected for normal tissues. The studies of Linder and Klein (1960) point in the same direction. It will be recalled that they found that strain A/Sn skin differed from its IR partners by a number of histocompatibility factors and that the latter were heterogeneous in this respect. Tumors, however, behaved as if the IR partners differed at a single locus, even if preimmunized hosts were used, and it seems as if they must have lost a number of H-factors. It is possible that the tumors originally used by Snell were really more complex and this may have militated against obtaining a difference due to a single antigenic locus. Doubts on this point emphasize the need for establishing IR lines by serological means.

The direct demonstration of isoantigenic loss in mice is limited to the H-2 system. References to earlier work can be obtained from Gorer (1956) and Amos (1959). Loss of H-2 antigens may be complete in some cases but in others this is not so. During the study of the cytotoxic reactions, O'Gorman and Mikulska (1960) found that Sarcoma 1 growing in our A strain had apparently lost antigen E. Antigens F and K were diminished in amount while D was apparently unaffected. Amos (personal communication, 1960) did not find these variations in Sarcoma 1 tested in Buffalo. It appeared that Sarcoma 1 was not completely compatible with our A's, but it seems difficult to see why these particular antigenic changes should have occurred.

The foregoing losses were all observed in homozygotes, and the Kleins and various associates have studied a number of F_1 tumors particularly those derived from crosses among the IR quartette A/Sn, A.SW., A.BY, and A.CA. Their material included methylcholanthrene-induced sarcomata, leucoses, mammary carcinomata, and a few estrogen-induced interstitial cell testicular tumors. The last have not yet given rise to any variants. Several types of variants were seen. Some tumors would grow in a variety of genotypes unless the hosts were preimmunized. The most interesting variants were those in which a loss of the H-2 antigens from one parental strain could be demonstrated. The liability to produce variants is not the same for all tumors. Some produced no variants, others might produce variants in which either parental type might be lost, while in others there was a tendency for one particular parental type to be lost. This was particularly so in those crosses where strain A was a parent in which case H-2a antigens tended to be lost more frequently than either H-2s, H-2b, or H-2f. Bayreuther and Klein (1958) studied a sarcoma arising in the $(A \times A.SW)F_1$ that produced a number of variants compatible with A.SW because of loss of H-2a antigens. From a study of the karyotypes of variants, they came to the conclusion that each new variant was the result of some independent change. In other words, these variations were not as a rule to be regarded as examples of the repeated selection of a single variant although this did appear to have occurred once. If one set of H-2 antigens are lost, the variant should be able to grow in immunized hosts. This was not always so, sometimes they needed to be passaged first in nonimmunized hosts before they could do so (Klein and Klein, 1959; Klein *et al.*, 1960). It is not clear why this should be so, possibly additional variants had to be selected before full compatibility was established.

It should be emphasized that from the beginning of the work rigorous serological tests were performed to make sure that antigens had really been lost. More recently Hellström (1960) has reported on variants

occurring in F_1 leucoses. With "solid" tumors, antigenic loss may be shown by failure to absorb antibodies together with failure to induce them. With leucoses, the loss may be demonstrated directly by their loss of susceptibility to the cytotoxic activity of pertinent antibodies.

The nature of these changes has been carefully considered. We have no acceptable examples of genetic mutations in the H-2 system (Amos, 1959; Gorer and Mikulska, 1959). It is not inconceivable that a chain reaction should occur in the chromosome changing the whole system. However, until we have such knowledge, it seems unwise to call the losses seen "mutational." Further as Klein *et al.* (1960) point out, true mutations should result in antigenic change, not simple loss. Experiments designed to detect variants with new components were performed by Klein and Klein (1959) but none were successful. It has been known since the earliest work on H-2 antigens (Gorer, 1938) that they show dosage effects in heterozygotes. Hellström (1960) found that his leucotic variants that had lost one parental H-2 combination gave strong reactions with antibodies directed against the remaining combination, suggestive of homozygosity. This could be brought about as a result of somatic crossing over. It is not clear how loss of H-2 components in homozygous stocks can be explained, especially partial losses. These, unlike the losses found by Hellström (1960) with leukemias arising in F_1 mice, are always associated with a high degree of heteroploidy, and some possible explanations of loss due to heteroploidy have been given previously (Gorer, 1956).

Barrett and Deringer (1950) found that if a tumor arising in a pure line were passaged once through an F_1 between it and another pure line, its genetic behavior was permanently changed. In their original work, this change could be interpreted as antigenic simplification. It was difficult to interpret this in terms of mutation and selection since all H-systems should be present in the F_1 host. Klein and Klein (1956b) investigated the nature of the change. A selective change became more unlikely when it was found that an apparent increase in complexity might sometimes occur. The selection of a mutant demands the presence of a large initial population, but the Barrett-Deringer type of change could be rapidly obtained with as few as 70 cells inoculated into newborn mice. The use of diffusion chambers showed that contact with host cells was not necessary and the change could sometimes be obtained by exposure to serum *in vitro*. As we have mentioned before, the change is a stable one. It may be that quantitative antigenic changes occur but deletion does not, since genetic tests performed in preimmunized hosts abolish manifestation of the modification (see Hellström, 1960).

The types of changes just outlined occur under conditions that may be described as "artificial," but few if any experimental procedures can avoid this charge. At least they show that malignant cells may undergo more than one type of modification. Orthodox "mutation selection" has been used to account for drug resistance (Law, 1958) and the emergence of ascitic forms from "solid" tumors (see Klein, 1959, 1961; Klein and Klein, 1956a), but it may not explain all modifications found in the evolution of autochthonous neoplasms.

VI. Intrastrain Immunity and Allied Problems

There are a number of claims concerning the detection of "tumor-specific antigens" in various species. Some of these are certainly isoantigens, but some others are probably perfectly genuine. Until fairly recently geneticists were extremely skeptical of such claims other than those that could be associated with viral infection, and attention was focused upon the question of antigenic loss.

Foley (1953a,b) was the first to use inbred strains successfully to demonstrate antigenic change other than simple deletion. In his first paper, he tried to immunize C3H mice against isogenic grafts of mammary tumors. The circulation was gradually occluded by tightening a ligature and after the graft had sloughed, animals were rechallenged with the same tumor. In no case was immunity demonstrated. However, immunity was demonstrated when he performed similar experiments with methylcholanthrene-induced sarcomata. The earlier experiments with mammary carcinomata served as good controls for the experiments with sarcomata. Baldwin (1955) obtained confirmatory evidence in rats. A very thorough investigation was done by Prehn and Main (1957) in mice. They showed that simple excision was as effective as strangulation of the blood supply. They found that tumors induced within a given strain might induce some cross immunity to other tumors of the same strain. Spontaneous sarcomata, histologically indistinguishable from induced sarcomata, did not induce intrastrain immunity. Animals immunized against a chemically induced tumor would accept skin grafts from tumor donors. After several transfers the ability to induce intrastrain immunity might be lost.

The study of chemically induced sarcomata has recently been carried further by the Stockholm group. They have made use of cells irradiated with doses of the order of 5000 r, which remain viable but incapable of cell division, to study immunity. They have also made use of a frozen tumor bank. In the earlier experiments, if an animal was immunized against the first transfer of a tumor, it had to be challenged with a tumor

from the second or later transfer, which is unnecessary if a tumor bank is available.

Using irradiated cells, Revesz (1960) had no difficulty in inducing immunity against methylcholanthrene-induced sarcomata but was unable to do so with recently arisen mammary carcinomata or leucoses. It was possible with one very old leucosis and one very old mammary tumor but this only serves to emphasize the need for caution in the interpretation of data in such cases. The situation was finally clinched by Klein *et al.* (Klein, 1961) who showed that immunity could be obtained with autochthonous tumors. Methylcholanthrene was inoculated into the thigh muscles and when a tumor appeared the limb was amputated. Subsequent challenge showed that the animal had become immune. Usually the immunity was less pronounced in primary hosts than in isogenic hosts but occasionally the reverse was found.

Klein and Sjögren (1960a, b) studied the nature of the immune reactions involved. In allogeneic hosts, as is known, lymph node or spleen cells can induce immunity if injected with the tumor or remote from it. The effects of antibodies on such hosts have already been described but enhancement can almost invariably be demonstrated. In isogenic hosts, Klein and Sjögren could detect no kind of effect with serum. Cells were only effective if mixed with the tumor. Strangely enough normal lymph node cells might have some effect.

Evidence of a different type has accrued from a study of leucoses. Neoplasms of this class are highly susceptible to the cytotoxic activity of isoantibodies *in vivo*. Gorer and Amos (1956) found no difficulty in demonstrating passive immunity to the chemically induced C57BL leucosis E.L.4 in allogeneic hosts. It came as a surprise to find that passive immunity could also be demonstrated in isogenic hosts provided E.L.4 was used as antigen. Sera prepared against normal tissues and against a C57BL mammary carcinoma were capable of protecting allogeneic but not isogenic hosts. It appeared, therefore, that we were dealing with two sets of antibodies, those against H-2 antigens and those against another antigen(s) which we called X. Since E.L.4 had been transplanted for many years before our experiments were performed, we naturally considered the possibility that the C57BL had been H-X/H-X when E.L.4 arose, but since that date a mutation had occurred from H-X to H-x and the "X effect" was due to the fact that E.L.4 had remained H-X while C57BL were now H-x/H-x. If this were so, it might be expected that C57BL or any F_1 hybrids derived from it could be actively immunized against E.L.4 but this proved impossible. A second apparent objection against the mutation hypothesis was obtained from a genetic

experiment. In a genetic study of E.L.4 in a cross (C57BL × BALB/c) × BALB/c mice were typed into two groups, H-2b and not H-2b. The latter group as expected formed anti-H-2b and anti-X. The former group formed antibodies against other H-systems that gave some protection in allogeneic hosts but did not form anti-X. We deduced that, if C57BL mice were now H-2x/H-2x, both groups should be able to form anti-X. Our reasoning may have been faulty here but fortunately our conclusions have not been upset. Admitting that "X" was not simply the result of an H-mutation, it still remained to be seen whether E.L.4 was a freak or whether X-components were common in leukemic cells. Amos and Day (1957) demonstrated X-components in three other leukemias in strains A, C3H, and DBA/2. They also showed that anti-X was in the α and β-globulin fraction whereas anti-H-2 was to be found in the γ-globulin fraction. This result was encouraging, but the authors did not investigate the antigenic relationship between the different X components.

Gorer (1959, 1961) studied four further leucoses in C57BL and one in strain A. Three of the former (E.L.5, 7, and 8) were chemically induced, while the C57BL E.L.6 and the A strain A.L.3 were spontaneous. Sera produced against E.L.4 were absorbed with the other C57BL leucoses in their earliest transfers. Of these both E.L.5 and E.L.7 absorbed some protective activity against E.L.4 but in neither case as effectively as the homologous antigen. Neither E.L.6 nor E.L.8 did so to an appreciable extent. Subsequent passive immunity experiments tended to confirm the results obtained with absorption experiments. The X-component of E.L.5 is of very low antigenicity; in fact, it is easier to protect with sera prepared against E.L.4 than with those against the homologous leucosis. In all other cases, protection is strongest against the homologous antigen. The E.L.4 seems to have a far higher proportion of X than any other leucosis. One may obtain a definite delay in the onset of lesions of E.L.4 with as little as 0.05 ml. of serum and exceptionally complete protection with 0.1 ml. With all other leucoses, 0.5 ml. is used as the standard test dose. Good anti-E.L.4 sera will give some protection against any of the others. This is weak in the case of E.L.6 and E.L.8 and is fairly strong in the case of E.L.5 and E.L.7, but in no case is protection as good as against E.L.4 itself. Although E.L.6 and E.L.8 show weak cross reactions with anti-E.L.4, they are distinct from one another and from the other three C57BL cases. It would seem that these X-components are antigenically related but no two seem to be exactly the same. The A strain A.L.3 was successfully tested for the presence of an X antibody at the fifth transfer. It is obvious that the X component must have been present at the fourth transfer at the latest. This, in conjunc-

tion with the absorption experiments, encourages the conclusion that X-components are not transplantation artifacts. Recently Miller (1961) has described X-components in two leukemias induced in C3H mice by Gross's Passage A virus. It is possible that Goldner *et al.* (1959) have discovered an X-component in a rat tumor but more data are needed in this species.

Before discussing the nature of the X-components it will be of help to consider some further data on the conditions under which anti-X's may be formed. We have already mentioned the work of Revesz (1960) with heavily irradiated leukemic cells. Boyse (unpublished) performed a similar experiment with A.L.3 after its X-component had been demonstrated. The result was precisely the same as that obtained by Revesz. It is possible that X-components are sensitive to the doses of radiation given, but it does not appear that this is the correct explanation. We have studied the various C57BL cases in our crossover stocks. As we have pointed out earlier, of these H-2G and H-2H have a minority of C57BL genes. Both produce good anti-X sera; indeed, some have been extremely good. However H2I which is predominantly C57BL would produce neither anti-E.L.4X nor anti-E.L.8X. This is unlikely to be due to the portion of the H-2b complex against which it can react. Dr. M. Simonsen kindly let us have some STA mice which are H-2b. They formed a good anti-E.L.4X. It may well be that anti-X is formed more easily in the presence of H-2 antagonism than in its absence, but this does not appear to be of final importance.

At one time I was inclined to favor the idea that "X" was a viral antigen. It may be a viral product but is not likely to be a viral component. It may be that all mouse leukemias are of viral etiology, but it seems rather unlikely that every leukemia arising in a small nucleus of one inbred strain should be caused by an antigenically distinct virus. It would seem, therefore, rather more likely that X components are modified normal tissue components, and it is not unlikely that all the E.L.X's derive from some common precursor.

There is a certain family resemblance between the X-components of mice and the human macroglobulins. There is dispute whether these are simply quantitative increases or whether qualitative changes occur as well, particularly in myelomatosis. The subject has been recently reviewed by Owen and Gott (1960) and cannot be considered here in full detail. The situation differs from that given in the foregoing in that antibodies were always formed in foreign species (usually rabbits). Not all people used the same techniques but there are other reasons for disagreement. It is possible that some macroglobulins have acquired new

determinant groups whereas others have not. It is further possible that not all rabbits have responded to the differential groups. Not all X-components are equally antigenic and different batches of sera show different degrees of specificity, so we should probably give more weight to positive than to negative results. The main point of resemblance is that abnormal macroglobulins may be shown to have individual specificity. Korngold and van Leeuwen (1957a,b) conclude that abnormal macroglobulins may lack certain determinant groups but have others peculiar to each patient. Mehrotra (1960) has published a preliminary report on macroglobulins found in patients with the cold agglutinin syndrome which also show individual specificity. This individual specificity is reminiscent of the X-components.

Curtain (1959) investigating the problem of macroglobulins made rabbits tolerant to normal human plasma and found that they were also tolerant to pathological macroglobulins. Cinader and his co-workers have investigated tolerance to diazotized serum proteins. Among rabbits made tolerant to normal human serum albumin the majority were also tolerant to diazotized human serum albumin (Cinader and Dubert, 1956; Cinader and Pearce, 1958); thus Curtain's work is far from conclusive. It may well be that the X-components of leucotic cells result from modifications of normal tissue components that are not sufficiently great to overcome the natural tolerance to the normal precursor.

It is possible that the methylcholanthrene (m-c) antigens are similar in nature to the X-components but represent a more extreme modification of a normal constituent. Prehn and Main (1957) in discussing the significance of the m-c antigens regard them as epiphenomena since they are not essential to malignancy and, even if present initially, may be lost during transplantation. In their view, the antigenic changes are merely one of many modifications brought about by carcinogenic chemicals. They are able to persist in such tumors because methylcholanthrene has a strong depressive action on the reticulo-endothelial system. In the case of mammary tumors, etc., no such depression precedes the appearance of tumors and such antigenic variants would be destroyed before they could become established. This has the great merit of being the only rational explanation put forward to explain the difference between spontaneous and induced tumors. However it does not explain why active immunization has failed with induced leukemias nor does it explain why m-c antigens are so common. Even if the reticulo-endothelial system is depressed, one would expect selection to favor the less extreme variants. Perhaps the more extreme variants determine some other selective advantage that overrides the disadvantage of potential incompatibility.

If it is true that the X-components represent a less extreme modification than the m-c antigens, the question naturally arises whether other spontaneous tumors have something akin to X. Unfortunately we have no ready means of testing for them. It is true that large doses of serum may inhibit solid tumors in allogeneic hosts, but even here it is not of universal occurrence. Immunological enhancement can only occur if there is a potential resistance to be depressed and this does not apply here. Mitotic stimulation may accompany enhancement, but it is not a manifestation of depressed host resistance. It has been seen from time to time with weak anti-X sera but is of irregular occurrence. A few attempts to demonstrate it with mammary tumors in the strain of origin were unsuccessful. It may be that foreign species will have to be used where neither active nor passive immunization of the home strain are possible. Zilber (1959, 1961) has studied the antigenic constitution of a C3H hepatoma by gel diffusion techniques with antibodies produced by rabbits. He has succeeded in detecting a tumor specific antigen by this means. It is not possible to say to what category the antigen belongs. The tumor was induced by dimethylaminoazobenzene, and it may, therefore, be homologous with the m-c antigens. On the other hand, we do not know if the substance is antigenic for mice.

It will be seen that the concept of the malignant cell as outlined by immunogenetics has changed in the last few years. Antigenic losses occur and some degree of loss may be universal. On the other hand, qualitative changes are also common and, in fact, have been shown in all types of tumor where suitable techniques have been available. Lastly some types of tumor have been shown to be potential autoantigens. How far these findings may be applied to man remains to be seen but at least the picture is no longer one of unrelieved gloom.

VII. Some Problems of Human Tumors

The immunology of human tumors presents problems that differ from those encountered in animals. Some of these are self-evident but others are less obvious. Unless one uses autopsy material, the mass of tumor one can obtain is often very small and this makes it very difficult to produce antibodies against the tumor of a live patient. It is also difficult to obtain normal homologous tissue from the same patient with the tumor or in some cases from any source. With the exception of lactation cancer, it is virtually impossible to obtain normal mammary gland from a patient with mammary carcinoma. The best tumors to use from this point of view would seem to be hepatomata which are rare in Europe and America or

skin tumors which are usually removed when small. In spite of these difficulties a considerable amount of work has been done. Some of the earlier studies have been previously considered by the writer (Gorer, 1938), and the Russian work has been reviewed by Zilber (1958, 1959). Here we will concentrate on more recent work.

Makari (1955) adopted an original approach to the problem. He did not look for antibodies or did he search for cellular antigens but instead sought a circulating antigen. He inoculated female guinea pigs with normal human plasma and plasma from cancer patients. As expected, the uteri from both groups gave a positive Schultz-Dale test with normal plasma. However, those that had been sensitized with plasma from cancer patients continued to react with their plasma after being desensitized to normal plasma. This work was confirmed by Burrows (1958), by Hauss (1960), and with partial success by Hackett and Gardonyi (1960). McEwen (1959) did not confirm Makari's results. Burrows (1958) found that plasma from nephrotic patients gave the same reactions as those from cancer patients. In work of this kind it is fair to place more weight upon confirmation than failure. Even within a species one may not always obtain the antibodies one wants and this is more likely to occur with foreign species as anybody with experience can testify. The finding of Burrows is of theoretical interest but does not necessarily detract from the potential value of the test. It remains to be seen whether it is of practical use. It is of interest that Miller and Bernfeld (1960) have found a new serum component in C3H mice with mammary carcinomata by a gel diffusion technique.

Björklund and his co-workers have obtained results which if authenticated have revealed an entirely novel immunological situation. Björklund (1956) immunized a horse with pooled tumors obtained at autopsy from 56 different patients. Using a gel diffusion technique he found that a number of antibodies were formed against components of plasma. In addition four "strictly cellular antigens" were found in the tumor pool. Of these three were shared by 16 normal human tissues but one was found in the tumor pool and in spleen, lung, kidney, and brain. In some ways these results are not unlike others reported by Korngold (1957). Subsequently Björklund *et al.* (1957) tested the same horse serum absorbed with plasma on fresh cultures of cervical carcinomata and normal vaginal and endometrial epithelia in the presence of guinea pig complement. It was not clear from the aforementioned paper how many tumors were tested, but a subsequent paper by Björklund and Björklund (1957) gives the number as 6. It is surprising that no antibodies directed against the "strictly cellular antigens" of Björklund (1956) showed any

cytotoxic activity against normal tissues. It is also unusual in a cytotoxic system to find changes develop slowly, but in this case they were observed first after 24 hours. Björklund and Björklund (1957) immunized two further horses but dispensed with guinea pig complement. They again absorbed with plasma before testing on fresh cultures of 4 tumors, on HeLa cells and on certain normal tissues. The antibodies also appeared specific for malignant cells. The authors point out that the immunological responses of the horses were unusual. The highest cytotoxic titers to be obtained were 1:40 and this fluctuated during immunization. The fact that neutralization with plasma removed all species-specific cytotoxic antibodies is not commented upon. Absorption with normal tissues did not abolish the cytotoxic action on HeLa cells whereas absorption with the tumor pool did so readily. The slowness of the cytotoxic effects makes it seem unlikely that complement is involved and according to Björklund (1960) it is not. According to him, both human and horse serum contain a thermostable substance that is lytic for established cell lines of both normal and neoplastic origin but not for recently isolated cultures of normal cells. This lytic substance is normally combined with an inhibitor and instructions are given for separating the two by curtain electrophoresis. It is suggested that antibody after combination with antigen dissociates the lysin–inhibitor complex. The immunization of horses takes some time and not all may respond in the same way, but it should not be too difficult to obtain independant evidence on the existence of this new lytic system.

The possibility of autoimmune reactions in cancer has always fascinated students of the disease. Previous to the work discussed in the preceding section, the only experimental data to support the idea came from the observed regressions in virus-induced neoplasms (see Gorer, 1938). Spontaneous regression of human neoplasms is unfortunately too rare to form a basis for investigation. Remissions in acute leukemia are commoner, and Bernard *et al.* (1954) found that patients in remission might give skin reactions with extracts of leukemic cells. It is well known that some neoplasms are accompanied by intense inflammatory reactions. Grace and Kondo (1958) investigated eight such cases. Positive skin tests were given by extracts of autochthonous tumors but not by extracts of autochthonous normal tissues. The sensitivity could be transferred by the patient's serum. This is an extremely interesting study, but it must be remembered that tumors of this type are exceptional and we do not know the nature of the antigens concerned. Graham and Graham (1955, 1959) have attempted to demonstrate autoimmune reactions principally in patients with carcinoma of the cervix. In twelve of forty-eight patients with

"gynecological" cancer, complement fixation was found with aqueous extracts of autochthonous tumors. Most of the reactors had a favorable prognosis. Later Graham and Graham (1959) gave a series of patients intradermal inoculations of partially disintegrated malignant cells in Freund's adjuvant. In only a few cases did nodules occur at the inoculation site. They were inclined to think that some patients benefited, particularly those with incompletely excised lesions. They also thought that the vaccine might improve the response to X-ray therapy. It cannot be said that the results obtained are particularly impressive but they have obtained some support from the work of Finney *et al.* (1960). They studied two groups of patients with advanced malignant disease. The first group of five patients had undergone X-ray therapy. The second group of nine patients were given intramuscular injections of their own tumors in Freund's adjuvant after the material had been frozen and thawed three times. The test for humoral antibodies involved the employment of the tanned red cell technique of Boyden (1951). In the irradiated group, three patients showed a rise in titer (up to 640 in one case). Two of these had reticulum-celled sarcomata and one lymphosarcoma. Both of the former were alive at the time of publication, approximately 2 years after irradiation while the fate of the third was unknown. The other two patients with carcinomata had died. Of particular interest in the second group were three patients with subcutaneous metastases, one of whom had malignant melanoma, the second reticulum celled sarcoma, and the third Hodgkin's disease. All nine patients showed a marked rise in the titer of agglutinins. In those patients with subcutaneous lesions, the authors noted marked inflammatory changes from 15 to 25 days following the primary injection which lasted from 2 to 7 days and this was followed by softening and shrinkage of the deposits. Antibody was subsequently injected into the subcutaneous deposits which showed shrinkage. All three patients were dead within 4 months.

Anticancer sera are always with us. However, it is unlikely that they will ever be of therapeutic value. Amos (1955) found that antibody administered to allogeneic hosts with E.L.4 had no effect upon the growth of an *established* tumor although establishment can be prevented (Gorer and Amos, 1956). Gorer *et al.* (1959) showed that very little antibody reaches an established tumor until an inflammatory reaction occurs. This happens automatically about the sixth day in most homograft responses. We are unable to elicit such reactions around autochthonous tumors. It would be of great benefit if we were able to do so (see, however, Finney *et al.*, 1960).

VIII. General Discussion

It is sometimes said that there are two alternative causes of cancer, viruses and somatic mutation. Schultz (1960) points out that malignancy involves a change in cellular heredity and that such changes involve some type of nucleic acid and, therefore, some form of mutation hypothesis is obligatory. Somatic mutation is favored by a number of very eminent investigators but some feel a certain reserve because it tells us nothing of the nature of the differences between normal and malignant cells. The simplest form of malignancy is that shown by a rodent ulcer which shows unlimited growth and local invasiveness. It is known that uncontrolled growth can occur without invasiveness, and malignancy must, therefore, depend on at least two modifications and ability to metastasize would appear to involve a third change. Other important changes are loss of response to hormones, resistance to X-rays, etc. By the time that a patient succumbs to a neoplasm, the cells will have undergone a number of changes since they first departed from normality. The work of the Kleins, already described, makes us doubtful if all these changes should be described as mutations. This problem is of less importance than that of discovering how these changes modify cellular behavior.

Within recent years, a number of workers have tended to regard malignant cells as defective cells. Pre-eminent among those who have helped to establish this view are James and Elizabeth Miller (Miller and Miller, 1952). They found that the livers of rats treated with dimethylaminoazobenzene gradually lost the power to bind the dye and that malignant cells were unable to do so. It would appear that uncontrolled growth could be due to the loss of certain cellular constituents that enable normal cells to respond to regulative stimuli. Local invasiveness is less easy to understand in terms of deficiency, but metastasis is probably due to the failure of malignant cells to adhere to one another as strongly as normal cells. The attractiveness of this concept is that it enables us to see how malignancy could be brought about by a number of different agents. One can envisage a virus such as the Rous No. 1 depriving the cells of essential receptors immediately. In the case of dimethylaminoazobenzene, the deprivation appears to be gradual and may be prevented by various dietary supplements (see Miller and Miller, 1952). It is also possible that the essential deprivations may be brought about by changes in one or more genes or at some other level in the cell. A car may fail to stop because something has gone wrong with the driver, the brakes may fail, or the throttle may not close properly.

We have seen that loss of isoantigens is extremely common and may be universal but it is impossible to say what the significance of the loss might be in terms of cellular behavior. Since it is likely that some of the receptors are organ specific, it is tempting to seek whether loss of any organ-specific substance is associated with malignant change. Seligmann *et al.* (1955) have shown that leukemic cells may lack certain constituents present in normal leucocytes. A more extensive investigation has been done by Weiler (1952, 1956a, b, 1959) in rat hepatomas and estrogen-induced renal tumors in hamsters. In a series of papers, King *et al.* (1958) have expressed doubts that the results found by Weiler are valid. Weiler's controls seem to be adequate and his results have been confirmed by Zilber (1961) and to some extent by Nairn *et al.* (1960). However, doubts may be felt as to whether Weiler's antigenic deletions are essentially connected with malignant change since he has shown (Weiler, 1959) that the renal antigen of hamsters is rapidly lost in tissue culture. The term "organ-specific antigen" is probably a gross oversimplification and we are very far from knowing if any essential deletion has yet been detected by serological means. H. N. Green (1958) lays great stress upon the loss of organ-specific antigens in his immunological theory of cancer. Even if lack of some cell component had been identified by immunological techniques, we should not be justified in advancing an immunological theory of carcinogenesis. The detection of γ-globulin deficiency by electrophoresis does not justify an electrophoretic theory of immunity. An immunological theory of carcinogenesis could be justified if it could be shown that regulation of normal growth depended on some type of antigen–antibody reaction, and we have no reason to suppose that this is so. It might also have some validity if it could be shown that some kind of antigenic gain were an essential feature of malignant change but that clinical malignancy only occurred after resistance had broken down. It is conceivable that this is true in chemical carcinogenesis in mice but this does not afford a very solid basis for any theory at the present time.

While it seems most probable that malignancy does depend on some type of cellular deficiency, it does not seem that we can explain all phenomena in such terms. Autonomy may sometimes result from loss of receptors but apparently not always. Klein and co-workers (Klein, 1961) found that testicular tumors showed hormone dependence when small, but none when larger. A somewhat similar result was found in a genetic experiment with a DBA/2 leucosis by Hauschka and Amos (1957). It showed no dependence in the strain of origin, but did show some if its growth was slowed by being transplanted to partially incompatible hosts. These would appear to be examples of the throttle sticking rather

than loss of brakes. Immunology does not encourage the idea of malignancy being simply an example of deficiency as we have seen; we seem to be dealing with antigenic change rather than pure deletion.

As was pointed out in Section I, immunology can contribute to cancer research in numerous different ways. As a research tool it has uses for studying differences between malignant cells and their normal precursors and hence of tumor progression. The possibility that autoimmune reactions may be of clinical importance is certainly worthy of further investigation. Experience with murine tumors suggest that not all human growths may be influenced by such reactions. Anticancer vaccines do not appear to offer a very promising prospect and anticancer sera still less so. In the study of new antigens in tumors, it may be advisable to follow Björklund in using polyvalent antigens. The use of single tumors has shown that most have determinant groups unique to the individual tumor. Polyvalent antigens might reveal a number of shared determinant groupings. It is possible that knowledge of novel antigenic components may ultimately be of help to chemists in designing therapeutic substances.

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AUTHOR INDEX

Numbers in italics show the page on which the complete reference is listed.

C

D

E

I

J

Y

Z

SUBJECT INDEX

T