ADVANCES IN IMMUNOLOGY

Volume 2

W. H. Taliaferro &
J. H. Humphrey

ADVANCES IN

Immunology

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Immunology

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VOLUME 2

1962

ACADEMIC PRESS New York and London

ACADEMIC PRESS INC.
111 FIFTH AVENUE
NEW YORK, NEW YORK 10003

United Kingdom Edition
Published by
ACADEMIC PRESS INC. (LONDON) LTD.
BERKELEY SQUARE HOUSE, LONDON W. 1

Library of Congress Catalog Card Number 61-17057

First Printing, 1962
Second Printing, 1965

PRINTED IN THE UNITED STATES OF AMERICA

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PREFACE

The second volume of *Advances in Immunology* continues the main objective of the series—to present timely reviews on different aspects of immunology, broadly defined to include not only immunochemistry but all biological aspects of acquired and innate immunity.

Two reviews in the present volume are of especial interest to immunochemists. In the first one on "Immunologic Specificity and Molecular Structure," Fred Karush formulates the basic concepts for understanding immunologic specificity and stresses the probable importance of apolar interactions in the antigen-antibody reactions. In the second one on "Heterogeneity of γ-Globulins," John L. Fahey gives a detailed physicochemical and immunologic characterization of the four classes of globulins in normal and neoplastic plasma cells. Three reviews cover widely different aspects of antibody formation. In "The Immunological Significance of the Thymus," J. F. A. P. Miller, A. H. E. Marshall, and R. G. White discuss the effect of neonatal thymectomy in suppressing subsequent immunologic responsiveness and the extent to which immunologically competent cells originate in the thymus. In addition, they draw attention to some clinical conditions in which thymus cells may be involved in auto-allergic responses. Chapters on the "Cellular Genetics of Immune Responses" by G. J. V. Nossal and on "Antibody Production by Transferred Cells" by Charles G. Cochrane and Frank J. Dixon review the rapidly increasing knowledge concerning the dynamic interrelationship of precursor cells, antibody-forming cells, and the time-sequence of antibody production. Two reviews are concerned with mechanisms of immunity. In "Phagocytosis," Derrick Rowley considers the factors involved in phagocytosis and intracellular digestion, and in "Antigen-Antibody Reactions in Helminth Infections," E. J. L. Soulsby reviews a field that has been too largely neglected by immunologists. The review by Reed A. Flickinger on "Embryological Development of Antigens" is not only of great intrinsic interest, but illustrates the recent increasing use of immunologic methods in other biological disciplines.

Again, the editors wish to express their appreciation to the contributors. To write comprehensive reviews at any time requires much effort and judgment, and this is especially true for a subject that is developing as fast as is immunology at the present time.

November, 1962

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CONTENTS

Immunologic Specificity and Molecular Structure[1]

FRED KARUSH

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I. Introduction

The origins of the concept of immunologic specificity lie buried in the distant past. The notion of specificity was nurtured by the common-sense observations made over many centuries that individuals acquired immunity against a particular disease, e.g., smallpox, following recovery from that disease. It was not, however, until the last decade of the 19th century that the phenomenon of immunity was subjected to significant experimental exploration. The efforts of this period resulted in a number of fundamental observations which provided the basis for the scientific inquiries that were to follow. The unequivocal association of immunity with substances contained in blood serum emerged from the findings of von Behring and Kitasato (1890) that antisera obtained from animals immunized with bacterial toxin protected normal animals from the otherwise lethal effect of the toxin. Many years later these substances were

[1] The preparation of this article has been assisted by a research grant (H-6293) from the National Institutes of Health, Public Health Service.

shown to be protein in nature and to belong to the γ-globulin fraction of serum proteins. The observation of von Behring and Kitasato was quickly extended to plant toxins by Ehrlich (1891). The generality of the immune response was secured through the discoveries of Bordet (1899) and Tchistovitch (1899) that antibodies appeared in the sera of animals after injection with innocuous materials, such as the serum proteins of another species. Thus it became clear that the formation of antibody was a biological phenomenon quite separate from the immunity against disease with which function this process was often associated.

This generalization, together with the demonstration by Ehrlich (1897) that antibodies act by combining with the antigen and the observations of Kraus (1897) that precipitates are formed when filtrates of several bacterial cultures are mixed with their specific immune sera, provided the basis for the recognition of immunologic specificity as a molecular problem. One could now formulate the following question: What properties does the antibody molecule possess which confers upon it the capacity to recognize and to combine selectively with the antigen which had induced the antibody? That the chemical structure of the reactants is an essential aspect of this problem was, of course, vaguely realized at the time and this recognition found its expression in the lock-and-key analogy of Ehrlich (1906). The same analogy had previously been used by Fischer (1894) to account for the specificity of enzymes.

The fundamental importance of the chemical structure of antigens in relation to the specificity of their antibodies was given a substantial experimental basis and further conceptual clarification by the studies of Wells and Osborne (1913). From their examination of the antigenic specificity and cross reactivity of a variety of highly purified plant proteins, they were led to infer that the specificity of the induced antibodies was dependent on the chemical structure of portions of the protein antigen molecule.

The most decisive discovery for the later development of the problem was made by Landsteiner and Lampl (1917) through their successful preparation of azoantigens. By the use of these conjugated proteins, it was established (Landsteiner, 1945) that antibodies could be formed against small haptenic groups of known chemical structure, embracing a wide variety of chemical types, and could combine with them. The demonstration of specific inhibition of precipitation by the homologous hapten together with the capacity of the conjugated antigen to elicit antihapten antibody provided the experimental foundation for almost all of the subsequent studies of the relation between immunologic specificity

and molecular structure. From this work there also emerged the formulation of the problem posed by this relation. For the purpose of this review, this formulation may be given in the following way: In what ways and to what extent does the selectivity (or recognition) involved in the reaction of antigen and antibody depend on the structure of these molecules? By structure of a molecule we mean, of course, the three-dimensional arrangement of the atoms of which the molecule is constituted.

Because of experimental limitations, the bulk of the research in this area has been limited to the study of the structure of the antigen in relation to the specific reaction. Consequently this review will be largely concerned with an examination of recently acquired, quantitative information which relates the molecular structure of the antigenic determinant to various aspects of its interaction with antibody. The intensive study of the chemical nature of the combining region of the antibody molecule has begun only very recently, and it does not appear appropriate to attempt to evaluate here the limited results which are at present available. Although we shall, therefore, omit the discussion of this problem, we shall nevertheless consider some features of the antibody combining site insofar as these have been inferred from the structure and interaction of the antigenic group with antibody.

II. General Considerations

A. The Molecular (Organic) Crystal as a Model *(Marrack, 1938)*

The structural relation of an antigenic group and its homologous antibody site cannot be studied directly on an atomic scale. However, a useful model of this relation can be found in the structure of crystalline solids, particularly molecular crystals (Erlenmeyer and Berger, 1932). The crystallization process, like the antigen–antibody reaction, shows a high degree of selectivity, and, as we shall see later, an interesting parallelism. Indeed, this process is even more specific than the immunologic reaction and, accordingly, the structural factors which govern this specificity may be expected to be discernible more readily in the case of the crystal than in the antigen–antibody complex. Of greatest significance in this comparison is the fact that by X-ray diffraction methods the detailed structure of many organic crystals has been worked out (Nyburg, 1961). Since the same intermolecular forces would be expected to be operative in both systems, an examination of the structure of such crystals should provide us with a basis for the structural interpretation of the antigen–antibody interaction.

The use of the organic crystal as a prototype for the antigen–antibody interaction may be illustrated by reference to the structure of acetamide shown in Fig. 1. In this structure the arrangement of the molecules is such as to allow maximum formation of hydrogen bonds. Thus, each nitrogen atom is bonded through its hydrogen atoms to two oxygen atoms and each oxygen atom, consequently, forms its maximum of two

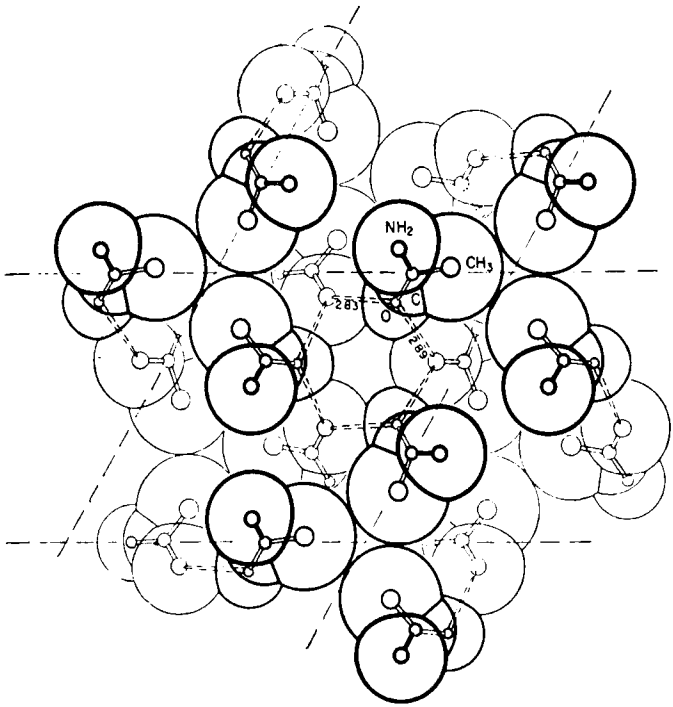


FIG. 1. Acetamide crystal viewed along the threefold axis showing two layers of molecules knit together by hydrogen bonds between oxygen atoms and $-NH_2$ groups. (From Corey, 1948.)

hydrogen bonds. At the same time, there is close-packing of the methyl groups so that the interaction among them also contributes to the stability of the crystal. From the point of view of any particular molecule, its environment is such as to allow the fullest attractive interaction with its nearest neighbors. Now let us carry out the hypothetical removal of a single acetamide molecule from the interior of the crystal. In the region vacated we would recognize a cavity which can be regarded as an idealized version of a larger and less sharply delineated antibody cavity.

The cavity thus generated can be described for geometrical purposes in terms of the boundaries, given by their van der Waals radii (discussed later), of the neighboring atoms. That is, the location and size of these atoms serve to define the contour of the cavity. In addition to its geometry, the cavity is also characterized by the chemical nature of the atoms that bound it. The complementary relationship between the cavity and the acetamide molecule has, thus, a geometrical aspect and a chemical aspect. Both aspects set the conditions that determine what kind of molecule will be most firmly held in the cavity. It is obvious, of course, that the acetamide molecule will be most compatible for the cavity, since it is space-filling without introducing steric distortion and at the same time fully satisfies its potential for attractive interaction and that of the adjoining atoms. It is in terms of these considerations that the selectivity of the crystallization process finds its natural explanation. The specificity of the immunologic reaction is also most reasonably interpreted in the same terms, though allowance must be made for the heterogeneity of antibodies and for the likelihood that the antibody molecule possesses sufficient flexibility to allow a distortion of the antibody site or cavity not to be expected in a crystal. These qualifying factors and their effect on specificity will be treated in more detail later.

B. The Haptenic Group as a Molecular Probe

We have already noted the decisive role played by the discovery of Landsteiner and Lampl (1917) that antibodies could be formed against haptenic groups of known structure. The availability of such antibodies and their ability to combine with haptens of low molecular weight provided a powerful experimental tool for examining important aspects of the molecular structure of the combining region of the antibody molecule. Thus, an antibody prepared against a particular group could be studied with respect to its capacity to discriminate between this group and other haptens which differed from it in terms of selected chemical and structural variations. For example, the effect of the addition of a methyl group to the original hapten or the shift of a substituent from the *ortho* to the *meta* position could be evaluated quantitatively. From the fineness of the chemical and geometrical sensitivity exhibited by the antibody molecule, significant inferences could be drawn regarding the nature of its combining site.

The use of haptenic groups conjugated to a protein carrier is not without its limitations. One of these arises from the nonrigidity of many of the haptens which have been used in specificity studies. Because of the

rotation possible around single bonds in the hapten, a variety of configurations may be assumed by the molecule in solution. For example, in the case of haptenic groups derived from monosaccharides, such as *p*-azophenyl-β-glucoside, the directions of the bonds between the oxygen and hydrogen atoms of the hydroxyl groups are not fixed relative to the pyranose ring. Even if one conformation is energetically favored in solution, there is no assurance that the antibody will be directed against it and not against other accessible conformations. Thus with many haptens a knowledge of their covalent structure does not suffice to define completely the three-dimensional pattern which is recognized by the homologous antibody.

Additional ambiguity resulting from the employment of conjugated proteins stems from the uncertainty as to the size of the antigenic determinant. Since the haptenic group is always covalently linked to the protein carrier, the amino acid side chains with which the linkage is established may comprise part of the antigenic group. In the case of azoproteins, for example, the residues, tyrosyl and histidyl, may be thus implicated. Some indication that this is, indeed, the case with the haptenic-group *p*-azophenylarsonic acid has been furnished by Hooker and Boyd (1933), although their evidence is far from conclusive. A more subtle aspect of this problem concerns the possible participation in the determination of the antibody specificity of amino acid residues not covalently linked to the haptenic group. The reality of this complication is suggested by the common experience that antisera to azoproteins often contain antibody which can be precipitated only by the immunizing antigen, even after absorption with the protein alone.

The limitations which we have noted here can be circumvented to a considerable extent under favorable conditions. Substantial simplification in the identification of the antigenic group may be achieved by the use of macromolecules made up of a single repeating unit of low molecular weight. The most useful class of substances of this kind has been the polysaccharides and the simplest subclass, the dextrans, made up only of glucosidic units. These have been exploited to great advantage by Kabat (1956). Another device which will probably contribute to our improved definition of the antigenic determinant involves the use of haptenic groups of substantially larger size than those which have been commonly employed in the past. In this connection, it appears highly desirable, regardless of the size of the haptenic group, to employ methods for linking it to the carrier protein which permit attachment to only one kind of amino acid residue. In this way the antigenic group can be specified in terms both of the hapten used and of a protein side chain.

C. Complex Formation as the Basic Process

In the study of immunologic specificity, the process which is basic and therefore common to such investigations is the formation of a complex between the antibody-combining site and selected haptens or antigens. Aside from kinetic observations, which have been few in number, the information which is potentially available is thermodynamic in nature. It may find expression, for example, in the form of an association constant, if appropriate quantitative analytical measurements are made, and will often include the effect of a variety of experimental variables on the extent of association.

The quantitative study of specificity has come into prominence only within the past 20 years. It found its first extended and fruitful utilization in the investigations of Pauling and Pressman and their associates starting about 1940, although the basis for such work had been provided 20 years earlier by the discovery that haptens, small molecules structurally similar to the haptenic group used for conjugation with the immunizing protein, could specifically inhibit the precipitation reaction (Landsteiner, 1920). From this discovery have emerged two main methods for the study of specificity involving the combination of hapten and antibody. The more direct of these methods requires the measurement of the extent of association of a hapten with antibody and was first used by Marrack and Smith (1932). This method, which generally utilizes the technique of equilibrium dialysis,[2] is most useful when purified antibody is available. Partly for this reason, the second method, which depends on the inhibition of precipitation, has, until recent years, been used almost exclusively.

One of the most serious problems encountered in the study of specificity arises from the nonspecific factors which affect the extent of the reaction of the antibody with the hapten or haptenic group. Such a complication is most apparent when precipitation is involved in the reaction under study. Thus, if one seeks to obtain thermodynamic data about the specific reaction from the solubilities of specific precipitates formed, for example, with conjugated antigens, then the solubility will depend not only on the free energy for the specific interaction but also on the free energy change arising from the transfer of protein molecules from a solution to a solid phase. This ambiguity can be avoided, but not without penalty, in the hapten inhibition method. What is done here is to deter-

[2] Recently, a novel technique of measuring the combination of hapten and antibody was introduced by Velick *et al.* (1960). This method utilizes the quenching of the protein fluorescence when the hapten complexes with the antibody.

mine for a variety of haptens the concentrations which are required to reduce the amount of specific precipitate obtained in the absence of hapten to one-half of this value. A value of 1 for the association constant is assigned to one of the simpler haptens and relative values are then calculated for the others. From these constants changes in the free energy of combination of the haptens with antibody can be correlated with their structural differences. It is not possible in the inhibition method to obtain the standard free energy for the combination of antibody and hapten. There is, furthermore, the assumption in this method that the composition of the precipitates at 50% inhibition is the same for all of the haptens. There is no evidence to indicate that this assumption is seriously incorrect but it has been shown that hapten inhibition of precipitation does lead to a substantial change in the ratio of antibody to antigen in the precipitate (Woolf, 1941).

For the unambiguous determination of the free energy of the specific combination, recourse must be had to the direct method, in which the reaction studied depends upon the combination of hapten and antibody to form a soluble complex. The difficulties associated with precipitation as well as the unknown contribution from protein-protein interaction are thus avoided. These considerations are important if the hapten is conjugated to a protein molecule or even if a monovalent antigen or antigen in large excess is used.

D. The Free Energy of the Specific Interaction

In recent years much of the work dealing with the specific reactivity of the antibody molecule has provided a thermodynamic description of the process in which antibody combines with hapten or antigen. This information can be expressed in terms of the free energy change (ΔF) for the process which may be written (assuming a monovalent hapten)

$$H + S \rightleftharpoons HS \tag{1}$$

In this equation H represents the hapten and S is a combining site of the antibody. Since the association constant, K_A, for this process is almost invariably calculated using molar concentrations, the free energy deduced from the equation

$$K_A = e^{-\Delta F/RT} \tag{2}$$

is the usual standard free energy (ΔF^0). It represents the change in free energy of the system resulting from the formation of 1 mole of the complex HS in an infinite volume of a hypothetical solution in which the concentration of H, S, and HS are each 1 molal, and the temperature,

pressure, pH, ionic strength, and concentrations of other components are the same as they were in the experiment. The concentrations of hapten and antibody are usually small enough so that activity corrections are unnecessary.

The exponential dependence of K_A on ΔF^0 makes possible the measurement of relatively small changes in ΔF^0. For example, if the ratio of the association constants for the binding of two haptens is 1.3, a difference which can often be detected experimentally, the corresponding difference in free energy is only 0.15 kcal./mole. Because of this situation the effect of slight structural variations in a hapten are accessible to experimental evaluation. On the other hand, it appears that this sensitivity has tended to limit the study and recognition of the full extent of the antigenic determinant and of the contributions to the specific affinity which are produced by its various portions.

The interpretation of free-energy data in terms of the interaction between the antigenic group and the antibody site is subject to serious limitations. Chief among these is the fact that ΔF for complex formation is the measurement of the net change in free energy for the transfer of a hapten, for example, in an aqueous environment to the antibody site, and depends, therefore, on the interaction of the hapten with water molecules. The energetics of this interaction plays a dominant role in fixing the measured value of ΔF, because the magnitude of this interaction is similar to that involved in hapten–antibody complex formation. The participation of water molecules in the process of formation of a complex may be represented by a modification of Eq. (1) in the following way:

$$H_{(x+n)H_2O} + S_{(y+m)H_2O} \rightleftharpoons H_{nH_2O}S_{mH_2O} + (x+y)H_2O \qquad (3)$$

Explicit reference is made here to the possibility that the unoccupied antibody site may also interact with water molecules. The number of molecules of water which become part of the bulk liquid as a result of complex formation is then given by $(x + y)$. The free-energy change resulting from the release of this "bound" water is included in the measured value of ΔF along with the contribution resulting from the formation of the complex.

The quantitative significance of the interaction of antigenic groups with water may be gathered from an examination of the energetics of solutions of acetamide and of benzene. The acetamide molecule, which serves as an example of a polar group with the capacity to form hydrogen bonds, is extensively hydrogen bonded in the crystalline state, as we have already noted. On solution in water, at infinite dilution, there is an ab-

sorption of heat to the extent that the change in enthalpy, ΔH, is 2.0 kcal./mole of solute (Speyers, 1896). From measurements of the vapor pressure of crystalline acetamide (Aihara, 1952), the value of ΔH for the vaporization of the solid was found to be 13.66 kcal./mole. These figures, together with the correction for the work done in the sublimation process, yield a value for the energy of solvation of the acetamide molecule of −11 kcal./mole. That is, on transfer of 1 mole of acetamide from the gaseous state to the solvated one there is a decrease of 11 kcal. in the internal energy. The major contribution to this fairly large stabilization energy arises, of course, from hydrogen bond formation between acetamide and water.

In contrast, the solvation of the benzene molecule, which serves to illustrate the behavior of apolar groups, is characterized by $\Delta H = 0$ (at 18°C.) and by a value for the unitary entropy change (ΔS_u; see following) of −14 e.u. [calculated by Kauzmann (1959) from data of Bohon and Claussen (1951)]. These figures relate to the transfer of 1 mole of benzene from the liquid state to the aqueous solution. The resulting unitary free-energy change (ΔF_u; see following) for this process is 4.1 kcal./mole. The low affinity of benzene for water, which is reflected in this figure, has an important implication for the antigen–antibody reaction. It may be anticipated that whenever the antigenic determinant contains an apolar group, the interaction of this group with the antibody region will make a large contribution to the stability of the complex.

The free-energy change for the combination of antigen and antibody is composed of an enthalpic term and an entropic term expressed as follows:

$$\Delta F = \Delta H - T\Delta S \tag{4}$$

The value of ΔH for the process is usually determined from measurement of the temperature dependence of the association constant. Since very dilute solutions of the reacting solutes are generally used, the experimental value of ΔH corresponds to that for an infinitely dilute solution and can be equated to ΔH^0. In contrast to ΔH, the values of ΔS and, therefore, ΔF depend, for an association reaction, on the concentration units employed in the calculation of the equilibrium constant. The conventional use of molarity for dilute solutions as the concentration unit is equivalent to the selection of the hypothetical unit molal solution as the standard state of the solutes to which the symbols ΔF^0 and ΔS^0 refer.

The interpretive value of free-energy data lies in relating it to the secondary bonds formed by antigen and antibody with water and between each other. However, for the association process represented by

Eq. (1), the value of ΔS contains a contribution which is statistical in nature and whose magnitude depends on the concentration unit. This term arises from the fact that, in the forward direction of the reaction, two kinetic units combine to form one, with the result that there is a decrease in the entropy of mixing. The effect is a decrease in the association constant and a value of ΔF which does not fully reflect the extent of the stabilization of the complex arising from the new intermolecular bonds represented by the right side of Eq. (3).

The elimination of the entropy of mixing can be readily and unambiguously achieved by the use of the concept of unitary entropy (Gurney, 1953). For a sufficiently dilute solution, the partial molal entropy of a solute is given by

$$\bar{S} = \bar{S}' - R \ln x \tag{5}$$

where x is the mole fraction of the solute and $\bar{S}'$ is the unitary entropy. This equation simply separates the entropy into a term which is structure dependent and another which is the entropy of mixing. If we now define the unitary entropy change for the reaction of Eq. (1) as follows:

$$\Delta S_u = \bar{S}'_{HS} - \bar{S}'_H - \bar{S}'_S \tag{6}$$

then it easily follows that (Kauzmann, 1959)

$$\Delta S_u = \Delta S^0 + R \ln 55.6 = \Delta S^0 + 7.98 \tag{7}$$

In accordance with Eq. (7) the unitary free-energy change ΔF_u, defined as $\Delta H - T \Delta S_u$, is given by

$$\Delta F_u = \Delta F^0 - 7.98T \tag{8}$$

The utility of unitary entropy and unitary free energy as the basis for discussing secondary bonding and association reactions is illustrated in Kauzmann's recent analysis of protein denaturation (1959).

Two final points should be made regarding the significance of ΔF_u. In the first place no correction has been made for the possible loss of rotational and translational entropy associated with complex formation. The difficulty here is that the rotational and translational entropy of the solute molecule in water is greatly reduced compared to that in the gaseous phase. The effect of our failure to make a quantitative estimate of its contribution in solution therefore is, perhaps, sufficiently small to be neglected. Secondly, there may be, of course, other changes in translational entropy resulting, for example, from the release of "bound" water. However, since these arise from the interaction between the

solvent and the reactants and are consequently dependent on their structure, they may appropriately be included in the unitary entropy change.

E. Intermolecular Forces in Specific Reactions

The stability of the antigen–antibody complex is provided by the same kinds of intermolecular or secondary bonds which serve to maintain the specific configuration of native proteins. An excellent discussion of these bonds and their significance for the structure of the protein molecule has been given recently by Kauzmann (1959). For this reason it will be sufficient for our purposes to summarize the main features of those intermolecular forces which predominate in immunologic reactions.

1. The Apolar Bond

Of major importance among the secondary bonds of immunologic significance is that arising from the interaction of apolar molecules and substituents with their aqueous environment. As we have already noted

TABLE I

Thermodynamic Changes in the Transfer of Hydrocarbons from a Nonpolar Solvent to Water[a]

Process	Temp. (°K.)	ΔS_u (e.u.)	ΔH (kcal./mole)	ΔF_u (kcal./mole)
CH_4 in benzene → CH_4 in H_2O	298	—18	—2.8	+2.6
CH_4 in ether → CH_4 in H_2O	298	—19	—2.4	+3.3
CH_4 in CCl_4 → CH_4 in H_2O	298	—18	—2.5	+2.9
C_2H_6 in benzene → C_2H_6 in H_2O	298	—20	—2.2	+3.8
C_2H_6 in CCl_4 → C_2H_6 in H_2O	298	—18	—1.7	+3.7
C_2H_4 in benzene → C_2H_4 in H_2O	298	—15	—1.6	+2.9
C_2H_2 in benzene → C_2H_2 in H_2O	298	— 7	—0.2	+1.9
Liquid propane → C_3H_8 in H_2O	298	—23	—1.8	+5.0
Liquid *n*-butane → C_4H_{10} in H_2O	298	—23	—1.0	+5.8
Liquid benzene → C_6H_6 in H_2O	291	—14	0	+4.1
Liquid toluene → C_7H_8 in H_2O	291	—16	0	+4.6
Liquid ethyl benzene → C_8H_{10} in H_2O	291	—19	0	+5.5
Liquid *m*- or *p*-xylene → C_8H_{10} in H_2O	291	—20	0	+5.8

[a] Taken from Kauzmann (1959).

for benzene, the transfer to water of an apolar molecule from the liquid state or from an apolar solvent is associated with a decrease in the unitary entropy (ΔS_u) of approximately 14 e.u. (Table I). The entropy decrease is ascribed to the formation of a quasi-crystalline structure of the water molecules in the immediate neighborhood of the apolar molecule. This structure is different from that of ordinary ice and is presumed to

resemble the polyhedral crystalline hydrates, known as the clathrate compounds, formed by methane, ethane, and propane as well as other molecules. For aliphatic molecules, the entropy change is accompanied by a decrease in the enthalpy, ΔH, ranging from –1 to –3 kcal./mole whereas, for aromatic molecules, ΔH is approximately zero at room temperature. At this temperature the unitary free energy for the transfer process ranges approximately from +3 to +6 kcal./mole. The physical consequence of this situation is that apolar molecules in water tend to acquire a nonaqueous environment. This tendency is manifested, for example, in the immiscibility with water of apolar liquids and, more directly relevant for our purpose, in the formation of micelles by soaps and detergents in aqueous solution. This inclination for apolar groups to adhere to each other in water is called hydrophobic bonding. Its stability, it should be emphasized, arises from the structural alteration which the apolar molecule imposes on its aqueous environment and on the consequent gain in entropy which results from the withdrawal of the molecule from this environment. Thus for the hydrophobic bond to be established between molecules carrying apolar groups, these groups must approach each other by a distance not much greater than the sum of their van der Waals radii.

In view of the range of values of the unitary free energy described in the foregoing, we may expect that hydrophobic bonding will make a large contribution to the affinity between antibody and ligand (hapten). Since the hapten (or haptenic group) may contain more than one apolar substituent, the total effect from this source is probably sufficient in many instances to account entirely for the unitary free energy of complex formation. We are here evaluating the contribution of the apolar substituents of a hapten on the basis of the thermodynamics of corresponding molecules (e.g., phenyl and benzene) in aqueous solution. In doing so, the unitary free energy should be used since the presence of the substituent on the hapten does not alter the number of kinetic units involved in the reaction of hapten and antibody.

Hydrophobic bonding, furthermore, is not restricted to hydrocarbon molecules. For the lower alcohols, for example, there is also a decrease in the unitary entropy on transfer of an alcohol molecule from the liquid state to a dilute aqueous solution. This is likewise true for many other liquid aliphatic derivatives that are not miscible with water, such as diethylketone, ethylacetate, *n*-butanol, and ethyl bromide (Kauzmann, 1959). Indeed, a comparison of the isoelectronic molecules C_2H_6 and CH_3OH demonstrates that the polarity of the molecule need not interfere with its entropic effect and presumably, therefore, with its capacity

or organize a clathrate structure about it. These inferences are based on the observation that the entropies of vaporization of these substances from dilute aqueous solution are almost identical and from the similarity in the volume changes associated with their transfer to water. At 25°C. the entropies of vaporization for methanol and ethane are 35.0 and 35.4 e.u., respectively (Frank and Evans, 1945).[3] The polarity of the methanol molecule does, however, manifest itself in the enthalpies of vaporization. For methanol at 25°C., ΔH is 11.24 kcal./mole compared to 4.43 for ethane; the difference undoubtedly reflects hydrogen bonding between the methanol molecule and water.

2. *The Hydrogen Bond*

Another source of stabilization of the specific complex is to be found in hydrogen bonding between appropriate groups of the reactants. This bond is formed by the interaction of a hydrogen atom covalently linked to one electronegative atom with the unshared electron pair of another electronegative atom. The particular atomic combinations which will be of most significance in the antigen–antibody reactions are O–H–O, O–H–N, and N–H–N. In this case, unlike the apolar bond, the aqueous polar solvent plays a competitive role which serves to reduce greatly the quantitative significance of hydrogen bonding. We have just noted from the enthalpy of vaporization of methanol that this molecule is hydrogen-bonded to water. The binding, therefore, of a hydrogen-bonding ligand to the antibody with such a bond will, in general, require that one or more hydrogen bonds be broken between the ligand and water and perhaps between the antibody site and water. The energetic gain will then represent the net effect of these processes and may be expected to be relatively small compared to apolar bonding.

Although the intrinsic strength of the hydrogen bond (i.e., in the absence of water) is known from experimental measurements to correspond to a ΔH in the neighborhood of −5 kcal./mole (Pimentel and McClellan, 1960), its contribution to the unitary free energy (ΔF_u) for the formation of the antibody–ligand complex is still uncertain. Experimental observations of solubility, specific salt effects on pH, and concentration dependence of conductance with several model systems of low molecular weight clearly indicate that the hydrogen bonds of interest will provide a ΔF_u which is not more negative than −2 kcal./mole and may be less negative than −1 kcal./mole (Kauzmann, 1959). Re-

[3] These values are based on a unit mole fraction of the solute for the standard state of the solution and a pressure of 1 atm. for the standard state of the gaseous form of the solute.

cently, studies have been made by Klotz and Franzen (1961) of the dimerization of *N*-methylacetamide through the formation of the hydrogen bond N—H---O, by measuring the infrared absorption spectrum in the overtone region as a function of concentration in aqueous solution. Dimers were only formed when the concentration of the solute reached 7 or 8 *M*. The temperature dependence of the dimerization process gave a ΔH of about −1 kcal./mole.

On the basis of the information we have just summarized, an individual hydrogen bond only makes a small contribution to the stability of the antigen–antibody complex. For those antigens, such as polysaccharides, whose binding to the antibody is predominantly the result of hydrogen bonding, a multiplicity of such bonds would be required to provide the necessary stabilization.

3. *Ionic Interaction*

The coulombic attraction between oppositely charged groups appears to contribute to the stability of many antigen–antibody complexes. Contrary to what seems to be a widespread belief, a charged group is not an essential condition for antigenicity nor is it necessary for strong binding of antigen and antibody. Although the unitary free energy contribution cannot be reliably estimated for ionic interaction, it cannot dominate the energetics of the antigen–antibody complex. If, for example, the antigenic determinant contained a carboxylate ion, the homologous antibody site might possess a cationic group provided by a lysyl residue or an arginyl residue. On the assumption that ionic interaction is the main stabilizing force, simple carboxylic acids, such as acetic acid, would be expected to dissociate such antigen–antibody complexes by their effective competition for the antibody site. As we shall see, this prediction is not borne out with charged determinants. We can anticipate, therefore, on the basis of observations with antibodies to such determinants, that ionic interactions do not play the predominant role.

The quantitative significance of the ionic interaction is enhanced by virtue of the fact that at least one of the charged groups is linked to a matrix, the protein molecule, of low dielectric constant. The effect of this feature on the interaction is to strengthen the ionic bond relative to the energy of attraction to be expected between two small oppositely charged molecules in aqueous solution.

The juxtaposition of oppositely charged groups in an antibody–ligand complex, when it does occur, may be expected to contribute to the free energy of the process by a positive contribution to the entropy change. Because of the electrostrictive effect of the separated charges on the sur-

rounding solvent molecules, the reduction of the electric field resulting from their contact leads to an increased freedom of movement of the constrained water. This physical alteration manifests itself as an entropy increase as well as an increase in volume.

4. Dispersion Forces

A more general source of attractive interaction between atoms and groups of atoms arises from the London dispersion forces. These have their origin in the orbital movements of the extranuclear electrons and in their quantum-mechanical properties. The dispersion force depends less on the chemical nature of the interacting groups than any of the others we have discussed. It is, therefore, less significant for specificity and in general may not be expected to be important energetically. The latter conclusion follows from the consideration that the dispersion contribution represents a difference which depends on the dispersion force between a ligand and its water envelope compared to the corresponding force between the ligand and its nearest neighbors in the antibody-combining region.

In the dispersion force the energy of attraction between interacting groups depends on the product of their electronic polarizabilities. Inasmuch as the polarizability of the antibody molecule is greater than that of water, some dispersion contribution to the stability of the antibody–ligand complex is to be expected. Its extent will depend on the nature of the substituent under consideration and will, for example, be greater for the iodine atom than for the methyl group because of the difference in their electronic polarizabilities. Quantitative estimates of this effect by Pauling and Pressman (1945), assuming the most favorable condition, indicate that the contribution will not be more negative than about –1 kcal./mole of substituent.

5. Steric Repulsion

The forces that have been discussed share the general property that their intensity increases with decreasing distance between the interacting components. The resulting tendency for increased proximity is countered by a repulsive force between nonbonded atoms, which arises from the interpenetration of their electron clouds. This force, often referred to as steric repulsion and as the steric factor, is much more sensitive to the distance between the interacting atoms than are the intermolecular attractive forces. It varies inversely with the twelfth power of the distance compared to the sixth power which appears to be the maximum dependence of the attractive forces on distance. The consequence of this

difference is that atoms can be approximated as hard spheres and their size described in terms of their van der Waals radii.

The values of the van der Waals radii are obtained from a knowledge of the detailed X-ray structure of molecular crystals. The distances between nearest-neighbor atoms which are not bonded are the distances of closest approach and these atoms are said to be in intermolecular contact. With a sufficient variety of such distances, a consistent set of radii can be formulated and these can then be employed as a description of the effective sizes of the atoms. It is also convenient to specify van der Waals radii for small groups of atoms, such as the methyl, azo, hydroxyl,

TABLE II
SELECTED VAN DER WAALS RADII[a]

Atom	Radius (Å.)	Atom or atom group	Radius (Å.)
H	1.2	Br	1.95
O	1.4	As	2.0
N	1.5	I	2.15
Cl	1.80	OH	1.6
S	1.85	NH_2	1.8
P	1.9	CH_3	2.0

NOTE: Half-thickness of aromatic ring, 1.70 Å.
[a] From Pauling (1960).

and amino groups, and to treat them as units in the discussion of steric effects. Values for the radii of the more familiar atoms and groups of atoms are given in Table II.

6. *The Affinity of the Antigen–Antibody Complex*

The stability of the antigen–antibody complex or of the antibody–hapten complex as expressed, for example, in an association constant, involves the simultaneous interaction of the residues of the combining region of the antibody with several substituents (or groups of atoms) of the antigenic determinant or a closely related ligand. Not only are a variety of forces significant in the determination of the affinity, but the interactions of the separate components with their aqueous environment as well as with each other play a decisive role. The affinity which can be experimentally established, then, is the net and integrated result of the variety of simultaneous interactions of which an aqueous system with two reacting solutes is capable.

To disentangle these various factors and to establish their quantitative significance is the task of investigation concerned with the explana-

tion of immunologic specificity in terms of molecular structure. It is apparent that a minimum condition for this type of study is the prior knowledge of the structure of the antigenic determinant. This condition is usually met by working with antibodies specific for haptenic groups that have been coupled to a carrier protein to form the immunizing antigen. To a lesser extent, simple polysaccharides have been used to provide a determinant of known structure. In the following discussion we shall present the results of studies of this kind designed to clarify the structural basis of immunologic specificity.

III. Aspects of the Specific Interaction

A. The Complementary Relationship

The concept of molecular complementarity as the basis for immunologic specificity emerged as a consequence of the extensive investigations carried out with antihaptenic sera. Following the discovery by Landsteiner and Lampl (1917) that simple chemicals when attached to a protein carrier through an azo linkage can induce antihapten antibodies, the cross reactions of these antisera with azoproteins carrying a wide variety of haptenic groups were studied in great detail. The scope of these inquiries and the facility with which they could be executed were greatly increased by the introduction of the method of hapten inhibition of specific precipitation (Landsteiner, 1920).

It is striking that despite the serious quantitative limitations of the bulk of this work, e.g., the quantity of specific precipitates was estimated visually, a wealth of detailed structural inferences could be drawn. For example, it was clearly established that an antibody induced by an antigenic determinant terminating in the *p*-azobenzoate group could distinguish *para*-substituted benzoates from the corresponding *ortho*- and *meta*-substituted compounds. In the case of carbohydrate determinants, also, small structural differences were detectable in the appropriate cross-reacting systems. Thus antisera produced against the haptenic groups of *p*-azophenyl-β-glucoside and *p*-azophenyl-β-galactoside differentiate sharply between these groups, although they differ only in the reversal of H and OH on carbon-4 of the glycoside. A scholarly discussion and summary of the results obtained during the "golden era" of immunology, lasting until about 1940, is to be found in Landsteiner's monograph (1945).

The next stage in the development of the concept of molecular complementarity was provided largely by the extensive series of investigations conducted by Pauling and Pressman and associates from about

1940 to 1949. [A summary of these will be found in an article by Pressman (1953).] These studies were carried out with antihapten sera in which the inhibition of precipitation of the homologous antigen by a wide variety of haptens was quantitatively assessed. In addition to the advantages of the hapten inhibition method, the quantitative character of the work, involving protein assays of the specific precipitates, and the use of a wide range of hapten concentrations have permitted estimates of the free-energy contributions of the various interactions underlying the stabilization of the antibody–hapten complex.

More recent efforts have been directed toward the thermodynamic characterization of the antibody–hapten complex using purified antibody and avoiding the formation of a second phase—the specific precipitate. Although binding of hapten by antibody was first described by Marrack and Smith (1932) almost 30 years ago, this method for the study of immunologic specificity, in spite of its theoretical superiority, has only been revived within the past 10 years. The use of purified antibody is of great advantage, and even often necessary, in studying the combination of hapten and antibody by such methods as equilibrium dialysis (Karush, 1956) and fluorescence (Velick *et al.*, 1960). Further progress in elaborating the details of antibody–ligand interactions will undoubtedly require that the reactions in solution contain defined reactants and products.

In the following discussion we shall attempt to assess the quantitative significance in immunologic specificity of the various kinds of interactions which have already been described briefly. We are limited in this task to the results of the last 20 years because of the lack of quantitative data in the earlier investigations. Before we proceed with our analysis it will be useful to attempt to obtain a general picture of the affinities which are encountered in immunologic reactions.

B. The Affinity of the Specific Complex

In Table III we have summarized the thermodynamic information presently available that describes the formation of the antibody–hapten complex. The system insulin–human anti-insulin has also been included for comparative purposes and because insulin appears to behave as a univalent antigen (Berson and Yalow, 1959).

An inspection of Table III indicates that the association constants for haptens span more than a 1000-fold range. This variation may be attributed partly to the possibility that the antibody site was formed against a determinant larger than the ligand itself. In this event the measured value of the antibody–hapten affinity would not reflect the

TABLE III

THERMODYNAMIC FUNCTIONS FOR THE FORMATION OF THE ANTIBODY–HAPTEN COMPLEX

Haptenic group	Ligand (hapten)	K_A (liter/mole)	ΔF_u (kcal./mole)	ΔH (kcal./mole)	ΔS_u (e.u./mole)	T (°C.)	Ref.[a]
p-Azophenylarsonate	*p*-(*p*-Hydroxyphenylazo)-phenylarsonate	3.5×10^5	—10.1	—	—	29	1
ε-*N*-2,4-Dinitrophenyllysyl	ε-*N*-2,4-Dinitrophenyllysine	2.3×10^5	— 9.0	—	—	5	2
p-Azophenylarsonate	Terephthalanilide-*p,p'*-diarsonate	3×10^5	—9.8 ± 0.2	—0.8 ± 2.6	30 ± 9	23	3
D-Phenyl-(*p*-azobenzoyl-amino)acetate	D-Phenyl-[*p*-(*p*-dimethylamino-benzeneazo)benzoylamino] acetate	3.1×10^5	— 9.88	—7.3	8.7	25	4
p-Azophenyl-β-lactoside	*p*-(*p*-Dimethylaminobenzene-azo)phenyl-β-lactoside	1.57×10^5	— 9.47	—9.7	—0.8	25	5
p-Azobenzoate	*p*-Iodobenzoate	5×10^4	— 8.2	—	—	5	6
p-Azobenzoate	*p*-(*p*-Hydroxyphenylazo)-benzoate	6.0×10^4	— 8.3	—	—	5	7
ε-*N*-2,4-Dinitrophenyllysyl	ε-*N*-2,4-Dinitrophenyllysine	2×10^8	—13.7	—8.6	17	26	8
p-Azophenylarsonate	3-(*p*-Azophenylarsonate)-tyrosine	1×10^6	—10.6	—	—	26	8
Insulin	Insulin	1×10^9	—15.4	—3.6	38.0	37	9

[a] 1—Eisen and Karush (1949); 2—Carsten and Eisen (1955); 3—Epstein *et al.* (1956); 4—Karush (1956); 5—Karush (1957); 6—Nisonoff and Pressman (1958a); 7—Nisonoff and Pressman (1958b); 8—Velick *et al.* (1960); 9—Berson and Yalow (1959).

potential affinity of the antibody for a structure equivalent to the original antigenic determinant. Beyond this possibility, however, the average affinities of the purified antibody preparations employed in these studies may still cover a wide range. This conclusion is particularly supported by a comparison of the results of the two investigations with the ε-*N*-2,4-dinitrophenyllysyl group. In all probability the 1000-fold difference in the association constants is related to the difference in the immunization procedures. In the earlier study (Carsten and Eisen, 1955), multiple intravenous injections of the soluble antigen were used, whereas in the more recent work (Velick *et al.*, 1960), a much smaller amount of antigen in Freund's adjuvant was administered in the foot pad. A discussion of the significance of the immunization procedure for the production of high-affinity antibodies has been given elsewhere (Karush and Eisen, 1962).

Of particular interest is the large value of K_A, 2×10^8, for antibody to the ε-*N*-2,4-dinitrophenyllysyl group. It is comparable to that for the anti-insulin antibody and we may infer that the antigenic determinant of insulin, and probably of other proteins, is not much larger than the synthetic one under consideration. In contrast, much smaller values of K_A, about 1000-fold, for most of the other haptenic groups have been observed. We are inclined to attribute this difference less to any special property of the dinitrophenyl antigenic determinant than to an important role played by the method of immunization. In view of the energetic heterogeneity of the antibody (see following), the use of multiple injections of antigen and the rather large amounts thereby introduced would be expected to result in the selective removal from the circulation of the antibody of highest affinity. This factor together with the further selection involved in the purification procedures would lead to antibody preparations which are hardly representative of the full range of affinities generated by the antibody-forming cells. It may be anticipated that more attention to these considerations together with the employment of somewhat larger haptenic groups will lead to the discovery of much greater values of K_A than have been generally found previously.

With respect to the relative roles of the enthalpic and entropic contributions to the unitary free energy, the results are too small in number to allow any generalizations. It is clear, however, that the entropy term plays an important role—a fact which indicates a consistently significant role for the apolar bond for antigens other than carbohydrates.

An important aspect of the interaction between antibody and ligand as well as of the production of antibody is the energetic heterogeneity of the antibody. By this we mean that in the antibody population induced against a single antigenic determinant there will be a range of

affinities with which the antibody molecules will complex with the homologous ligand. This heterogeneity appears to be a phenomenon unique to the immune response since it does not, for example, apply to the behavior of enzymes in the formation of complexes with their substrate molecules (cf. Cohn, 1957). The inference can hardly be avoided that some distinctive aspect of the biosynthetic process underlies this phenomenon.

Antibody heterogeneity emerged initially most clearly in terms of the cross-reacting properties of antihaptenic sera. It could be shown that absorption with cross-reacting antigen A, for example, would remove a portion of the antibody but leave in solution antibody which could not react with A but would precipitate with cross-reacting antigen B. In both cases, however, the homologous antigen would remove all of the precipitable antibody (Landsteiner and van der Scheer, 1936). On the basis of such observations Landsteiner (1945) was led to the following view:

"One may conclude that the antibodies formed in response to one determinant group are, though related, not entirely identical but, as evident from their cross reactions with heterologous antigens, vary to some extent around a main pattern, and that what ordinarily is spoken of as an antibody is generally a mixture of specifically different components."

The attempt to provide a quantitative description of the energetic heterogeneity of antibody was first made by Pauling *et al.* (1944) in order to interpret quantitatively the hapten inhibition of the precipitation of antihapten sera. The basic assumption was made that the heterogeneity in the combination of hapten with antibody could be described by an error function in the free energy of complex formation expressed as follows:

$$w(\Delta F) = \frac{1}{\sigma\sqrt{\pi}} \exp\left[-(\Delta F_0 - \Delta F)^2/(RT\sigma)^2\right] \tag{9}$$

in which ΔF_0 is an average free energy corresponding to the maximum in the normalized distribution function w. The degree of heterogeneity is denoted by σ, the heterogeneity index, and the other symbols have their usual meaning. Expressed in terms of association constants (K), the distribution function has the form:

$$w(K) = \frac{1}{\sigma\sqrt{\pi}} \exp\left\{-[\ln(K/K_0)]^2/\sigma^2\right\} \tag{10}$$

The dependence on σ of the distribution function is shown in Fig. 2.

When this distribution function is applied to a description of the

effect of energetic heterogeneity on the combination of hapten and antibody to form a soluble complex, it can be shown (Karush, 1949) that the fraction of antibody sites occupied as a function of the concentration of free hapten is given by

$$r/n = 1 - \frac{1}{\sqrt{\pi}} \int_{-\infty}^{\infty} \frac{e^{-\alpha^2}}{1 + K_0 c e^{\alpha\sigma}} \, d\alpha \qquad (11)$$

in which α is equal to $\ln(K/K_0)/\sigma$. The number of combining sites per antibody molecule is n, and r is the average number of sites occupied at the concentration c of the free hapten. Since in Eq. (11) $r/n = \frac{1}{2}$

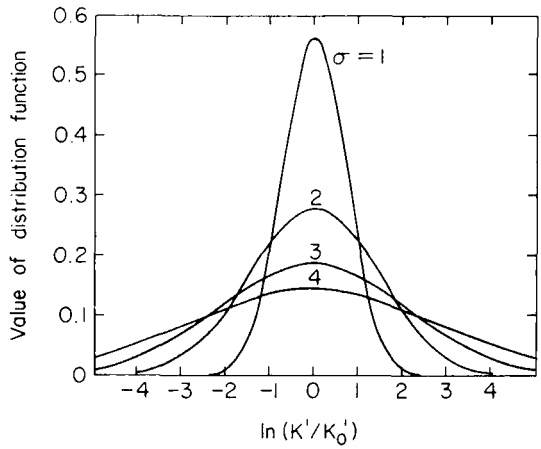


FIG. 2. *Distribution function plotted for several values of the heterogeneity index* σ. (From Pauling *et al.*, 1944.)

when $K_0 c = 1$ for all values of σ, the value of K_0 is given directly by by the reciprocal of the concentration, c, at which the antibody is half-saturated. The determination of the value of σ for any particular system involves the laborious procedure of numerical integration over a range of c with selected values of σ and the matching of the experimental results with the theoretical curves thus obtained.

Within the limits of the data available, Eq. (11) has provided an adequate description of the heterogeneity in the two systems to which it has been applied. These involved purified antibody preparations induced by the haptenic groups D-phenyl-(*p*-azobenzoylamino)acetate (D-Ip) and *p*-azophenyl-β-lactoside (Lac). The thermodynamic characteristics of the reactions studied have already been listed in Table III. The best values of σ were 2.3 for the anti-D-Ip antibody and 1.5 for anti-Lac

antibody. The binding curves of Figs. 3 and 4 demonstrate the excellent agreement which can be achieved between the experimental points and the theoretical curves. One qualification needs to be emphasized here, however, to the effect that small values of r were not experimentally accessible. Consequently the suitability of the assumed distribution function for the complete description of the antibody heterogeneity cannot be claimed to have been established.

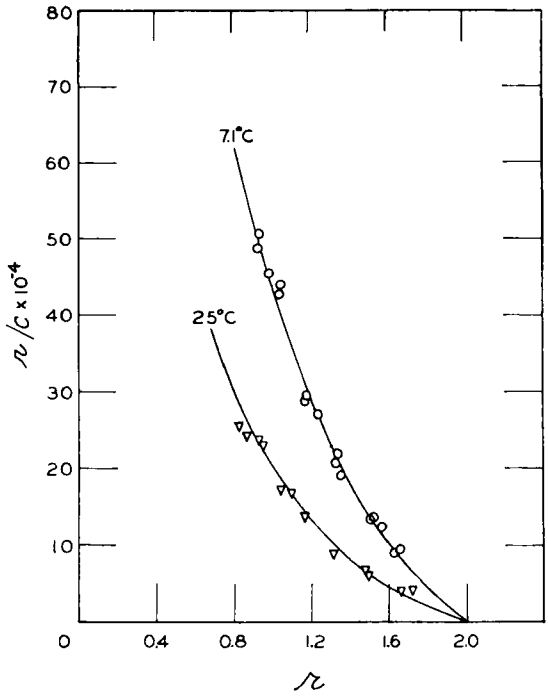


FIG. 3. Binding results at 25° and 7.1°C. for the reaction between D-Ip dye and purified anti-D-Ip antibody. The points are experimental and the curves are theoretical. (From Karush, 1956.)

The physical significance of the values of σ may be represented in several ways. Expressed in terms of the standard deviation of the free energy, one obtains values of 0.96 and 0.63 kcal./mole for σ equal to 2.3 and 1.5, respectively. The distribution functions for these numbers have been plotted in Fig. 5 to illustrate the difference in the widths of the distributions of the antibody affinity. In Fig. 6 are shown plots of the integrated form of the distribution function. From these curves, one can readily ascertain what fraction of the sites possess affinities which are

within any selected difference from the average free energy, ΔF_0. For example, for 2.3, it is found that 70% of the sites interact with the azo dye with values of ΔF which do not differ from the average value by more than 1 kcal./mole. For $\sigma = 1.5$, the narrower distribution is reflected in the fact that 89% of the sites fall within the range of $\Delta F_0 \pm 1$ kcal./mole.

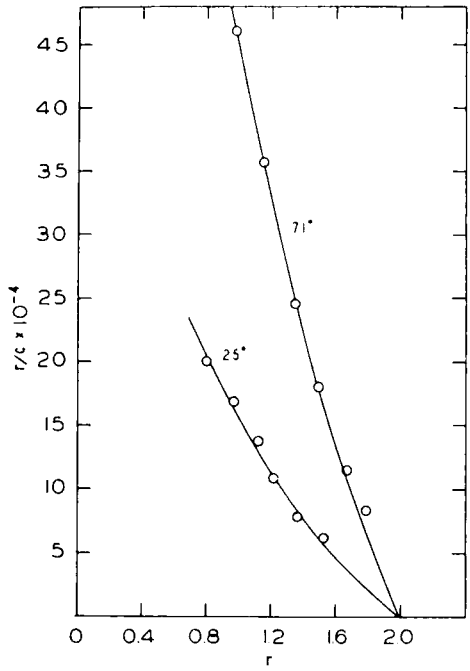


FIG. 4. Binding results at 25° and 7.1°C. for the reaction between Lac dye and purified anti-Lac antibody. The points are experimental and the curves are theoretical. (From Karush, 1957.)

More recently, Nisonoff and Pressman (1958a,b) have employed a different equation, relating r and c, which also contains a parameter that reflects the heterogeneity of the antibody population. This equation is based on a distribution function suggested by Sips (1948) which is very similar to the error function already described. By using Sips' function, the equation relating r and c corresponding to Eq. (10) can be integrated analytically. The resulting binding equation, in our notation, may be written:

$$r/n = \frac{(K_0 c)^a}{1 + (K_0 c)^a} \tag{12}$$

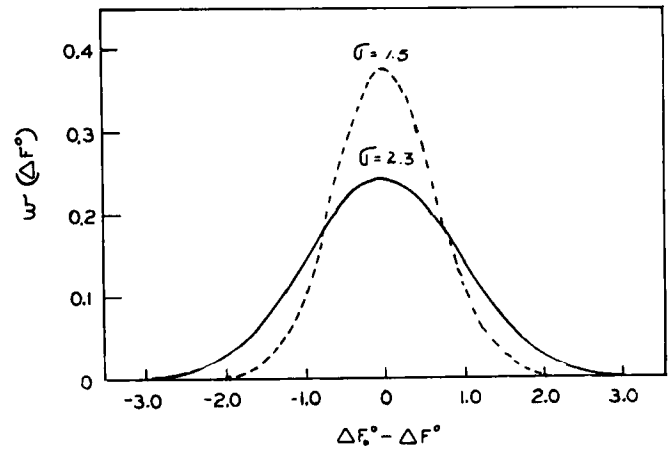


FIG. 5. Plots of the distribution function $w(\Delta F^0)$ for two values of σ. (From Karush, 1959.)

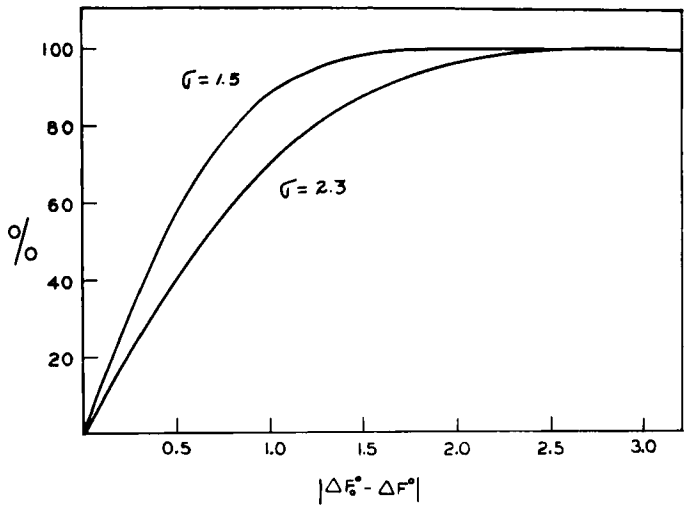


FIG. 6. Plots of the integrated form of the distribution function $w(\Delta F^0)$ for two values of σ. (From Karush, 1959.)

in which a, the index of heterogeneity, may range from 0 to 1. For $a = 1$, Eq. (12) reduces to the familiar form for noninteracting identical sites. Corresponding values of the indices of heterogeneity, α and a, are given by Nisonoff and Pressman (1958a).

The experimental suitability of Eq. (12) and the determination of

a can be facilitated by the use of the following equation readily derived from the above:

$$\log \left(\frac{r}{n-r} \right) = a \log c + a \log K_0 \tag{13}$$

A plot of $\log [r/(n-r)]$ vs. $\log c$ over a sufficient range of c will test the adequacy of the Sips' distribution. If the plot is linear then the values of a and K_0 can be directly established.

C. The Contribution of Apolar (Hydrophobic) Interaction

The quantitative energetic significance of apolar interaction in the formation of the antibody–hapten complex, has, until recently, gone almost unrecognized. This oversight probably stems primarily from the paradoxical situation that the contribution of an apolar group, such as the phenyl group, is so great that its experimental evaluation was not readily feasible with the usual procedures employed in hapten inhibition of specific precipitation. The neglect of the apolar bond was also strengthened by the earlier qualitative observations which indicated that apolar haptenic groups exhibited a lower degree of specificity than did groups of a polar or ionic nature (Landsteiner, 1945, p. 163).

TABLE IV

The Inhibition of D-Ip Dye Binding by Structurally Related Molecules at 25°C.[a,b]

Inhibitor	$K_I \times 10^{-4}$	$-\Delta F_u$ (kcal./mole)
D-Phenyl-(*p*-nitrobenzoylamino)acetate	12.8	9.36
L-Phenyl-(*p*-nitrobenzoylamino)acetate	0.0657	6.23
D-Phenyl-(benzoylamino)acetate	8.77	9.12
Phenylacetate	0.285	7.10
Benzoylaminoacetate	0.00776	4.96

[a] Data taken from Karush (1956).

[b] D-Ip dye is D-phenyl-[*p*-(*p*-dimethylaminobenzeneazo)benzoylamino]acetate; K_0 for the D-Ip dye is 29.4×10^4; and ΔF_u is —9.86 kcal./mole.

The most direct evidence bearing on the quantitative contribution of apolar bonding is provided by two recent studies with purified anti-hapten antibody. In the first (Karush, 1956), the association constants were determined for the binding of several haptens of related structure to antibody homologous to the haptenic group D-phenyl-(*p*-azobenzoylamino)acetate. The association constants and unitary free energies, together with the compounds studied, are shown in Table IV. The constants for these compounds were determined indirectly by measuring

their inhibitory action in the binding by the antibody of the anionic azo dye, D-phenyl-[*p*-(*p*-dimethylaminobenzeneazo)benzoylamino]acetate.

The contribution of the phenyl group to the antibody–hapten interaction may be taken as the difference between the free energies for the binding of D-phenyl-(benzoylamino)acetate and benzoylaminoacetate. The value of –4.1 kcal./mole computed in this way is in striking, and perhaps coincidental, agreement with the value of ΔF_u (= –4.1 kcal./mole) for the transfer of 1 mole of benzene in an aqueous environment to liquid benzene (Table I). We may infer, therefore, that the phenyl group induces a clathrate-like structure of water molecules in its immediate vicinity when the hapten is free, but is entirely bounded by, and in intermolecular contact with, apolar side chains of the protein when the hapten is complexed with the antibody.

It may also be of interest to note that the contribution of the benzoylamino group, calculated in the same way, is –2.0 kcal./mole. The structural interpretation of this figure is complex because, in addition to apolar interaction, hydrogen bonding of the benzoylamino group with water may modify substantially its energetic role in the formation of the complex.

The second study bearing on our discussion is that of Velick *et al.* (1960) with purified antibody homologous to the ε-*N*-2,4-dinitrophenyllysyl group. These authors found, with the use of their fluorescence quenching method, that the binding constants for the haptens 2,4-dinitrophenylacetate and 2,4-dinitrophenolate were from 100- to 1000-fold less than that for the homologous hapten ε-*N*-2,4-dinitrophenyllysine. Thus it appears that the $(CH_2)_4$ portion of this hapten may contribute at least about –3 kcal./mole to the free energy of complex formation. As may be seen from Table I this figure is much less negative than the value of the unitary free energy, namely, –6 kcal./mole, for the transfer of butane from aqueous solution to pure butane. It is not to be expected that the $(CH_2)_4$ group as part of a larger molecule need influence the structure of the solvent as much as the butane molecule. However, the potential contribution of this group is probably considerably greater than that observed. In the particular investigation under discussion, the antibody, which had been precipitated with ε-*N*-2,4-dinitrophenyllysyl-bovine γ-globulin, was purified by extraction of the specific precipitate with 2,4-dinitrophenol. Since only 50% of the precipitated antibody was solubilized, it appears quite likely that the antibody with the strongest interaction with the $(CH_2)_4$ group was selected against by this procedure. This interpretation is strongly supported by the observation of Farak *et al.* (1960) that, relative to ε-N-2,4-dinitrophenyllysine, 2,4-dinitro-

phenol is much more effective in the dissolution of specific precipitates formed with purified antibody than with those prepared directly from the antiserum.

The interpretation of the energetic significance of apolar groups in terms of their structural effect on the solvent does not take into account the contribution of the London dispersion force. Previous discussions of the role of apolar groups in the stabilization of the antibody–hapten complex have attributed this effect to the enhanced dispersion interaction between such a group and its nearest neighbors in the antibody site compared to its interaction with water molecules (see, for example, Pressman *et al.*, 1948). If there is a dispersion contribution to the free energy of binding, it would appear in the ΔH term. Aside from the quantitative difficulty which arises, it appears from the properties of aqueous solutions of apolar molecules that there is counteracting contribution to ΔH. Since the values of ΔH in Table I are not greater than zero, this contribution is at least sufficient to cancel the dispersion factor and, in some cases, to favor dissociation of the antibody–hapten complex. It appears questionable, therefore, that a dispersion effect is involved in the important role played by apolar groups. For clarification of this matter, however, it would be desirable to measure the temperature dependence of the apolar contribution to ascertain the respective values of the entropic and enthalpic terms.

D. The Contribution of Coulombic Interactions

The qualitative significance of ionic interaction in the formation of the antibody–hapten complex received recognition almost immediately after the introduction of haptenic groups as antigenic determinants. Its role was emphasized by virtue of the high degree of specificity associated with the presence of acid substituents, e.g., carboxylate, sulfonate, and arsonate, in these groups. Antisera prepared against determinants differing only in the acid group could readily distinguish between the homologous and heterologous test antigens. More directly, antibody against *p*-azobenzoate did not precipitate with test antigens linked to azobenzene, *p*-azonitrobenzene, or *p*-azotoluene (Landsteiner, 1945).

The unambiguous determination of the quantitative significance of the ionic interaction has not yet been provided. Experimental difficulty is often encountered because of the insolubility of the uncharged haptens which must be employed for comparison. Nevertheless some observations by Pressman *et al.* (1946) with antiserum directed against the *p*-azophenyltrimethylammonium group have furnished quantitative data of interest. These authors compared the inhibitory activity of the "H-acid"

(1-amino-3,6-disulfonic acid–8-hydroxynaphthalene) derivatives of the homologous haptenic group and the isosteric *p*-azo-*t*-butylbenzene on the precipitation of the antibody with an azoantigen prepared with horse serum albumin. These haptenic groups are very similar in size, shape, and electric polarizability but differ in that one is neutral and the other charged. After correction for albumin-binding, Pressman and Siegel (1953) found that the free energy of binding of the charged group was more negative by 1.2 kcal./mole.

In a later study, Nisonoff and Pressman (1957) have attempted to evaluate the electrostatic contribution arising from the carboxylate substituent in the haptenic group *p*-(*p*-azophenylazo)benzoate (X'p). This was done, as in the foregoing, by comparing the inhibitory activities of the *p*-azobenzoate and *p*-azonitrobenzene derivatives of H-acid in the precipitation of anti-X'p antibody with X'p-ovalbumin. It was found that the binding constant for H-acid *p*-azobenzoate was 89 times as large as that of benzoate, whereas the constant for H-acid *p*-azonitrobenzene was at least 100-fold less. The inference was drawn that the negative charge of the carboxylate contributes an amount to the combining energy more negative than –4.8 kcal./mole. There is reason, however, to doubt the validity of this figure as a measure of the electrostatic interaction. In the first place, such a large coulombic contribution would lead to the expectation that inhibition of precipitation could be effected with the acetate ion. Nevertheless a concentration of about 0.01 *M* acetate did not cause any decrease in the amount of specific precipitate. It would be desirable to evaluate the inhibition at concentrations at least up to 1 *M*. Secondly, the substitution of a nitro group may have introduced a steric factor in the binding of H-acid *p*-azonitrobenzene by the antibenzoate antibody. This possibility is suggested by the consideration that the charged group of the antibody which interacts with the carboxyl in the homologous case is undoubtedly hydrated. When the homologous haptenic group fits into the cavity of the antibody, the strongly bound water molecules are displaced by virtue of the charge neutralization. The accommodation of the nitro haptenic group in the site, on the other hand, would be prevented by the presence of solvent molecules. Thus, this hapten would cause a loss of affinity not only from the absence of an electrostatic interaction but also from the failure of the nonionic portions of the group to interact most effectively with the antibody.

It is quite apparent that the evaluation of the energetic significance of the various substituents of a haptenic group is best executed with haptens which are deficient in the substituent under study, but which are otherwise as closely similar to the antigenic determinant as is possible.

E. The Contributions of Hydrogen Bonding and the London Dispersion Force

These contributions are conveniently discussed under one heading since the scant quantitative information available for both kinds of interactions comes largely from an investigation carried out by Pauling and Pressman (1945). These authors studied the inhibition of precipitation of rabbit anti-*p*-azobenzoate antibody by a variety of *p*-substituted benzoates, some of which are listed in Table V. It was assumed that since

TABLE V

Comparison of Values of ΔF_{rel} and ΔW_A for the Anti-*p*-azobenzoate System

Hapten	$(R_A - R_H)$	$-\Delta F_{rel}$ (obs.)[a] (cal./mole)	$-\Delta W_A$ (calc.) (cal./mole)
p-Acetaminobenzoate	13.0	1800	1270
p-Nitrobenzoate	6.58	1400	643
p-Methoxybenzoate	6.86	1095	670
p-Bromobenzoate	7.79	890	761
p-Chlorobenzoate	5.00	720	488
p-Methylbenzoate	4.92	520	480

[a] Values of ΔF_{rel} taken from Pressman (1953).

the substituent occupied the same position as the azo group of the immunizing antigen no steric factor would be introduced by its presence in the hapten. Consequently the inhibitory contributions of the substituents could be compared and related to their physical and chemical properties.

In order to relate the observed relative binding constants of the inhibiting haptens to their dispersion interaction with the antibody an approximate equation was derived from which could be calculated the increments in the affinity (ΔW_A) resulting from the replacement of the *para* hydrogen of the benzoate ion with various substituents (Pauling and Pressman, 1945). In this equation, with ΔW_A expressed in calories per mole of substituent and given by

$$\Delta W_A = -\frac{400{,}000}{r_{AB}^6}(R_A - R_H) \qquad (14)$$

r_{AB} is the distance in angstroms between the interacting groups and is given by the sum of the van der Waals radii. A value of 2.0 Å. is used for the radius of the antibody groups which are adjacent to the hapten. The same figure may be used, without serious error, for the radius of the *para* substituent. The factor $(R_A - R_H)$ is the difference in mole

refraction between the substituent (A) and the hydrogen atom. Actually, for the calculation of ΔW_A, it is convenient to use the difference in mole refraction between benzene and the monosubstituted benzenes.

Since R_A is almost always greater than R_H, ΔW_A will be a negative quantity and *para* substituents, if not excessively large, would therefore enhance the binding of hapten to the anti-*p*-azobenzoate antibody. The basic reason for this enhancement is to be found in the fact that the average electronic polarizability of the protein molecule, hence its index of refraction, is greater than that of water. This provides an increased dispersion force for a substituent when its aqueous environment is replaced by the side chains of the antibody-combining region.

Values of ΔW_A have been calculated by the use of Eq. (14) for the six haptens shown in Table V together with their free energies of binding relative to benzoate (ΔF_{rel}) taken from a compilation by Pressman (1953). For the last three haptens there is, in view of the approximations involved, satisfactory correlation between the observed and calculated values. Thus, the dispersion contribution for substituents in the size range under consideration may be expected to be less negative than –1 kcal./mole.

The disparity between ΔF_{rel} and ΔW_A for the first three haptens has been attributed by Pauling and Pressman (1945) to the participation of a hydrogen bond in the binding of these haptens to the antibody. It is argued that in the formation of the antibody a hydrogen-bonding group which can donate a proton will be placed in juxtaposition to the azo group of the antigenic determinant. This proton will then form a hydrogen bond with the acetamino, nitro, and methoxy groups and increase their affinity beyond that which arises purely from the dispersion interaction. On this basis it can be inferred that, in view of the extent of the difference between ΔF_{rel} and ΔW_A, a hydrogen bond between antibody and ligand will contribute less than 1 kcal./mole to the stability of the complex.

F. The Steric Factor

Aside from determining the distance of closest approach of the homologous hapten to the various groups of the antibody which define its combining region, steric repulsion operates to decrease the affinity of the antibody for structurally dissimilar ligands. This is, indeed, the basis for the discrimination which the antibody exhibits in its selective combination with antigen. A nonhomologous ligand will, in general, not be able to achieve maximum affinity for the antibody site because the steric repulsion of some portion of the ligand will prevent the remainder from

establishing intermolecular contact without distortion of the antibody site. This restriction is similar to that which operates in the specificity of the crystallization process and which accounts for the occasional formation of mixed crystals. This similarity was established by the experimental demonstration by Erlenmeyer and Berger (1932) that organic molecules which form mixed crystals, e.g., *p*-dibromobenzene and *p*-bromotoluene, are immunologically equivalent. A more detailed discussion of this matter is given by Marrack (1938).

Although the foregoing discussion implies a considerable degree of rigidity of the antibody site, its capacity to complex with heterologous ligands demonstrates that the antibody possesses a flexibility with respect to the architecture of its combining region which is absent in the

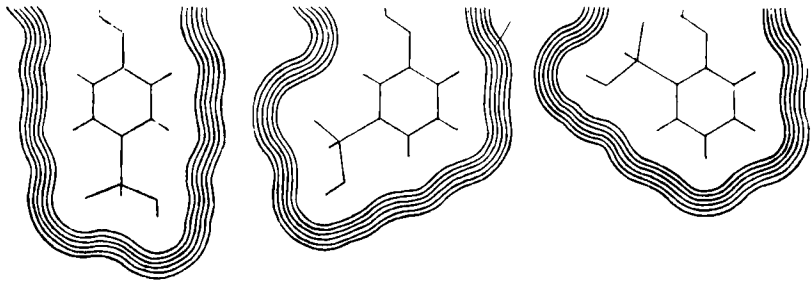


FIG. 7. Scale drawings of the antibody cavity specific to the *o*-, *m*-, and *p*-azophenylarsonic acid haptenic groups, with circumferential contours corresponding to close fit and to radial dilatation in increments of 0.2 Å. (From Pauling and Pressman, 1945.)

molecular crystal. A quantitative energetic evaluation of the role of distortion of the antibody associated with its binding of heterologous ligands cannot be made. However, Pauling and Pressman (1945) have attempted to correlate the affinities of related haptens to the degree of dilatation required for their accommodation in a cavity defined by the antigenic determinant.

This analysis was applied to the binding of a variety of monosubstituted benzenearsonates by antibody induced with the haptenic groups *o*-, *m*-, and *p*-azobenzenearsonate (R_o, R_m, and R_p). The assumption was made that the homologous azophenylarsonate fits into a cavity and makes van der Waals contact with the protein groups which define the contour of this cavity. The shape of this cavity in the plane of the benzene ring is represented in Fig. 7 by the innermost contour line for each of the three groups. Additional circumferential contours were drawn corresponding to increasing dilatation in increments of 0.2 Å. Scale drawings

of the haptens, prepared with the usual bond distances and van der Waals radii, were superimposed on the drawings shown in Fig. 7 with the arsonate group of the hapten in the same position as shown for the immunizing group. The degree of dilatation required to accommodate the hapten was estimated. These estimates are given in Table VI for a

TABLE VI

RADIAL DILATATION IN ÅNGSTROMS OF ANTIBODY MOLECULES FOR COMBINATION WITH HETEROLOGOUS SUBSTITUTED BENZENEARSONATES[a]

	Anti-R_o		Anti-R_m		Anti-R_p	
Substituent	*m*	*p*	*o*	*p*	*o*	*m*
Nitro	0.9	1.6	0.8	1.0	1.0	0.8
Methyl	0.7	1.2	0.6	0.9	0.8	0.7
Chloro	0.8	1.2	0.7	0.9	0.8	0.7
Amino	0.8	1.2	0.6	0.9	0.8	0.6

[a] From Pauling and Pressman (1945).

group of four selected haptens, for which the required dilatation ranges from 0.6 to 1.6 Å.

The correlation between the degree of dilatation required for a particular substituent in a given position and its effect on binding affinity is apparent from a comparison of Tables VI and VII. In the first place,

TABLE VII

FREE-ENERGY CONTRIBUTIONS OF SUBSTITUENTS ON BENZENEARSONATE FOR BINDING TO ANTIBODY AGAINST *o*-, *m*-, AND *p*-AZOBENZENEARSONATES[a]

	Anti-R_o			Anti-R_m			Anti-R_p		
Substituent	*o*	*m*	*p*	*o*	*m*	*p*	*o*	*m*	*p*
Nitro	— 970	340	1520	250	—940	580	280	—220	—950
Methyl	— 690	80	980	370	10	770	860	140	—350
Chloro	—1160	—260	145	65	—140	510	670	—140	—430
Amino	— 180	480	720	330	— 25	410	750	320	—110

[a] Values (in calories per mole) were taken from Pressman (1953) and had been calculated from data given by Pauling and Pressman (1945) after correction for albumin binding.

a substituent in the same position on the benzene ring as the azo group of the immunizing antigen invariably enhances the binding, with the questionable exception of the *m*-methyl group in the anti-R_m system. When present on the other two positions of the ring, the substituent either shows a smaller enhancement or an actual decrease of binding. The differential effect between the homologous and nonhomologous substitution exhibits a consistent correlation with the corresponding degree

of dilatation. Thus for the anti-R_o antibody, the dilatations are greatest for the *para* substituents, and these cause the maximum decrease in binding affinity. With respect to the anti-R_m system, the *para* substituents require somewhat greater dilatations than the *ortho* groups and again cause greatest reduction in binding. For the anti-R_p antibody, finally, the maximal dilatation of the *ortho* substituents leads to the greatest decrease of binding.

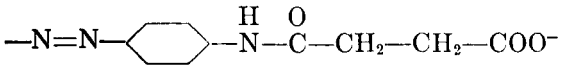
The consistency of this correlation strongly supports the notion that the steric factor plays an important role in the selectivity of the antibody molecule as well as the assumption that the antibody cavity is closely fitted to the shape of the haptenic group as expressed in its van der Waals contour. Indeed it may be inferred from the values in Table VI that this fitting is within considerably less than 1 Å.

G. The Size of the Antibody-Combining Region

The extent of the combining region of the antibody is an aspect of immunologic behavior that has an important bearing on such properties of the antibody–hapten reaction as affinity, heterogeneity, and cross reactivity. The combining region can be defined, of course, only with respect to the ligand with which it interacts. Because of the nature of our experimental methods, we can at best only specify the extent of the ligand which is energetically relevant in the combination of antigen and antibody. From this kind of information, inferences may then be drawn regarding the shape and extent of the combining region.

Since the discovery of antihapten antibodies, it has been clear that the combination of antibody with a test antigen, to yield, e.g., a specific precipitate, need involve the interaction of only a small portion of the antibody molecule. Thus, for example, the haptenic group *p*-azobenzoate linked to a tyrosyl or a histidyl residue of almost any protein is sufficient to precipitate antibody induced by this group. Nevertheless, such observations coupled with the results of hapten inhibition provide only a picture of the minimum extent of the combining region. Because neither the size nor the chemical nature of the effective antigenic determinant in azoantigens was known, it was not possible to explore fully the extent of the antibody site. The early experiments of Hooker and Boyd (1933) did serve to show that, for a small haptenic group, such as *p*-azophenylarsonate, the antibody site extended beyond this to include at least the residue to which the azo group was attached. On the other hand, from a hapten inhibition study with antibody homologous to the *p*-azosuccinanilate group, Pressman *et al.* (1948) concluded that "the combining region of the antibody is complementary in structure to the

succinamate group and also to the benzene ring, but that it does not extend much farther along the haptenic group."



The uncertainty regarding the definition of the antigenic determinant may be minimized either by the use of large haptenic groups in the case of protein-coupled antigens or of polymeric antigens, such as polysaccharides, made up of a single, small, repeating unit. The first of these two alternatives has been used to a limited degree by the author in

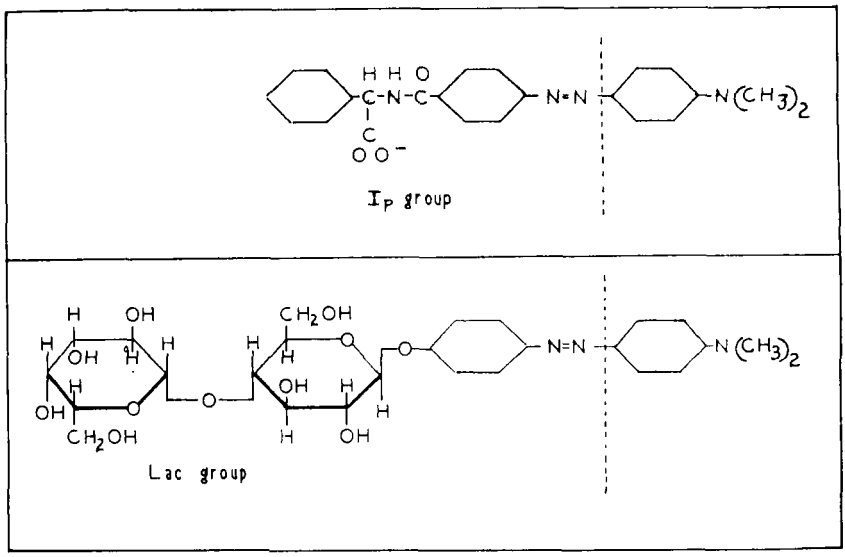


FIG. 8. Formulas for the haptenic groups and the corresponding dyes.

recent years (Karush, 1956, 1957). The studies provide significant information although they were not designed primarily to explore the extent of the combining region.

These investigations were carried out with purified antibody specific for the haptenic groups shown in Fig. 8. Association constants for the binding of the corresponding dyes (Fig. 8) were established by the method of equilibrium dialysis. The association constants for a variety of more weakly bound haptens were obtained by evaluating their inhibitory effect on the binding of the azo dyes. The results for the D-Ip group are shown in Table IV and those for the Lac group in Table VIII. What appears to be of particular significance for the anti-D-Ip antibody is that the affinity of the binding of the D-Ip dye is only 0.5 kcal./mole more

negative than that for the smaller hapten D-phenyl-(*p*-nitrobenzoylamino)acetate. From this it was inferred that the energetically effective volume of the combining region of the antibody is probably not larger than that required to make contact with the entire dye molecule. A parallel conclusion was reached for the anti-Lac antibody from the fact that the affinity of *p*-nitrophenyl-β-lactoside for the antibody was only 0.4 kcal./mole more positive than that of the Lac dye. Here too it could be concluded that the energetically effective region of the antibody is probably not larger than that required to accommodate the Lac dye. Thus very similar estimates were obtained for two haptenic groups of very different chemical nature.

TABLE VIII
THE INHIBITION OF LAC DYE BINDING BY STRUCTURALLY RELATED MOLECULES AT 25°C.[a,b]

Inhibition	$K_I \times 10^{-4}$	$-\Delta F_u$ (kcal./mole)
p-Nitrophenyl-β-lactoside	6.75	8.98
Methyl-β-lactoside	2.02	8.26
Lactose (64% β)	1.10	7.90
Cellobiose (66% β)	0.00275	4.34
Methyl-β-D-galactoside	0.00747	4.94
Methyl-α-D-galactoside	0.00134	3.92
Methyl-β-D-glucoside	0.00054	3.35

[a] Data taken from Karush (1957).

[b] Lac dye is *p*-(*p*-dimethylaminobenzeneazo)phenyl-β-lactoside; K_0 for the Lac dye is 13.4×10^4; and ΔF_u is —9.38 kcal./mole.

The second alternative for the elucidation of the extent of the antibody site has been exploited by Kabat (1954, 1956, 1960) through the use of the hapten inhibition method applied to human antidextran antiserum. These studies were largely concerned with human antidextran of 1 ⟶ 6 specificity and the inhibition of its precipitation with oligosaccharides of the isomaltose series ranging from glucose to isomaltoheptaose. Since the antibody was induced by an antigen consisting of long chains of 1 ⟶ 6 linked α-D-glucopyranose units, it was possible to explore the size of the antigenic determinant with these homologous oligosaccharides.

From a comparison of the hapten inhibition curves obtained with six individual human antidextran sera and the five compounds—isomaltoheptaose, isomaltohexaose, isomaltopentaose, isomaltotetraose, and isomaltotriose—a number of interesting conclusions can be drawn (Kabat, 1961). Increasing inhibition per mole of compound was observed be-

tween the triose and the hexaose, but the incremental increase declined with increasing length. Maximum inhibition was observed with the hexaose indicating that this represented the limit of the size of the antigenic determinant to which the antibody can be complementary. The extent of the antibody-combining region inferred from this result fits in well with the more approximate estimates made in the foregoing. A marked heterogeneity was revealed by differences in the relative effectiveness of the homologous inhibitors among the several human antisera. This heterogeneity may be due to the existence of mixed populations of antibody with combining regions of different size or to the overlapping notion that the degree of complementarity to each of the six units varies from one antibody molecule to another. A choice between these extreme alternatives is, of course, not possible presently and, indeed, some combination of them may prove to represent a more useful description of the observed heterogeneity.

Notwithstanding its insufficiency, the information we have summarized regarding the extent of the antibody-combining region does provide a reasonable and useful picture in spite of its tentative and approximate character. Particularly desirable for the extension of our knowledge in this regard would be studies with high-affinity antibodies, with association constants of the order of 10^{10}. Such studies could not only sharpen our picture of the antibody site, but might also provide important information about the energetic contributions of the various portions of the homologous hapten.

Even on the basis of present knowledge we can estimate roughly that the antibody utilizes between ten and twenty amino acid residues for the definition of one combining region. The corresponding approximate figure of 1% of the residues of the protein for each site serves to emphasize the small fraction of the total number of residues which interact directly with the antigen. On the basis of this figure the view (Haurowitz, 1957) that three or four residues of an antibody molecule are sufficient to establish immunologic specificity appears unreasonable and interpretations of immune phenomena based on this assumption (Burnet, 1961) must be considered irrelevant.

IV. Closing Statement

Although the basic concepts for the understanding of immunologic specificity appear to be in hand, a satisfactory physicochemical description of this phenomenon is yet to be achieved. Much additional information regarding the quantitative energetic importance of the several at-

tractive interactions discussed here must be obtained. In the search for this information, particular emphasis should be placed on the induction and purification of high-affinity antibody since only with such antibody will these interactions reveal their full significance. In this connection the use of relatively large haptenic groups attached to a single kind of protein side chain will provide important advantages. In this way also it may be possible to ascertain the upper limits of the affinity of antigen and antibody and to provide, thereby, the quantitative basis for elucidating many biological immune processes.

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Heterogeneity of γ-Globulins

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I. Introduction

The history of γ-globulins is still relatively short, since they were defined only 25 years ago by Tiselius (1937) as the slowest migrating electrophoretic group of serum proteins. Unique characteristics of the γ-globulins are their antibody activity and an extensive heterogeneity not seen in other serum proteins.

Since the only serum proteins so far shown to be formed in the plasmacytes and lymphoid cells are the electrophoretically identifiable γ-globulins and related proteins, the author of this paper has used the term "γ-globulins" to include all the serum proteins known to be formed in plasmacytes and lymphoid cells. The term γ-globulins has more biological and physicochemical meaning when used in this sense, where it applies to proteins with related properties originating in lymphoid organs, than when the term is restricted to an electrophoretically limited group of proteins. This broader use of the term γ-globulins has been developing for the past 10 years and seems preferable to introducing a new term to apply to these proteins most of which, in any case, migrate more slowly than the electrophoretically defined β-globulins.

Just as electrophoresis served initially to distinguish γ-globulins from other serum proteins, additional physicochemical and immunochemical procedures have made it clear that the γ-globulins are not a single protein group. Most γ-globulins have a molecular weight of about 160,000, but ultracentrifugal studies have shown that approximately 10% of the γ-globulins in man are γ-macroglobulins, i.e., 18 S globulins with molecular weights of about 1,000,000 (Deutsch *et al.*, 1946; Müller-Eberhard *et al.*, 1956). At the other end of the molecular scale, the Bence-Jones proteins were shown to be microglobulins with sedimentation coefficients of about 3.4 or 2.2 S and molecular weights of 20,000–50,000 (Svedberg and Pedersen, 1940; Rundles *et al.*, 1951; ten Thije, 1956; and Putnam and Miyake, 1957). Finally, immunochemical studies revealed an additional class of serum proteins, the β_{2A}-globulins, which were related to the 6.6 S γ-globulins but which differed significantly in antigenic and physicochemical properties from the 6.6 S γ-globulins and γ-macroglobulins in man (Burtin, 1960; Heremans *et al.*, 1959).

At the present time, the four major classes of proteins formed in plasmacytes and lymphoid cells can be termed (1) 6.6 S γ-globulin, (2) β_{2A}-globulins, (3) γ_1-macroglobulins, and (4) γ-microglobulins or Bence-Jones proteins. At least the first three classes coexist in normal serum. Independent recognition of the protein groups by different analytic techniques in different laboratories has resulted in an understandable overlap

in terminology: γ_1-macroglobulins are frequently referred to on immunoelectrophoresis as β_{2M}-globulins (Burtin *et al.*, 1957), and the β_{2A}-globulins have also been designated γ_{1A}-globulins (Waldenström, 1961). Heremans (1959) has suggested "immunoglobulins" as an inclusive term for 6.6 S γ-globulin, β_{2A}-globulin, and macroglobulins. This term avoids the problem inherent in using "γ-globulins" for a specific electrophoretic group as well as for the whole collection of proteins formed in plasma cells. Reservations regarding the term are based on uncertainty as to whether all normal γ-globulins are part of the immune system, whether other serum proteins participating in immunity, such as components of complement which may not be formed in plasma cells, are to be included as immunoglobulins, and whether myeloma proteins and Bence-Jones proteins which are products of malignant plasma cells, should be included even though they have no known immune function. Also present in the γ-globulin region of normal human serum is an 11 S, thermolabile component described by Müller-Eberhard and Kunkel (1961). This component precipitates soluble γ-globulin aggregates and participates in hemolytic reactions but is not identified with the classical components of complement. This protein does not have antigenic determinants in common with γ-globulins and its site of formation is unknown.

Identification of the major groups of γ-globulins has brought a new appreciation of the complexity of antibody response. Some antigens apparently give rise to macroglobulin antibodies, others largely to 6.6 S γ-antibodies. Serum fractionation has revealed that the antibody response may depend on the duration and route of immunization. Furthermore, genetically determined globulin properties may be evident only in one group of the γ-globulins, i.e., the Gm groups are features only of 6.6 S γ-globulins. Recognition of the several classes of γ-globulins also raises questions about their cellular sites of origin and the mechanisms controlling synthesis and metabolism of these proteins. Because recognition of the major γ-globulin groups is a prerequisite for the molecular characterization of antibodies and related molecules, the principal features of these groups will be considered before reviewing antibody properties and the details of γ-globulin substructure.

Proteins produced by malignant plasmacytes or lymphoid cells fall into each of the major γ-globulin categories. Those formed in malignant plasma cells (γ- and β_{2A}-myeloma proteins, macroglobulins, and Bence-Jones proteins) are receiving greater attention. They comprise accessible and readily identifiable products of plasma cell metabolism. In addition to their clinical and biological importance, they are of particular value in structural studies of γ-globulins since they provide the only source of

relatively homogeneous γ-globulins. For these reasons, the question of whether the proteins formed in malignant plasma cells are normal or abnormal globulins is considered in detail.

Heterogeneity is characteristic of each class of γ-globulins with respect to electrophoretic mobility, antibody activity, hexose content, antigenic determinants, and genetic properties. Some of these molecular parameters vary independently indicating considerable variety in internal γ-globulin structure. Since, ultimately, variations in γ-globulin properties will be understood in terms of the chemical and physical nature of the molecules, a brief review of what is known of the structure of γ-globulin molecules will be presented before considering in more detail the various forms of γ-globulin heterogeneity and their possible significance.

II. Four Classes of Proteins Formed in Plasma Cells

A. 6.6 S γ-Globulins

The major features of the γ-globulins, which have been reviewed recently by Porter (1960) and are summarized in Table I, serve as a useful point of departure for discussing proteins formed in plasma cells. The 6.6 S γ-globulins have molecular weights of about 160,000 and may vary somewhat in size and shape (Cann, 1953). On electrophoresis, they migrate as a continuous spectrum of electrophoretically heterogeneous proteins extending from the slowest γ-globulin region to the α-globulin region of serum (Wallenius *et al.*, 1957; Williams and Grabar, 1955b). Electrophoretic heterogeneity as revealed by immunoelectrophoresis is shown in Fig. 1. Most γ-globulin preparations isolated from serum pools have a hexose content of about 1.2% and a total carbohydrate content of about 3% (Rosevear and Smith, 1961). γ-Globulins have multiple antigenic determinants, some of which are specific for this group of proteins.

The 6.6 S γ-globulins form the bulk of the electrophoretic γ-globulin group in man, in rabbit, and probably in many other species as well. They carry the genetically determined Gm(a), (b), and (x) factors (Grubb, 1959). Most antibodies studied in detail have been 6.6 S γ-globulins. The γ-globulins are fragmented to 3.5 S components by treatment with papain and cysteine (Porter, 1958). The detailed structure of these globulins will be considered in Section V.

B. 18 S γ-Macroglobulins (γ_{1M}, β_{2M})

γ-Macroglobulins were first identified in the sera of horses hyperimmunized to pneumococcus polysaccharide (Heidelberger and Pedersen, 1937). Waldenström (1944) discovered large amounts of γ-macroglobulins

TABLE I

PROPERTIES OF FOUR CLASSES OF HUMAN γ-GLOBULINS

Properties	6.6 S γ-Globulins	β_{2A}-Globulins	γ_{1M}-Globulins	Microglobulins (Bence-Jones)
Physicochemical[a]				
Mol. wt. (approx.)	160,000	160,000 and more	1,000,000	45,000 (22,000)
Ultracentrifuge ($s_{20,w}$)	6.6 S	6.6–13 S	18 S (24 S, 32 S)	3.4 S (2.2 S)
Electrophoretic mobility (predominant region)	mid- γ	slow β	fast γ	γ and β
Carbohydrate content (%)	2.6	10.7	12.2	0
Hexose content (%)	1.2	4.8	6.2	
Hexosamine (%)	1.1	3.8	3.3	
Sialic acid (%)	0.2	1.7	2.0	
Antigenic[b]				
Class specific antigen	+ (γ)	+ (β_{2A})	+ (γ_{1M})	...
Common determinants	+	+	+	+
Immunological				
Specific antibodies present	+	...	+	
Cross placenta[c]	+	0	0	
Skin fixation[d]	+	0	0	
React with rheumatoid factors[e]	+	0	0	
Genetic				
Gm factors[f]	+	0	0	
InV factors[g]	+	+	+	+
Quantity in normal serum				
Man (gm %)[h]	1.2	0.4	0.1	

[a] Physicochemical data for 6.6 S γ-globulins and γ_1-macroglobulins from Müller-Eberhard *et al.* (1956) and Müller-Eberhard and Kunkel (1959); for β_{2A}-globulins from Heremans *et al.* (1959) and Heremans (1960); and for Bence-Jones proteins from Putnam (1957), Osserman (1957), and Müller-Eberhard (1962).

[b] See Section IX.

[c] Hitzig (1957).

[d] Referred to by Franklin and Stanworth (1961).

[e] Fudenberg and Kunkel (1961).

[f] Grubb (1959), Mårtensson (1961), Fahey and Lawler (1961).

[g] Harboe *et al.* (1962), Franklin *et al.* (1962).

[h] Based on the inhibition of labeled antigen precipitation (Weiler *et al.*, 1960) using antisera specific for 6.6 S γ-globulin, β_{2A}-globulin, or γ_1-macroglobulin to test sera from 15 normal adults with a mean γ-globulin level of 1.3 gm. % by paper electrophoresis.

in the serum of several patients with a malignant disease of lymphocytoid cells, and Deutsch *et al.* (1946) described similar components in the rapidly migrating γ-globulin fractions of normal human serum. Pedersen (1945) and Deutsch *et al.* (1947) identified isohemagglutinin activity in a γ_1-macroglobulin fraction and subsequently a number of antibody activities have been found among the macroglobulins (cf. Section III). γ-Macroglobulins also occur in the horse (Kabat, 1939), rabbit (Talmage *et al.*, 1956a, b; Thorbecke and Franklin, 1961), mouse (Rask-Nielsen

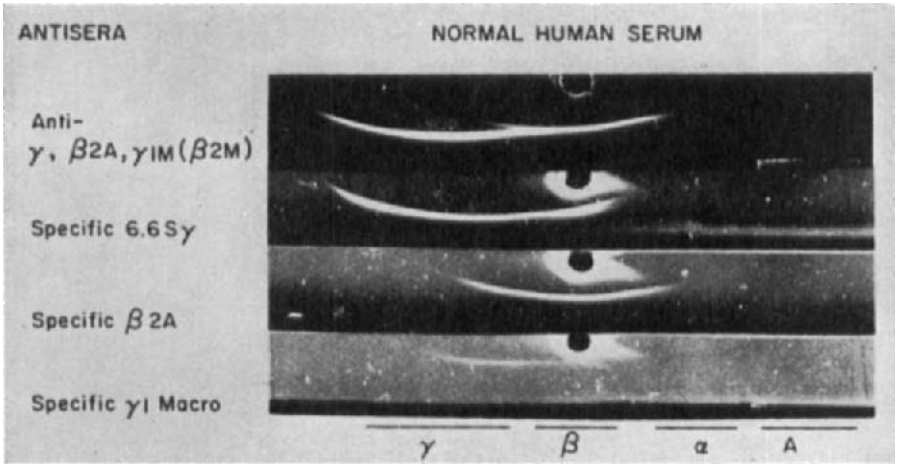

FIG. 1. Immunoelectrophoretic identification of γ-, β_{2A}-, and γ_{1M} (β_{2M})-globulins in normal human serum. Composite illustration of four analyses of normal human serum using rabbit antisera (from top to bottom) reacting with γ-, β_{2A}-, and γ_{1M}-globulin and antisera specific for γ-, β_{2A}-, or γ_{1M}-globulins. Specific antisera were prepared by absorption with appropriate purified proteins (i.e., anti-γ antiserum was absorbed with purified β_{2A}-globulins and γ_1-macroglobulins, etc.).

et al., 1960; Fahey and Humphrey, 1962), and chicken (Makinodan *et al.*, 1960), but the γ_1-macroglobulins of man have been most extensively characterized (Kunkel, 1960) (Table I).

Most γ_1-macroglobulins sediment as 18 S (19 S) components, but even larger components of 24 and 32 S are also present. Although these latter components have not been isolated and compared with the 18 S γ-macroglobulins, they are generally regarded as complexes of 18 S components (Kunkel, 1960). The γ-macroglobulins are electrophoretically heterogeneous with mobility throughout the γ-globulin region and extending into the β-globulin region (Wallenius *et al.*, 1957) (cf. Fig. 2). They contain about 6% hexose (i.e., five times the content of 6.6 S

γ-globulins) and a total of about 12% carbohydrate (Müller-Eberhard and Kunkel, 1959). Many macroglobulinemic macroglobulins are euglobulins, precipitating from solution at low ionic strength. The normal serum content of γ_1-macroglobulins is about 0.1 gm. %, i.e., approximately 1–2% of the total serum protein.

Specific antigenic determinants are present on γ_1-macroglobulins derived from normal serum (Franklin and Kunkel, 1957; Korngold and van Leeuwen, 1957) or from macroglobulinemic serum (Habich, 1953; Kanzow *et al.*, 1955). Detection of these globulins in normal serum by immunoelectrophoresis with specific antisera is illustrated in Fig. 1. In

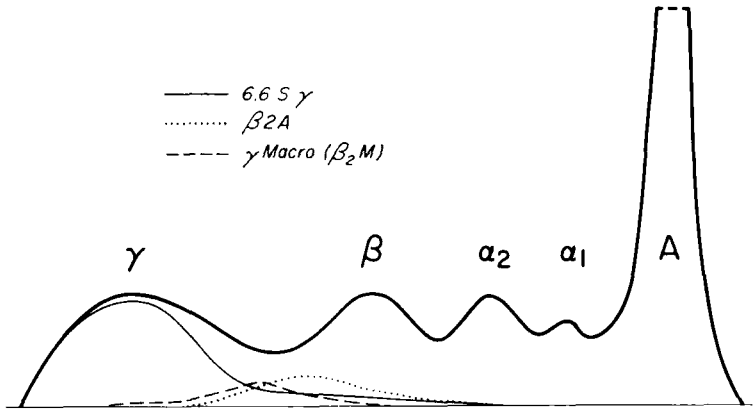

FIG. 2. Schematic representation of the distribution of γ-globulins, β_{2A}-globulins, and γ_1-macroglobulins on zone electrophoresis. The distribution is based on quantitative analysis of the amount of each globulin group in normal serum and on semiquantitative analysis of serum fractions prepared by zone electrophoresis in polyvinyl copolymer blocks.

addition to having specific antigenic determinants, the γ_1-macroglobulins possess antigenic determinants which are present also on 6.6 S γ-globulins and the other protein classes formed in plasmacytes and related cells (Franklin and Kunkel, 1957; Heremans, 1960). The antigenic features of these globulins will be presented in detail in Section IX.

The γ_1-macroglobulins are probably composed of six protein units, approximately the size of 6.6 S γ-globulins, polymerized through disulfide bonds to form the 18 S protein. Deutsch and Morton (1958) and Glenchur *et al.* (1958) showed that γ_1-macroglobulins can be dissociated into 6.6 S units by treatment with sulfhydril compounds. The macroglobulin subunits retain many features of the 18 S molecule, including the high hexose content (Reisner and Franklin, 1961) and specific antigenic determinants (Morton and Deutsch, 1958a). Korngold and van Leeuwen

(1959) and Reisner and Franklin (1961) found that some antigenic determinants of the 18 S molecules were lost after mercaptoethanol dissociation. Comparison of the 7 S units from 18 S γ_1-macroglobulins with 6.6 S γ-globulin molecules reveals some similarities, but also some differences, such as the slightly greater sedimentation velocity of macroglobulin subunits (Reisner and Franklin, 1961), differences in antigenic determinants, in hexose content, and in the ability of macroglobulin subunits to reaggregate on removal of the sulfhydril reagent (Deutsch and Morton, 1958).

The macroglobulin antibodies of man lose antibody activity on reduction to 7 S units by mercaptoethanol (Grubb and Swahn, 1958; Chan and Deutsch, 1960). These observations raise the question of whether mercaptoethanol has produced molecular rearrangement within the 7 S units (Jensen, 1959) and has thus destroyed antibody activity, or whether the integrity of the 18 S molecule is required in order to provide the specific stereochemistry needed for antibody activity. The observations of Petermann and Pappenheimer (1941) who treated 19 S horse antipneumococcus polysaccharide with pepsin and obtained antibody active products with a molecular weight of about 100,000 indicate that for some antibodies, at least, the larger structure is not required.

C. β_{2A}-Globulins

This component, initially detected by Williams and Grabar (1955a) as β_2-globulin in the region between the γ- and β-globulin zones on immunoelectrophoresis of normal human serum, was subsequently designated β_{2A} (Burtin *et al.*, 1957) and the immunoelectrophoretic terminology is generally applied to this globulin group. The β_{2A}-globulins comprise about 20% of the total γ-globulins in man (Table I). Much of our knowledge about β_{2A}-globulins is based on the work of the Heremanses, —which has been summarized recently (Heremans, 1960).

The β_{2A}-globulins are an electrophoretically heterogeneous globulin group migrating in the fast γ- and β-globulin region in human serum (Figs. 1 and 2). They migrate nearer the mid-β region in the mouse (Fahey, 1961a). Most β_{2A}-globulins sediment as 6.6 S components in the ultracentrifuge, but larger components have been detected in concentrates of normal β_{2A}-globulins (Heremans *et al.*, 1959); 9, 11 and 13 S components are characteristically found with β_{2A}-myeloma proteins. The hexose content of β_{2A}-globulins is reported to be 3.2% (Schultze, 1959) or 4.9% (Heremans *et al.*, 1959). Purified β_{2A}-globulin fractions have been prepared by Heremans (1960) by taking advantage of differences in the solubility of γ- and β_{2A}-globulins in the presence of zinc ions.

The β_{2A}-globulins have distinctive antigenic determinants which permit their identification as a separate protein group (Fig. 2). Burtin (1960) has emphasized the antigenic differences between β_{2A}-globulins and the 6.6 S γ-globulins and γ_1-macroglobulins. Williams and Grabar in their initial report (1955a) recognized that the β_2(β_{2A})-globulin probably cross reacted with γ-globulin. Heremans (1960) and Heremans, J. F. and M.-T. (1961), however, systematically investigated the relationship between the β_{2A}-globulins and other γ-globulin groups and showed that the β_{2A}-globulins shared several antigenic determinants with 6.6 S γ-globulins and with γ_1-macroglobulins.

D. Microglobulins (γμ) (Bence-Jones Proteins)

1. Normal Components

Low molecular weight γ-globulins from normal human urine which cross react with normal serum γ-globulins have been described by Webb *et al.* (1958b), Franklin (1959), and Berggård (1961a). A similar component in normal serum, which cross reacted with 6.6 S γ-globulin but also was antigenically deficient in relation to normal γ-globulin, has been described by Berggård (1961b). The serum and urinary microglobulins are heterogeneous on electrophoresis (Berggård, 1961b). These urinary proteins have been said to have molecular weights of about 10,000 (Webb *et al.*, 1958b) or 30,000 (Franklin, 1959). Evidence that such proteins from normal urine have the heat solubility properties of Bence-Jones proteins has been presented by Stevenson (1960).

The serum of the newborn pig contains globulins with sedimentation coefficients of about 5.1 S which share antigenic determinants with adult pig 6.6 S γ-globulin (Franěk *et al.*, 1961). These microglobulins are only present in small amounts in the newborn pig (20 μg./ml.), and such quantities would be difficult to detect in normal adult pig serum with its relative abundance of 6.6 S γ-globulins.

Some γ-microglobulins may be breakdown products of serum 6.6 S γ-globulins. Webb *et al.* (1958a) and Franklin (1959) injected radioiodinated γ-globulin intravenously and found protein-bound radioactivity in the urine, but it is uncertain how much of the urinary microglobulin is derived from breakdown of larger serum γ-globulins.

On the other hand, the quantity of urinary microglobulin may not reflect the amounts of microglobulins that are synthesized normally especially if these components undergo renal tubular reabsorption or have a high fractional rate of catabolism within the body. The possibility

that γ-microglobulins are formed normally by the plasma cells is worthy of further investigation, utilizing the characteristics of Bence-Jones proteins in multiple myeloma as a guide to the general properties of corresponding normal components.

2. Bence-Jones Proteins Associated with Malignant Plasma Cells

Small globulins with molecular weights of 50,000 or less ($s_{20,w}$ of 2–4 S) are frequently found in association with the malignant proliferation of plasma cells and most of these proteins exhibit reversible heat insolubility as originally described by Bence-Jones (1847). The need for careful control of the pH, ionic strength, and protein concentration to detect the reversible heat insolubility of Bence-Jones proteins has been emphasized recently by Putnam *et al.* (1959). The physicochemical basis for this distinctive property, however, remains unknown.

Two classes of Bence-Jones proteins have been described by Putnam *et al.* (1959) on the basis of quantitative pH heat precipitation curves. The "α"-type proteins had asymmetrical precipitation curves, maximum about pH 5 but broadly spread on the alkaline side. The "β"-type proteins, in contrast, showed symmetrical precipitation curves (maximum about pH 5) without protein precipitation above pH 6.5. Furthermore, the finding that α-type Bence-Jones proteins lacked N-terminal aspartic acid, in contrast to the β-type proteins, provided additional evidence of structural differences between the two types of proteins (Putnam and Miyake, 1957).

Differences in antigenic determinants are the basis for two major classes of Bence-Jones proteins described by Korngold and Lipari (1956b) and by Burtin *et al.* (1956). Korngold's antigenic types A and B correspond to Putnam's physicochemical type α and β. Two types of Bence-Jones proteins are seen in Fig. 3 where Ouchterlony diffusion analysis reveals crossing of the precipitin lines formed by each protein.

More extensive heterogeneity of Bence-Jones proteins is revealed by viewing collectively the electrophoretic properties of proteins from a number of patients (Osserman, 1957; Putnam, 1957). In these, the mobility ranged from the slow γ-globulin region to the α-globulin region. Variability in antigenic determinants between individual Bence-Jones proteins has been emphasized by ten Thije (1956), by Korngold and Lipari (1956b), and by Burtin *et al.* (1956). Starch gel electrophoresis has revealed heterogeneity of the Bence-Jones proteins in most patients (Engle *et al.*, 1961).

Molecular analyses indicate that Bence-Jones proteins may occur in

two sizes with molecular weights of about 45,000 ($s_{20,w}$ = 3.4 S) or 22,000 ($s_{20,w}$ = 2.0–2.9 S) (Putnam, 1957). There have been no reports, however, showing that these size differences are related to other differences among the Bence-Jones proteins.

Low carbohydrate content for Bence-Jones proteins, lower than for γ-globulins or γ-myeloma proteins, has been reported (Osserman and Lawlor, 1954; Putnam and Miyake, 1957; Maiorca and Scarpioni, 1961).

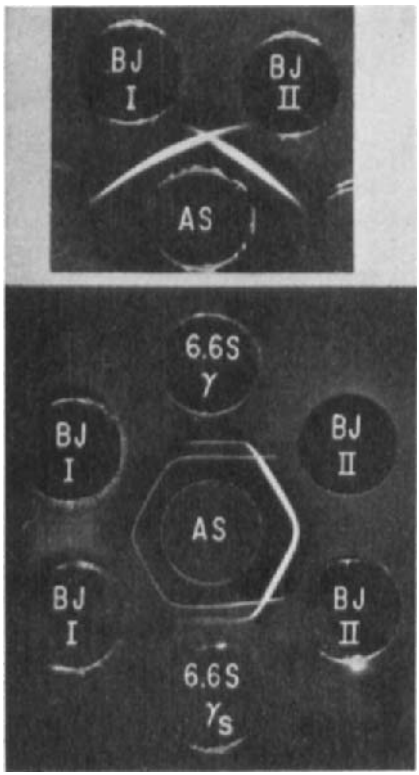

FIG. 3(A). Two types of Bence Jones proteins. Differences in antigenic determinants on Type I and Type II Bence Jones proteins were seen on Ouchterlony analysis using rabbit anti-NHγ antiserum (AS).

FIG. 3(B). Two types of normal 6.6 S γ-globulins. Two precipitin lines formed by NHγ in the upper part of the Ouchterlony plate are seen to fuse with lines formed by Bence Jones proteins Types I and II. In the lower part, two lines formed by S (γ_s) fragments (from a papain digest) of NHγ are seen to differ antigenically in the same manner as the intact NHγ molecules above. Rabbit anti-antiserum *vs.* NHγ-S fragments was used in the center cell (AS).

Studies of Vis and Crokaert (1956) and Müller-Eberhard (1962) indicate that Bence-Jones proteins may be devoid of carbohydrate.

The Bence-Jones proteins share many features with the other γ-globulin groups as revealed by a similarity in amino acid composition (Putnam, 1957), in physicochemical organization (Jirgensons, 1958a,b), and in antigenic determinants (ten Thije, 1956; Korngold and Lipari, 1956b; Burtin *et al.*, 1956). The properties of Bence-Jones proteins have been reviewed by Putnam (1957, 1960) and the major features of these proteins are tabulated for comparison with other classes of γ-globulin in Table I. The relationship between Bence-Jones proteins and the γ-globulins, β_{2A}-globulins, and γ_1-macroglobulins will be considered further in Section XI.

E. Fractionation of the Serum γ-Globulins

A variety of fractionation procedures has been used to separate the γ-globulins from other serum proteins and to obtain γ-globulin subgroups for detailed study, as well as to characterize specific antibodies and determine whether antibody activity is present in more than one globulin group. The most commonly used fractionation procedures are zone electrophoresis, ultracentrifugation, anion-exchange or cation-exchange cellulose chromatography, alcohol or ether fractionation, ammonium sulfate fractionation, or precipitation with specific antigen and subsequent separation of antibody (Singer *et al.*, 1960).

For a fuller discussion of these fractionation procedures the reader is referred to reviews by Kunkel and Trautman (1959), Kunkel (1960), Peterson and Sober (1960), Pennell (1960), and Cooper (1960), but some observations on γ-globulin separations by several of these methods are included in this section. In many procedures, conditions suitable for one sample size cannot be arithmetically converted successfully to conditions involving appreciably larger or smaller samples. For this and other reasons identification of the major components as well as any contaminant proteins in protein fractions is usually carried out by determining electrophoretic mobility on zone and starch gel electrophoresis, by immunochemical tests such as Ouchterlony gel diffusion analysis or immunoelectrophoresis with appropriate antisera, by the ultracentrifugal sedimentation behavior, and by measuring the recovery of antibody activity in the fractions.

Most of the γ-globulins can be separated from other serum proteins under the mild conditions of zone electrophoresis carried out on blocks of starch (Kunkel, 1954), on polyvinyl chloride or polyvinyl copolymer particles (Müller-Eberhard and Kunkel, 1956; Müller-Eberhard, 1960),

on columns of treated cellulose (Flodin and Porath, 1954), on continuously flowing systems supported by packed inert materials (Brattsten, 1955) or by paper curtains (Durrum, 1955). Partial separation of the 6.6 S γ-globulins, β_{2A}-globulins, and γ_1-macroglobulins is obtained by zone electrophoresis, the latter two components being concentrated in the slower migrating β-globulins and the faster migrating γ-globulin fractions, respectively, as shown in Fig. 2. Purification of the individual γ-globulin subgroups and separation of the faster migrating γ-globulin from other components migrating in the β-globulin region, however, require combination of zone electrophoresis with some other fractionation procedure. Block electrophoresis can be used for serum samples from 0.5 to 20 ml. volume provided that the block is adequately cooled (restricting block thickness to about 6 mm. helps in this regard) and the width of the block allows adequate sample loading. It is relevant to note that polyvinyl chloride preparations may vary markedly in their settling properties when a block is prepared. Zone electrophoresis on paper strips is limited by the small quantity of protein that can be applied. Starch gel electrophoresis (Smithies, 1955, 1959) is similarly limited by its small capacity as well as by the difficulties of recovering protein from the gel.

A pure 6.6 S γ-globulin preparation can be obtained by diethylaminoethyl (DEAE) cellulose chromatography of whole serum (Sober *et al.*, 1956; Levy and Sober, 1960; Fahey and Horbett, 1959; Stanworth, 1960). The distribution of γ-, β_{2A}-, and γ_{1M}-globulins on DEAE-cellulose chromatography of normal human serum and of electrophoretically prepared γ-globulin are illustrated in Fig. 4. Most of the 6.6 S γ-globulins (over 90%) were eluted in the first third of the chromatogram. Subsequently the β_{2A}-globulins were eluted and, finally, the γ_1-macroglobulins, with the bulk of each γ-globulin group in separate chromatogram fractions. Small amounts of 6.6 S γ-globulin of progressively faster mobility, however, are eluted throughout the chromatogram.

Subfractionation of the 6.6 S γ-globulins and partial purification of β_{2A}-globulins and γ_1-macroglobulins can be obtained if DEAE-cellulose chromatography is combined with fractionation by zone electrophoresis (Fahey and Horbett, 1959; Fahey, 1962a). The 6.6 S γ-globulins can be further subfractionated by carboxymethyl (CM) cellulose chromatography (Sober and Peterson, 1958). Conditions for chromatographic absorption and elution of many proteins are cited by Peterson and Sober (1960), but it must be borne in mind that different lots of substituted celluloses may vary greatly in their capacity to absorb and release protein. Thus with some preparations of DEAE cellulose, the ionic strength

of the initial buffer has to be lowered, and the serum protein load has to be reduced to obtain a first fraction containing only 6.6 S γ-globulin.

Antibody distribution between 6.6 and 18 S globulins can be determined by density gradient ultracentrifugation (Kunkel, 1960) or by measuring differential rates of sedimentation in a Waugh-Yphantis cell (1953) or in a preparative ultracentrifuge tube using the procedure out-

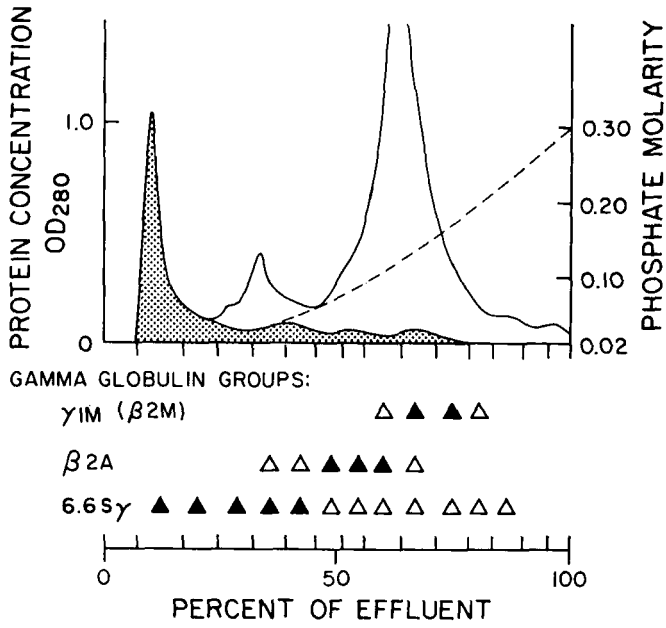

FIG. 4. Distribution of the γ-globulins and of the γ-, β_{2A}-, and γ_{1M}-globulin groups on DEAE-cellulose column chromatography. The protein distribution was determined by optical density measurements at 280 mμ, the γ-globulin distribution by zone electrophoresis (stippled area), and specific globulins by gel diffusion analysis of fractions using specific antisera. Chromatography of 1 ml. of serum was conducted on 2 gm. of DEAE cellulose using a gradient elution from 0.02 to 0.30 *M* phosphate pH 8 buffer.

lined by Robbins *et al.* (1954). β_{2A}-Globulins remain in the region of the 6.6 S γ-globulins. Similar sedimentation techniques have been used to prepare γ-macroglobulins (Müller-Eberhard *et al.*, 1956) but poor recovery of macroglobulin from the sedimented protein pellets was encountered. Density gradient techniques have limited capacity when applied to serum. Macroglobulins in macroglobulinemic sera often can be purified by repeated low ionic strength precipitation. With further develop-

ment, gel filtration techniques for separating macromolecules (Porath, 1960) may be of great aid in the preparation of 18 S γ-macroglobulins.

F. Sites of Synthesis

Formation of γ-globulins in plasmacytes and related cells was inferred from observations that increased amounts of γ-globulin components were associated with the malignant proliferation of plasma cells and by a correlation of the plasma cell content of tissues with the serum antibody and γ-globulin levels in human disease and experimental immunization (Fagraeus, 1948). This was supported by immunofluorescent localization of specific groups of γ-globulins, including 6.6 S γ-globulin (Ortega and Mellors, 1957; Vasquez, 1958), macroglobulins (Mellors *et al.*, 1959; Curtain, 1959a; Dutcher and Fahey, 1960), and β_{2A}-myeloma proteins and Bence-Jones proteins (Solomon *et al.*, 1962). Biosynthetic techniques utilizing C^{14}-labeled amino acids permitted the direct demonstration of specific γ-globulin and antibody synthesis in rabbit lymph nodes and other tissues (Askonas *et al.*, 1956; Thorbecke, 1960). Studies of malignant plasma cells have shown 6.6 S γ-myeloma protein, β_{2A}-myeloma protein, and Bence-Jones protein synthesis in appropriate mouse plasma cell tumors (Nathans *et al.*, 1958; Askonas, 1961; Askonas and Fahey, 1961b), and recently γ-globulin, β_{2A}-globulin, and γ-macroglobulin formation in human lymph nodes and other tissues has been reported (Asofsky and Thorbecke, 1961; Levine *et al.*, 1961). In sum, these studies clearly indicate that the 6.6 S γ-globulins, β_{2A}-globulins, γ_1-macroglobulins, and Bence-Jones proteins are formed in plasmacytes, in lymphocytoid-plasma cells, and in immature cells present in the lymph nodes, spleen, bone marrow, and related tissues of the lymphoid system.

III. Antibody Heterogeneity

A. Distribution of Antibodies among γ-Globulin Classes

1. 6.6 S γ-Globulins

Antibodies to viruses, bacteria, and animal cells, as well as to proteins, carbohydrates, and a variety of chemical haptenes may be 6.6 S γ-globulins. A number of 6.6 S γ-antibodies in man are listed in Table II, and antibody characteristics in other species have been tabulated by Porter (1960) and Kabat (1961). The immunochemistry of antibodies has been reviewed recently by Porter and Press (1962).

Antibody activity to specific antigens may be associated with 6.6 S γ-globulins of widely different electrophoretic mobility in hyperimmunized states (Cann *et al.*, 1950; Williams and Grabar, 1955b; Askonas

et al., 1960; Velick *et al.*, 1960). It is not known, however, whether electrophoretic heterogeneity is as extensive in the earliest states of 6.6 S antibody response. Neither is it known whether all antigens elicit an immune response in electrophoretically identical 6.6 S antibody molecules. Sober and Peterson (1958) with Martin, utilizing CM-cellulose

TABLE II
MOLECULAR DISTRIBUTION OF ANTIBODIES IN MAN

Antibody	6.6 S Globulin[a]	18 S γ_{1M}-Globulin[a]
Antipneumococcus[b]	+	
Antidextran[c]	+	
Antimumps virus[d]	+	
Antihistoplasma[d]	+	
Antityphoid H[d]	+	
Isohemagglutinins		
A and B[e]		
Saline	+	+ +
Antihuman glob.	+	
Anti-Rh (D)[f]		
Saline		+ +
Albumin	+	
LE cell factor[g]	+	
Antithymus nucleoprotein[h]	+	
Antiliver nucleoprotein[h]	+	+ +
Anticytoplasmic antigens[i]	+	+
Antithyroglobulin[j]	+ +	+
Rheumatoid factors[k]		+
Wassermann reagins[l]	+	+
Cold hemagglutinins[m, n]		+
Warm hemagglutinins[n]	+	

[a] + = Presence of antibody activity; ++ = antibody most frequently present in greatest activity in this fraction.
[b] Kabat (1939).
[c] Kabat (1954).
[d] Fahey (1960).
[e] Pedersen (1945), Deutsch and Chan (1958), McDuffie *et al.* (1958), Fahey and Morrison (1960), and Rawson and Abelson (1960a).
[f] Campbell *et al.* (1955), Chan and Deutsch (1960), and Abelson and Rawson (1959b).
[g] Holman and Kunkel (1957), Willkens *et al.* (1958), and Fallet *et al.* (1958).
[h] H. C. Goodman *et al.* (1960).
[i] Deicher *et al.* (1960).
[j] Pressman *et al.* (1957), Korngold *et al.* (1959), and Fahey and Goodman (1960).
[k] Kunkel *et al.* (1959), Lospalluto and Ziff (1959), and Svartz *et al.* (1958).
[l] Davis *et al.* (1945) and A. B. Laurell and Malmquist (1961).
[m] Gordon (1953) and Christenson *et al.* (1957).
[n] Fudenberg and Kunkel (1957).

chromatography to subfractionate γ-globulins, found differences in the relative chromatographic distribution of antibodies to toxoplasma, histoplasma, brucella, typhoid H, and streptolysin O antigens. The basis for the differences is uncertain since sera from different individuals were pooled for the experiment. Further studies of the possibility of physicochemical differences between specific antibodies are needed.

Martin *et al.* (1957) found that specific antibodies differed in their rate of degradation when given to patients with severe hypogammaglobulinemia. The antibodies used were pooled from many donors, and determining the basis for the metabolic differences requires further information about the physicochemical properties of specific antibodies, especially in view of the observations of Taliaferro and Talmage (1956) and Cohen and Freeman (1960) that 6.6 S γ-globulin and 18 S γ_1-macroglobulin are metabolized at different rates.

Assignment of antibody activity to a specific class of γ-globulins as in Table II is done on the basis of the predominant form of serum antibody and is not to be taken as indicating that antibody activity is only in one globulin group. Several antibodies have been found in both 6.6 S γ- and 18 S γ_{1M}-globulin groups. The antigenic substances involved, however, are complex, and it is not certain that antibodies in different groups are directed at the same antigenic sites. Furthermore, individual subjects may vary in their antibody response, and many antibody characterization studies have been done on relatively few serum samples. Understandably, the detailed characterization of antibodies is undertaken with the most potent antisera available because of the problems of assay of the serum fractions. The most potent antisera, however, may not be representative of all sera or situations. For example, a normal human subject (with group O red blood cells) immunized with blood group substance A will have most anti-A antibodies among the 6.6 S γ-globulins, but a nonimmunized normal subject may have most of his anti-A activity among the 18 S γ-macroglobulins (Rawson and Abelson, 1960b).

2. *γ-Macroglobulins*

Macroglobulin antibodies were identified by Heidelberger and Pedersen (1937) in horses immunized with pneumococcus. Subsequently, Pedersen (1945) identified isohemagglutinin activity among the rapidly sedimenting proteins of human serum, and Deutsch *et al.* (1947) found isohemagglutinin activity in γ-globulin fractions rich in 18 S macroglobulin. Many, but by no means all, antibodies against red blood cells have been found subsequently among the γ_1-macroglobulins (see Section III, B).

Macroglobulin antibodies have received increasing attention as the techniques of protein chemistry have been applied in studies of human disease factors (Table II). In some patients with chronic thyroiditis, γ_1-macroglobulin antithyroglobulin antibodies have been identified (Pressman *et al.*, 1957; Korngold *et al.*, 1959; Fahey and Goodman, 1960), although these seem to be less common than 6.6 S antithyroglobulin antibodies.

Patients with lupus erythematosus (LE) may have multiple serum factors reacting with cellular components. The serum factors responsible for formation of the LE cells (Hargrave cells) are 6.6 S γ-globulins (Table II). In some patients with LE, however, factors reacting with human liver nucleoprotein preparations (Goodman *et al.*, 1960) or with liver cytoplasmic preparations (Deicher *et al.*, 1960) have been found to be predominantly 18 S γ_1-macroglobulins.

Wassermann reagins are heterogeneous—activity having been identified in γ_1-macroglobulin fractions as well as in 6.6 S γ-globulins (Davis *et al.*, 1945; A. B. Laurell and Malmquist, 1961). Indirect evidence that the Paul-Bunnel antibodies and typhoid O antibodies are macroglobulins was obtained by treatment of antisera with mercaptoethanol (Grubb and Swahn, 1958), which caused a loss of activity of these antibodies and of known 18 S antibodies but only a slight reduction in activity of 6.6 S γ-antibodies.

Rheumatoid factors, substances present in the serum of patients with rheumatoid arthritis and related diseases and which react with 6.6 S γ-globulins, have been identified as 18 S γ_1-macroglobulins (Kunkel *et al.*, 1959; Lospalluto and Ziff, 1959). A variety of rheumatoid factors, all of which are macroglobulins, have been described on the basis of ion-exchange chromatographic distribution (Lospalluto and Ziff, 1959), absorption with specific precipitates (J. H. Vaughan *et al.*, 1958), and specific reactivity in the Gm system (Harboe, 1959; Grubb, 1959). Multiple rheumatoid factors are usually present in the serum of individual patients (Fudenberg and Kunkel, 1961).

3. β_{2A}-*Globulins*

Antibody activity that is present only in the β_{2A}-globulins has not been reported as yet. Schultze (1959) prepared serum fractions composed largely of β_{2A}-globulin from pooled human serum and identified antibodies against seven antigenic materials. It is uncertain, however, how much of this activity was due to β_{2A}-globulins for the same antibody activities were also present in 6.6 S γ-globulin and 18 S macroglobulin fractions and the β_{2A}-globulin preparations contained other

globulin components. The role of β_{2A}-globulins in immunity remains to be determined.

A number of antibodies, which cannot be characterized as being slow migrating 6.6 S γ-globulins nor as 18 S macroglobulins, may be β_{2A}-globulins. In a study of normal serum antibody activities against enteric pathogens by Turk (1959), the major 6.6 S γ-globulin fraction (Fraction II) prepared by ethanol fractionation procedures had little activity, whereas Fraction III contained most of the activity and sedimented as 6.6 S globulins. This contrasts with the reported properties of antibodies after immunization with enteric antigens where the activity is largely in the 6.6 S γ- and 18 S γ_1-globulin fractions (Schultze, 1959) and indicates that the properties (or at least the relative distribution) of "natural" antibodies may differ from antibodies present after immunization. Some of the allergic reaginic antibodies (Humphrey and Porter, 1957; Stanworth, 1959; Augustin and Hayward, 1960), Wassermann reagins (A. B. Laurell and Malmquist, 1961) and isohemagglutinins (Abelson and Rawson, 1959a) are found in anion-exchange cellulose chromatogram fractions which contain β_{2A}-globulins as well as relatively rapidly migrating γ-globulins. Further studies will be required, however, to determine whether the antibody activities are associated with β_{2A}-globulins or with another subgroup of the γ-globulins.

B. Antibodies against Red Blood Cells

The isohemagglutinins present in normal human serum exist both as 18 S γ_1-macroglobulins and as 6.6 S γ-globulins (Rawson and Abelson, 1960a; Fahey and Morrison, 1960). The 18 S γ-macroglobulins are active as saline agglutinins and the 6.6 S γ-globulins are most active in the presence of antihuman globulin (Coombs) serum (Rawson and Abelson, 1960a). It is not known whether β_{2A}-globulin possess isohemagglutinin activity. Wurmser and Fillitti-Wurmser (1957) summarized a series of studies indicating that a variety of anti-B isohemagglutinins may occur, depending on the blood group of the individual. Individuals with blood group A_1O had 15.7 S anti-B isohemagglutinins, whereas A_1A_1 subjects had 8.4 S and OO subjects had 5.7 S isohemagglutinins. In addition, Abelson and Rawson (1959a) found isohemagglutinin activity in an intermediate chromatogram fraction which might contain β_{2A}-globulins but, since 6.6 S γ-globulins are present as well, it is not yet possible to be specific about the molecular form (γ or β_{2A}) of isohemagglutinin activity in these fractions.

Rh antibodies have been found in the 18 S γ_1-macroglobulins (saline agglutinins) and in the 6.6 S γ-globulins (active in the presence of albu-

min) by Campbell *et al.* (1955). Saline agglutinating anti-M, anti-N, anti-P, and anti-K activities have also been identified as γ_1-macroglobulins (Fudenberg and Kunkel, 1958; Abelson and Rawson, 1961). The relationship between 6.6 S and 18 S Rh antibodies was investigated by Chan and Deutsch (1960) who found distinct chemical and immunochemical properties in each group. These two forms of antibodies did not appear to be dissociation or aggregation products of each other.

Antibodies that agglutinated erythrocytes only on refrigeration (cold agglutinins) were identified as 18 S γ-macroglobulins by Gordon (1953). This result was confirmed by Christenson *et al.* (1957) and Fudenberg and Kunkel (1957). Fudenberg and Kunkel also showed that hemolytic antibodies active at 37°C. were 6.6 S γ-globulins.

The molecular form of anti-red-cell antibodies may be an important factor in determining the production of erythroblastosis fetalis. Abelson and Rawson (1961) point out that hemolytic disease of the newborn is more likely to be associated with a predominance of 6.6 S antibodies than with a predominance of 18 S antibodies. The ready transference of 6.6 S antibodies across the placental barrier (Brambell *et al.*, 1960), in contrast to the poor transfer of 18 S globulins (Franklin and Kunkel, 1958), would be a contributing factor to erythroblastosis fetalis due to 6.6 S antibodies.

Heterophile antibodies, in some species at least, appear to be 18 S γ_1-macroglobulins (Kunkel, 1960). Administration of heterologous red blood cells into rabbits, however, induces a complex assortment of 6.6 S and 18 S antibodies, as shown by Talmage *et al.* (1956a,b), Taliaferro and Talmage (1956), Stelos (1956) and their associates. Briefly, these workers have identified two Forssman as well as two isophile antibodies. Some of the distinguishing characteristics are mentioned in the following section.

C. Factors Determining the Physicochemical Properties of Antibodies

The physicochemical properties of antibodies may be influenced by (1) the chemical properties of the antigen, (2) route and duration of immunization, (3) age of recipient, (4) site of antibody formation, (5) genetic factors, and (6) the species injected. The fact that some antigens induce 6.6 S γ- and others 18 S γ_1-antibodies indicates that chemical properties play an important role in the antibody response, but the nature of antigenic configurations stimulating predominantly a macroglobulin, rather than 6.6 S antibody response is obscure. Whether the significant factor is the chemistry of the antigen or the manner in which it is

presented to the immune system (many of the macroglobulin-evoking antigens are attached to large molecules or cells) remains to be determined. Also, those antigenic substances which react both with 6.6 and with 18 S antibodies are large and complex molecules, i.e., erthrocytes, nucleoprotein extracts, and thyroglobulin preparations, and whether the different antibodies react at the same or different antigenic sites is not known. Clarification of these points would be of great interest, especially where both 6.6 and 18 S antibodies are present in a single individual.

The rate and duration of antigen administration influences antibody properties. Porter (1955) and Humphrey and Porter (1956) studied the partition chromatographic behavior of antibodies from rabbits immunized intravenously with pneumococcal polysaccharide, influenza virus, and ovalbumin. Antibodies were found in the slower running fractions during the early stages of immune response, but later predominantly in the middle chromatogram fractions. The antibodies in the different partition chromatogram regions, however, were not characterized as to size.

Duration of immunization may affect antibody characteristics in man. Subjects immunized with Rh antigens by pregnancy or by transfusion first develop saline-active agglutinins, but later incomplete (Coombs'-active or albumin-active) antibodies predominate (Diamond, 1947; Abelson and Rawson, 1961). Since the former antibodies are largely 18 S γ_1-macroglobulins and the incomplete antibodies are 6.6 S γ-globulins (Campbell *et al.*, 1955), it would appear that the duration of antigen exposure influences the γ-globulin features of the antibody. Immune response to certain antigens may normally pass through a stage in which 18 S antibodies are formed to one in which 6.6 S γ-globulin antibodies are predominantly produced. Analogous observations were made in syphilis by A. B. Laurell and Malmquist (1961) who obtained evidence that primary syphilis may be associated with Wassermann reagin activity in the macroglobulin fractions and that more low molecular weight antibody develops as the disease progresses.

Of the four types of hemolysins induced by sheep red cells in rabbits, one Forssman and one isophile antibody migrate in the γ_1-region and sediment as 18 S macroglobulins, whereas the other Forssman and the other isophile antibody migrate in the γ_2-region and sediment as 6.6 S γ-globulins (see review by Taliaferro, 1957). The larger Forssman globulin predominates early both in primary and in secondary immunizations, whereas the smaller ones arises later (Talmage *et al.*, 1956a; W. H. and L. G. Taliaferro, 1961). It is also interesting that the large molecule equilibrates at a concentration of about 80% in the serum and 20% in the tissues and that the smaller one equilibrates at an equal concentra-

tion in the two sites. The two Forssman antibodies have also been studied following the injection of human type A red cells and guinea-pig and horse kidney (Stelos and Taliaferro, 1959; Stelos *et al.*, 1961).

Bauer and Stavitsky (1961) reported that 18 S γ_1-antibodies are formed first and predominate at least until the tenth day following primary immunization of rabbits by foot-pad injections with hemocyanin, bovine γ-globulin, human serum albumin, or diphtheria toxin. Thereafter, they decline as the level of 6.6 S antibodies increases. In C3H mice, however, antibody against pneumococcal polysaccharide and hemocyanin on day 6 following primary intravenous antigen injection (2–3 days following appearance of detectable antibody in the serum), was found to be predominantly 6.6 S γ-globulins (Fahey and Humphrey, 1962). The differences between these observations and those of Bauer and Stavitsky may be the result of different routes of immunization, an instability of the 18 S mouse antibodies, or species differences.

Age or immunological maturity may affect the type of antibody response evoked. R. T. Smith (1960) has shown that newborn infants react to typhoid H antigen by producing specific 18 S macroglobulin antibodies but older children and adults respond to this antigen with 6.6 S γ-globulin antibodies.

Genetic factors also influence the molecular form of the antibody response. Qualitative, as well as quantitative, antibody response to immunization with A and B substances is under genetic control and is related to the erythrocyte blood group of the antigen recipient. Rawson and Abelson (1960b) found that individuals with blood group O responded with a predominance of 6.6 S isohemagglutinins, but that individuals with blood groups A and B responded to immunization with a greater increase in the 18 S macroglobulin group of isohemagglutinins.

Interspecies differences in response to antigens have been demonstrated for pneumococcal polysaccharides—horses often responding with macroglobulin antibodies and rabbit and other species producing 6.6 S γ-antibodies (Kabat, 1939). Comparison of different inbred mouse strains (Fahey and Lawrence, 1962) has also revealed intraspecies differences in electrophoretic mobility and chromatographic behavior of antihemocyanin antibodies. Since the mouse antibodies were largely 6.6 S globulins, these studies provided evidence that genetic factors may influence the characteristics of 6.6 S γ-globulin antibodies, as well as the distribution between 6.6 and 18 S classes of γ-globulins.

Additional evidence for differences in some of the factors controlling the serum γ-globulin groups is seen in the observations of pathologic

dissociation in the synthesis of 6.6 and 18 S γ-globulins (dysgammaglobulinemia). Patients, with normal levels of 6.6 S γ-globulins but with a marked deficiency of 18 S γ_1-macroglobulins, were described by Giedion and Scheidegger (1957). The converse abnormality, low levels of 6.6 S γ-globulins (and β_{2A}-globulins) but greater than normal levels of 18 S γ_1-macroglobulins, was described by Rosen *et al.* (1961). Discrepancies in 6.6 and 18 S γ-globulin levels were also noted by Shohl *et al.* (1962) in a study of isohemagglutinins and 6.6 S γ-globulins in a variety of malignant diseases. The observations now available, however, only indicate that the different classes of γ-globulins are, in part, under separate control. Little is known of the nature of these controls.

Factors regulating maturation of plasmacytes and related cells play an important role in determining the production of 6.6 and 18 S γ-globulins and β_{2A}-globulins. The synthesis of all three protein groups appears to be slow in the newborn and all three classes of proteins are deficient in congenital agammaglobulinemia.

Although disease may alter the quantitative antibody response, there is no evidence that disease influences the physicochemical qualities of specific antibody. The presence of 18 S antithyroglobulin activity in chronic thyroiditis and 18 S antinucleoprotein activity in LE have not been associated with any clinical features which can be distinguished from the presence of only 6.6 S disease antibodies (Fahey and Goodman, 1960; Goodman *et al.*, 1960). Furthermore, evidence that disease may not cause exclusive formation of one or another class of antibody was obtained in a patient with LE who had 18 S macroglobulin antibodies against a liver nucleoprotein extract and 6.6 S antibodies against thyroglobulin (Goodman *et al.*, 1959).

The evolutionary significance of the various forms of the humoral antibody response seems to have received little attention. The observations of macroglobulin antibody formation in premature and newborn infants (R. T. Smith, 1960) and of macroglobulin antibodies preceding the appearance of 6.6 S antibodies (Bauer and Stavitsky, 1961; also see in the foregoing) indicates that the macroglobulin antibody may represent the older form of response. However, more information on the composition and immunochemistry of the γ-globulins as well as on the immunological role of various antibodies will help to indicate whether the six-unit 18 S macroglobulin is a more primitive and less efficient form of antibody than the divalent 6.6 S γ-globulin, and whether the β_{2A}-globulins are residual evidence of evolutionary development between the 6.6 and 18 S γ-globulins or have evolved more recently and have been retained because of some specific function.

IV. Myeloma Proteins and Macroglobulinemic Macroglobulins

A. General Features

Proteins formed in malignant plasmacytes and lymphoid cells are notable for their individuality and the discreteness of their physicochemical properties. They can readily be classified as γ-myeloma proteins, β_{2A}-myeloma proteins, or macroglobulinemic macroglobulins (or Bence-Jones proteins) on the basis of distinctive immunochemical or physicochemical features which have been summarized in studies employing a variety of techniques to characterize large numbers of myeloma proteins and macroglobulinemic macroglobulins (Putnam, 1960; Roulet *et al.*, 1961; C.-B. Laurell *et al.*, 1961; Fahey, 1962c). Studies of human myeloma proteins have recently been reviewed by Putnam (1960) and C.-B. Laurell *et al.* (1961). The *collective properties* of each group of anomalous proteins are summarized in Table III and are briefly discussed in the following.

γ-Myeloma proteins migrate as distinct peaks on zone electrophoresis, which appear anywhere in the γ-globulin region or, rarely, in the β-globulin region. More than half the γ-myeloma proteins in man are heterogeneous and contain multiple components differing slightly in net charge as revealed by starch gel electrophoresis (Fig. 5). In the ultracentrifuge, however, single peaks sedimenting at 6.6 S are characteristically seen. The hexose content varies from 1 to 2%. The γ-myeloma proteins possess specific antigenic determinants characteristic of 6.6 S γ-globulins, but they may be antigenically deficient in relation to the normal γ-globulin group.

β_{2A}-Myeloma proteins usually migrate in the fast γ-globulin region or among the β-globulins on zone electrophoresis. In the ultracentrifuge, although some β_{2A}-myeloma proteins are seen to be largely 6.6 S components and a few are almost entirely 9 S components, many β_{2A}-myeloma proteins contain 6.6, 9, 11, and 13 S components. On starch gel electrophoresis several widely spaced bands are seen (Fig. 5). This fact indicates a polymer-type heterogeneity with the larger (9, 11, and 13 S) components being retarded to a progressively greater extent by the interstices of the gel structure. The hexose content varies from 2 to 5%. The β_{2A}-myeloma proteins possess the specific antigenic determinants of β_{2A}-globulins which permit their identification by immunoelectrophoresis or other immunochemical techniques.

Macroglobulins from patients with primary macroglobulinemia usually migrate among the slow γ-globulins on paper electrophoresis, although macroglobulins of fast γ-globulin or β-globulin mobility have been ob-

TABLE III

MAJOR PROPERTIES OF MYELOMA PROTEINS IN MAN AND MOUSE

Properties	Man			Mouse	
	6.6 S γ (60)[a]	β_{2A} (15)	γ_{1M} (20)	6.5 S γ (5)	β_{2A} (25)
Electrophoretically discrete components	+	+	+	+	+
Electrophoretic mobility	γ	β γ	γ β	γ	β α-3
Ultracentrifuge ($s_{20,w}$)	6.6 S	6.6 S 9, 11, 13 S	18 S 24, 32 S	6.5 S	6.5 S 9, 11, 13 S
Starch gel electrophoretic heterogeneity	Electrophoretic	Polymer		Electrophoretic	Polymer
Hexose content (%)	1–2	2–5	6	1	2–4
Antigenic determinants					
Common to γ-Globulins	+	+	+	+	+
Specific for γ, β_{2A}, or γ_{1M}	γ	β_{2A}	γ_{1M}	γ	β_{2A}
Antigenic individuality	+	+	+	+	+

[a] Number of proteins examined is indicated in parentheses.

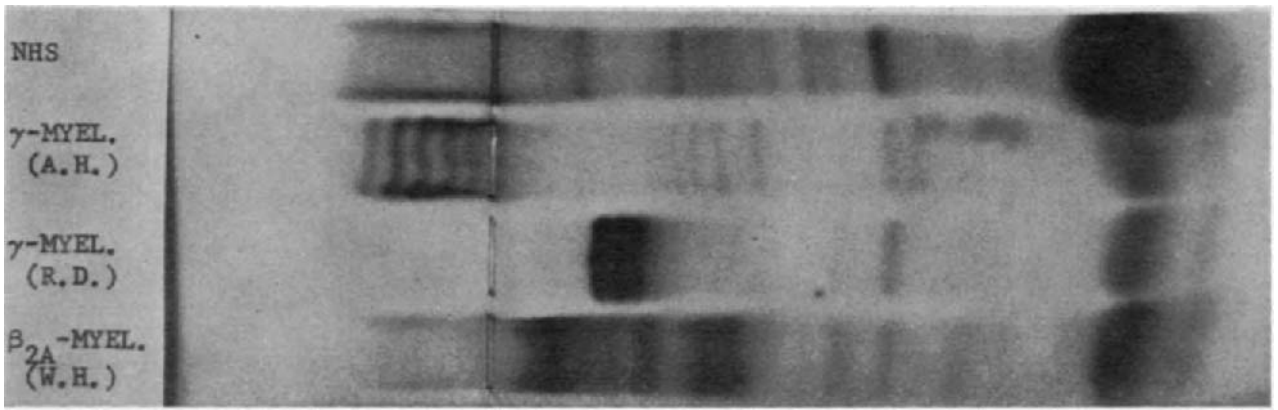

FIG. 5. Starch gel electrophoretic analysis of γ- and β_{2A}-myeloma proteins. A normal human serum (NHS) is included for reference. Two myeloma sera (A. H. and R. D.) contain γ-myeloma proteins differing slightly in electrophoretic charge (electrophoretic heterogeneity). Myeloma serum W. H. contains a β_{2A}-myeloma protein which is composed of several components widely separated on starch gel electrophoresis because of differences in size (polymer-type heterogeneity).

served. Most macroglobulins do not migrate into the gel on starch gel electrophoresis, but a few with a β-globulin mobility migrate a short distance into the gel. In the ultracentrifuge, relatively discrete components of 18, 24, and 32 S are typically seen, the latter two components being present in progressively smaller amounts. The hexose content is usually about 6%. The specific antigenic determinants of γ_1-macro (β_{2M}) globulins can be detected by immunochemical techniques, and individual macroglobulinemic macroglobulins have been shown to share many but not all antigenic determinants with normal γ-macroglobulin preparations (Franklin and Kunkel, 1957; Korngold and van Leeuwen, 1957; Morton and Deutsch, 1958a).

Individuality of myeloma proteins and macroglobulinemic macroglobulins is well recognized, and two identical proteins have not been reported. Whether this individuality represents differences present among the normal γ-globulin population or abnormalities of γ-globulin structure are considered in the following. As long as comparisons could be made only in man, myeloma protein individuality might have represented genetic differences between individual patients. Studies of myeloma proteins from many plasma cell tumors in inbred strains of mice, however, reveal that a variety of myeloma proteins, similar to that seen in man, may occur in a genetically uniform animal population.

B. Myeloma Proteins of Inbred Mice

Transplantable mouse plasma cell tumors in inbred mice have enlarged the opportunities for study of γ-globulin production by plasma cells. The first tumors found had apparently developed spontaneously (Potter *et al.*, 1957; Rask-Nielsen, 1958; Potter and Fahey, 1960), but subsequently many plasma cell tumors have been induced in BALB/c mice by Merwin and Algire (1959) and Merwin (1962), using intraperitoneal placement of plastic chambers or particles, and by Potter and Robertson (1960) and Potter (1962), using intraperitoneal injection of Freund's adjuvant or mineral oil. Almost all transplantable plasma cell tumors produce an electrophoretically discrete serum myeloma protein or urinary Bence-Jones protein. The serum myeloma proteins can be classified as γ- or β_{2A}-myeloma proteins on the basis of immunoelectrophoretic and physicochemical properties. They thus correspond to γ and β_{2A} components of normal mouse serum (Clausen *et al.*, 1959; Fahey, 1961a,b; Rask-Nielsen *et al.*, 1961; Potter and Kuff, 1961). The analogous properties of γ- and β_{2A}-globulins in mouse and man are seen by comparing the characteristics of 75 human and 30 mouse myeloma proteins listed in Table III.

All of the myeloma proteins from 25 plasma cell tumor lines in BALB/c mice studied in the author's laboratory have been different. This individuality reflects the individuality of each clone of malignant plasma cells (each tumor line) and may indicate the heterogeneity of the normal plasma cell population from which the different malignant cell lines arose (Fahey, 1962b). The constancy of the myeloma proteins through many tumor transplant generations indicates that the production of myeloma protein is a relatively stable, heritable characteristic of malignant plasma cells (Potter and Fahey, 1960; Fahey, 1962b).

C. Heterogeneity

Although initial studies indicated that myeloma proteins might be homogeneous (Putnam, 1957), recent observations employing starch gel electrophoresis alone (Owen *et al.*, 1958; Engle *et al.*, 1961) or in combination with chromatography and ultracentrifugation (Askonas, 1961; Fahey, 1961b) have shown that most myeloma proteins are heterogeneous. Two types of heterogeneity have been observed—one based on differences in net electrical charge and the other on differences in size of the molecular components of myeloma proteins.

Heterogeneity due to electrophoretic differences was clearly shown by Askonas (1961) who chromatographically separated components of the γ-myeloma protein 5563 and found them to differ in starch gel electrophoretic mobility although these components had the same sedimentation coefficient and antigenic determinants. Electrophoretic heterogeneity has been found with other γ-myeloma proteins of the mouse (Fahey, 1961b) and in about two-thirds of the γ-myeloma proteins in man (Fahey, 1962c). This type of heterogeneity has been found only with 6.6 S γ-myeloma proteins and not with β_{2A}-myeloma proteins or intact macroglobulins.

Myeloma protein components of differing size are frequently seen on ultracentrifugal analysis of β_{2A}-myeloma proteins which may have 9, 11, and 13 S as well as 6.6 S components (A. H. F. Laurell, 1961; Fahey, 1961b; Imhof and Ballieux, 1961). On starch gel electrophoresis (Fig. 5), these are seen as relatively widely separated bands, the larger components migrating less far through the gel matrix (Fahey, 1961b). Because the 9, 11, and 13 S components can be reduced to 6.6 S components by treatment with mercaptoethanol (Putnam, 1960; Fahey, 1961b), the larger components are probably formed of 6.6 S polymers. γ-Myeloma proteins do not exhibit this type of heterogeneity. Polymer-type heterogeneity, however, is found with macroglobulinemic sera which characteristically contain 18, 24, and 32 S macroglobulin components on ultra-

centrifugation (Kunkel, 1960; Putnam, 1960), and the larger components are thought to be complexes of 18 S globulins.

The frequency of heterogeneity among myeloma proteins, though a limited heterogeneity to be sure, indicates that clones of plasma cells (if the tumors are, indeed, single clones of plasma cells, a still unproved assumption) may produce several closely related molecular forms which in turn are related to a small part of the total normal γ-globulin population. Also, myeloma protein heterogeneity will complicate γ-globulin structural studies in which heterogeneous myeloma proteins are used, although the heterogeneity can be used advantageously when components from the same myeloma protein are separated and compared.

D. Relationship of Myeloma Proteins to Normal γ-Globulins

Are myeloma proteins to be regarded as normal or abnormal proteins?

The question is not trivial, since myeloma proteins provide the only relatively homogeneous γ-globulins available for the study of γ-globulin structure. All evidence indicates a very close similarity of myeloma proteins to normal γ-globulins as shown by the data in Table I for normal γ-globulins and in Table III for myeloma proteins and macroglobulins. While, however, some investigators have emphasized the possibility that myeloma proteins are normal components present in excessive amounts (E. L. Smith *et al.*, 1955a; Deutsch *et al.*, 1956), others have viewed myeloma proteins as abnormal (Putnam, 1957, 1960; Korngold and Lipari, 1956a; Roulet *et al.*, 1961).

The major difficulty in deciding whether individual myeloma proteins are abnormal or normal is the lack of knowledge about *individual* normal γ-globulin molecules. Studies relating individual myeloma proteins to normal γ-globulins, with few exceptions, used a large group of normal γ-globulins as the basis for comparison. The result is that the properties listed for normal γ-globulins are, in fact, mean values for many differing globulin molecules. It is conceivable that individual normal γ-globulins differ as much as do individual myeloma proteins. Certainly, groups of normal γ-globulins and groups of γ-myeloma proteins contain molecules varying widely and similarly in electrophoretic mobility. Where γ-globulins have been subdivided, i.e., on the basis of electrophoretic mobility (Putnam and Udin, 1953; Müller-Eberhard and Kunkel, 1956) or of chromatographic behavior (Fahey, 1962a) and hexose content (C.-B. Laurell *et al.*, 1957, 1961), and the fractions compared to myeloma proteins, similar values have been obtained. Alternatively, analytic data for myeloma proteins may be pooled for comparison with normal γ-globulin

preparations, as in Tables I and III. For example, pooling of Putnam's (1958) data on the N-terminal amino acids of 31 myeloma proteins, as has been done in Table IV, indicates that the N-terminal amino acid differences between a myeloma protein pool and a normal γ-globulin pool are not striking.

TABLE IV

COMPARISON OF N-TERMINAL AMINO ACID VALUES OBTAINED FOR A GROUP OF MYELOMA PROTEINS AND FOR GROUPS OF NORMAL γ-GLOBULINS[a]

Substance	Moles of N-terminal amino acid/mole of protein			Ratio Asp/Glu[b]
	Glu[b]	Asp[b]	Other	
Normal				
γ_2-globulin	1.8	1.1	—	0.61
Fraction II-1,2	1.8	1.1	—	0.61
Fraction II-3	1.1	1.0	—	0.91
Myeloma (31 proteins)[c]	1.35	0.87	0.19	0.64

[a] From Putnam (1958).

[b] Glu = glutamic acid; Asp = aspartic acid.

[c] Mole ratios of 0.5 or more were considered as whole units.

Some myeloma proteins and macroglobulins are cryoglobulins, i.e., precipitate or gel on cooling. This property cannot be called abnormal, however, since detection of cold precipitability is concentration-dependent (at a sufficiently low concentration, cryoglobulins cannot be detected by cooling), and such globulins have been found in human diseases, without any evidence of plasma cell malignancy (Lerner and Watson, 1947), and in hyperimmunized rabbits (Askonas *et al.*, 1960). Cryoglobulins detected in hyperimmunized mice (Askonas, 1962) have been shown to have specific antibody activity.

Immunochemical studies by Wuhrmann *et al.* (1950), Kunkel *et al.* (1951), Lohss and Hillman (1953), Slater *et al.* (1955), and Korngold and Lipari (1956a) showed clearly that there were two antigenically distinct groups of myeloma proteins. One of these groups was closely related to normal 6.6 S γ-globulins, but the second group was notably different. Slater *et al.* (1955) demonstrated that this second group of faster migrating myeloma proteins was antigenically related to a normal serum protein component which migrated in the fast γ-globulin region on zone electrophoresis, and differed from the slow migrating globulins. However, Slater *et al.* (1955) did not give this component a name, although they evidently independently discovered the serum proteins identified on immunoelectrophoresis as the β_{2A}-globulins.

Many immunochemical studies have shown (1) that myeloma proteins differ in their antigenic properties and (2) that individual myeloma proteins are antigenically deficient when compared with the large family of normal γ-globulins (cf. review by Putnam, 1960). It is not certain whether these observations reflect heterogeneity among the normal γ-globulin population or represent deletions from the normal molecular structure of γ-globulin.

Evidence that myeloma proteins and macroglobulinemic macroglobulins have antigenic determinants that are not present on normal γ-globulins has been presented by Korngold and co-workers (1956a, 1957). On the other hand, E. L. Smith *et al.* (1955a) and Deutsch *et al.* (1956) could find no antigenic groups in myeloma proteins that were not present in normal serum. Part of the difficulty in detecting antigenic determinants among normal γ-globulins may relate to the quantitative limitations of analytic techniques employing precipitation. Curtain (1959b) took advantage of the induced tolerance theory to test for antigenic abnormalities among myeloma proteins. Rabbits made tolerant to normal human serum by injection of these proteins at birth were subsequently challenged with three myeloma proteins and failed to make detectable antibodies against the myeloma globulins although they responded to foreign globulins. This experiment cannot be regarded as conclusive, partly because of the variability in rabbit response to human serum proteins, but it is in accord with the view that myeloma proteins are normal proteins.

In view of the evidence that myeloma proteins represent heritable characteristics of malignant plasma cells (Potter and Fahey, 1960; Fahey, 1962b), the properties of the myeloma proteins can be considered as reflecting the normality or abnormality of genetic components controlling plasma cell metabolism. Myeloma proteins could be (1) abnormal proteins resulting from mutation in a *structural gene* controlling γ-globulin structure, (2) normal proteins present in excessive amounts because of mutation in a *regulatory gene* controlling the rate of specific protein synthesis, or (3) normal proteins formed at normal rates in a clone of plasma cells present in abnormally large numbers because of mutation in a gene-determining cell proliferation.

Much evidence favors the third possibility, i.e., proliferation of a clone of plasma cells from the normal heterogeneous plasma cell population, as the major cause of myeloma protein in serum. An abnormal increase in plasma cell numbers is characteristic of multiple myeloma, and the quantity of myeloma protein has been shown to be directly related to the quantity of tumor (Fahey, 1962b). In those rare cases

where proteins such as myeloma proteins are found in the serum in the absence of malignant plasma cell proliferation, it is possible that mutation in a *regulatory gene* is responsible for increased protein synthesis in a clone of otherwise normal plasma cells. Mutation in a *structural gene* would cause abnormal proteins or perhaps an absence of protein. As already noted, physicochemical and immunochemical observations are inconclusive on the question of whether myeloma proteins are normal or abnormal globulins. There seems, however, to be no compelling reason why a cellular alteration causing malignant proliferation should invariably be accompanied by mutation in the genes controlling specific globulin synthesis. Tumors of many different types—malignant melanomas, carcinoid tumors, and pituitary, adrenal, thyroid, and ovarian tumors—may form large amounts of normal metabolic products. It seems reasonable to believe that many plasma cell tumors are producing normal components and that many myeloma proteins represent large amounts of protein normally present in small amounts. It is by no means certain, however, that all myeloma proteins are normal. Identification of individual myeloma proteins (and macroglobulinemic macroglobulins) as normal or abnormal will be difficult until more is known of the range of structural variations among the normal γ-globulins. In any event, the myeloma proteins and macroglobulinemic macroglobulins are probably closely related to the normal products of plasmacytes and related cells.

V. γ-Globulin Structure

A. Intact γ-Globulin Molecules

The 6.6 S γ-globulins from many species are composed of a small amount of carbohydrate and approximately 1500 amino acids, ranging from 13 methionine to 166 serine residues per molecule [approximate concentrations for human γ-globulin from Hsiao and Putnam (1961) and Deutsch *et al.* (1961)]. This large number of amino acids greatly complicates the problem of relating a specific property of γ-globulin to specific amino acid composition. There is, furthermore, evidence that γ-globulins are heterogeneous both in primary structure and in secondary and tertiary structure. In conformance with the terminology of Linderstrøm-Lang and Schellman (1959), "primary structure" refers to the number and sequence of amino acid residues linked together by peptide bonds, "secondary structure" to the folding of petide chains brought about by linking carbonyl and imide groups by means of hydrogen bonds, and "tertiary structure" to the configuration resulting from links between side groups of several peptide chains or of adjacent helices of a single peptide chain.

1. *Primary Structure*

Primary structure of γ-globulin molecules is determined by the amino acid composition and sequence in the peptide chains since, as noted in Section VIII, all or almost all of the carbohydrate is present in single polysaccharide units attached through an aspartyl residue to a peptide chain. Amino acid analyses of whole proteins have indicated differences between individual myeloma proteins (Grisolia and Cohen, 1953; E. L. Smith *et al.*, 1955b) and between macroglobulins and γ-globulins (Pernis *et al.*, 1954), but subgroups of normal 6.6 S γ-globulins have not been compared. Fried and Putnam (1959) prepared tryptic digests of a normal γ-globulin pool and were able to separate numerous peptide fragments by ion-exchange chromatography. γ-Myeloma proteins, similarly treated with trypsin, showed many peptides in the same chromatographic positions, several of which were compared and found to have the same amino acid composition. These authors also found peptide chromatogram differences between individual myeloma proteins and normal γ-globulin, but, in preliminary reports (Fried and Putnam, 1959; Putnam, 1960), no correlation was made between the properties of the intact molecules and the tryptic digest results. Gitlin and Merler (1961) observed differences in the petide "finger prints" when rabbit antibodies against pneumococcal capsular polysaccharides of types II, III, VI, VII, and XII were digested by subtilisin, chymotrypsin, or trypsin, but these differences could not be related with certainty to the immunological specificity of the antibody. Although these studies indicate probable amino acid differences beween γ-globulins, amino acid composition has not as yet been correlated with a specific γ-globulin property.

2. *Secondary and Tertiary Structure*

Heterogeneity of secondary or tertiary structure among normal 6.6 S γ-globulins is indicated in the studies of Edelhoch *et al.* (1962) and Steiner and Edelhoch (1962) on the physicochemical changes produced by partial denaturation of γ-globulins. Edelhoch *et al.* (1962) measured changes occurring in solubility, optical rotation, and other physicochemical parameters during gradual structural disorganization produced in rabbit and bovine γ-globulin by urea, alkali, detergents and other agents. The broad range of conditions under which progressive alterations occurred indicated that marked internal molecular heterogeneity was present in the γ-globulin preparations. Phelps and Cann (1957) and Steiner and Edelhoch (1962) further observed that 6.6 S γ-globulins treated so as to dissociate all or almost all noncovalent bonds would regain most of the original molecular configuration with removal of the

dissociating reagent. These observations indicate that at least some part of the secondary structure of γ-globulin molecules is determined by the primary structure.

The number of peptide chains in 6.6 S γ-globulins has been estimated at three for human γ-globulin on the basis of the number of N-terminal amino acids per mole of protein (McFadden and Smith, 1953; Phelps and Putnam, 1960). Although only one N-terminal amino acid was found with rabbit γ-globulin (Porter, 1950a), analysis of the C-terminal amino acids in rabbit γ-globulin by Silman *et al.* (1962) revealed from three to five COOH-terminal groups per mole. This finding indicates that multiple peptides are present in rabbit γ-globulins.

Additional evidence for multiple subunits (peptide chains) in γ-globulins was obtained by reduction of disulfide bonds under conditions unlikely to cause peptide bond cleavage (Edelman and Poulik, 1961; Franěk, 1961). The minimum number of subunits produced by these methods is three for human and rabbit γ-globulins. Although the exact number of peptide subunits composing γ-globulins is not known, it is evident that disulfide bonds are major forces binding the peptide chains together.

B. Chemically Produced Subunits of γ-Globulin

Disulfide bonds in γ-globulins from several species have been cleaved by mercaptoethanol addition (Edelman, 1959) or S-sulfonation (Franěk, 1961) in 8 *M* urea, with a reduction in the sedimentation coefficient from 6 to about 2 S. Edelman and Poulik (1961) reported the reduction of seven to twelve disulfide bonds in the cleavage of 7 S γ-globulins, but it is not certain how many of these bonds are actually involved in binding separate peptide chains.

Starch gel electrophoresis of the reduced material revealed at least two major groups of components which could be separated by CM-cellulose chromatography (Edelman and Poulik, 1961). One component appeared to have a molecular weight of about 17,000; the others were larger. The contribution of these subunits in various γ-globulin functions remains to be determined, and the problem of assessing their physiological role is complicated by the insolubility of the chemically produced subunits in water or saline. The chemically produced subunits are, however, of great importance for they are probably identical in primary structure to the subunits of native γ-globulin. The extent to which secondary structure is altered by reduction and alkylation remains to be determined, although the lack of solubility in aqueous solvents may reflect changes in secondary structure.

C. Enzymatically Produced Subunits of γ-Globulin

Fragmentation of 6.6 S γ-globulins by enzymatic techniques has successfully produced subunits that are soluble and that retain many, if not all, of the physiological properties of γ-globulins (Petermann, 1942, 1946; Porter, 1950b, 1958; Burtin, 1961). The enzymatic fragments, however, are probably not identical with any natural subunit of γ-globulin, and it is difficult at the present time to relate the enzymatically produced fragments to the different peptide chains of native γ-globulin. Nonetheless, several functional properties of γ-globulins have been identified on separate enzymatically produced fragments, and studies of enzymatically produced fragments have contributed considerably to current views on the composition of γ-globulins.

Porter (1958, 1959) extended his earlier studies of γ-globulin fragmentation (Porter, 1950b) and, treating rabbit antibody with 1% papain and 0.01 *M* cysteine, obtained fragments which could be separated by CM-cellulose chromatography into three groups. Groups I and II had molecular weights of about 50,000 and Group III, of about 80,000 (Charlwood, 1959). Antibody activity was found in Groups I and II (Porter, 1958). γ-Globulins from many species are similarly affected. Rabbit, human, mouse, guinea-pig, horse, and bovine γ-globulins are reduced from 6.6 to 3.5 S components by papain and cysteine.

The chain of events following treatment of γ-globulin with papain and cysteine was analyzed by Nisonoff and by Cebra and associates. Cebra *et al.* (1961) exposed rabbit γ-globulin briefly (5 minutes) to insoluble papain, opening from three to five peptide bonds, but without reducing γ-globulin size ($s_{20,w}$ = 6.2 S). Subsequent addition of sulfhydril reagents cleaved the molecule to 3.27 S fragments. Deutsch *et al.* (1961) similarly observed that 1–2 hours digestion with papain without cysteine produced only a partial reduction in human γ-globulin size, although subsequent addition of mercaptoethanol converted all the molecules to 3.5 S fragments. Treatment with 0.01 *M* sulfhydril reagents alone, however, causes no detectable changes in native γ-globulin. These observations show that papain is required only to split certain peptide bonds and that the remainder of the fragmentation process results from reduction of disulfide bonds by sulfhydril reagents.

Pepsin digestion similarly prepares rabbit γ-globulin for conversion by cysteine to 3.5 S units (Nisonoff *et al.*, 1959). Pepsin appears to act largely by destroying the Group III piece and by converting γ-globulin to a 5 S component which is reduced to 3.4 S by addition of cysteine. This report was the first to indicate that reduction of disulfide bonds as

well as hydrolysis of peptide bonds occurred during γ-globulin fragmentation by papain and cysteine. Nisonoff *et al.* (1961) using papain-digested rabbit antibody, further showed that a single disulfide bond joined the two fragments having the antibody activity. This disulfide bond could be reduced to form nonprecipitating univalent 3.5 S antibody fragments and could be reoxidized to convert 3.5 S fragments into 5 S complexes capable of precipitation reactions with antigen (Nisonoff and Rivers, 1961).

Brief exposure to papain is generally sufficient to initiate splitting of some γ-globulin molecules, but there is evidence that γ-globulins are heterogeneous in their susceptibility to papain, some being quite resistant to hydrolysis by papain and cysteine (Hsiao and Putnam, 1961). Prolonged exposure to papain, on the other hand, may cause alteration or destruction of some of the fragments, particularly those related to Porter's group III. Studies of human γ-globulin (Hsiao and Putnam, 1961; Deutsch *et al.*, 1961) indicate that in addition to three major fragments, a fourth fraction was produced with a molecular weight under 5000 and represented from 2 to 5% of the total protein. This fraction was shown to be a mixture of peptides, which was present even after a 10-minute digestion (Hsiao and Putnam, 1961) and increased in quantity as digestion was continued (Deutsch *et al.*, 1961). Hsiao and Putnam (1961) viewed these peptides as products of a random degradation of denatured protein by papain.

1. *Physicochemical Properties of Papain-Produced Fragments*

γ-Globulin (6.6 S) molecules from rabbit (Porter, 1958, 1959), man (Edelman *et al.*, 1960; Stiehm *et al.*, 1960; Franklin, 1960; Hsiao and Putnam, 1961; Deutsch *et al.*, 1961), and mouse (Askonas and Fahey, 1961a; Fahey and Askonas, 1962) are split into three fragments, approximately one-third the size of the original molecule. Two fragments are similar and contain the antibody activity whereas the third fragment is distinctly different. The two fragments which are similar and contain antibody activity will be referred to as Groups I and II in the case of rabbit γ-globulin (Porter, 1958) and as S (slow) fragments in the case of human (Edelman *et al.*, 1960) and mouse γ-globulins (Askonas and Fahey, 1961a). The third fragment with distinctly different properties is designated Group III for rabbit and F (fast) fragments for man and mouse. The properties of γ-globulin fragments of rabbit, man, and mouse are compared in Table V.

Conflicting terminologies for the fragments of human γ-globulin arose because simultaneous work in several laboratories revealed that the

TABLE V

COMPARISON OF PAPAIN PLUS CYSTEINE FRAGMENTS OF γ-GLOBULINS[a]

Properties	Rabbit			Man and Mouse	
	I	II	III	S	F
Physicochemical					
Net charge (relative mobility toward anode at pH. 8.6)	Fastest	Intermediate	Slowest	Slower	Faster
$s_{20,w}$	3.6 S	3.55 S	3.4 S	3.5–3.9 S	3.4–3.9 S
Mol. wt.	50,000	53,000	80,000	34,000–50,000	...
Portions of total protein represented in each group of fragments	1/3	1/3	1/3	2/3	1/3
NH_2-terminal amino acids	Alanine	Alanine	(several)	...	...
Hexose content (% of protein)	0.6	0.15	0.9	0.1–0.3	2.5–3.1
Antigenic					
γ and β_{2A}-globulins[b]:					
Distinctive determinants	...		...	0	+
Common determinants	...		...	+	0
Immunological					
Antibody activity	+	+	0	+	0
Complement fixation[c]	0	0	+	...	...
Cross placenta[d]	0	0	+	...	...
Skin fixation[e]	0	0	+	...	...
Rheumatoid factor reaction[f]	0	0	+	0	+
Genetic factors					
Rabbit allotype[g]	+	+	0		
Human Gm[h]				0	+
Human InV[h]				+	0

[a] The data on rabbit γ-globulin are assembled from the reports of Porter (1958, 1959), Charlwood (1959), and Cebra *et al.* (1961). The data on human γ-globulin are from Edelman *et al.* (1960), Franklin (1960), Stiehm *et al.* (1960), Hsiao and Putnam (1961), and Deutsch *et al.* (1961) and that on mouse γ-globulin are from Askonas and Fahey (1961a) and Fahey and Askonas (1962). The S and F terminology suggested by Edelman *et al.* (1960) has been followed for human and mouse γ-globulin. The nomenclature of other authors is indicated in Section V.

[b] Franklin and Stanworth (1961) and Askonas and Fahey (1962).

[c] Amiraian and Leikhim (1961b).

[d] Brambell *et al.* (1960).

[e] Ovary and Karush (1961).

[f] Franklin (1961) and J. W. Goodman (1961).

[g] Kelus *et al.* (1961).

[h] Harboe *et al.* (1962), Franklin *et al.* (1962).

digestion products of human γ-globulin differed sufficiently from rabbit γ-globulin to make it advisable not to apply to human γ-globulin fragments the terminology used by Porter (1958) for rabbit fragments. A comparison of the published data indicates that the fragments of human γ-globulin designated S fragments (Edelman *et al.*, 1960) are the same as those termed C and A (Franklin, 1960), C (Stiehm *et al.*, 1960; Deutsch *et al.*, 1961), and A (Hsiao and Putnam, 1961).

The description of F fragments given subsequently is based in large part on the description of fragments termed B by Franklin (1960), by Deutsch *et al.* (1961), and by Hsiao and Putnam (1961).

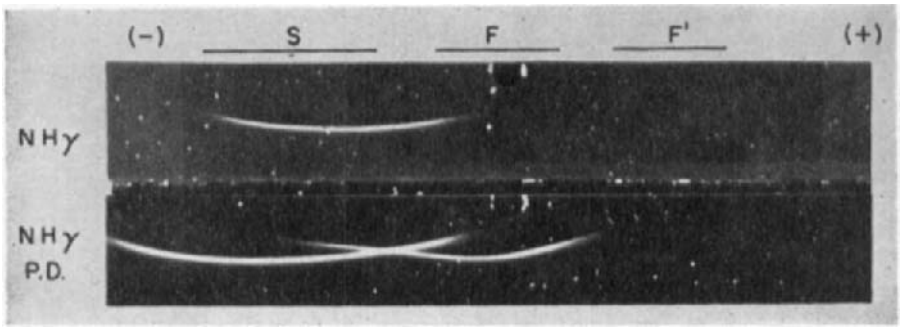

FIG. 6. Immunoelectrophoresis of the papain digest of normal human γ-globulin. The papain- and cysteine-treated normal human γ-globulin is seen to contain three precipitin arcs (S, F, and F′) when reacted with rabbit antisera against normal human γ-globulin. The broad S precipitin arc includes components migrating more slowly than the intact γ-globulin. Intersection of the S and F precipitin arcs indicates antigenic as well as electrophoretic differences between these two classes of fragments obtained from human γ-globulin. F′ precipitin arc extends from the F arc.

S fragments and rabbit Groups I and II fragments sediment at approximately 3.5 S (3.5–3.9 S) and have been found to have molecular weights of 50,000–53,000 for rabbit (Charlwood, 1959) and 34,000–55,000 for man and mouse (Franklin, 1960; Deutsch *et al.*, 1961; Fahey and Askonas, 1962).

The S fragments have little if any hexose [0.1–0.3% according to Franklin (1960)] but some corresponding fragments in the rabbit (Group II) have a relatively high hexose content (Porter, 1959). This interesting difference remains to be explained, especially if all of the carbohydrate in rabbit γ-globulin is present in a single polysaccharide as in human γ-globulin (Rosevear and Smith, 1961).

S fragments of human and mouse γ-globulin on electrophoresis at pH 8.6 typically migrate more slowly than, or at the same rate as, intact

γ-globulins (Fig. 6). The electrophoretic heterogeneity of the S fragments is at least as marked as in normal 6.6 S γ-globulins. Rabbit fragment Groups I and II are similarly heterogeneous but migrate more rapidly than, or at the same rate as intact rabbit γ-globulins.

F fragments represent approximately one-third of the total digest. This fraction is more susceptible than the S fractions to proteolytic digestion and the observed properties depend, in part, upon the duration of papain action. The electrophoretic mobility of this fraction is faster than the S fraction from human or mouse γ-globulin, although in digests of normal γ-globulin there is a region of overlapping mobility containing both types of fragments. In rabbit γ-globulin digests, however, Group III fragments migrate more slowly at pH 8.6 than do the antibody-containing fragments. F fragments and Group III fragments sediment at approximately 3.5 S (3.4–3.9 S). Most of the hexose in human γ-globulins (about 90%) is present in this fraction (Franklin, 1960). Digests of human and mouse γ-globulin indicate the presence of two groups of F components (F and F′) differing in electrophoretic mobility and in antigenic composition (Fig. 6). It remains to be determined whether the faster moving F′ component results from progressive degradation of F fragments or whether F and F′ components may reflect differences in the original molecules, perhaps in the sites most susceptible to papain cleavage.

2. *Antigenic Properties of Papain-Produced Fragments*

Papain digests appear to contain many but not all of the antigenic determinants present on the original molecule. In Porter's studies (1959) 70–80% of precipitating activity of whole γ-globulin was retained in the digests, and Stiehm *et al.* (1960) found that digests contained 30 and 74% of the precipitating capacity of two γ-myeloma proteins. Knowledge of the exact effects of papain and cysteine fragmentation requires further study.

Porter (1959) showed that Group III fragments of rabbit γ-globulin differed from Groups I and II in antigenic activity. Most of the antibody activity of a goat antirabbit serum was directed at antigenic determinants on Group III fragments. A rat, antirabbit globulin antiserum which reacted with all three groups of fragments revealed an antigenic similarity between Group I and II fragments and confirmed that they differed antigenically from Group III fragments. Studies with human γ-globulin fragments (Edelman *et al.*, 1960; Stiehm *et al.*, 1960; Franklin, 1960) and mouse γ-globulin fragments (Askonas and Fahey, 1961a) confirmed that two antigenically different classes of fragments resulted from treatment of γ-globulin with papain and cysteine. The S and F classes of

fragments differ in antigenic as well as in electrophoretic and other physicochemical properties. This fact is demonstrated by the immunoelectrophoretic analysis of human γ-globulin papain digests. As seen in Fig. 6, the intersection of the S and F precipitin arcs indicates that the S and F fragments bear different antigenic determinants.

It is not known whether all S fragments from normal γ-globulin pools have the same antigenic composition although many must share common antigenic determinants to produce the single precipitin arc shown in Fig. 6. Antigenic differences among the F fragments are indicated by the appearance of two F lines (the faster termed F′, Fig. 6) and by differences in antigenic composition between these two groups as revealed by the use of different antisera on immunoelectrophoresis (Edelman *et al.*, 1960; Fahey and Askonas, 1962).

The finding of two or three groups of fragments in digests of γ-globulins raised the question whether these came from the same molecules or from different molecules. Heremans (1960) and Edelman *et al.* (1960), on the basis of immunoelectrophoretic observations on human γ-globulin digests suggested that F fragments might come from one group of γ-globulin molecules and S fragments from another group. Askonas and Humphrey (1961) subsequently demonstrated that mouse F and S fragments were from the same γ-globulin molecules by showing that 95% or more of normal γ-globulin or γ-myeloma protein labeled with I^{131} was precipitated by antisera rendered specific for the S or the F antigenic determinants, as well as by unabsorbed antiserum. It can be concluded that F and S fragments are present in all γ-globulin molecules.

3. *Immunological Properties of Papain-Produced Fragments*

Many of the properties of γ-globulins and antibodies have been localized on one or another of the papain-produced fragments (Table V). Perhaps most important, the antibody activity is located on rabbit globulin fragments of Groups I and II (Porter, 1958, 1959) and in the S fragments of human (Franklin, 1960) and mouse (Fahey and Askonas, 1962) γ-globulin molecules. Each of these fragments contains a single antigen-binding site (Porter, 1959; Nisonoff *et al.*, 1961). These data are in accord with evidence that antibody molecules are bivalent and indicate that the antibody active sites are on separate but similar portions of the γ-globulin molecule.

The Group III or F fragments of the γ-globulin molecules, on the other hand, carry the part of the original molecule responsible for complement fixation (Amiraian and Leikhim, 1961b), transfer across the placental barrier (Brambell *et al.*, 1960), and binding to skin (Ovary and

Karush, 1961). The part of 6.6 S γ-globulin responsible for the reaction of rabbit or human γ-globulin with the rheumatoid factor is also present on the Group III or F fragment of the molecule (J. W. Goodman, 1961; Franklin, 1961).

D. Schematic Summary of γ-Globulin Properties

A schematic synthesis of information on 6.6 S γ-globulin molecules is of necessity highly speculative, but Fig. 7 serves the useful purpose

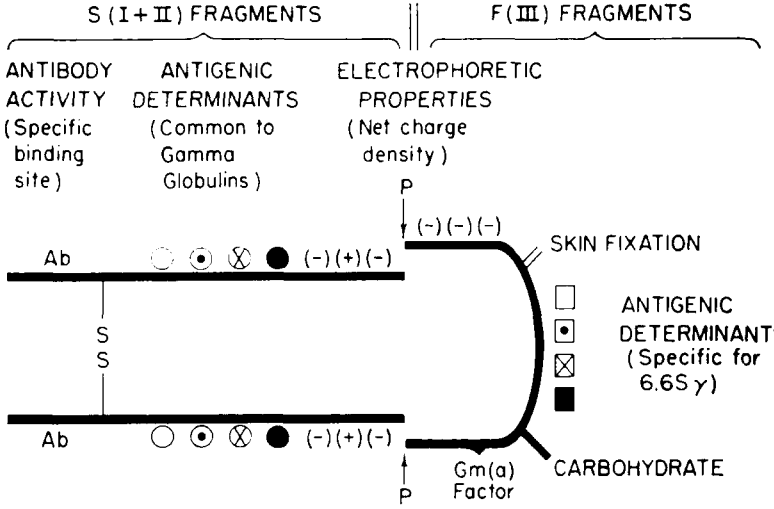

FIG. 7. Schematic representation of the structure of a γ-globulin molecule. Possible peptide chains are indicated by the solid lines. Only one disulfide bridge is drawn. The diagram is arranged so that the S fragments (obtained by papain digestion) are indicated on the left and the F fragments on the right. The sites of antibody activity (Ab), antigenic determinants (○ ⊙ ⊗ ●), and structural variability accounting for electrophoretic heterogeneity (− + −) are located on the S fragments, although not necessarily on the same peptide chain (see Sections V and XI). Two S fragments of similar or identical character are indicated for the molecule. Possible sites of papain hydrolysis are indicated by P.

of relating much of what is known about γ-globulin properties. It must be understood that the illustrated relationship between the peptide chains of γ-globulin molecules is largely diagrammatic. Three globulin pieces are illustrated by continuous lines. Possible loci of papain action (P) are arbitrarily indicated in order that the peptide fragments on the left side of Fig. 7 can be used to represent S fragments (rabbit Groups I and II) and those on the right side F fragments (rabbit Group III).

Two similar and perhaps identical S fragments are obtained from each γ-globulin molecule. Each S fragment (rabbit I and II fragments) has one antibody (Ab) site; both S fragments have approximately the same net electrical charge (− + −); and both have similar sets of antigenic determinants (○ ⊙ ⊗ ●) indicating clearly that some antigenic determinants are present at two sites on each intact γ-globulin molecule. Each of these properties (antibody activity, net electrical charge, antigenic determinants) of S fragments has been placed on a separate portion of the fragment, because evidence reviewed later indicates that these properties may be independent of one another.

The F fragment has a greater negative charge (− − −) than the S fragments, except in the case of rabbit fragments. Separate antigenic determinants (□ ⊡ ⊠ ■) are present on this fragment, including those specific for 6.6 S γ-globulin. Skin-binding, placental transfer, complement-fixing sites, and Gm(a) factors are properties of the F piece. The major portion of globulin carbohydrate is present as a polysaccharide bound to the F fragment.

Most of the preceding data are based on measurements made with heterogeneous normal γ-globulin preparations. Such studies do not indicate, however, whether individual γ-globulin molecules yield two identical or two different S fragments. Porter (1959) inclined to the view that each rabbit γ-globulin molecule contained fragments I, II, and III (i.e., two different S-type fragments). The possibility, however, that fragments I and II come from different populations of γ-globulins has not been ruled out. Amiraian and Leikhim's (1961a) finding that some rabbit γ-globulin preparations yield more Group I than Group II fragments is compatible with a mixed molecule population, one of which contains I–I–III fragments, while others contain II–II–III or I–II–III combinations.

In an effort to obtain homogeneous γ-globulins, Askonas and Fahey (1961a) separated the closely related components of a heterogeneous γ-myeloma protein and prepared S and F fragments by treatment with papain and cysteine. One of the γ-myeloma protein components yielded two S fragments that were identical on ultracentrifugal and starch gel electrophoretic analysis. Other components of the myeloma protein provided two S fragments differing in chromatographic behavior and in starch gel electrophoretic mobility. All the S fragments, however, had the same antigenic determinants. Although further studies are needed, the results indicate that some γ-globulin molecules may yield two identical S fragments on treatment with papain and cysteine and other molecules may yield two closely related but not identical S fragments.

VI. Electrophoretic Heterogeneity

Normal serum 6.6 S γ-globulins extend in a spectrum of electrophoretic mobility from the slowest γ-region through the β-region and into the α-globulin region (Williams and Grabar, 1955b; Wallenius *et al.*, 1957). The β_{2A}-globulins and γ_1-macroglobulins have similar but less extensive electrophoretic heterogeneity (cf. Figs. 1 and 2). This physicochemical heterogeneity, which is far greater for γ-globulins than for any other serum protein, is characteristic of γ-globulins in all species (Engle and Woods, 1960).

Electrophoretic heterogeneity of proteins may be based on variations in primary structure, i.e., in the amino acid sequence, or in secondary or tertiary structure, i.e., in the folding of polypeptide chains and the relations of polypeptide chains to each other [see Linderstrøm-Lang and Schellman (1959) and M. Vaughan and Steinberg (1959) for reviews of other protein systems], but the important question concerning how much each of these several levels of structure contributes to the electrophoretic heterogeneity of γ-globulins remains to be determined.

The chemical basis of electrophoretic heterogeneity has been attributed to the carbohydrate content as well as to the amino acid composition of the molecules. Since increases in carbohydrate content of myeloma proteins were observed (Müller-Eberhard and Kunkel, 1956; C. B. Laurell *et al.*, 1957) to parallel roughly the increases in electrophoretic mobility, a relationship between carbohydrate content and electrophoretic mobility was suggested. The relationship between hexose content and electrophoretic mobility is less evident when γ-myeloma proteins alone, without β_{2A}-myeloma proteins, are compared (C.-B. Laurell and Heremans, 1961). In a study of DEAE-cellulose chromatogram fractions of human γ-globulins, the globulins with higher mobility had a greater proportion of carbohydrate. Subsequently (Fahey, 1962a), β_{2A}-globulins have been identified in several of the later chromatogram fractions, and the higher carbohydrate of the β_{2A}-globulins (Heremans *et al.*, 1959) probably contributed to the carbohydrate levels in these fractions. Fractions of rabbit γ-globulin having different electrophoretic mobility were found to have the same hexose content (Askonas *et al.*, 1960). It would seem that carbohydrates play little if any role in the electrophoretic heterogeneity of 6.6 S γ-globulins. Sialic acid probably plays a role in determining the electrophoretic mobility of γ-globulins and the higher sialic acid content of β_{2A}-globulins and γ_{1M}-globulins may help to produce the generally faster mobility of these proteins. It is unlikely, however, that sialic acid differences play any role in the heterogeneity of 6.6 S γ-globulins since

the low level of sialic acid in these proteins [1 mole sialic acid per mole γ-globulin; Rosevear and Smith (1961)] does not permit sufficient variability to account for the wide range of γ-globulin electrophoretic heterogeneity.

γ-Globulin subunits produced by papain fragmentation have been examined to determine whether electrophoretic variability is a property of only one group of fragments. In studies with normal γ-globulin fractions (Edelman *et al.*, 1960; Franklin, 1960; Hsiao and Putnam, 1961; Fahey and Askonas, 1962), the mobility of S fragments roughly paralleled that of the parent molecule. Slow migrating γ-globulins yielded slower migrating S fragments than fast γ-globulins.

Evidence that both S and F fragments play a role in determining electrophoretic mobility of intact molecules was obtained by comparing fast and slow migrating 6.5 S components of an electrophoretically heterogeneous γ-myeloma protein. Initial observations (Askonas and Fahey, 1961a) showed that papain digestion of two slower migrating components produced two electrophoretically identical S_2 fragments, whereas faster myeloma protein components yielded fragments with a greater net negative charge (S_3 fragments) as well as S_2 fragments. Approximately five F fragments were obtained in these digests and the proportion of fast migrating F components was greater from the faster myeloma protein components. Askonas (1962) subsequently clarified the contribution of F fragments to the mobility differences by reducing the extent of papain degradation and showed that faster migrating F fragments are obtained from faster migrating myeloma globulin components. These studies indicate that the mobility of intact γ-globulin molecules is related to the properties of the molecular pieces represented in the F as well as the S fragments.

Molecular variations can occur in intact γ-globulins (Fig. 1) or in S fragments (Fig. 6) that are sufficient to produce extensive electrophoretic heterogeneity, and yet these differences do not affect the antigenic determinants or the molecular configurations responsible for antibody activity. Since all species have electrophoretically heterogeneous γ-globulins (Engle and Woods, 1960), it is not surprising that the structural variations responsible for electrophoretic heterogeneity are not recognized as foreign by animals injected with heterologous γ-globulin. It seems probable that electrophoretic heterogeneity in different species is accomplished by similar molecular variations.

Variations in the molecular sites determining electrophoretic mobility and antibody specificity apparently can occur independently. This is shown by the fact that (1) antibodies against single haptenes can extend

throughout the γ-globulin electrophoretic spectrum (Askonas *et al.*, 1960; and Velick *et al.*, 1960) and, conversely, that (2) different antibodies may have the same electrophoretic mobility. These observations indicate that antibody specificity is segregated on a portion of the γ-globulin molecule not affected by the molecular variations responsible for electrophoretic heterogeneity.

VII. Heterogeneity by Formation of Polymers and Complexes

A. Macroglobulins and β_{2A}-Globulins

The larger proteins formed in plasmacytes and related cells, whether normal or malignant, show a fairly constant stepwise progression in sedimentation coefficients of 6.6, 9, 11, 13 and 18, 28, 44 S. The 9, 11, and 13 S components are characteristically present and may be the major components in β_{2A}-myeloma proteins (Fahey, 1961b; A. H. F. Laurell, 1961; Imhof and Ballieux, 1961). Components of this size have been seen in fractions of normal serum (Wallenius *et al.*, 1957; Heremans *et al.*, 1959), but their levels are low in view of ultracentrifugal analysis of whole normal serum. The regular progression in sedimentation coefficients and in discrete ultracentrifugal peaks of β_{2A}-myeloma proteins having 9, 11, and 13 S components and the fact that these components are reduced to 6.6 S by treatment with mercaptoethanol (Putnam, 1960; Fahey, 1961b) indicate that these components may be polymers of 6.6 S protein units and may represent two, three, and four 6.6 S units, respectively. The 6.6, 9 S, and larger components of β_{2A}-myeloma proteins upon separation by anion-exchange cellulose chromatography have the same antigenic determinants (Fahey, 1962c).

The 18 S γ_1-macroglobulins, dissociated by Deutsch and Morton (1958) into 6.6 S units by treatment with sulfhydryl compounds such as 0.1 *M* mercaptoethanol, appear to be polymers composed of six smaller units about the size of 6.6 S globulins. Disulfide bonds appear to provide the major link between the constituent 6.6 S γ_M-globulin units, because removal of the sulfhydryl reagent permits partial reaggregation and blocking of the sulfhydryl groups with iodoacetamide prevents reaggregation (Deutsch and Morton, 1958). The observation that macroglobulins are 18 S globulins and not a spectrum of macromolecules indicates that the cellular synthetic mechanisms can specifically make the 18 S macroglobulin. It is not known, however, whether the 18 S globulins are polymers in the sense of being aggregations of identical or different 6.6 S components.

In addition to the 18 S γ_1-macroglobulins of normal serum, larger

components of 24 and 32 S may be seen when the concentration of macroglobulins is raised as in macroglobulinemic serum (Putnam, 1957; Kunkel, 1960) or by fractionation of normal serum (Wallenius *et al.*, 1957). Although the largest components have not yet been isolated, their resemblance to 18 S macroglobulins in electrophoretic mobility and their response to mercaptoethanol (Deutsch and Morton, 1958) indicate that they may be aggregates of 18 S globulins.

B. γ-Globulin Complexes in Serum

γ-Globulins may react with other proteins to form soluble complexes which are detectable in the serum. The reaction of the rheumatoid factors (18 S γ_1-macroglobulins) with 6.6 S γ-globulins to form soluble 22 S components is the best known reaction of this type (Franklin *et al.*, 1957). Further study may reveal reactions between γ_1-macroglobulins and other components. The author has observed a patient with macroglobulinemia whose γ_1-macroglobulin formed a soluble complex with β-lipoprotein.

γ-Globulins (6.6 S) may form soluble complexes with sedimentation coefficients from 10–16 S as shown by Kunkel *et al.* (1961). The complexes were dissociated to 7 S components by treatment with 6 *M* urea or pH 3 buffer (in contrast to 18 S macroglobulins which are not dissociated under these conditions) and had immunological and chemical properties of 6.6 S γ-globulins. These complexes were found in patients with rheumatoid arthritis or idiopathic purpura but, as pointed out by Kunkel *et al.* (1961), they may be present in sufficiently high concentration to form relatively discrete protein peaks on zone electrophoresis and in the ultracentrifuge and may be mistaken for macroglobulinemic or myeloma proteins. β_{2A}-Globulins also may form complexes with serum proteins—Heremans (1960) has shown β_{2A}-globulin reaction with albumin.

The pathological significance of the γ-globulin complexes is unknown. The association of some of these complexes with rheumatoid arthritis and with purpura raise questions about the rôle of these substances in the development of pathological lesions. However, there appears to be no evidence that the rheumatoid factor complexes cause arthritic lesions although 22 S complexes are found in largest amounts in patients with severe rheumatoid arthritis. It is not certain that these complexes reflect the presence of new antibodies (Burnet, 1961) or abnormal proteins, or whether the conditions imposed by disease have produced quantitative rather than qualitative alterations in the γ-globulin components. The identification of genetic factors controlling the reactions between rheumatoid factors and 6.6 S γ-globulins (Grubb, 1959) and the discovery of

γ-reactive macroglobulins (rheumatoid factors) in normal individuals (Ropartz *et al.*, 1960) indicate the need for further inquiry into the nature and significance of complexing γ-globulins (see also Section X).

VIII. Carbohydrate Heterogeneity

Proteins formed in plasma cells contain tightly bound carbohydrate, composed of the hexoses, galactose and mannose, and glucosamine, fucose, and sialic acid (Winzler, 1960; C.-B. Laurell *et al.*, 1957). Rosevear and Smith (1961) showed that normal human γ-globulin contains eight or nine residues of hexose with a 3/5 molar ratio for galactose to mannose, and approximately ten hexosamine, two fucose, and one sialic acid residues. These carbohydrates appear to be present in a single polysaccharide unit attached to the protein molecule through an aspartyl residue (Rosevear and Smith, 1961). In this respect γ-globulin resembles other proteins such as ovalbumin where a single polysaccharide unit is attached to the protein through an aspartyl residue (Cunningham *et al.*, 1957; Johansen *et al.*, 1958; Jevons, 1958).

The site of polysaccharide attachment in γ-globulins is in the F fragment produced by papain digestion. The S fragment has relatively little carbohydrate (Franklin, 1960). The question whether S fragments have any carbohydrate is of interest since the presence of carbohydrate would imply at least one additional site of carbohydrate binding on some γ-globulin molecules.

Proteins formed in plasma cells are not uniform in carbohydrate content and measurement of the hexose content of purified proteins has indicated the carbohydrate heterogeneity of these globulins. Normal γ-globulin preparations have a hexose content of about 1.2% and γ-myeloma proteins range from 1 to 2% hexose (A. H. F. Laurell, 1961). β_{2A}-Globulin preparations are reported to have 3.2–4.9% hexose (Schultze, 1959; Heremans *et al.*, 1959), β_{2A}-myeloma proteins contain 2–5% hexose (Laurell and Heremans, 1961; Fahey, 1961b), and macroglobulins about 6% hexose (Müller-Eberhard and Kunkel, 1959). Analyses of myeloma proteins and macroglobulins offer opportunities to assess a relatively limited number of globulin molecules, in contrast to normal γ-globulin studies which reflect median values for a large group of globulins.

The differences in hexose content of these proteins could be caused by (1) multiple polysaccharide units attached separately to the protein—if this were the case stepwise increases in carbohydrate content would be observed—by (2) extension or branching or shortening of the single polysaccharide unit, or by (3) attachment of carbohydrate at sites other than the major polysaccharide unit.

It would be of interest to know if the high (6%) hexose content of γ-macroglobulins is due to multiple (5 or 6) polysaccharide units attached to each 6.6 S subunit of the macroglobulin. Müller-Eberhard and Kunkel (1956) found that the hexosamine/hexose ratio of macroglobulins (0.55) differs from that of 6.6 S γ-globulins (0.95). Thus, qualitative as well as quantitative carbohydrate differences exist between these protein classes. The wide range of hexose contents observed with β_{2A}-myeloma proteins presents an additional opportunity to investigate the basis for carbohydrate differences among proteins formed in plasma cells.

Measurement of carbohydrate levels in intact proteins by colorimetric tests is complicated by color development produced by protein alone which may give incorrectly high values. Therefore, when testing proteins with low carbohydrate content, the simultaneous testing of a protein having no carbohydrate (such as carefully purified human serum albumin) is a necessary control. Chromatographic separation of carbohydrates from other chromogens after protein hydrolysis (Müller-Eberhard and Kunkel, 1959) should provide material for accurate carbohydrate measurements.

The hexose content of proteins formed in plasma cells correlates in a general way with size and with electrophoretic mobility. The smallest proteins, i.e., the Bence-Jones proteins, have the least carbohydrate and the macroglobulins the most. It would be of interest to know if carbohydrate affected the structure of 6.6 S units so as to influence polymerization. (The relation of carbohydrate and γ-globulin electrophoretic mobility were discussed in Section VI).

IX. Antigenic Determinants

A. Antigenic Multiplicity

γ-Globulins which are similar in many properties have been shown by immunochemical techniques to differ in structural details. Injection of γ-globulin from one species into another excites the formation of antibodies reactive with structural configurations (antigenic determinants) not present on the immunized animal's γ-globulins. For example, antibodies (antisera) against human γ-globulins have been evoked by injecting these proteins into chickens, mice, rats, guinea pigs, rabbits, goats, sheep, and horses. Such antisera are very useful in identifying the several classes of γ-globulins, in identifying myeloma proteins and macroglobulinemic macroglobulins, and in measuring γ-globulin levels, especially where small amounts are present as in cerebral spinal fluid (Kabat *et al.*, 1950). These antisera contain a mixture of antibodies against a

variety of γ-globulin antigenic determinants (i.e., polyvalent antisera as shown in Fig. 6), but the number of antigenic determinants detected and the potency of the antisera will differ from one animal to another as well as between species. For these reasons, the immunochemical characteristics of γ-globulins revealed by anti-γ-globulin antisera depend greatly on the specific system used, i.e., on the properties of the antisera and the capacity of the immunochemical techniques. In spite of this reservation, by taking advantage of the antigenicity of γ-globulins in foreign species, immunochemical studies have provided considerable information about the interrelationships of γ-globulin subgroups and γ-globulin fragments.

γ-Globulins exhibit antigenic heterogeneity at three levels: (1) the antigenic differences between the major (6.6 S γ, β_{2A}, and γ_{1M}) γ-globulin classes (cf. following section); (2) the antigenic differences between molecules within major groups; and (3) the multiple antigenic determinants present on individual γ-globulin molecules. Multiple antigenic determinants have been revealed in human, mouse, and rabbit γ-globulins. Evidence for two antigenic determinants on γ-globulin molecules is clearly seen in Fig. 6 where the S and F components in a papain digest of 6.6 S γ-globulin were separated by immunoelectrophoresis and were tested with rabbit antisera against human γ-globulin. The resulting S and F precipitin arcs intersect and indicate that each type of fragment has at least one antigenic determinant not present on the other. Since the S and F fragments share these different antigenic determinants with the intact γ-globulin molecule, the whole molecule must have at least two different antigenic determinants. In addition, the fusion of the F′ arc with the F band (Fig. 6) indicates that the F component has an antigenic determinant in addition to the one(s) shared with the F′ component, i.e., a third antigenic determinant in the whole γ-globulin preparation. By techniques such as this and by immunochemical comparisons using myeloma proteins and Bence-Jones proteins with fewer antigenic determinants, multiple antigenic determinants have been shown to be present in normal human γ-globulin preparations (Korngold and Lipari, 1956a; Scheidegger and Buzzi, 1957; Edelman *et al.*, 1960; Heremans, 1960; Augustin and Hayward, 1961). Korngold and Lipari (1956a) calculated a minimum of seven antigenic determinants and probably more exist. Similar studies with mouse γ-globulins indicate the presence of at least five antigenic determinants in this species (Askonas and Fahey, 1962).

Not only do γ-globulin molecules have several different antigenic determinants, but some antigenic determinants of the S fragments are

present on at least two sites in the intact molecule. The evidence that two S fragments are obtained from each γ-globulin molecule (Section V), that the S fragments from normal γ-globulins are antigenically similar, and that different S fragments from the same myeloma proteins share the same antigenic determinants (Askonas and Fahey, 1961a) indicates that the antigenic determinants on the S fragments are present at two sites on each γ-globulin molecule.

Interspecies antigenicity depends on the species investigated. Although Porter's (1959) initial work indicated that interspecies antigenicity lay largely with rabbit fragment III (F fragment in other species) subsequent studies indicated that both S and F fragments from human and mouse γ-globulin may possess considerable interspecies antigenicity (Edelman *et al.*, 1960; Franklin, 1960; Stiehm *et al.*, 1960; Askonas and Fahey, 1961a). Recognition of species differences in response to γ-globulin injection is important in assessing preparations of "despeciated" antibodies. Tests of such preparations in animals may be misleading as to their effects in man.

Little is known of the chemical configurations in γ-globulin molecules responsible for interspecies antigenicity. Actually, most studies have been carried out with polyvalent antisera, i.e., antisera with antibodies against variety of antigenic determinants. There is need, however, for studies using antisera specific for single antigenic determinants. With such reagents it would be possible to ascertain the extent to which all 6.6 S γ-globulin molecules share the same antigenic determinants and also to subdivide the 6.6 S γ-globulins (and perhaps the β_{2A}- and γ_{1M}-globulins as well) on an immunochemical basis into subgroups based on distinctive antigenic determinants. Evidence already exists of differences in antigenic composition among the normal human 6.6 S γ-globulins (Dray, 1960) and γ-myeloma proteins (cf. Putnam, 1957, 1960). Identification of subgroups in the normal 6.6 S γ-globulins should help to shed light on the γ-globulin responses to immunization and disease.

B. Antigenic Properties of 6.6 S γ-, β_{2A}-, and γ_{1M}-Globulins

1. Specific Antigenic Determinants

Each class of γ-globulin (6.6 S γ, β_{2A}, and γ_{1M}) can be identified by specific antigenic determinants. These are revealed by separate precipitin lines on immunoelectrophoresis (Fig. 1) and by lines or spurs on gel diffusion comparison (Korngold and Lipari, 1956a; Korngold and van Leeuwen, 1957). Injection of 6.6 S γ-globulin into heterologous species does not necessarily produce an antiserum specific for 6.6 S γ-globulins.

Such an antiserum may contain antibodies which react with antigenic determinants shared in common with β_{2A}- and γ_{1M}-globulins, and the antiserum will have to be absorbed with β_{2A} and macroglobulins to be rendered specific for 6.6 S γ-globulins. Similar precautions regarding shared antigenic determinants also apply to the preparation of antisera specific for β_{2A}-globulin or γ-macroglobulin.

Antisera that are truly specific for 6.6 S γ-globulin react only with F fragments in the γ-globulin papain digest of γ-globulins (Franklin and Stanworth, 1961; Askonas and Fahey, 1962). Some β_{2A}-myeloma globulins can be split by papain and cysteine into S and F portions and the specific β_{2A} determinants shown to be on the F fragments (Askonas and Fahey, 1962). Localization of the specific γ_{1M} antigenic determinants on papain digest fragments of macroglobulins has not been reported.

2. *Common Antigenic Determinants*

Proteins formed in plasmacytes and related cells share common antigenic determinants which can be identified by immunochemical tests (Franklin and Kunkel, 1957; Korngold and van Leeuwen, 1959; Heremans, 1960). Studies of the papain digest fragments of γ-globulins and β_{2A}-globulins reveal that the common antigenic determinants reside largely, if not entirely, on the S fragments (Franklin and Stanworth, 1961; Askonas and Fahey, 1962).

Two antigenically different types of S fragments can be identified in human γ-globulins. Two types of 6.6 S γ-globulins are shown in Fig. 3 where Type I and II Bence-Jones proteins react with two different 6.6 S γ-globulin components. Two antigenically different classes of S fragments are found in the papain digest of normal 6.6 S γ-globulin (Fig. 3). F fragments, however, do not possess the antigenic determinants responsible for the two components seen in Fig. 3.

Type I antigenic determinants appear to represent a specific polypeptide chain and type II antigenic determinants a second polypeptide chain. Studies of myeloma proteins demonstrate that these molecules contain either type I or type II polypeptide chains, but not both types, indicating that each γ-myeloma protein molecule (and, probably, each normal γ-globulin molecule) contains two S fragment polypeptide chains of the same antigenic type.

Types I and II molecules are seen among β_{2A} and γ_{1M}-globulins in Fig. 8 (Fahey, 1962d). These observations and those of Mannik and Kunkel (1962) and Franklin (1962) identify Types I and II molecules in all classes of γ-globulins. The identification of antigenic types I and II indicate that polypeptide chains responsible for these antigenic

determinants are represented among the β_{2A}-globulins and γ_1-macroglobulins.

These studies indicate that there may be no antigenic determinant which is common to all γ-globulin molecules. Although type I and II antigenic determinants are present in all γ-globulin groups (6.6 S γ-globulins, β_{2A}-globulins, γ_1-macroglobulins and Bence-Jones proteins), individual molecules appear to have either type I or type II antigenic

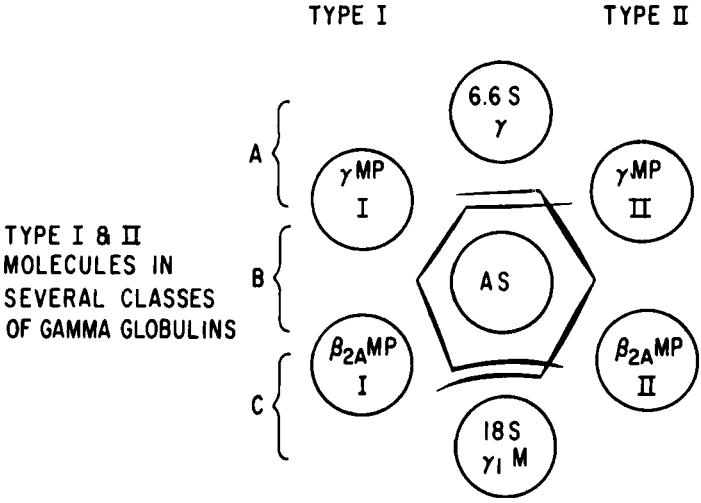

FIG. 8(A). Drawing of Ouchterlony test showing two types of 6.6 S γ-myeloma proteins related to two types of normal 6.6 S γ-globulins.

FIG. 8(B). Types I and II γ-myeloma proteins are seen to correspond to Types I and II β_{2A}-globulins.

FIG. 8(C). Two types of normal 18 S γ_1-macroglobulins related to Type I and Type II gamma globulins in other systems. Rabbit antiserum *vs.* NHγ-S fragments was used in the center well (AS).

determinants. A study of 75 myeloma proteins, Bence-Jones proteins and macroglobulins revealed that each had either type I or II antigenic determinants (Fahey, 1962d), and in this sense the type I and II antigenic determinants are common to all γ-globulin groups but not to all molecules.

X. Genetic Factors

A. Allotypes

Intraspecies differences in γ-globulins have been identified and shown to be under genetic control. By injecting γ-globulins from one animal into others of the same species, antibodies have been evoked against

different forms of γ-globulin or allotypes within the same species. Allotypes have been described in γ-globulins of the rabbit (Oudin, 1956; Dubiski *et al.*, 1959; Dray and Young, 1959), guinea pig (Benacerraf and Gell, 1961), and mouse (Kelus and Moor-Jankowski, 1961). Many factors are important in allotype detection, not the least of which is a characterization of the allotype as being a property of γ-globulin, β_{2A}-globulin, or macroglobulin.

The most extensive investigations of allotypes have been made in the rabbit where Dray and Young (1959) and Dubiski *et al.* (1959) found two determinants and Oudin (1960b) described seven main allotypes. These investigators will publish shortly a comparison of the independently discovered components in which a correspondence has been established between several allotypes. Oudin (1960b) has indicated that allotypes comprise two groups (b,c,d and a,f,g) each of which is controlled by three allelic genes (Oudin, 1960b). Recent observations of Kelus *et al.* (1961) indicate that the rabbit allotypes are properties of papain digest fragments I and II.

When several different allotypes are present in the same serum, it is of interest to know whether they were on the same or on different molecules. Oudin (1960a) obtained evidence—in the form of multiple precipitation zones in selected sera—that the allotypes are on different molecules. Further studies (Oudin, 1961) indicated that in some animals, b and f allotypes might be on the same molecules. Quantitative studies are needed to determine what per cent of the γ-globulin molecules contain each of the allotypes and whether heterozygous as contrasted to homozygous rabbits have half as many molecules with allotype characteristics.

γ-Globulin allotypes in man have not yet been reported although individuals receiving multiple transfusions or γ-globulin injections might be expected to develop antibodies against γ-globulins of other individuals.

B. Gm and InV Groups in Man

Hereditary γ-globulin specificity in man was demonstrated by Grubb (1956) and Ropartz *et al.* (1960). This activity was termed the Gm factor (Grubb, 1956) when first detected, but is now known as Gm(a) since additional related factors have been described. Harboe (1959) described Gm(b), and Harboe and Lundevall (1959) discovered Gm(x). The Gm-like factor was identified by Steinberg *et al.* (1960) and is common in Negro but not in white populations. Frequency and family data indicate that Gm(a) and Gm(b) may reflect allelic genes in Caucasian but not in Negro populations (Grubb, 1959; Harboe and Lundevall, 1959).

Gm(a) and Gm(x) are closely related, nearly all a+ persons being x+ (Harboe and Lundevall, 1961).[1]

The Gm factors (a), (b), and (x) are properties of 6.6 S γ-globulins (Grubb, 1959) but not of 18 S macroglobulins or β_{2A}-globulins (Mårtensson, 1961; Fahey and Lawler, 1961). The absence of reactivity in β_{2A}-myeloma proteins and macroglobulins could not be attributed to a masking of Gm activity by the polymerization of 6.6 S globulins since dissociation with mercaptoethanol and iodoacetate did not uncover any Gm activity (Fahey and Lawler, 1961). Subfractions of 6.6 S γ-globulin differing in electrophoretic mobility have approximately the same content of Gm(a), (b), and (x) components (Fahey and Lawler, 1961), but the question remains whether individual normal γ-globulin molecules carry multiple Gm factors or only one. Since individual normal γ-globulin molecules cannot be isolated, γ-myeloma proteins were studied.

Since Gm(a) and (x) have been identified together in individual γ-myeloma proteins (Mårtensson, 1961; Fahey and Lawler, 1961), they probably coexist on the same molecules. Gm(b) was identified on other myeloma proteins (Mårtensson, 1961) but further work is needed to settle the question of whether Gm(a) and (b) normally exist on separate molecules.

Comparison of whole myeloma serum and purified myeloma protein indicated that many myeloma proteins contained only a portion of the Gm factors present in the whole serum, and some myeloma proteins had neither Gm(a) nor (b) activity. As pointed out by Mårtensson (1961), complete absence of Gm(a) and (b) on myeloma proteins contrasts with the findings of Harboe and Lundevall (1961) that Gm(a) or (b) was present in all of 680 sera. Either the Gm(a-b-)myeloma proteins represent a subgroup of normal γ-globulins or else they reflect alterations due to malignancy. The phenomenon of antigenic loss by neoplastic cells, as compared with normal cells, may apply to the protein products of malignant cells. Antigenic deletion appears to be an irregular event with malignant cells (Gorer, 1961) and might influence myeloma proteins

[1] The Gm factors are usually detected by an agglutination–inhibition system in which appropriate 6.6 S γ-globulin is attached to red cells for visualization of the agglutination reaction (Rh-positive red cells are coated with Gm (+) incomplete (6.6 S) anti-Rh globulin) and agglutination is caused by adding appropriate 18 S macroglobulin, usually obtained from rheumatoid sera. The sample to be tested for Gm activity is added first to the rheumatoid sera and the Gm-positive γ-globulin will react with the rheumatoid factor and reduce the agglutination of globulin-coated red cells added subsequently. Gm (−) samples do not inhibit agglutination. In such an assay system, the 6.6 S γ-globulin reagent (incomplete Rh antibody) and the reactive rheumatoid factor have to be critically selected.

with similar uncertainty and unpredictability. If, however, the γ-myeloma proteins represent normal γ-globulins in their Gm features, some of the normal γ-globulin molecules may not have Gm(a) or (b) factors although they probably have some other distinctive feature.

Studies of papain-produced fragments of human γ-globulin and γ-myeloma protein revealed Gm(a) and (b) activity to be solely on the F fragments (Harboe *et al.*, 1962; Franklin *et al.*, 1962). The finding of Gm activity on that portion of the γ-globulin molecule containing other γ-specific properties is in accord with the absence of Gm properties from β_{2A}-globulins and γ-macroglobulins.

The InV factors were identified by Ropartz *et al.* (1961) using an inhibitor system. These factors were found to be under genetic control but independent of the Gm system. Harboe *et al.* (1962) have shown that InV factors are properties of β_{2A}-globulins and γ-macroglobulins as well as of 6.6 S γ-globulins. Thus the InV factors differ from the Gm groups by their presence in all the major γ-globulin groups and in being on the S fragments of γ-globulins. These observations provide additional evidence that features common to all molecules of the γ-globulin domain are properties of the molecular subunits represented in the S fragments of γ-globulins.

A corollary to the identification of different 6.6 S γ-globulin groups by the Gm tests is the demonstration of different γ-macroglobulins. Detection of Gm(a), (b), (x), and Gm-like factors requires 18 S macroglobulins of different serological specificity. Additional differences in rheumatoid factors have been demonstrated by using a variety of γ-globulins from a large number of sources in tests for rheumatoid factor specificity (Fudenberg and Kunkel, 1961; J. H. Vaughan *et al.*, 1958).

The basis for the presence of macroglobulins reacting with specific γ-globulins is unknown. It has been suggested that they represent autoantibodies (Burnet, 1961). There is little evidence for the view that Gm-reactive macroglobulins are antibodies against Gm configurations (see comments by Grubb, 1961). The possibility that they may be isoantibodies, analogous to the isohemagglutinins, is pointed out by Fudenberg and Kunkel (1961) who note, however, that the evidence of universal autospecificity of rheumatoid factors, i.e., formation of 22 S complexes with 6.6 S γ-globulins of the same patient, is difficult to reconcile with an isoantibody concept. Increased levels of serum rheumatoid factors can occur in other diseases, and Gm-active macroglobulins have been found in normal subjects (Ropartz *et al.*, 1960; Audran and Fine, 1961). The disease processes may then be responsible for the detecta-

bility rather than the specificity of γ-reactive macroglobulins. Further studies are needed to determine whether the specificity and heterogeneity of the γ-reactive 18 S macroglobulins (including rheumatoid factors) are under genetic control.

XI. γ-Globulin Interrelationships

A. Comparison of Subunits

The characteristics of intact γ-globulin molecules reflect the structure and properties of the γ-globulin polypeptide chains in somewhat the same manner as hemoglobin characteristics are determined by the polypeptide chains of that molecule (Ingram, 1959). γ-Globulin subunits, however, are not yet well defined although there is considerable evidence that at least two types of polypeptides and a total of three or more peptide units are present in each γ-globulin molecule. The extensively studied S (rabbit Groups I and II) and F (Group III) fragments of γ-globulins produced by papain and cysteine *cannot* be directly equated with the natural polypeptides of γ-globulin because papain hydrolysis of two or more peptide bonds (and breaking of polypeptide chains) is necessary to produce the S and F fragments. However, the presence on the S fragments of N- and C-terminal amino acids identical with those of the whole molecule (Porter, 1958; Silman *et al.*, 1962) indicates that each S fragment probably contains one native polypeptide chain. Furthermore, the studies of Harboe *et al.* (1962) indicate that parts of the S and F fragments of human γ-globulin are under separate genetic control—the S fragments carries the InV factors and the F fragments, the Gm factors. Thus, the papain fragments serve as tentative guides to the submolecular components in which the interrelationships between different γ-globulin groups, different γ-globulin molecules, and different γ-globulin functions are based.

The antigenic interrelationship of the major γ-globulin groups is schematically presented in Fig. 9. The relationships are illustrated in terms of present knowledge of the γ-globulin subunits. γ-Globulin molecules of all types have been found to have either type I or type II polypeptides. Studies of proteins formed in malignant plasma cells and quantitative studies of normal 6.6 S γ-globulins indicate that all, or almost all, molecules have either type I or type II polypeptide chains. Thus, it seems probable that each γ-globulin molecule has two identical (or very similar) polypeptide chains, which are either type I or type II.

In addition to the two type I or II polypeptide chains, each molecule (excepting the Bence-Jones proteins) has one or more polypeptide

chains, which determine whether the molecule is in the 6.6 S γ-globulin, β_{2A}-globulin or γ_1-macroglobulin class. At the present time, it is not clear whether the specific polypeptide chains within one class are identical, or vary in a manner analogous to the type I and II polypeptide chains.

The polypeptide localization of the variable part of the γ-globulin molecules responsible for electrophoretic heterogeneity, however, is not

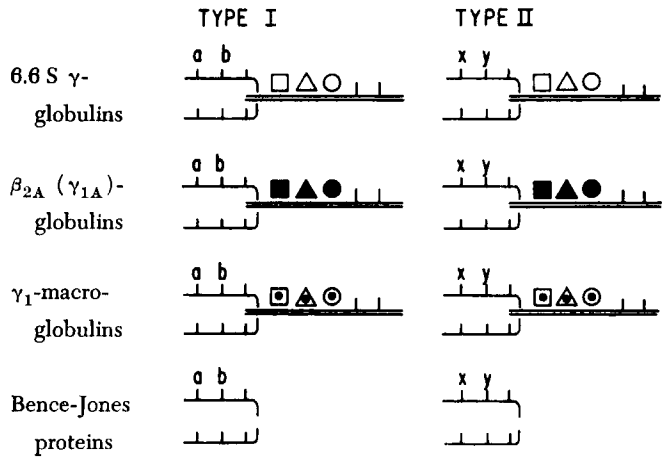

FIG. 9. Schematic diagram of the antigenic relationship between several classes (6.6 S γ, β_{2A}, γ_{1M}, BJ) and types (I and II) of gamma globulins. Multiple antigenic determinants are present on each molecule. The antigenic determinants common to all classes of gamma globulins are divided into Types I and II, and these are illustrated on the left of each diagram. Antigenic determinants specific for each class (6.6 S γ, β_{2A}, γ_{1M}) are illustrated on the right. All 6.6 S γ-globulins, whether Type I or II, appear to share some common antigenic determinants. β_{2A}-globulins share another group of specific antigenic determinants, and γ_1-macroglobulins have another set of determinants. The close antigenic relationship between Bence-Jones proteins and Type I or Type II globulin pieces is also shown. The diagram is not intended to indicate the limits of antigenic variability among γ-globulins; indeed, differences within each class are to be expected.

known. Although electrophoretic properties are determined by the net charge on each molecule, heterogeneity in electrophoretic mobility probably reflects variation in only a part of each molecule. Antibody specificity, Gm factors, and certain antigenic determinants are not affected by the molecular variations responsible for electrophoretic heterogeneity. Furthermore, localization of antibody activity and electrophoretic heterogeneity on the S fragments indicates that each S fragment may have

two parts, one with antibody specificity and another differing from one molecule to another and accounting for electrophoretic heterogeneity.

The 17,000-mol.-wt. polypeptide chain described by Edelman and Poulik (1961) is probably present in the S fragments. N- and C-terminal amino acid analyses indicate that the S fragment contains a complete polypeptide chain as noted in the foregoing. Also the demonstration of a similarity between the small polypeptide chains and components derived from reduced and alkylated Bence-Jones proteins (Poulik and Edelman, 1961) is compatible with a complete polypeptide chain comprising part of the S fragment. Since the polypeptide chain described by Edelman and Poulik (1961) accounts for about half of the S fragment (mol. wt. = 40,000), the remaining piece of the S fragment should be about the same size. Which of these two parts of the S fragment is responsible for antibody activity and which is responsible for electrophoretic variability is not clear. Edelman *et al.* (1961) found that specific antibody preparations from guinea pigs varied in the starch gel electrophoretic properties of the 17,000-mol.-wt. polypeptides. On the basis of the relationship between antibody specificity and the starch gel pattern of the reduced and alkylated guinea pig antibodies, Edelman *et al.* (1961) suggested that antibody specificity lay in the 17,000-mol.-wt. polypeptide. However, because of the insolubility of the fragments in aqueous solutions, they could not be directly tested for antibody activity. Further investigation of this interesting possibility using different antibodies from the same guinea pig as well as studies in other species should be of great interest.

Deductions about the structure of γ-globulins are necessarily tentative. The schematic drawings in Figs. 7 and 9 do not indicate the physicochemical relationship between the polypeptide chains from which the S and F fragments are derived. More information about the number and properties of the peptides in γ-globulin molecules is needed in order to be more explicit about the structural bases for the several forms of γ-globulin heterogeneity.

B. Bence-Jones Proteins as Subunits of γ-Globulins

γ-Globulin formation in plasma cells presumably proceeds by the incorporation of amino acids into separate polypeptide chains which are then assembled into a 6.6 S globulin product. Most studies of γ-globulin substructure have attempted to disassemble the final globulin product. The Bence-Jones proteins, however, offer the opportunity to examine polypeptides formed in plasma cells which may represent peptide subunits of γ-globulin molecules.

Many studies, which have been reviewed by Putnam (1957, 1960), indicate that Bence-Jones proteins have some of the properties of the larger γ-globulins. Bence-Jones proteins closely resemble the S fragments of γ-globulins in antigenic determinants, in range of electrophoretic mobility, and in low carbohydrate content. In spite of the similarity of Bence-Jones proteins to the S fragments, they are not identical. Studies of Bence-Jones protein and S fragments from the same plasma cell tumor (Askonas and Fahey, 1962) revealed significant differences in electrophoretic mobility and in response to papain and cysteine, and Stiehm *et al.* (1960) showed that Bence-Jones proteins from two patients with multiple myeloma were not antigenically identical to either the S or F (C or B) fragments of myeloma globulins from the same patients. It is not surprising that Bence-Jones proteins differ from enzymatically produced γ-globulin fragments, but the close antigenic similarity of the type I and II Bence-Jones proteins to the corresponding S fragments of γ-globulins indicate that the S fragments include polypeptides closely related to the Bence-Jones proteins.

Additional evidence that Bence-Jones proteins may represent polypeptides present in γ-globulins was obtained by Poulik and Edelman (1961) who compared subunits obtained by reduction and alkylation of myeloma proteins and Bence-Jones proteins from two patients. The finding that some components from the Bence-Jones proteins and myeloma protein of each patient had the same mobility on starch gel electrophoresis encouraged these authors to emphasize the possibility that Bence-Jones proteins may be composed of peptide chains of the same type as those present in myeloma proteins.

Bence-Jones proteins are not breakdown products of γ-globulin or of myeloma proteins (Putnam and Hardy, 1955; Osserman *et al.*, 1957), but it is not certain whether they represent intermediate stages in the synthesis of larger globulins or whether they are synthesized separately from myeloma globulins. *In vitro* isotope incorporation studies with the mouse plasma cell tumor, MPC-2, demonstrated that Bence-Jones protein obtained in saline extracts of the tumor was not a precursor in the synthesis of myeloma protein (Askonas and Fahey, 1961b). Although these experiments did not include a study of cell-bound Bence-Jones protein, they did show that free Bence-Jones protein in this transplantable plasma cell tumor was not a precursor in the synthesis of the larger globulin.

XII. Heterogeneity of the Normal Plasma Cell Population

To account for the heterogeneity of the γ-globulins, either the individual plasma cells are multipotential and synthesize all of the normal forms of γ-globulin or they are limited to the formation of only a part of the γ-globulin spectrum and thus comprise a heterogeneous population. Heterogeneity of γ-globulins does not refer to a single property, but to at least three separate domains (i.e., class-specific properties, electrophoretic properties, and antibody activities) which seem to be properties of separate parts of the molecule. Therefore, individual plasma cell capacities at each of these levels of molecular structure can be considered separately.

The observations that antibody response may appear first among the macroglobulins and then later in the 6.6 S γ-globulins (Section III,C) raise the question whether these two phases are brought about by the same cells or by two cell groups. Evidence that each clone of plasma cells synthesizes only one class of γ-globulin (6.6 S γ, β_{2A}, or γ_{1M}) is seen in the observations with clones of malignant plasma cells where (excluding the Bence-Jones proteins) only one class of γ-globulin is found, and malignant plasma cell tumors do not alter their protein product from one class to another.

Measurements of the electrophoretic properties of proteins produced in individual normal cells have not been reported. Heterogeneity in many serum myeloma proteins (Section IV,C) may reflect the capacity of each malignant plasma cell to form several closely related peptides with slightly different charge properties. Alternatively, the tumor cell population may represent a stable polymorphism with several malignant clones synthesizing only one type of molecule. In either case, the electrophoretic properties of individual myeloma proteins and macroglobulins represent only a small part of the normal electrophoretic γ-globulin spectrum. These findings indicate that the malignant plasma cells synthesize, at most, only a few closely related sets of peptides. How completely these observations on malignant plasma cells can be transferred to normal plasma cell population is matter of conjecture but, as noted in Section IV,D, many malignant plasma cells may have retained unaltered the globulin synthetic mechanism of normal plasma cells.

Considerable evidence has been assembled (Nossal, 1958; Burnet, 1959) that individual plasma cells may be limited to the formation of one or, in some cases, two antibodies (Attardi *et al.*, 1959), i.e., antibody peptides with only one or two configurations. Although it is certain that single plasma cells can synthesize two forms of antibody-specific peptide,

cells apparently do not mix the antibody peptides but incorporate into each antibody molecule two antibody peptides of the same specificity. The genetic and biochemical features making possible this fine discrimination by plasma cells have not been elucidated. It seems probable, however, that individual plasma cells are limited to the synthesis of one or only a few polypeptides for each part of the γ-globulin molecule and that normally there exists a heterogeneous and, apparently, highly specialized cell population for the maintenance of immunity.

XIII. Concluding Comment

The serum γ-globulins in many species are now known to include three major groups of proteins (γ, β_{2A}, γ_{1M}). A fourth group, the γ-microglobulins (Bence-Jones proteins), may be formed in normal as well as in malignant plasmacytes and related cells. A dual relationship exists between these groups of proteins. Each group of γ-globulins has distinctive physicochemical and immunochemical properties, yet all of the γ-globulins share certain common features. Furthermore, each group possesses an extensive electrophoretic heterogeneity. Thus, the γ-globulins comprise three and, perhaps, four families of related but not identical protein molecules. Recognition of the existence of the major groups of γ-globulins has helped to clarify questions about the genetic factors determining γ-globulin properties and about the functional role of the γ-globulin groups. The 6.6 and 18 S γ-globulin antibodies may be produced at different times during the immune response.

Structural studies indicate that γ-globulin molecules are composed of several polypeptide subunits. Although satisfactory separation of these subunits with retention of biological function has not been reported, enzymatic fragmentation with papain plus cysteine has produced γ-globulin pieces with distinct differences in biological and chemical properties. Studies at the submolecular level of the interrelationships between different γ-globulin groups, between different γ-globulin molecules, and between different γ-globulin functions have begun, and a better understanding of the physicochemical and immunological heterogeneity of the γ-globulins is beginning to develop. This rapidly moving area of investigation, taking advantage of developments in other fields, is, in turn, contributing to advances in many areas of immunology and other biological sciences.

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The Immunological Significance of the Thymus

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I. Introduction

The faculty of immunological response is generally considered to be a function of some of the mesenchymal cells of the body. The lymphocyte is the commonest of mesenchymal cell types. Although there is evidence that members of the plasma cell series are responsible for synthesizing antibody (Coons *et al.*, 1955; Nossal, 1959), there are clear indications that the lymphocyte can also function as an immunologically competent cell. Neil and Dixon (1959), utilizing immunohistochemical methods for tracing the fate of cells producing antibody after their transfer into recipient animals, have obtained data which suggest that lymphocytes from donors are transformed into plasma cells; that the development of plasma cells corresponds to the appearance of cells con-

taining antibody; and that secondary or anamnestic responses can be elicited in animals passively sensitized with cells of the lymphocytic series. Also, White (1960) has shown that the cells which comprise the germinal centers of the cortical lymphoid nodules within a lymph node can, under certain circumstances, acquire a content of antibody in response to an antigenic stimulus. Medawar (1944), Billingham *et al.* (1954), and Weaver *et al.* (1955) have all reported findings which suggest that allogeneic skin grafts evoke an immune cellular reaction, predominantly of the lymphocytic and plasmocytic cell types. Gowans *et al.* (1961) report experiments indicating that small lymphocytes are capable of initiating an immunological reaction, and, in doing so, appear to be transformed into pyroninophilic cells. It is thus reasonable to assume that the lymphocyte may bear the brunt of immunological defenses. It has been suggested that the main function of the lymphocyte is to carry immunological information (Burnet, 1959a). Consequently, it might be expected that any interference with or disturbance of the mechanism responsible for the development of the lymphoid system would be associated with serious consequences: interference may be expected to result in immunological failure; disturbance could conceivably lead to the failure of production of competent cells with consequent defects in immunological performance (as in hypogammaglobulinemia) or to the production of cells capable of reacting against the individual's own tissue, the "forbidden clones" of Burnet (1959a), which would be responsible for some autoimmune conditions.

II. Role of the Thymus in Lymphocyte Production

The most effective lymphocyte-producing tissue is the thymus. This is evident from studies of cell production in individual lymphoid organs and in thymectomized animals as described in the following sections.

A. Lymphopoiesis in the Thymus

1. Origin of Thymic Lymphoid Cells

Two schools of thought have developed concerning the origin of thymus lymphocytes. The Maximow-Hammar group (Maximow, 1909; Hammar, 1921, 1938; Maximow and Bloom, 1957) maintained that thymic lymphocytes originated from mesenchymal cells which migrated into the epithelial portion of the thymus rudiment prior to morphogenetic changes within the rudiment. The Maurer-Stöhr group (Maurer, 1885; Stöhr, 1906), on the other hand, claimed an epithelial origin for thymus lymphoid cells believing also that they had the potentiality of reversion to

their original epithelial form. Dustin (1913) and Grégoire (1932) also considered that thymocytes were derived by mitosis from the original epithelial structure of the thymus and denied the immigration of lymphocytes from outside or differentiation of mesenchymal cells. Further experimental evidence supporting either theory was obtained mostly by histogenetic studies of the degenerative and regenerative changes that follow transplantation of thymus tissue into various species of animals (Nusbaum and Prymak, 1901; Dudgeon and Russell, 1905; Jonson, 1909; Pinner, 1915; Danchakoff, 1916; Renton, 1916; Gottesman and Jaffé, 1926; Baillif, 1949).

Ackerman and Knouff (1959) and Burnet (1961) have pointed out that there is morphological evidence that, in the chick, the entodermal epithelium gives rise to the cells of the lymphoid nodules of the bursa of Fabricius. The mammalian thymus and avian bursa both have an epithelial origin from the gut, a similar ontogenetic behavior, progressing to a peak size and regressing slowly, and somewhat similar functions (Jolly, 1915; Ackerman and Knouff, 1959; Mueller *et al.*, 1960).

Auerbach (1961), using tissue culture techniques developed by Grobstein (1955), was able to analyze experimentally some of the morphogenetic events in thymus development. Thymic rudiments were obtained from 12-day mouse embryos and separated into epithelial and mesenchymal components by the use of trypsin. It was shown that the epithelial component of the rudiment was capable of forming the lymphocytes of the thymus, the mesenchyme providing the initial inductive stimulus and serving to furnish the stromal elements of the gland. Neither host migrating cells, nor thymic mesoderm, nor generalized mesenchyme appeared to contribute significantly to the initial lymphoidal cell population of the thymus.

2. *Cell Multiplication in the Thymus*

The thymus at birth is by far the major lymphoid organ—the lymphoid tissue in the spleen being then only very poorly developed. In very elegant studies, Axelrad and van der Gaag (1962) have described the postnatal growth of the thymus of normal C3H mice. The number of cells in the thymus increased to as much as 30 times its value at birth (from about 5 million to about 150 million cells per lobe) during the first 2 weeks of life. Thereafter, it remained approximately constant until 5 to 6 weeks of age when it began to fall. Two simultaneous phenomena appeared to account for the growth of the thymus in the first two weeks: (1) multiplication involving great numbers of "large free round" cells, particularly those concentrated in the outer zone of the cortex (see also

Grégoire, 1932; Kaplan, 1960; Smith and Kaplan, 1961); (2) transformation of these large cells into smaller lymphoid cells which were themselves capable of intense mitotic activity. After 2 to 3 weeks of age, the large multiplying round cells had been replaced by smaller lymphoid cells which now formed the outer zone of the cortex, and the growth of the thymus as an organ had ceased. However, the smaller cells still continued to exhibit intense mitotic activity.

A quantitative cytological study of the thymus from birth to the age of 28 weeks was performed by Nakamura and Metcalf (1961). In C3H mice, the percentage of medium lymphocytes fell progressively with age and the over-all mitotic index was maximal at 1 week and fell sharply, by about 50%, at the onset of age involution between 3 and 8 weeks. After this time, the mitotic index remained relatively constant.

It can be calculated from mitotic data on thymuses of normal rats 15 and 20 days old (Kindred, 1940), 28 days old (Andreasen and Christensen, 1949), and 80 days old (Kindred, 1942) that the rate of lymphocyte production in the thymus cortex in all these animals was about 2 million per hour. From histological studies involving differential cell counts and mitotic indices, Leblond and Sainte-Marie (1960) suggested that throughout the thymus the small lymphocyte was the end point of a series of eight successive generations, from reticular cells and large lymphocytes through smaller and smaller lymphocytes. Since few large lymphocytes were observed in the medulla, it was postulated that the evolution of reticular cells into lymphocytes was exceptional in that region and that the overproduction of reticular cells was balanced by their degeneration into Hassall's corpuscles. The presence, in the medulla, of many more small lymphocytes than had been expected from the number of medium ones present, suggested that small lymphocytes had migrated from the cortex into this zone. Diapedesis of small lymphocytes was commonly seen across the walls of lymphatic channels and blood vessels in the medulla but not in the cortex. The conclusion was drawn that cortex-formed small lymphocytes migrated through the medulla in order to reach the circulation.

The thymus has the greatest lymphopoietic activity of all the lymphoid organs. Mitotic indices of thymic lymphoid cells are 4–10 times higher than those of other lymphoid cells (Table I) (Kindred, 1940, 1942; Andreasen and Christensen, 1949; Bierring, 1960; Metcalf, 1962) and deoxyribonucleic acid (DNA) turnover is 2–5 times as active in the thymus as it is in lymph nodes (Andreasen and Ottesen, 1944, 1945). Thymic lymphoid cells are subject to the same hormonal influences as other lymphoid cells (Dougherty, 1952), but such general regulators

cannot account for regional differences in lymphopoietic activity. Subcutaneous thymus grafts display the high proliferative activity characteristic of thymus tissue and increase in size rapidly even in hosts whose own thymuses are undergoing age involution weight loss during the same period (Metcalf *et al.*, 1961; Metcalf, 1962). It appears, therefore, that the factor determining the high proliferative activity of thymic lymphoid cells must be intrinsic to the thymus itself. The constant presence of epithelial cells in thymus tissue, whether in its natural location or in a subcutaneous site, hints at the possibility of some functional interrelationship between these epithelial cells and thymus lymphoid cells.

TABLE I
MITOTIC INDICES OF LYMPHOID TISSUES OF THE MOUSE AND RAT

Tissue	Mean number of mitoses/1000 lymphoid cells in:	
	Mouse (Metcalf, 1962)	Rat (Bierring, 1960)
Thymus	7.0 ± 0.1[a]	6.85
Peyer's patches	1.2 ± 0.3	0.85
Mesenteric lymph nodes	1.0 ± 0.6	0.65
Subcutaneous lymph nodes	0.5 ± 0.2	0.60

[a] Standard deviation, ±.

Recently, Metcalf and Ishidate (1961) have described periodic acid-Schiff (PAS) positive, giant cells in the mouse thymus cortex which may be responsible for the high mitotic rates characteristic of thymus lymphoid cells. The PAS cells were frequently surrounded by tightly packed lymphocytes one or more of which were in mitosis. This close association suggested that PAS-positive cells, through some cell contact mechanism, stimulated mitosis in neighboring lymphoid cells. The uniqueness of thymus tissue might then rest on the existence of large numbers of these PAS-positive cells in the thymus cortex.

3. *Relation between "Thymocytes" and "Lymphocytes"*

There has been some controversy as to whether lymphocytes produced in the thymus are similar in nature to lymphocytes found elsewhere (see Yoffey and Courtice, 1956). This is because, in some experimental situations, the behavior of "thymocytes" (i.e., thymic lymphocytes) seemed to differ from that of "lymphocytes" (i.e., lymphocytes found in other lymphoid organs). Differences in the behavior of thymocytes and lymphocytes may be summarized as follows:

1. Thymocytes are more actively mitotic than lymphocytes (as already mentioned).

2. There are quantitative differences in the responses of thymocytes and lymphocytes to various hormonal and nutritional influences (Dougherty, 1952) and to ionizing radiations and various chemicals (Trowell, 1958).

3. Thymocytes appear to be more susceptible to leukemic transformation than lymphocytes (Miller, 1961b, 1962a).

4. Thymocytes, unlike lymphocytes, appear to play little part in the response to foreign antigens (see Section III).

5. Thymocytes are less sensitive to the action of isoimmune sera than are lymphocytes (Winn, 1962).

On the other hand, there are no morphological differences between small lymphocytes in the thymus and elsewhere (Kindred, 1940). There is evidence that after radiation-induced involution, the thymus can be repopulated by nonthymic lymphoid cells, which subsequently behave as typical thymic cells (Gengozian *et al.*, 1957), and there is indirect evidence that lymphocytes produced in the thymus are distributed elsewhere (see following). This would suggest that there are no fundamental differences between thymocytes and lymphocytes and that the peculiar functional behavior of thymocytes is a reflection of the special conditions afforded by the thymic environment (e.g., the presence of epithelial and PAS-positive cells, as discussed in the foregoing, and the presence of a blood–thymus barrier as discussed in Section III).

4. Fate of Thymus Lymphocytes

The high DNA turnover and mitotic index characteristic of thymus tissue does not necessarily mean that the thymus contributes to the general pool of lymphocytes. It has been held, for instance, that the fate of thymic lymphocytes is local degeneration to release DNA to the circulation (Policard, 1950). There is, however, much evidence (mostly indirect) to suggest that lymphocytes produced in the thymus are released from the organ. This evidence can be summarized as follows:

1. Calculations by Kindred (1940) show that the thymus cortex produced far more small lymphocytes than were needed for local growth, whether the mitotic cycle was taken as of 1, 2, or 3 hours' duration. Under normal conditions, the rate of cell death in the thymus was slight amounting to no more than 15% of cell production (Kindred, 1942; Sainte-Marie and Leblond, 1958). Furthermore, there has been very little evidence that lymphocytes in the normal unirradiated animal enter the thymus from other parts of the body. Transfusion of P^{32}- or tritium-labeled small lymphocytes show that the cells have "homed" in large numbers to all the lymphoid tissues with the notable exception of the thymus (Gowans,

1959, 1961). Thus, the discrepancy among cell death, cell production, and total cell number in the thymus strongly suggests that some lymphoid cells produced in the organ are destined to leave it.

More recent calculations by Metcalf and Nakamura (1962) indicate that the thymus produces 66% of the total lymphocytes in 6-month-old AKR mice and 40% in 6-month-old C3H mice. From their data on thymus mitotic indices (Nakamura and Metcalf, 1961), it would appear that the percentage contribution of the thymus to total lymphocyte production in the baby mouse is much higher than in the adult animal. From histological observations, Kindred (1940) suggested that in very young, but not in older rats, the thymus provided lymphocytes necessary for the growth of lymph nodes.

2. Mature small lymphocytes can be seen in the lymphatics in every thymus, and sections of the medulla have revealed diapedesis of small lymphocytes across lymphatic channels and blood vessels (Leblond and Sainte-Marie, 1960). Similarly, in thymus grafts the lymphatics are always filled with small lymphocytes and the draining lymph nodes are larger than the opposite nodes although their mitotic index is not elevated (Metcalf *et al.*, 1961). It would appear, therefore, that the thymus graft is sending out cells some of which would tend to increase the size of the draining lymph nodes.

3. Transfused P^{32}-labeled thymus lymphocytes have been traced to the spleen, and the suggestion was made that the spleen might be an important destination for thymus lymphocytes (Fichtelius, 1960).

4. In neonatally thymectomized mice grafted with thymus tissue, it has been shown, with the aid of a cytological marker technique, that some of the cells dividing in the spleen had originated in the thymus graft (Miller, 1962c,d). This is certainly direct evidence that lymphocytes produced in thymus tissue can be distributed elsewhere, but this situation does not necessarily indicate what is occurring in the intact animal.

Further evidence that the thymus produces lymphocytes destined for other places has been obtained in studies of thymectomized animals.

B. Lymphopoiesis in the Thymectomized Animal

Thymectomy of the adult animal generally causes some diminution in the lymphocyte population of the body. Metcalf (1960) studied the peripheral blood lymphocyte levels and histology of the lymphoid tissues of mice thymectomized between 4 and 6 weeks of age. There was a slow progressive fall in circulating lymphocytes to a maximum of 30 to 40% below normal values. This low level persisted during an observation

period of 4 months. Lymph node and spleen weights fell by 25%. The lymphoid follicles were less tightly packed with lymphoid cells and the germinal centers were less prominent. There was a decreased mitotic activity (-28%) in the germinal centers and a decreased cell content (-30%) in the central areas of the follicles. Bierring (1960) performed similar studies on rats thymectomized at about 8 weeks of age. The lymphocyte output of the thoracic duct fell to about 40% below normal values (see also Reinhardt and Yoffey, 1956) and the blood lymphocyte content showed a similar drop. Only a minor decrease in weight of spleen and lymph nodes was recorded 65 days after thymectomy but the mitotic activity in these organs was the same as that found in control animals. There were no changes in the Peyer's patches or in the bone marrow. Lymphopenia and a certain degree of lymphoid atrophy have also been reported following thymectomy in other species of rodents (Nakamoto, 1957a; Comsa, 1957; Manning, 1959).

Thymectomy of the newborn animal is associated with a striking decrease in the lymphocyte population of the body. Schooley and Kelly (1958) performed thymectomy on 6-day-old rats and found that the thoracic duct lymphocyte output fell to 27% of the values in the control animals and that the weight of the cervical and mesenteric lymph nodes was markedly reduced. Waksman *et al.* (1962) thymectomized rats at birth and observed a depletion of small lymphocytes, strikingly expressed in the white pulp of the spleen and in the large lymphoid masses (tertiary nodules of Ehrich) in various lymph nodes, as well as a depression in the level of circulating small lymphocytes. On the other hand, germinal centers and plasma cells appeared normal.

A study of the lymphocyte population of mice thymectomized at birth has been performed by one of the authors (Miller, 1961a, 1962b,c,d). In sham-thymectomized mice, the lymphocyte/polymorph ratio rose progressively during the first 8 days of life to reach almost the normal adult ratio. In mice thymectomized at birth, this ratio did not increase significantly during that time and at 6 weeks of age was not much higher than at birth (Fig. 1). The total white cell count 6 weeks after birth was just over half that of sham-thymectomized littermate controls, the decrease being entirely owing to a lymphopenia. There was no significant difference in the levels of the polymorphonuclear neutrophiles, eosinophiles, and monocytes (Table II). The lymphocyte count fell to lower levels, reaching values as low as 500/mm.^{3} or 90% below control levels during the third and fourth months (Fig. 2).

Involution of the lymphoid tissues was a characteristic anatomical feature of neonatally thymectomized mice. At 6 weeks of age, the spleen

was greatly reduced in size, the average spleen weights being about half that of sham-thymectomized controls (Miller, 1962d). Microscopically (Miller, 1961a, 1962b,c,d), the spleen showed ill-defined, inactive follicles with little basophilia, poor cellularity, and few mitoses (Figs. 3–6). There were few germinal centers. The lymph nodes were also considerably diminished in size and showed inactive follicles and poor cellularity. The Peyer's patches were present but smaller and less cellular than in controls. This involution was even more striking during the third or fourth month when the mice developed a wasting

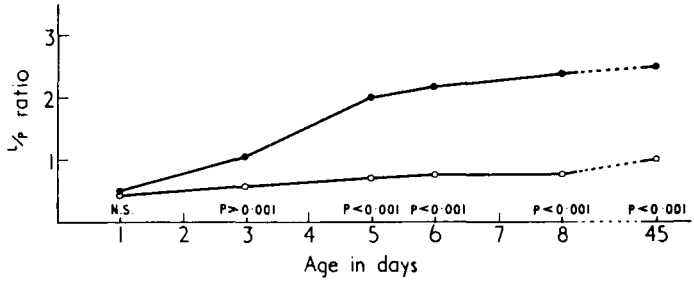

FIG. 1. Average lymphocyte/polymorph ratio of mice thymectomized in the neonatal period compared with sham-thymectomized controls. Statistical differences indicated. (o) Thymectomized mice; (•) sham-thymectomized mice. From Miller (1961a).

syndrome, described in the following. Many mice at that time showed minute lymph nodes with a poor cell content, a markedly shrunken spleen with no follicular structure, and either no or minute, poorly developed Peyer's patches in the intestine. These changes were found in the following strains and interstrain hybrids: Ak, C3H, CBA, C57BL, T6, (Ak × T6) F_1, and (CBA × T6)F_1. Mice of the BALB/c strain, however, did not consistently show diminution in the lymphocyte population after neonatal thymectomy.

The foregoing experiments indicate that (1) thymectomy is generally associated with a diminution in the lymphocyte population, and (2) the earlier in life the thymectomy is performed, the greater is the deficiency of lymphocytes in other lymphoid organs. Furthermore, while lymphocyte production continues, although at a lower level, in the animal thymectomized during adult life, it appears eventually to stop in the neonatally thymectomized mouse. Two possible mechanisms might account for this defect. (1) The thymus, through cell migrations, populates other lymphoid tissues and continually replenishes them. This cellular contribution would be of major importance in very early life and would

TABLE II

PERIPHERAL LEUCOCYTE LEVELS OF SIX-WEEK-OLD C3H AND (AK × T6)F_1 MICE THYMECTOMIZED AND SHAM-THYMECTOMIZED AT BIRTH

Strain	Treatment	Number of mice in group	Peripheral leucocytes/mm.^{3}			
			Total	Lymphocytes	Neutrophiles	Eosinophiles and monocytes
C3H	Thymectomy at birth	21	5150 ± 310[a]	2230 ± 380	1920 ± 240	750 ± 50
	Sham-thymectomy at birth	17	8110 ± 270	5260 ± 390	2140 ± 300	610 ± 80
	Statistical significance of difference	—	$P < 0.001$	$P < 0.001$	Not significant	Not significant
(Ak × T6) F_1	Thymectomy at birth	33	5640 ± 320	2880 ± 200	1970 ± 210	690 ± 60
	Sham-thymectomy at birth	22	8730 ± 260	6600 ± 720	1390 ± 140	700 ± 70
	Statistical significance of difference	—	$P < 0.001$	$P < 0.001$	Not significant	Not significant

[a] Standard error, ±.

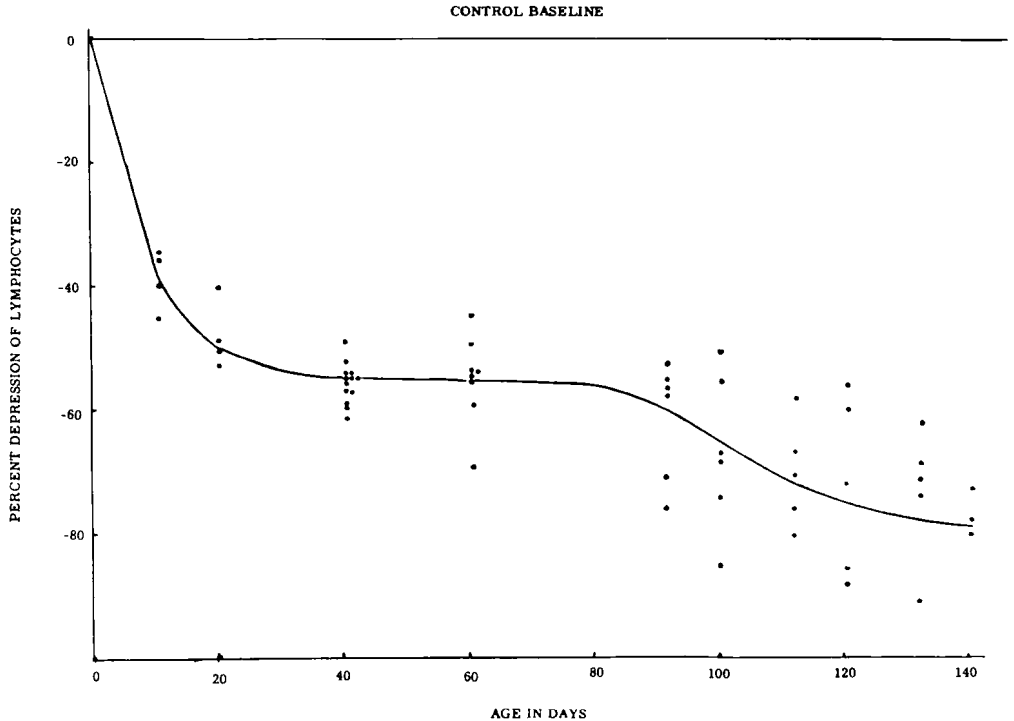

FIG. 2. Effect of thymectomy at birth on the peripheral blood of C3Hf/PW mice. Each point represents the value for one mouse compared with the value of a littermate sham-thymectomized control.

decrease with age. As pointed out in the previous section, there is much evidence of a cellular contribution from the thymus which is particularly important in early life. (2) The thymus produces a noncellular or humoral factor which regulates lymphocyte production and maturation, particularly during early life. Evidence for the existence of such a factor is

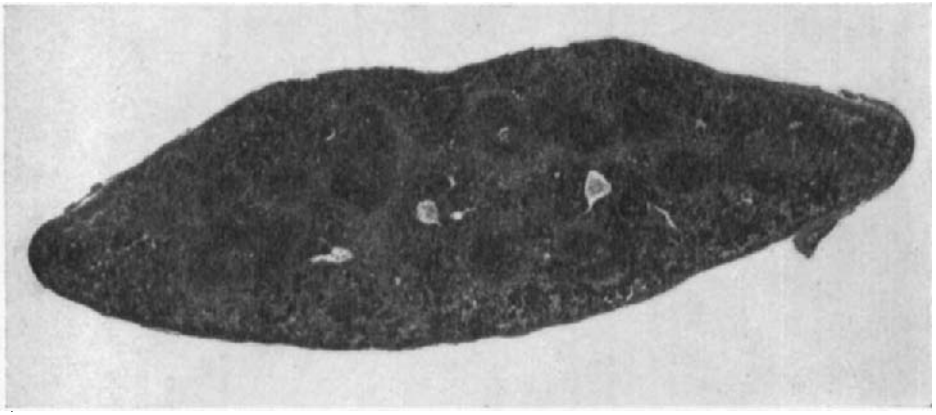

FIG. 3. Spleen of 6-week-old C3H mouse sham-thymectomized at birth. Magnification: ×20. From Miller (1961a).

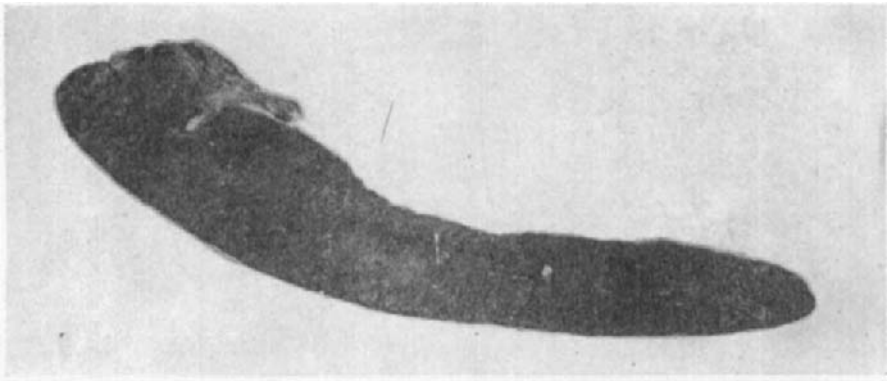

FIG. 4. Spleen of 6-week-old C3H mouse thymectomized at birth. Magnification: ×20. From Miller (1961a).

reviewed in the following. It is also possible that both cellular and noncellular thymic factors play a role in the proper development and maintenance of the lymphoid tissue.

Clues to the mechanism by which the thymus controls the development of the lymphoid system may be obtained from morphogenetic studies. When a thymus rudiment from a 14-day mouse embryo was iso-

FIG. 5. Spleen of 6-week-old (Ak × T6)F_1 mouse sham-thymectomized at birth. Magnification: ×120. From Miller (1962d).

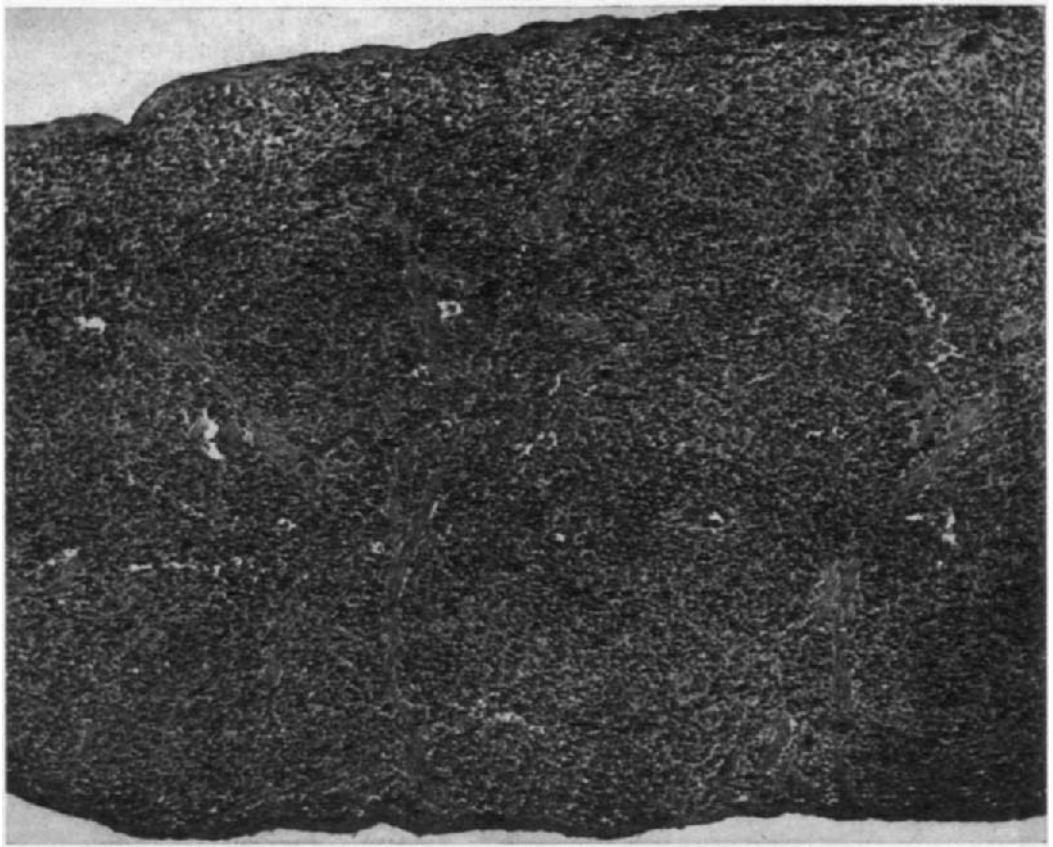

FIG. 6. Spleen of 6-week-old (Ak × T6)F_1 mouse thymectomized at birth. Note ill-defined follicles and poor cellularity. Magnification: ×120. From Miller (1962d).

lated and grown *in vitro* in tissue culture, it formed lymphocytes in characteristic fashion (Auerbach, 1961). When a 14-day embryonic spleen was isolated in this way, it failed to differentiate further. When the two were combined, a synergistic effect was obtained and discrete lymphoid nodules appeared. Spleen–thymus interaction was unique in that thymus–salivary gland mesenchyme and thymus–lung mesenchyme combinations did not show such synergism. Synergism was further evidenced by the enhanced long-term survival (more than 4 weeks) of the combination cultures, whereas thymus without spleen became decreasingly lymphocytic after 4 weeks (Auerbach, 1962). The histological picture of a thymus–spleen combination was virtually identical with that of normal spleen. The picture of an embryonic spleen grown by itself was similar to that of the spleen of mice thymectomized at birth, as already described (Auerbach, 1961, personal communication). Attempts were made to trace the lymphocytes which made up the splenic follicles in a thymus–spleen system (Auerbach, 1962). In chick–mouse combinations, the lymphoid cells appearing in the combined culture were judged, by morphological criteria, to have originated from the thymus rudiment, not from the spleen. This suggests that the spleen does not have the lymphoid primordial cells necessary for lymphoid differentiation and that these cells may be contributed by the thymus.

C. Lymphocytosis-Stimulating Factors

The exceedingly high lymphopoietic activity of the thymus could result from the elaboration of a specific lymphocytosis-inducing factor by nonlymphoid thymic cells, or by cells which are not usually found in other lymphoid tissues. Grégoire and Duchâteau (1956) reported that implants of thymus tissue depleted of lymphocytes by irradiation and, therefore, consisting mainly of the radioresistant epithelial stroma, could stimulate lymphopoiesis in lymph nodes. Implants of lymph nodes or muscle fragments, on the other hand, had no such effect. Other workers reported that thymus extracts produced a temporary lymphocytosis when injected into various animals (Comsa, 1956; Nakamoto, 1957b). Metcalf (1956a,b,c, 1958, 1959) claims to have demonstrated in the thymus a specific lymphocytosis-stimulating factor (LSF) the activity of which is apparently associated with the epithelial-reticular cell complex of the medulla. The factor is heat labile, filtrable, but nondialyzable. It is present in normal blood and its concentration both in blood and in thymus is increased in high-leukemic mouse strains and in mice or patients with lymphatic leukemia. On injection into baby mice or thymectomized adult mice, thymic LSF produced a temporary lymphocytosis reaching its

maximum point 3–7 days after injection. The mechanism by which lymphocytosis is produced is undetermined, but histological studies of the lymphoid tissues of thymectomized mice led to the suggestion that LSF might stimulate the maturation or division of primitive lymphocytes into mature small lymphocytes (Metcalf, 1960). It is possible, however, that the lymphocytosis produced in baby mice may not represent a stimulation of lymphopoiesis but an increase of cells released from the thymus.

One might postulate the existence of other LSF's since, even in the adult thymectomized animal, lymphocyte production continues, although at a lower level. The studies by Ernström and Gyllensten (1959) of thyroxine-treated guinea pigs appear to indicate that the thyroid hormone may be an important specific stimulator of the lymphoid tissues. Preliminary experiments (Miller, unpublished data, 1962) have shown that radioactive iodine uptake by the thyroid of neonatally thymectomized mice is low. This suggests that thyroid function may be depressed and that some feedback mechanism may possibly operate between thymus and thyroid.

III. Immunological Reactivity of the Thymus

It has often been debated whether the thymus plays a significant part in immunological reactions. On the one hand, there are hints from clinical observations that the thymus may play some role. For instance, in acute infections, when presumably the need for an immunological response with antibody production is great, the thymus undergoes rapid involution. In patients with acquired agammaglobulinemia there is often the simultaneous occurrence of benign thymomas (MacLean *et al.*, 1956). On the other hand, there are experimental studies which show that the intact thymus does not produce antibody in immunized animals. These studies will now be briefly reviewed.

A. Antibody Production in the Thymus

The demonstration of a high antibody titer within an organ after parenteral injection of antigen is usually taken as evidence that this organ plays a part in the formation of antibodies. This is so in the case of the spleen and lymph nodes. A significant antibody titer has not, however, been demonstrated in the intact thymus of an immunized animal (Bjornebøe *et al.*, 1947; Fagraeus, 1948; S. and T. N. Harris, 1954). During the production of antibodies, the spleen and lymph nodes characteristically show a profuse plasma cell proliferation (Bjornebøe and Gormsen, 1943; Fagraeus, 1948). There is strong evidence that antibody is synthesized in plasma cells (Coons *et al.*, 1955; Nossal, 1959). The thymus

contains few or no plasma cells and such cells do not appear in the organ under conditions of heightened antibody production (Fagraeus and Gormsen, 1953; Yoffey and Courtice, 1956). When thymus tissue, derived from immunized animals, was maintained *in vitro,* it showed a very low level of activity in the synthesis of specific antibody and γ-globulin (Askonas and White, 1956). It seems, therefore, that no significant production of antibody takes place in the intact thymus. This could be because of (1) the lack of a suitable phagocytic mechanism to segregate antigen in the gland; (2) the absence of immunologically competent cells; (3) the inability of immunologically competent thymus cells to function *in situ,* and/or (4) the presence of a barrier preventing the entry of antigens into the thymus.

The presence of large numbers of reticulo-endothelial cells in the normal thymus and the rapid phagocytosis of pycnotic thymocytes following the injection of adrenotropic hormone (A. White and Dougherty, 1946) or exposure to ionizing radiation (P. F. Harris, 1958) indicates that a phagocytic mechanism can operate in thymus tissue. After direct injection of antigen into the thymus, the histological changes are those usually associated with antibody production in other lymphoid tissues (Marshall and White, 1961). Under these conditions, specific antibody-containing plasma cells were shown to be present in the thymus by the fluorescent antibody "sandwich" technique of Coons *et al.* (1955). This is in contrast to the complete lack of reaction following intravenous, intraperitoneal, or subcutaneous injection of antigen. The failure of the thymus to react to circulating antigens suggests the existence of a barrier between the blood stream and thymus parenchyma that prevents entry of antigen into the thymus. Moreover, the reaction of the thymus to antigen introduced directly into the gland would appear to indicate that some cells in the thymus are capable of undertaking an immunological response. Further evidence for this is given in the following section.

B. Immunological Competence of Thymus Cells

Most studies suggest that, once outside the environment of the intact thymus, thymocytes are capable of taking part in immunological reactions. Stoner and Hale (1955) claimed to have demonstrated the production of tetanus antitoxin by thymus transplants within the anterior chamber of the eye of irradiated recipients. For reasons which are difficult to understand this production only occurred following subcutaneous and intraperitoneal stimulation of the donor, but did not occur following intravenous stimulation. The transplants exhibited a "recall antibody response" when the recipient mice were given an intravenous injection of

the specific antigen. The histological reactions associated with antibody formation were observed in these transplants (Williams *et al.*, 1958). In cell transfer studies, thymus cells were found to be capable of transferring antibody response to bovine serum albumin but were much inferior in this respect to lymph node cells or peritoneal exudate cells (Dixon *et al.*, 1957; see review by Cochrane and Dixon, 1962).

The competence of thymus cells to take part in homograft immunity reactions has also been demonstrated. Thymus cells were capable of producing graft-versus-host reactions such as runt disease in newborn mice (Billingham and Brent, 1959; Miller, 1960; Billingham and Silvers, 1961) and wasting disease in sublethally irradiated adult F_1 hybrid mice, although much less effectively than similar dosages of spleen cells (Cole and Ellis, 1958; Kaplan and Rosston, 1959). In chicks (Terasaki, 1959) and in rats (Billingham *et al.*, 1962), however, thymus cells were unable to cause such reactions. Thymocytes could confer adoptive immunity to transplantation antigens as shown by the prevention by syngeneic thymus cells of the therapeutic action of rat bone marrow in lethally irradiated mice (van Bekkum and Vos, 1957; Congdon and Duda, 1961). In the experiments of Congdon and Duda (1961) there was striking histological evidence, particularly in the spleen of the host animal, that pyroninophilic and plasma cells appeared in large numbers before the rat marrow graft was rejected. In the absence of cell markers, however, one cannot establish the relationship between these cells and the injected thymocytes. In the transfusion experiments of Fichtelius (1960), thymus cells were traced to the red pulp of the spleen, particularly perifollicularly, at the site where plasma cell proliferation is known to take place during antibody formation. This led to the suggestion that the thymus and spleen may together constitute a large type of central lymph node—the thymus producing lymphocytes and the spleen forming antibodies with their aid.

It may be concluded from the preceding observations that antibody formation does not take place in the intact thymus simply because antigen is unable to get there and that immunologically competent cells in the thymus have to migrate out of the organ before they can take part in immunological reactions. It may be, as Congdon and Duda (1961) have suggested, that stress reactions liberate thymocytes for dissemination to other tissues where they can supplement local immunologically competent cells.

IV. Longevity and Immunological Status of Thymectomized Animals

By removing an organ surgically it is possible indirectly to demonstrate whatever role it may play in immune reactions. Several investigators have reported that splenectomy is associated with a reduced ability to respond to antigenic stimuli (Wolfe *et al.*, 1950), the magnitude of the response depending on the amount, route, and frequency of administration of antigen (Rowley, 1950). The survival of allogeneic skin grafts was not significantly altered by splenectomy (Krohn and Zuckerman, 1954), or by removal of regional lymph nodes in mice (Billingham *et al.*, 1954). Studies of the response to standard antigens of animals thymectomized as adults have revealed either slight or no impairment of antibody production. Since the thymus is the major lymphoid organ in perinatal life, at a time when the response to antigen can easily be modified (Medawar, 1961), it is possible that thymectomy at an early age might be associated with some detectable effect on immunological response. Studies of the longevity and immune status of thymectomized animals will now be reviewed.

A. Longevity

Thymectomy after 3 weeks of age has been performed in many mouse strains and interstrain hybrids by workers interested in the genesis of leukemia. These studies have revealed no differences in weight curves, breeding behavior, or susceptibility to common laboratory infections in thymectomized and control groups of mice (Miller, 1961b). There were no significant differences in longevity, except in high-leukemia strains, in which life was prolonged as a result of thymectomy, preventing early death from leukemia (Furth, 1946).

Thymectomy of infant mice, however, was associated with significant mortality between 1 and 4 months of age (Miller, 1961a,c, 1962b,c,d). The appearance and body weights of mice thymectomized and sham-thymectomized in the neonatal period were very similar until 1 to 3 months of age when the majority of the mice in the thymectomized group developed a syndrome characterized by wasting, lethargy, ruffled fur, hunched posture, diarrhea, and death within 1 to 3 weeks. The incidence of death from this syndrome following thymectomy at different ages in early life and for different strains of mice is shown in Fig. 7 and the age at death for one particular F_1 hybrid thymectomized at birth, in Fig. 8. The characteristic anatomical feature of mice with wasting disease was marked involution of the lymphoid tissue as described in Section II. Similar findings have been reported by Parrott (1962a, b) in mice thymectomized within 24 or 36 hours after birth. It was of interest that T0 mice (an

outbred strain) thymectomized under the same conditions at or near birth showed a negligible incidence of wasting disease up to 24 weeks of observation.

The removal of the bursa of Fabricius from 1-week-old chickens was not associated with significant shortening of life. Prenatal inhibition of bursal differentiation by hormonal means caused some early postnatal mortality from cloacal constipation. Those hormone-treated birds which

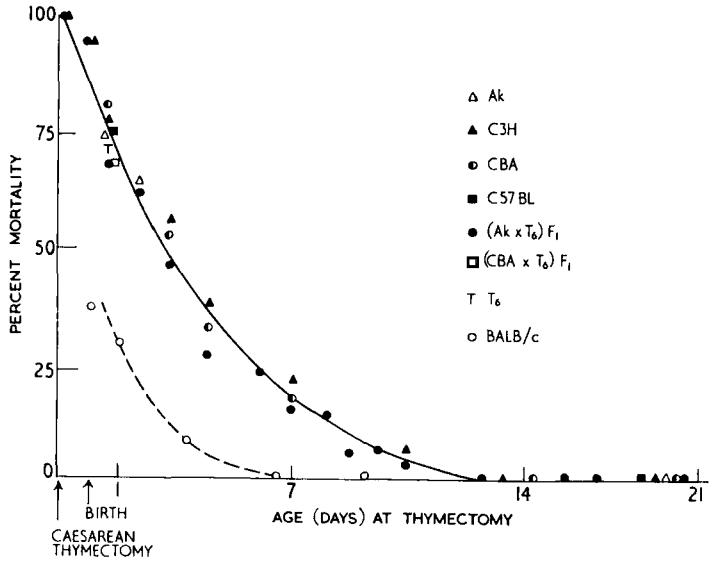

FIG. 7. Mortality from wasting disease in thymectomized mice.

survived this constipation developed a condition characterized clinically by diarrhea, poor growth, and poor survival, and anatomically by deficiency of the lymphoid system (Mueller *et al.*, 1960).

There are striking resemblances between the wasting syndrome associated with neonatal thymectomy (or bursectomy) and the diseases occurring during graft-versus-host reactions, such as runt disease in neonatal mice, homologous disease in F_1 hybrid mice, and secondary disease in radiation chimeras. The clinical and pathological features of these conditions are very similar. In many cases of graft-versus-host diseases, there is a complete absence of graft lymphocytes as well as host lymphocytes (Billingham, 1958; Billingham and Brent, 1959; Gorer and Boyse, 1959; Nisbet and Heslop, 1962). The close association between wasting disease and lymphoid atrophy in neonatally thymectomized mice in which antihost immune responses are not operative (unless one postu-

lates an autoimmune reaction for which there is no evidence) would suggest that the primary factor in the pathogenesis of all such syndromes is the inadequacy of the lymphoid system (see also Billingham, 1959). In graft-versus-host disease this would be superimposed upon or consequent

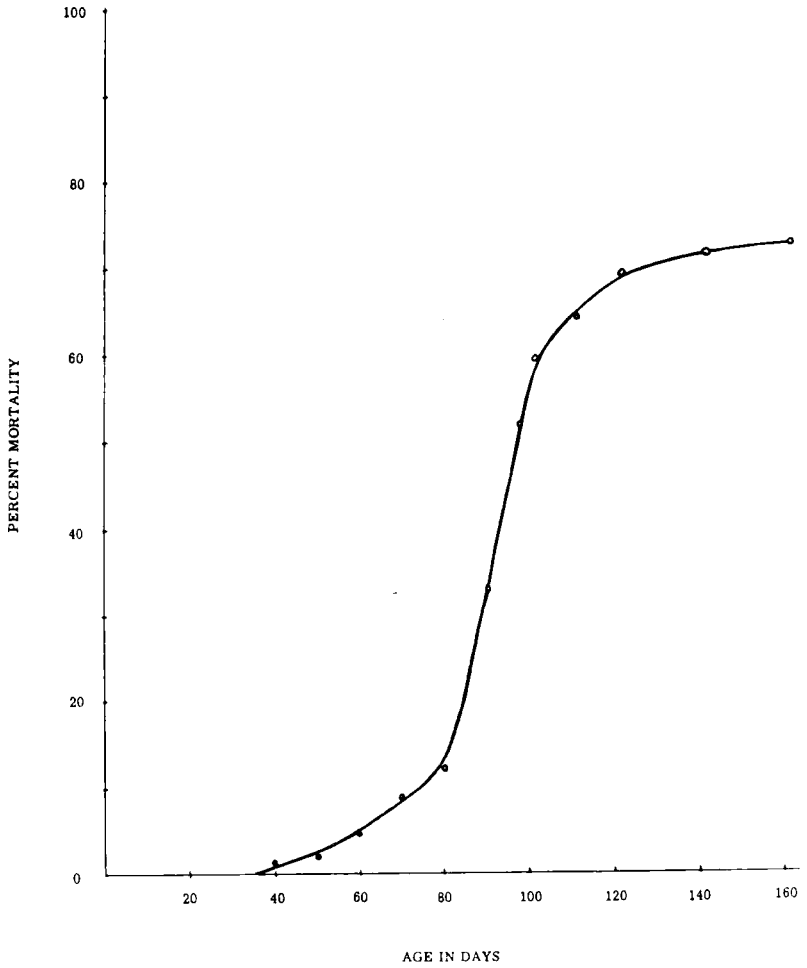

FIG. 8. Cumulative percentage mortality from wasting disease in 122 (Ak × T6)F_1 mice thymectomized at birth.

to tissue damage resulting from the immune action of the grafted cells. Such a hypothesis obtains support from recent work in which "secondary disease" has been observed in lethally irradiated mice restored with syngeneic hemopoietic elements obtained from fetal livers (Barnes *et al.*,

1962). Further support for the hypothesis would be obtained if it could be shown that the symptoms seen in graft-versus-host conditions could be prevented or alleviated by an adequate transplant of active lymphopoietic tissue such as neonatal thymus. The wasting disease associated with neonatal thymectomy does not develop in thymus-grafted mice (Miller, 1962c,d).

B. Humoral Immunity

Hammar (1938) could find no real difference in titers of antibody to *Salmonella paratyphi* B antigen injected intravenously in thymectomized and in nonthymectomized animals. T. N. Harris *et al.* (1948) found no significant differences between thymectomized and sham-thymectomized

TABLE III

Effect of Neonatal Thymectomy on the Formation of Influenza Virus (PR8) Hemagglutinins in (Ak × T6)F_1 Mice[a]

Operation	Titer of influenza virus hemagglutinins in individual mice[b]
None	320, 320, 280, 240, 200, 200, 200, 160, 160, 160
	160, 160, 160, 120, 120, 100, 80, 80, 80, 70
Sham-thymectomy	240, 160, 160, 120, 120, 120, 80
Thymectomy	160, 120, 50, 10, < 10, < 10, < 10, < 10, < 10, < 10

[a] Sera were kindly titrated by Dr. Alick Isaacs.

[b] Antihemagglutination titers (dilution of 0.25 ml. serum causing 50% inhibition of agglutination) measured against eight agglutinating doses of PR8 virus.

rabbits injected subcutaneously with sheep erythrocytes or *Shigella paradysenteriae*. MacLean *et al.* (1957) reported no differences between thymectomized and sham-thymectomized rabbits given intravenous injections of bovine serum albumin, and they interpreted their data as evidence that the thymus does not participate in the control of the immune response. Fichtelius *et al.* (1961) performed thymectomy and sham-thymectomy in guinea pigs immediately after intravenous injection of *Salmonella typhi* H antigen and reported somewhat lower antibody titers in the thymectomized group. There was no difference between the two groups after a secondary challenge with the same antigen. All these studies were performed on animals thymectomized during adult life.

The removal of the bursa of Fabricius from chickens at 10 weeks of age did not impair the ability of these birds to produce precipitins against bovine serum albumin. However, chicks bursectomized at 1 week had greatly reduced antibody responsiveness at 6, 12, and 22 weeks of age, and prenatal inhibition of bursal differentiation by testosterone treatment

caused an even greater interference with antibody production (Mueller *et al.*, 1960).

Thymectomy of rabbits in the first week of life was followed by subsequent failure to produce antibody to bovine serum albumin (Archer and Pierce, 1961). Thymectomy of the neonatal mouse was associated with serious impairment of the immune response of the mature animal to *Salmonella typhi* H antigen (Miller, 1961d, 1962d), to sheep erythrocytes (Miller and Davies, 1963), and to killed influenza virus (Table III) (Miller and Isaacs, 1963). In about 50% of rats thymectomized at birth, there was a depression of antibody production to bovine serum albumin. These findings could be largely duplicated in rats thymectomized as late as 3 weeks after birth (Arnason *et al.*, 1962).

C. Homograft Immunity

Thymectomy of some strains of mice at birth was associated with severe impairment of the ability to reject skin and tumor grafts not only from different strains but also from rat donors (Table IV) (Miller, 1961a,d, 1962a,b,c,d). All skin grafts grew luxuriant tufts of hair and remained intact in the majority of the mice until death which generally occurred within 4 months from wasting disease. Many of the grafts in those mice surviving beyond 4 months of age gradually diminished in size and the hair became progressively thinner until it eventually disappeared. There were no signs such as edema, reddening, thickening, bleeding, or scab formation. Such mice showed no evidence of active immunity to a second set graft of the same skin (Miller, 1962d).

Thymectomy of the mouse at 1 week of age was still associated with some impairment of the immune response to allogeneic skin grafts particularly when donor and hosts did not differ at the H-2 locus (Miller, 1962c,d). Female C57BL mice thymectomized at 2 weeks of age developed no immune response to syngeneic male skin grafts but could reject allogeneic skin grafts (Miller, 1962c,d). Thymectomy after 3 weeks of age was no longer associated with any significant impairment of the immune response to allogeneic skin grafts. Sham-thymectomy or splenectomy at birth had no effect on homograft immunity (Miller, 1961a, 1962c,d) nor did subtotal thymectomy leaving as little as one-quarter of one thymic lobe *in situ* (Miller, unpublished data, 1961).

Suppression of the skin-homograft rejection mechanism was also evident in rats thymectomized at birth (Arnason and Janković, 1962; Arnason *et al.*, 1962). Somewhat similar findings were recently reported in mice by Martinez *et al.* (1962) and by Trentin (1962).

TABLE IV

SURVIVAL OF ALLOGENEIC AND HETEROSPECIFIC SKIN GRAFTS ON THYMECTOMIZED, SHAM-THYMECTOMIZED, AND SPLENECTOMIZED MICE

Strain	Treatment	Donor skin	Number of mice in group	Number of mice showing skin graft survival for:		
				< 25 days	25–50 days	> 50 days
C3H	Thymectomy at birth	Ak	7	0	1	6
		BALB/c	5	0	1	4
		Rat	4	2	2	0
	Sham-thymectomy at birth	Ak	8	8	0	0
	Thymectomy at 1 week	Ak	14	1	8	5
		BALB/c	10	6	3	1
		Rat	5	5	0	0
	Splenectomy at birth	Ak	5	5	0	0
(Ak × T6)F_1	Thymectomy at birth	C3H	25	2	5	18
		C57BL	15	3	3	9
		BALB/c	15	3	3	9
		DBA/2	7	1	2	4
		Rat	16	9	4	3
	Sham-thymectomy at birth	C3H	8	8	0	0
		C57BL	5	5	0	0
		BALB/c	5	5	0	0
		DBA/2	5	5	0	0
		Rat	6	6	0	0
	Thymectomy at 3–4 weeks	C3H	14	14	0	0
	Splenectomy at birth	C3H	9	9	0	0
BALB/c	Thymectomy at birth	C3H	9	7	2	0
		C57BL	9	7	2	0
		DBA/2	9	3	3	3
	Sham-thymectomy at birth	C3H	12	12	0	0
		C57BL	8	8	0	0
		DBA/2	8	8	0	0
♀ C57BL	Thymectomy at 2 weeks	♂ C57BL	9	0	0	9
		♀ Ak	5	5	0	0
	Sham-thymectomy at birth	♂ C57BL	7	0	7	0
		♀ Ak	5	5	0	0

D. Delayed Hypersensitivity

There was partial to complete suppression of several types of delayed reaction in neonatally thymectomized rats, apparently varying with the intensity of the antigenic stimulus. There was a marked decrease in delayed skin reaction to bovine serum albumin and in tuberculin sensitivity (Table V) and a loss of the ability to develop allergic encephalomyelitis

TABLE V

Decrease of Tuberculin Sensitization in Rats Thymectomized at Birth[a]

Group	Number of rats	Day of test[b]	Number of rats with reaction at 48 hours		
			0–4 mm.[c]	5–9 mm.	10–14 mm.
Thymectomized	13	9–11	6	4	3
		20–21	6	7	0
Control	15	9–11	1	4	10
		20–21	2	9	4

[a] By courtesy of Dr. Byron Waksman.
[b] With original tuberculin 1:10, 0.1 ml. intradermally.
[c] Diameter of reaction area.

(Arnason and Janković, 1962; Arnason *et al.*, 1962). About 50% of the animals failed to develop an Arthus reaction, but the development of adjuvant arthritis was not affected by neonatal thymectomy. The depression of delayed sensitization in individual animals was correlated with diminution in their lymphocyte population.

E. Cell Transfer Studies

The effect of injecting lymphoid cells into neonatally thymectomized mice of the same or of a different strain, at birth or during adult life, has been investigated. The available results are summarized as follows.

1. Syngeneic thymus cells from 1-day-old donor mice given intravenously to newborn mice immediately after thymectomy did not prevent runting, lymphoid atrophy, or immunological failure in such mice (Miller, 1962c).

2. Syngeneic lymphoid cells from 8-week-old mice presensitized against Ak skin, on injection into 10-week-old neonatally thymectomized C3H mice which were carrying healthy Ak skin grafts for over 1 month, conferred adoptive immunity. The Ak skin was rejected within 12 days and the mice showed evidence of immunity to a second-set graft of Ak skin (Miller, 1962b,c,d). They did not develop wasting disease and their lymphoid tissues appeared normal when sacrificed 12 months later.

3. Syngeneic lymphoid cells from 8-week-old nonsensitized mice, on

injection into 10-week-old neonatally thymectomized C3H mice which were carrying healthy Ak skin grafts for more than 1 month, gave equivocal results. A few mice slowly lost their skin graft after several months, but the majority died from wasting disease at the usual age with their skin graft intact (Miller, unpublished data, 1961).

4. Allogeneic lymphoid cells from 2-month-old mice caused a severe graft-versus-host reaction when injected intravenously into newborn mice immediately after thymectomy (Miller, 1962d). In contrast to what was found in non-thymectomized mice (Davies and Doak, 1960; Howard *et al.*, 1961), a high proportion of donor-type cells was identified in the spleens of thymectomized animals. The ability of adult lymphoid cells to multiply in the spleen of neonatally thymectomized mice can be interpreted in several ways. It could be used as an argument against a humoral theory of thymus action. On the other hand, one might postulate that lymphoid cells from adult animals are not dependent for their proliferation on a humoral thymic factor in contrast to lymphoid cells from newborn animals.

The effect of injecting lymphoid cells from 2-month-old mice which had been thymectomized at birth into nonthymectomized newborn mice of a different strain is shown in Table VI. The absence of any significant graft-versus-host activity in these mice may be owing to the fact that the spleens of neonatally thymectomized adult mice contain a smaller proportion of immunologically competent cells than do the spleens of normal adult mice. This is in accordance with the histological appearance of such spleens and is consistent with the observation that C57BL marrow cells are relatively less harmful than spleen cells when administered to baby A-strain mice (Billingham, 1958). If this is true, any tissue damage caused by a graft-versus-host immune reaction in radiation chimeras would not be expected to occur if hemopoietic tissue from neonatally thymectomized mice was used. On the other hand, "secondary disease" might still occur in such cases owing to inadequate repopulation of the lymphoid tissue. This repopulation, however, might possibly be effected, as suggested in the foregoing, by a simultaneous graft of thymus tissue.

F. Immune Status Following Thymus Grafting

Mice thymectomized at birth and grafted subcutaneously within 3 weeks with whole intact thymuses from newborn donors did not develop wasting disease, had a normal life span, a normal peripheral blood picture, and normal lymphoid tissues and could reject foreign skin grafts (Miller, 1961a, 1962b,c,d).

When neonatally thymectomized F_1 hybrid mice were grafted with

TABLE VI

EFFECT OF INTRAVENOUS INOCULATION OF NEWBORN MICE WITH SIX MILLION ALLOGENEIC SPLEEN CELLS FROM NEONATALLY THYMECTOMIZED (TX) OR SHAM-THYMECTOMIZED (STX) ADULT MICE

Strain combination	Incidence of runt disease	Mice sacrificed for spleen assay				Mortality before weaning in mice allowed to survive
		Number	Age (days) at sacrifice	Body weight (gm. ± S.E.)	Spleen (mg.)/10 gm. body wt. (± S.E.)	
STx C57BL to CBA	15/15	5	10	3.7 ± 0.09	91.2 ± 6.4	10/10
Tx C57BL to CBA	0/10	5	10	5.2 ± 0.22	51.8 ± 5.1	1/5
STx C57BL to (Ak × T6)F_1	12/12	5	14	5.1 ± 0.23	115.4 ± 9.7	7/7
Tx C57BL to (Ak × T6)F_1	0/11	5	14	6.8 ± 0.09	55.4 ± 0.5	0/6

TABLE VII

SURVIVAL OF PARENTAL AND ALLOGENEIC SKIN GRAFTS ON NEONATALLY THYMECTOMIZED (AK × T6)F_1 MICE GRAFTED WITH NEWBORN THYMUSES

Treatment	Number of mouse	Survival of first-set skin grafts (days): Ak	C3H	C57BL	BALB/c	DBA/2	Condition of second-set skin grafts at 5 days: C3H	C57BL	BALB/c
Thymectomy at birth; newborn Ak thymus graft at 1 week	1	—	23	22	18	17	Ischemic; necrosis	—	—
	2	—	23	13	16	13	Ischemic; necrosis	Ischemic; necrosis	—
	3	—	29	27	20	18	Ischemic; necrosis	Ischemic; necrosis	—
	4	—	38	28	21	19	Ischemic; necrosis	Ischemic; necrosis	—
	5	—	29	24	22	22	Ischemic; necrosis	Ischemic; necrosis	—
	6	—	33	20	17	17	Ischemic; necrosis	Ischemic; necrosis	—
Thymectomy at birth, newborn C3H thymus graft at 1 week	7	> 150	> 150	21	14	—	Intact	—	Ischemic; necrosis
	8	> 150	> 150	25	16	—	Intact	—	Ischemic; necrosis
	9	> 150	> 150	—	20	—	Intact	—	Ischemic; necrosis
Thymectomy at birth; newborn C57BL thymus graft at 1 week	10	> 100	18	87	14	—	—	—	—
	11	> 100	23	> 100	14	—	—	—	—
	12	> 100	25	75	13	—	—	—	—
	13	> 100	45	63	19	—	—	—	—

parental thymus they rejected foreign skin grafts and showed evidence of immunity to a second set graft of the same skin (Miller, 1962c,d). When allogeneic thymus was grafted, neither parental skin nor skin from the donor of the thymus graft were rejected, but third-party skin was and the mice showed evidence of immunity to a second set graft of the third-party skin (Miller, 1962c). These results are summarized in Table VII.

An analysis was made of the chromosome constitution of cells in metaphase present in the spleens of these mice and is presented in Table

TABLE VIII

CYTOLOGICAL ANALYSIS OF THE SPLEENS OF (AK × T6)F_1 MICE THYMECTOMIZED AT BIRTH AND GRAFTED WITH NEWBORN THYMUSES

Treatment	Age at sacrifice (weeks)	Number of host cells found	Number of donor cells found	Host dividing cells (%)
Thymectomy at birth; newborn Ak thymus graft at 1 week	14	48	1	98
	16	38	7	85
	16	31	6	84
	16	29	7	81
	17	29	7	81
	17	41	2	95
	18	35	7	83
Thymectomy at birth; newborn C3H thymus graft at 1 week	17	30	5	86
	17	47	3	94
	17	27	8	77
	18	34	6	85

VIII. In 14- to 18-week-old mice, only 2–20% of the dividing cells in the spleen were of donor origin and hence derived from the thymus graft.

The implications of these results will be discussed in Section VI.

V. Systemic Disorders Associated with Primary Thymic Disease in Man

Abnormalities of the thymus in man may be associated with a large number of clinical syndromes (Table IX). These associations present a problem of remarkable interest both from the possible interpretations of the nature of such conditions and also from their significance in relation to the function of the normal thymus. The first of these associations was recognized by Weigert in 1901 in a classic description of the relationship of myasthenia gravis to thymic tumors. Since that time an increasing number of systemic disorders have been similarly recognized as associated with thymic neoplasms, and myasthenia gravis has been found to be associated with other thymic lesions in addition to tumors of the

gland. Few attempts have been made to interpret the nature of this relationship and it has, in fact, been passed over or dismissed as of a noncausal nature by some authors. It is considered here, however, that a common pathology probably underlies the associations hitherto described and is related to the immunological functions of the thymus gland in the normal body.

TABLE IX
THYMIC SYNDROMES IN MAN

Lesion of thymus	Associated systemic disorders
Hyperplasia with germinal center formation	Myasthenia gravis; rarely, aplastic anemia or agammaglobulinemia
Tumors	Myasthenia gravis
Lymphoepithelioma	Agammaglobulinemia
Spindle-celled thymoma	Hyperglobulinemia
	Erythroblastic aplasia
	Leucopenia
(Germinal center formation may	Thrombocytopenia
also be seen in association with	Acute hemolytic anemia (antihuman globulin
tumors.)	test positive)
	Myocarditis and myositis
	Myocarditis with L.E .cells
	Thrombotic thrombocytopenic purpura

A. PATHOLOGY OF THE THYMUS GLAND ASSOCIATED WITH SYSTEMIC DISORDERS

Two types of thymic lesions may be recognized: (1) thymic "hyperplasia" associated with myasthenia gravis and (2) thymic tumors.

1. Thymic "Hyperplasia" Associated with Myasthenia Gravis

This condition has been well described by Ringertz (1951) and Castleman (1955) and accompanies between 60 and 70% of cases of myasthenia gravis. A true hyperplasia of the gland is frequently absent as normal weight limits may not be exceeded. Histologically however, the condition is recognized by the presence of varying numbers of germinal centers, apparently identical with those commonly seen in the lymph nodes or spleen, scattered throughout the medulla of the gland. Such germinal centers may be in varying developmental phases, either as active centers with numerous mitoses containing numerous immature cells of lymphoblast morphology (Fig. 9) or as smaller, apparently atrophic structures with no mitoses and few immature lymphoid cells (Fig. 10).

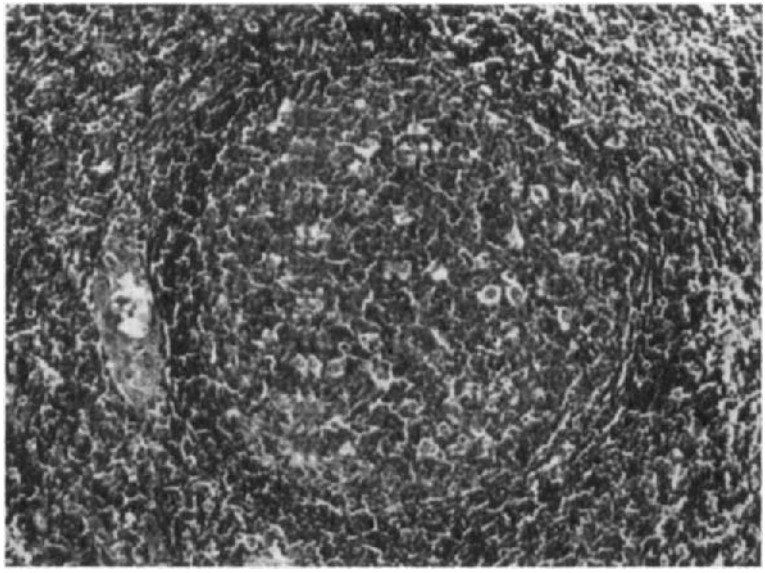

FIG. 9. Thymus hyperplasia in myasthenia gravis. Active germinal center in medulla of thymus containing numerous cells of lymphoblast type. Magnification: ×130.

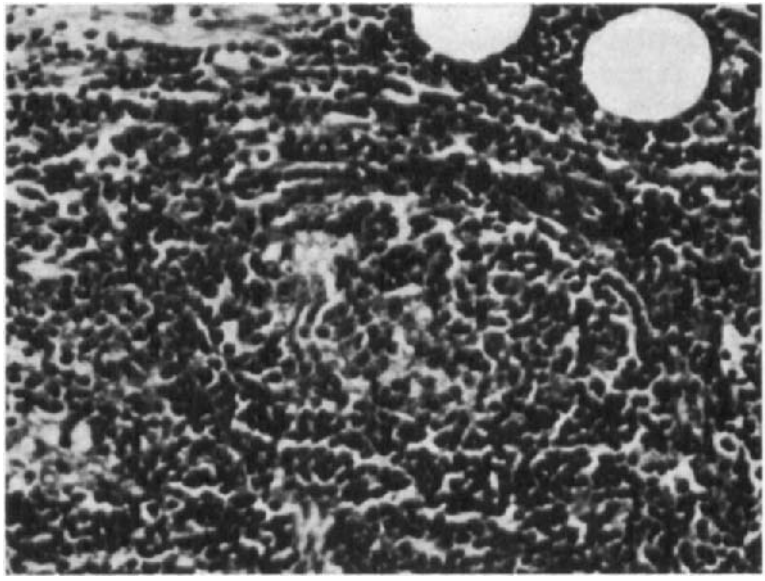

FIG. 10. Thymus hyperplasia in myasthenia gravis. Atrophic germinal center containing few immature lymphoid cells. Magnification: ×250.

The latter type of center closely resembles the "reaction centers" of Hellman commonly seen in lymph nodes. In addition to the presence of germinal centers, a variable number of cells of mature or immature plasma cell morphology may be scattered in the connective tissue surrounding the main vessels penetrating the gland, but are rarely seen in the medulla or cortex of the gland itself. No change is usually seen in the cortex of the gland which may, in fact, be reduced to a thin layer over a medulla expanded by germinal centers.

The germinal center reaction seen in the myasthenic thymus appears identical histologically with that seen in the spleen of an experimental animal following intravenous antigen injections and in the lymph nodes draining the site of locally deposited antigen. Lymphoid nodules with germinal centers can also occur at the local tissue site of antigen, and in Hashimoto's disease the presence of germinal centers in the thyroid presumably represents the immunological response to local escape of the thyroglobulin and other segregated thyroid antigens. It is of interest that Castleman (1955) drew a comparison on histological grounds between lymphadenoid goiter (Hashimoto's disease) and the thymus in myasthenia. The cells which are produced in the germinal centers of lymph nodes were shown to contain γ-globulin (Ortega and Mellors, 1957) in the case of nodes draining the sites of human carcinomata, and specific antibody (White, 1960) in the case of rabbit nodes draining the local sites of injection of ovalbumin and diphtheria toxoid. Also, in the case of the lymph nodes of cases of rheumatoid arthritis, which show a striking development of lymphoid nodules containing germinal centers, the center cells were shown to be the site of rheumatoid factor (19 S and 7 S γ-globulin) (Mellors *et al.*, 1959). In the thymus, however, interpretation is difficult owing to the complete lack of reactivity of the gland to antigens circulating in the blood stream. In order to claim that the germinal centers in the myasthenic thymus represent an immunohistological reaction connected with formation of antibody by the gland, it is necessary to show both that the normal thymus can produce similar germinal centers under antigenic stimulation and that the human thymus in myasthenia is forming antibody or γ-globulin. Marshall and White (1961) have shown that direct injection of antigens into the exposed thymus produced histological changes of antibody production including the local accumulation of plasma cells (Fig. 11) and the formation of germinal centers (Fig. 12), in contrast to the complete failure of reaction observed by others when antigens were injected by parenteral routes. White and Marshall (1962) in a survey of six thymus glands obtained at thymectomy for the treatment of severe myasthenia used fluo-

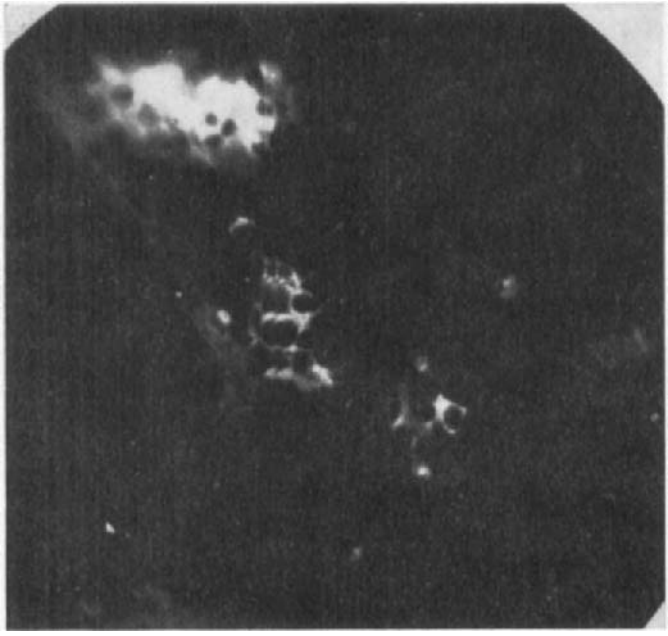

FIG. 11. Fluorescence micrograph. Antibody-containing cells in guinea pig thymus at margin of deposit of aluminum phosphate-precipitated diphtheria toxoid. Magnification: ×456.

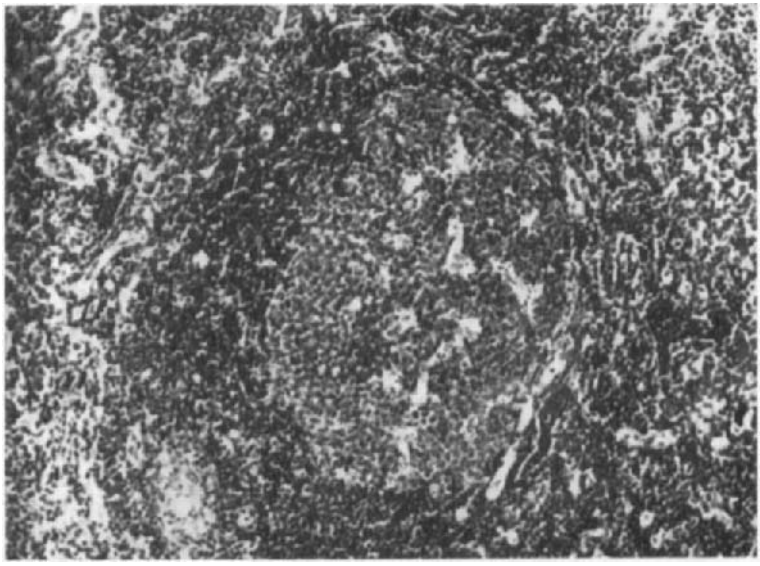

FIG. 12. Germinal center in medulla of guinea pig thymus following direct injection of typhoid-paratyphoid B vaccine. Magnification: ×150.

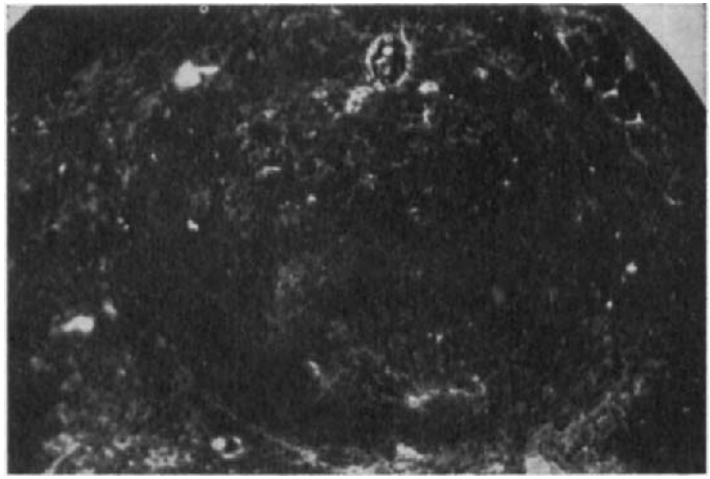

FIG. 13. Fluorescence micrograph. Germinal center in medulla of thymus obtained at thymectomy for myasthenia gravis (female, 18 years). The cells in the center show a faint specific fluorescence denoting a content of γ-globulin. Magnification: ×190.

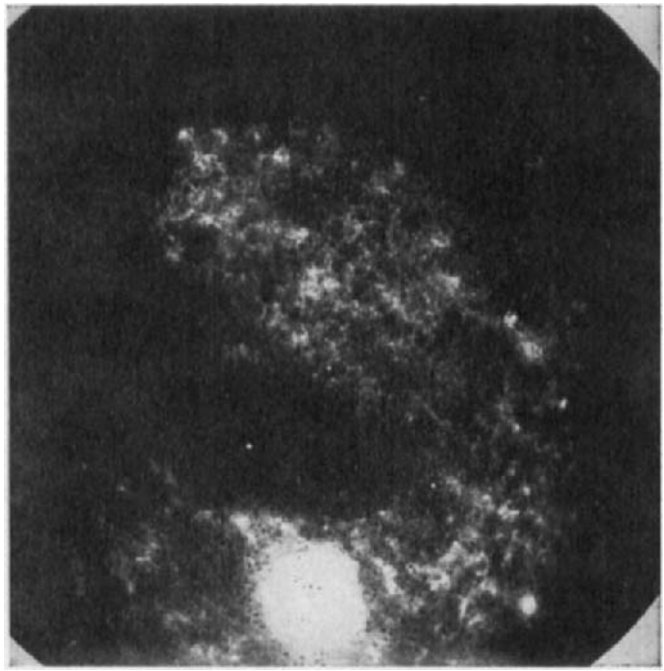

FIG. 14. Fluorescence micrograph. Germinal center in medulla of thymus obtained at thymectomy for myasthenia gravis (female, 20 years) showing a content of γ-globulin in the cells of the center. The brilliant fluorescent circle at bottom is a Hassall's corpuscle, showing a partially specific fluorescence for γ-globulin in its cystic center. Magnification: ×141.

rescein-conjugated antibody to human 7 S γ-globulin in order to detect and localize γ-globulin in frozen tissue sections. All specimens showed simple hyperplasia of the thymus with preponderance of medullary elements, including numerous lymphoid nodules with germinal centers. Some of the latter (Figs. 13 and 14) included γ-globulin containing cells with the morphology, in conventionally stained sections, of large and medium lymphocytes. No mature plasma cells were present. Other germi-

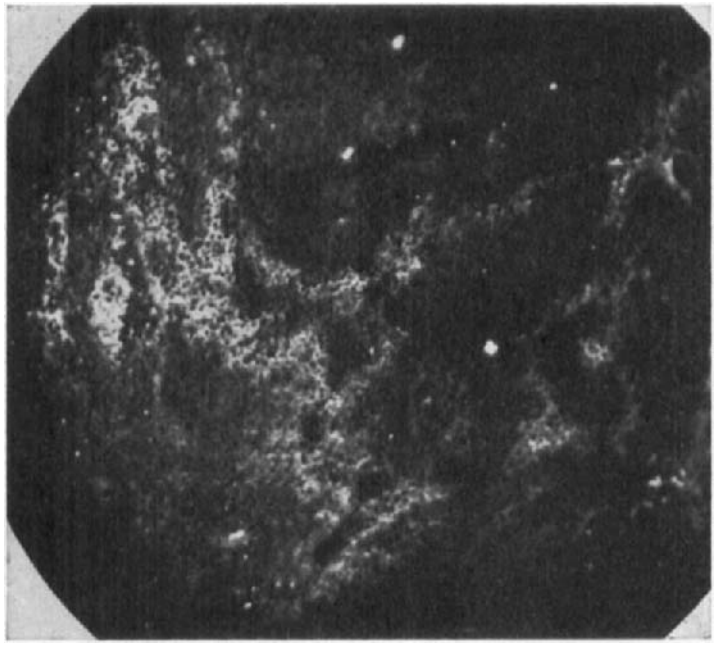

FIG. 15. Fluorescence micrograph. Low-power view of thymus (female, 18 years) obtained at thymectomy for myasthenia gravis. Strands of γ-globulin-containing cells, mainly accompanying the blood vessels and trabeculae, are present in the medulla. Magnification: ×141.

nal centers were relatively acellular, partly occupied by eosinophilic hyaline material, and empty of γ-globulin containing cells. A striking feature of the low-power view was the strands of lymphocytes and immature plasma cells, many containing γ-globulin in their cytoplasm (Fig. 15). Such strands appeared to accompany the connective tissue septae and blood vessels penetrating into the gland. They were narrow (30–80 μ) at the periphery, but, as they passed through the cortex to enter the medulla, they fanned out into broad leashes of cells. The mature-type plasma cell (Marshalkó type) was relatively uncommon and

Russell body formation was not present. Occasionally the medulla included dense nodular collections (Fig. 16) of γ-globulin-containing cells (immature and mature plasma cells). Small extracellular deposits of γ-globulin also appeared to be present in the midst of such cells. In normal human thymic tissue obtained as controls, γ-globulin-containing cells were observed in the immediate neighborhood of blood vessels but were not scattered in the substance of the gland.

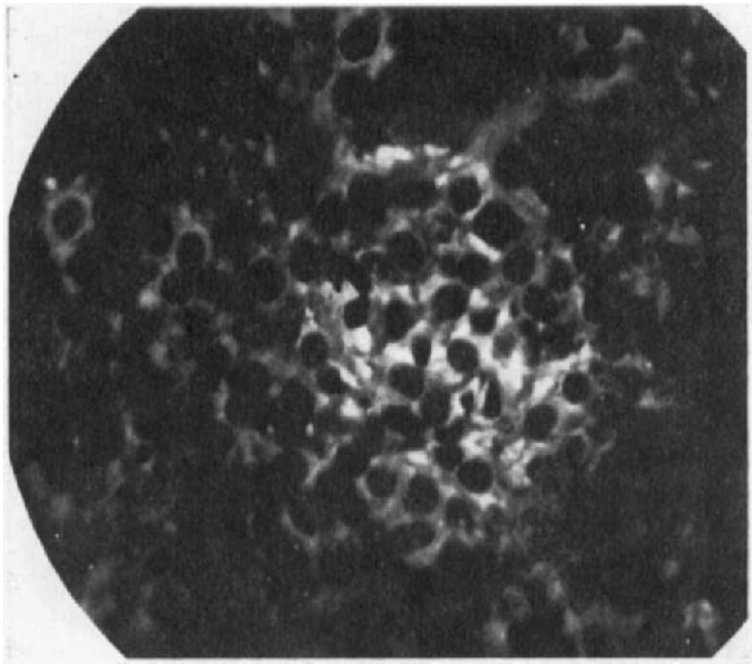

FIG. 16. Fluorescence micrograph. Area of thymic medulla of same patient as Fig. 15, showing an area of γ-globulin-containing cells, between which are extra cellular cytoplasmic fragments or deposited γ-globulin. Magnification: ×660.

Some of the Hassall's corpuscles of the human thymus gave a bright fluorescent reaction with fluorescein-conjugated antibody to human γ-globulin. Such fluorescence is partly produced by a gray natural fluorescence and some nonspecific reaction with the conjugated globulin, but in the main it is produced by the presence of γ-globulin, which occurs in relation to the laminated hyaline or degenerate central zone of a solid corpuscle, or within the lumen of a cystic one, but never inside the peripheral epithelial cells. Such γ-globulin-containing corpuscles are not, however, restricted to cases of myasthenia but occur in normal glands

(Gitlin *et al.*, 1953) and in the healthy normal thymuses of guinea pigs and rabbits.

The foregoing facts provide substantial evidence that the thymus in myasthenia gravis may have embarked on an immunological response against antigens present within it or have become the seat of an autochthonous reaction of a mutant clone or clones of cells. From the absence of any cellular immune response outside the thymus, it may be deduced that a circulating antigen cannot be responsible even if it succeeded in crossing the blood–thymus barrier.

The development of an autochthonous cellular response or "forbidden clone" may be envisaged in two ways: first, by mutation to an autonomous cell and, second, by a weakness of the immunological homeostatic mechanism (Burnet, 1959a) which normally operates to eliminate mutant cells that are reactive with self-components. In the case of the human diseases, lupus erythematosus (L.E.) and acute, acquired hemolytic anemia, there is evidence suggesting that the second alternative may be at work and that a model for this mechanism exists in the NZB/BL mouse strain of Bielschowsky *et al.* (1959; see also Holmes *et al.*, 1961). Moreover it has recently been claimed (Helyer and Howie, 1961) that some F_1 and F_2 hybrids of strain NZB/BL and another related and apparently healthy strain can develop the L.E. phenomenon in their blood cells, and lupus-like glomerular lesions in the kidney.

Reports of a clinical association between myasthenia gravis and systemic L.E. have been published by Harvey *et al.* (1954), Denny and Rose (1961), and White and Marshall (1962). The latter authors surveyed sixteen cases of myasthenia gravis. In one case, clinically manifest systemic lupus was evidenced by hyperglobulinemia, arthritis, pericarditis, a positive Latex agglutination test, and a positive direct L.E. phenomenon. In other cases, cutaneous L.E. and rheumatoid arthritis accompanied the myasthenia gravis. Moreover, tests of the patients' serum showed the presence of antinuclear factors by the fluorescent antibody method in six out of sixteen cases of myasthenia gravis.

2. *Systemic Disorders Associated with Thymic Tumors*

As given in Table IX, systemic disorders, apart from myasthenia gravis, fall into 3 groups: (a) those of serum protein formation, agammaglobulinemia or, rarely, hyperglobulinemia; (b) blood dyscrasias (erythroblastic aplasia, aplastic anemia, and hemolytic anemia); (c) myositis and myocarditis. These groups are not exclusive and conditions of more than one group may occur in the same patient.

a. Disorders of Serum Protein Formation. Agammaglobulinemia in

association with a thymic tumor was first reported by Good (1954); additional cases have been given by Martin *et al.* (1956), Ramos (1956), Lambie *et al.* (1957), and Soutter *et al.* (1957). These cases were complicated by erythroblastic aplasia in three instances and in two by previous autoimmune hemolytic anemia and hypergammaglobulinemia. Two cases, in which agammaglobulinemia developed after removal of the thymoma, resembled the reported examples of the development of myasthenia gravis after removal of a thymic tumor.

As in other reported cases of agammaglobulinemia plasma cells appeared to be very scanty or absent from the tissues of these patients.

b. Blood Dyscrasias. These have been well reviewed by Harvard and Bodley Scott (1960) who described a total of thirty-two cases. Of this series thirteen showed pure red cell anemia only; eleven of the remainder showed also pancytopenia, thrombocytopenia, or neutropenia. Two cases had an autoimmune hemolytic anemia, and five cases myasthenia gravis in addition to blood dyscrasias. Fourteen of the thymic tumors in the whole group were of the spindle-celled type: the remainder were lymphoepitheliomas in those cases where a definite diagnosis was made. Similar results are given in the earlier review of Fisher and Beyer (1959).

The response of these patients to thymectomy is uncertain; in six cases partial or complete remission of the blood disorders followed operation and in an equal number no improvement was obtained. It is suggested by Harvard and Bodley Scott (1960) that failure to respond to thymectomy may be owing to irreversible damage to the bone marrow. This is clearly possible, but dissemination from the main tumor, either locally, or in the form of colonization of other lymphoid organs by lymphocytes derived from the tumor may also account for a continuation of symptoms. In view of the low malignancy of most thymic tumors such colonization of lymphoid organs might be difficult to recognize.

c. Myocarditis and Myositis. These conditions may be considered under two headings: (1) muscle lesions associated with myasthenia gravis and also seen in myasthenia accompanying thymic hyperplasia and (2) granulomatous myocarditis and myositis accompanying thymic neoplasms.

The changes in voluntary and cardiac muscle in myasthenia gravis although originally thought to consist only of the focal infiltration by lymphocytes described as "lymphorrhages" are now recognized to involve marked inflammatory and degenerative changes in the muscle fibers themselves.

These changes are described by Russell (1953) as consisting of three types: (1) an acute necrosis of muscle fibers with associated inflamma-

tory cellular reactions commonly seen in the heart; (2) a progressive atrophy of individual fibers with the formation of lymphorrhages in the later stages; and (3) a simple atrophy of fibers or groups of fibers without alterations of staining reaction or inflammatory cellular infiltration. The cellular infiltrations present around degenerating muscle fibers usually consist of lymphocytes and histiocytes—plasma cells are rare.

In addition to the muscle lesions commonly associated with myasthenia gravis, a more severe form of myositis and myocarditis associated with thymic tumors has been described by several authors (Funkhouser, 1961; Waller *et al.*, 1957; Mendelow and Genkins, 1954; Langston *et al.*, 1959). In these cases marked destruction of muscle fibers was associated with a granulomatous inflammatory reaction including giant cell formation. In the case of Funkhouser (1961) a positive L.E. phenomenon was also obtained. The thymic tumor associated with these cases was of the lymphoepitheliomatous type.

B. Association of Thymic Hyperplasia with Disorders of Serum Proteins and Blood Formation

As already stated these disorders appear primarily associated with thymic tumors. Good (1954), however, described a case of agammaglobulinemia associated with thymic hyperplasia and Wintrobe (1951) a case of aplastic anemia also associated with thymic hyperplasia; no thymic histology was given. Further investigation of the γ-globulin levels and hemopoiesis in cases of myasthenia gravis might provide valuable information on this point.

C. Pathology of Thymic Tumors Associated with Systemic Disorders

Two varieties of tumor may be recognized: (1) lymphoepithelioma of the thymus (associated with myasthenia gravis in about 75% of cases and, more rarely, with other generalized disorders); and (2) spindle-celled thymoma, rarely associated with myasthenia gravis, but occurring with a large number of other generalized disorders (Table IX).

1. Lymphoepithelioma

This tumor shows the well-known picture in which (Fig. 17) large spheroidal cells resembling the normal epithelial reticulum of the thymus are intermingled with varying proportions of lymphocytes. The majority of such tumors are encapsulated and the clinical effects are due to the associated myasthenia, but in about 25% of cases (Castleman, 1955) local dissemination over the pleura, pericardium, and diaphragm may

occur. This pattern of behavior is clearly different to that seen in lymphomata, as widespread involvement of lymph nodes, spleen, and marrow does not occur, although it is possible that colonization of those sites by lymphocytes derived from the tumor may be present and may account for the persistence of myasthenic symptoms after the removal of the primary growth. In association with lymphoepitheliomas, germinal centers of similar type to those present in the "hyperplastic" thymus of

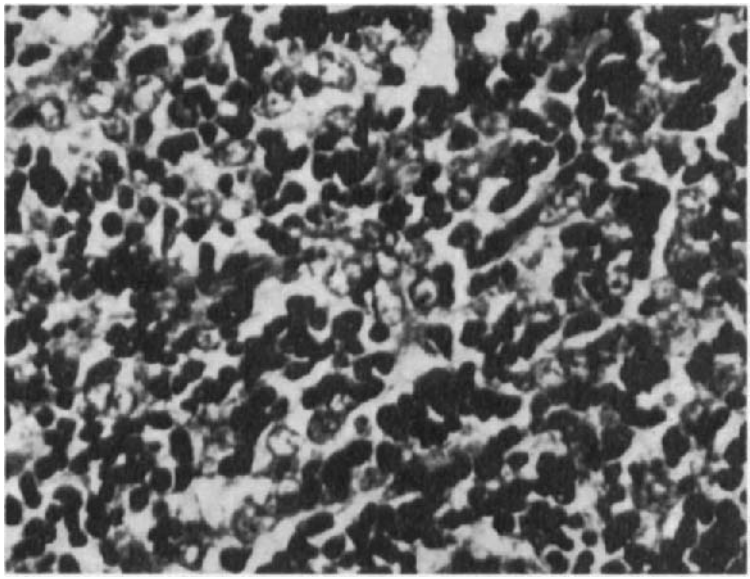

FIG. 17. Lymphoepithelioma of the thymus showing the typical structure of large, pale, epithelial cells mixed with lymphocytes. Magnification: ×700.

myasthenia gravis may occur in the substance of the tumor or the neighboring thymic tissue.

2. *Spindle-Celled Thymoma*

In this tumor the epithelial component shows a characteristic structure of thin spindle cells arranged either in parallel bundles or in whorls somewhat resembling the Hassall's corpuscles of the normal thymus (Fig. 18). Scattered among the epithelial components are varying numbers of lymphocytes, in some examples being exceedingly scanty. Tumors of mixed "lymphoepitheliomatous" structure and spindle-celled morphology may occur, and the latter cell type is probably only a variant of the normal epithelial reticulum, being commonly present in the normal thymus of older persons. Spindle cells of an apparently identical type to

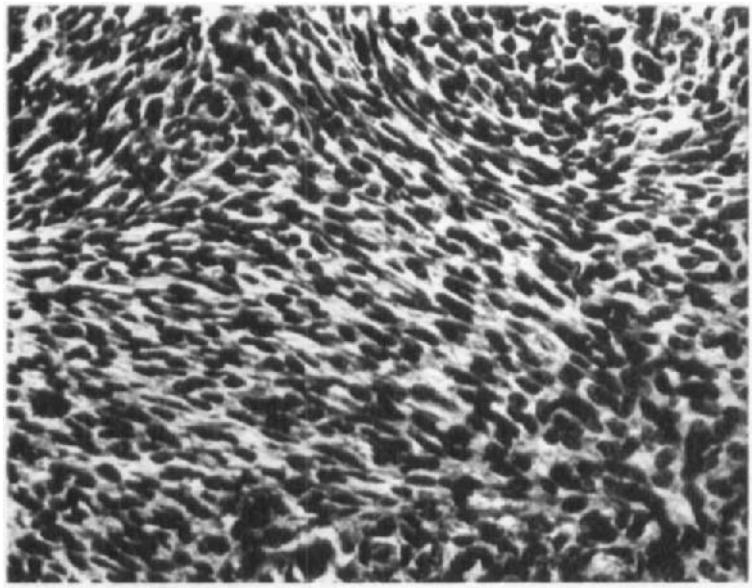

FIG. 18. Spindle-celled thymoma showing parallel bundles of tumor cells with adjacent whorled areas. Magnification: ×250.

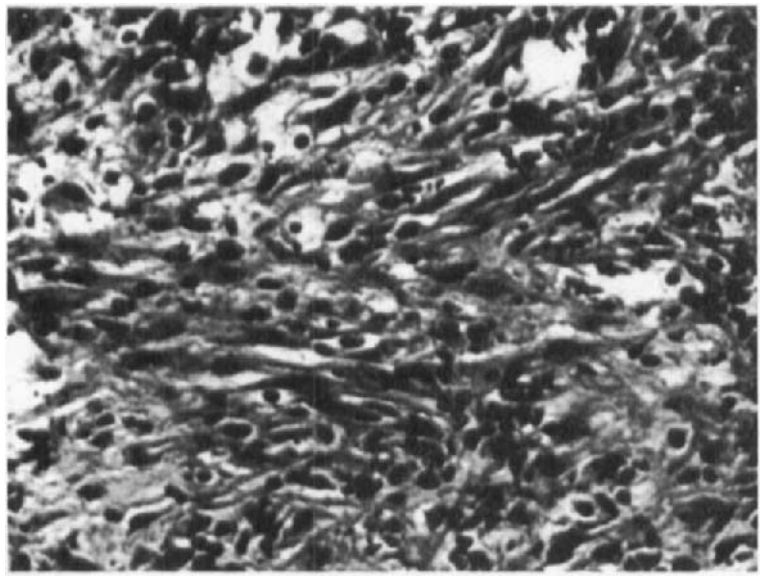

FIG. 19. Seven-day-old subcutaneous graft of rat thymus showing spindle-celled area. Magnification: ×250.

those seen in tumors may also be observed in the early stages of the growth of thymic transplants in animals (Fig. 19), and which at a later period in the organization of the graft become converted to the normal, spheroidal cell type (Fig. 20). As stated, myasthenia gravis is rarely observed in association with predominantly spindle-celled thymomas, but these tumors may be accompanied by a variety of other generalized disorders.

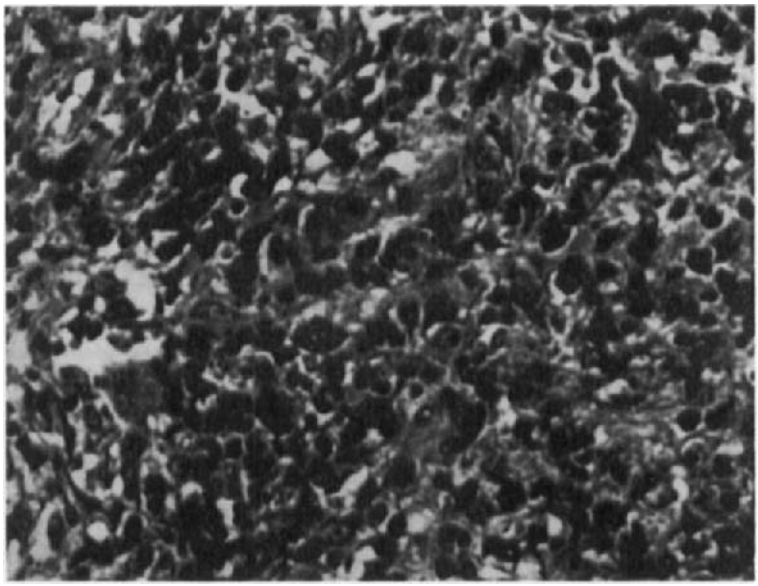

FIG. 20. Ten-day-old subcutaneous graft of rat thymus showing mixed structure of spheroidal epithelial cells and lymphocytes. Magnification: ×300.

D. The Pathogenesis of the Thymic Syndromes in Man

The nature of the relationship between the various types of thymic disorder and the associated clinical syndromes has received few explanations. Myasthenia gravis has frequently been regarded either as a primary disorder of the neuromuscular junction or as a consequence of an unknown pathological process affecting both the thymus gland and the motor nerve endings (Havard and Bodley Scott, 1960). Clinical evidence suggesting the presence of a curariform agent in the circulation of myasthenics, particularly that derived from the occurrence of neonatal myasthenia in infants born to myasthenic mothers, has proved resistant to experimental verification (Nastuk *et al.*, 1959).

All attempts to elucidate the pathogenesis of myasthenia gravis have

been defeated by the lack of knowledge of the normal function of the thymus gland. If it is accepted from the evidence previously given that the main function of the thymus is immunological and concerned with the provision or development of immunologically competent cells throughout the body at a time either shortly before or after birth, the pathology and symptomatology both of myasthenia gravis and of other clinical syndromes associated with thymic disease may be reinterpreted and may provide confirmatory evidence of the original immunological hypothesis.

A number of pathological and clinical features of myasthenia gravis suggest the operation of an immunological mechanism. The germinal center hyperplasia of the gland associated with 65–70% of cases of myasthenia has been shown to have both the histological features of immunological lymph node reactions, to be produced experimentally in the thymus by direct injection of antigens (Marshall and White, 1961), and to be associated in man with the formation of demonstrable γ-globulin. The lymphorrhages in the muscles of myasthenics, which also occur in the muscles only in rheumatoid arthritis, are similarly compatible with an immunological basis; as are the claims of Strauss *et al.* (1960) and Nastuk *et al.* (1960) of the demonstration of a muscle-binding, complement-fixing, globulin fraction in myasthenic serum and of marked variations in serum complement activity during the course of the disease. Among the clinical features of the disease, the predominant female sex distribution present in myasthenia gravis associated with thymic hyperplasia may be compared with the similar female preponderance constantly observed in autoimmune disease in general. Simpson (1960) has recorded a number of additional clinical features suggesting an immunological basis for the disorder.

The nature of clinical syndromes associated with thymic tumors presents greater difficulties in interpretation. No direct evidence has yet been obtained, either by extraction or by immunofluorescent methods, that thymic tumors may produce antibodies. A certain amount of indirect evidence, however, suggests this possibility: the association of thymic tumors with autoimmune hemolytic anemia, hyperglobulinemia, and the presence of L.E. cells; the presence of germinal centers both in thymic tumors and in adjacent thymus tissue; and the occurrence of an identical clinical syndrome (myasthenia gravis) associated both with a hyperplastic antibody-forming thymus and with thymic neoplasms. Further, if the normal thymus is considered as essentially an immunological organ, thymic tumors may probably produce immunological disturbances. Finally, in view of the clinical interrelationships of the syndromes associated with thymic disease and their multiple occurrence in the same

patient, it is impracticable with existing knowledge to assume varying etiologies for these conditions. If a single etiological basis is probable, no evidence exists for this outside the immunological field.

Certain features of these systemic disorders seem of particular interest. The variety of histological and serological abnormalities associated with thymic tumors or hyperplasia, and their relationship in some cases to the so-called "collagen diseases" is remarkable considering the rarity of the primary thymic disorders. Approximately 75% of lymphoepitheliomas of the thymus are stated to be associated with myasthenia gravis; if detailed examination of such cases were made it is probable that most thymic tumors would be found to be associated with some variety of the systemic disorders hitherto described. In spite of the far greater incidence of lymphomas affecting extrathymic lymphoid tissue, no examples of myasthenia gravis, erythroblastic aplasia, or myositis and myocarditis have been recognized in association with such conditions as Hodgkin's disease, follicular lymphoma, or reticulum cell sarcoma. If an immunological basis is accepted for the systemic disorders associated with thymic disease, these facts suggest that the thymus may possess a much greater capacity for the production of immunologically competent cells capable of reacting against an individual's own tissues than lymphoid organs elsewhere in the body. Such a capacity may reflect its original, normal immunological function (as discussed in Section VI), its high cellular proliferation rate, and its relative isolation from antigenic stimuli and possibly, also, from the homeostatic mechanisms affecting other lymphoid tissue.

VI. General Discussion

The general picture emerging from studies of the relation between the thymus and the remainder of the lymphoid complex is one of extremely active cell proliferation in the thymus associated with cell distribution to other lymphoid organs. These activities appear to be at their peak during early life, before the onset of age involution of the thymus. During the same time, the body is encountering antigens of all sorts and thus acquiring its immunological experience which it incorporates not only in the circulating globulin molecules but also in clones of cells accumulating in spleen, lymph nodes, bone marrow, Peyer's patches, and other localized accumulations of lymphoid tissue. Removal of the thymus during this early period is associated with some defects in immunological performance, and the earlier in life thymectomy is performed, the greater the degree of immunological inertia. The failure to effect an immunological response under these circumstances could be owing to (1) the lymph-

oid tissue-depleted state, (2) the loss of certain specific types of cells originating only in thymus tissue, or (3) to a combination of both.

Methods known to decrease the lymphocyte population usually produce a general, nonspecific, impairment of the capacity to develop an immune response. Among these are irradiation (Taliaferro, 1957), cortisone and other oxysteroids (Taliaferro, 1957; Dougherty and White, 1945), some folic acid antagonists (Uphoff, 1958), severe graft-versus-host reaction (Howard and Woodruff, 1961), serial transplantation of bone marrow in syngeneic hosts (Doak, 1962), and chronic thoracic duct fistula (McGregor and Gowans, 1961). It is possible, therefore, that the marked deficiency of lymphocytes following neonatal thymectomy might, per se, account for the failure of such animals to undertake immunological responses.

Recent work has shown that even newborn mice may be capable of effecting immunological responses provided they are stimulated by rather small doses of allogeneic cells (Howard and Michie, 1962) and that an antibody response can occur in a developing embryo as soon as lymphoid elements can first be identified (Kalmutz, 1962). This has led to the concept (cf. Brent and Gowland, 1961; Michie and Howard, 1962; Kalmutz, 1962) that immunological tolerance is not the result of an all-or-nothing effect of antigen on an "immunologically immature" population of cells but is, in reality, an unresponsive state analogous to that produced in mature animals by large doses of antigen (Felton, 1949; Dixon and Maurer, 1953, 1955). It is reasonable to assume that such a state can be produced more easily in the younger animal with its incompletely developed lymphoid system. The refractoriness of the adult, neonatally thymectomized animal to antigen stimulation could thus result from two factors: (1) the lymphoid tissue-depleted state (which itself results from the absence of a cellular contribution from the thymus or from the loss of a noncellular thymic factor inducing lymphopoiesis, or from both, as discussed in Section II), and (2) the ease with which a small population of immunologically competent cells can be paralyzed by antigen. Whether such mice can be actively immunized by minute doses of antigen, as is the case with newborn mice (Michie and Howard, 1962) and opossum embryos (Kalmutz, 1962), remains to be determined. If this proves possible, one will have to consider that cells capable of undertaking specific immunological reactions are present in the tissues of such mice. If, on the other hand, this proves impossible, then (1) either such cells are absent, or (2) they exist but not in sufficient numbers to give a detectable response—possibly because they cannot proliferate in the absence of a humoral contribution from the thymus.

Thymus grafting restores the neonatally thymectomized mouse to normal reactivity. When parental thymus was grafted to an F_1 hybrid host, the host eventually behaved as if it had not been thymectomized. When allogeneic thymus was grafted, the thymectomized host was, in many cases and for long periods of time, specifically tolerant of skin homografts from the strain of mice supplying the thymus graft. Clearly, there are two hypotheses that can account for this "tolerance." The simplest one is that immunological reactivity is of adoptive origin. Immunologically competent donor-type cells would arise in the thymus graft and colonize the lymphoid-depleted spleen and lymph nodes of their host. The host would be immunologically inert as a result of thymectomy but could now muster a number of immunological reactions by proxy. Third-party skin rejection would be mediated entirely by the immunologically competent cells originating in the donor thymus. The absence of antihost activity could be explained not only by the fact that the thymus grafted was from a newborn donor but also by the finding of Dubert and Kaplan (1961) that thymus grafts undergoing a sequence of necrosis and regeneration in the presence of foreign host antigens became immunologically unresponsive to these particular antigens. Against this theory may be cited the evidence that the majority of the cells dividing in the spleens of these thymus-grafted animals, which had been thymectomized at birth, were host type and not donor type.

The other hypothesis that may account for the tolerance of thymus-donor-type skin is that the host is specifically tolerant, in the classic sense, of donor strain tissue. After neonatal thymectomy, there would still be a population of immunologically competent cells, although small compared with that of an intact animal. Under the influence of the foreign thymus, these host cells would now be able to multiply to normal levels. The foreign thymus would act as a paralyzing source of antigens, causing a specific immune unresponsiveness in the developing lymphoid system of the host.

If it is shown that immunologically competent cells are present in the tissues of neonatally thymectomized animals, the hypothesis that the thymus, during embryogenesis, produces the originators of such cells (Miller, 1961a), is not necessarily invalidated. Some or many of these cells could have differentiated and migrated out of the organ before birth and would then be able to take part in immune reactions provided they were not paralyzed by excessive doses of antigen. Morphogenetic studies suggest that lymphocytes differentiate in the thymus region before they begin to appear elsewhere (see Kalmutz, 1962) and that lymphoid primordial cells necessary for lymphoid differentiation are not

produced in the spleen (see Section II). It is thus possible that, in the absence of a contribution from the thymus, the spleen would not become a lymphoid organ. The evidence available at present suggests only that such a contribution may be a cellular one.

There are indications from clinical and experimental material that immunologically competent cells may still be arising in the thymus in postnatal life. Burnet (1959a) has distinguished between autoimmune diseases derived from defects of antibody-forming cells and those depending upon some alterations in the distribution of normally inaccessible antigen. On the basis of clonal selection (Burnet, 1959b), "forbidden clones" of immunologically competent cells arise by somatic mutation (Burnet, 1959a) and survive presumably because of some intrinsic resistance to an otherwise normally effective homeostatic control (Holmes *et al.*, 1961). We suggest that such clones of cells may, in some cases, arise primarily in the thymus. This organ has the greatest lymphopoietic activity of all the lymphoid tissues (see Section II), thus allowing a greater opportunity for mutation to occur within its population of cells. The presence of centers of intense lymphopoietic activity in the thymus of patients with myasthenia gravis and the absence of analogous abnormal hyperplasia in other lymphoid organs (see Section V) would suggest that the thymus is the primary site of origin of pathogenic cells associated with this condition. The presence of a blood–thymic barrier excluding circulating antigenic protein (see Section III) would ensure that any "forbidden clones" would not be destroyed or functionally eliminated by contact with antigen, and could, therefore, become established and flourish.

Since normal animals in nature are not ordinarily called upon to make a distinction between compatibility and incompatibility of one another's tissues (except, perhaps, in the special case of the "engrafted" fetus), it has been suggested (e.g. Burnet, 1961) that the biological *raison d'être* of homograft immunity might be to provide against the danger of proliferation of cells carrying wrong molecular configurations. There are now a number of careful studies showing syngeneic and even autochthonous resistance to tumors in animals. It has become well established that chemically induced and virus-induced tumors have an antigenic individuality of their own (Foley, 1953; Prehn and Main, 1957; Révész, 1960; Klein *et al.*, 1960; Sjögren *et al.*, 1961; Habel, 1962; Klein *et al.*, 1962). The experimental evidence described in this review has suggested the possibility that immunologically competent cells, which are known to mediate homograft immunity, may originate in the thymus. Viewed in this light, the thymus would become part of a homeostatic mechanism,

an immunological homeostasis, which would allow the body to maintain a close watch over the chemical integrity of its cellular components and to eliminate antigenically foreign neoplastic clones before they could spread.

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Cellular Genetics of Immune Responses

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I. Introduction

Immune phenomena can be regarded in a genetic light from many points of view. For example, antibody is a highly specific protein. Believing that protein specificity is the result of specific gene action, we may ask how the lymphoid cells of the body came to acquire the genetic information necessary to synthesize the many antibodies manufactured by an animal throughout its life (Burnet, 1959; Lederberg, 1959). Alternatively, given that a cell has the capacity to form an antibody, how is it induced to express this potentiality by massive protein synthesis as the

[1] Supported by grants E-2700 and E-3958 of the National Institute of Allergy and Infectious Diseases, U.S. Public Health Service. Much of the work reported here was performed in the laboratories of Professor J. Lederberg, Stanford University School of Medicine, Palo Alto, California, in collaboration with Dr. O. Mäkelä.

need arises? It should be stressed that at least a formal distinction exists between these two questions. Further, we can attempt to construct a population genetics of immunologically competent cells in the body (Burnet, 1959) and to uncover the rules governing orderly functioning, proliferation, and homeostasis in this vast and complex population of largely autonomous microorganisms. Other genetic questions, outside the scope of the present article, are concerned with the evolution of the immune response, the inheritance of patterns of immunological competence within a species, the genetics of immunological diseases in man, and the control of the formation of homotransplantation antigens. It would, therefore, be very difficult to review the genetic aspect of immunology exhaustively. We will confine ourselves to the few recent studies in antibody synthesis and the homograft field which have most shaped the author's current concepts. Immunology now contains such a mass of information, gained by the use of such diverse methodologies, that it is becoming increasingly difficult to achieve a meaningful synthesis. As the immunologist widened his perspectives, general theories of immunity became more numerous and more complex. Some of these (Haurowitz, 1953; Burnet and Fenner, 1950; Schweet and Owen, 1957; Jerne, 1955, 1960; Talmage, 1957; Burnet, 1957b, 1959; Lederberg, 1959; Karush, 1961) have had a profound influence both in stimulating new experiments and in catalyzing new interpretations of old data. But the present review will not be primarily a discussion of theories. I believe it to be currently more important to decide which of the newer experimental conclusions are sufficiently proven to demand serious consideration in any theory of immunity and to outline specific questions that must be answered before a cellular genetics of immune responses can be constructed.

II. Immunologically Competent Cells as a Population of Microorganisms

There is little question that within the lymphoid and reticulo-endothelial systems, individual cells have different life histories, perform different tasks, and respond to different signals. Though a healthy defense mechanism undoubtedly depends on the coordinated action of all members of the population, each cell is an individual entity with its own, often measurable, contribution to make. One way to achieve a greater understanding of immunity is to gain more precise knowledge of the behavior of each of these individuals. We should, therefore, ask which are the immunologically competent cells of the body; where they come from; how long each lives; what becomes of each after its obvious tasks

are done; what each can do under different stimuli; and, perhaps most important from the genetic point of view, we must know which of the cells have progeny and how the capacities of the progeny relate to the capacities of the parents. These questions outline the scope of our genetic approach.

A. The Effector Cell Population

1. Antibody Formation

Certain cytological aspects of antibody formation will be covered by Dr. Miller *et al.* in another chapter of this volume. Suffice it to say here, that there now appears little doubt that the chief producers of circulating antibody belong to the plasma cell family. The chain of evidence is impressive, starting with inferential observations by Fagraeus (1948), continuing with studies on cellular antibody content by various ingenious techniques (Reiss *et al.*, 1950; Moeschlin and Demiral, 1952; Leduc *et al.*, 1955; Vazquez, 1961; Berenbaum, 1959), and concluding with studies on antibody formation *in vitro* by single plasma cells (Nossal and Lederberg, 1958; Nossal, 1958, 1959a,b, 1960a; Mäkelä and Nossal, 1961a,b; Attardi *et al.*, 1959). The newer techniques have shown that not all antibody-forming cells clearly belong to the plasma cell family. Various workers (Hayes and Dougherty, 1954; Leduc *et al.*, 1955; White, 1958; Gunderson *et al.*, 1958; Vazquez, 1961; Mäkelä and Nossal, 1961a) have drawn attention to an active cell morphologically similar to the small lymphocyte, but differing from typical small lymphocytes by possessing a discernible rim of deeply basophilic cytoplasm. These cells are active much less frequently than plasma cells; what one calls them is largely a matter of taste and personal prejudice. As they exhibit ribonucleic acid (RNA)-rich cytoplasm and appear as the specific result of antigenic stimulation, concurrently with typical plasma cells, I would consider them provisionally as atypical plasma cells. The earlier ideas on the relationships of these cells to various other connective tissue cells and to immunity have been reviewed by Taliaferro (1949).

2. Homograft Rejection

Mechanisms of homograft rejection have been frequently reviewed in recent years (Lawrence, 1959; Brent, 1958; Hašek *et al.*, 1961). Though it is established that homograft rejection is mediated by lymphoid cells, the exact nature of the effector cell type is not known. As thoracic duct lymph, a fluid virtually free of monocytes or other phagocytes, can be used to initiate homograft reactions (Billingham *et al.*, 1960; Schooley and Berman, 1960), it appears clear that the effector cell is a lymphocyte

or close derivative. As small lymphocytes predominate in all lymphatic tissues, and as about 10,000 cells appear to be effective in initiating a reaction (Terasaki, 1959; Burnet and Burnet, 1960), it is often stated that the response must be produced by the action of small lymphocytes. This conclusion is not justified from present data. All cell populations used so far to initiate a homograft phenomenon contain at least a few primitive cells. In view of the tremendous proliferative power of primitive lymphocytes correctly stimulated (Nossal and Mäkelä, 1962b), it is difficult to rule out the possibility that these few give rise to a new effector population, whose histological appearance is not clear. Certainly small lymphocytes do accumulate at the site of homograft reactions, but so, in smaller numbers, do plasma cells, histiocytes and other phagocytes, and unclassifiable intermediate and primitive cell forms (Medawar, 1958; Waksman, 1959; Porter and Calne, 1960; Isacson, 1959). This question is now in the same confused state as the cytology of antibody formation was 10–15 years ago. Obviously more precise and incisive tools are needed to supply the final answer as to which is the effector cell in graft rejection. Certainly there are homograft situations in which circulating antibodies are of greatest importance; others in which they play little or no role. By presuming that circulating antibodies are made by plasma cells, we can postulate that certain aspects of graft rejection may depend on plasma cells; we can guess that small lymphocytes, always present, also play some, unfortunately obscure, role.

B. The Origin of Plasma Cells

Undoubtedly we know more about the plasma cell than about any other immunologically competent cell. Abundant data is now available about its development (Fagraeus, 1948; Leduc *et al.*, 1955), ultrastructure (Bessis, 1961), antibody-forming capacity (reviewed by Nossal and Mäkelä, 1962a), and metabolic activities (Schooley and Berman, 1960; Schooley, 1961; Mäkelä and Nossal, 1962). For our genetic approach, exact knowledge of the origin of plasma cells and of the life history of their ancestors would be most fruitful. After antigenic stimulation, particularly in a secondary response, the most primitive members of the plasma cell family can be clearly recognized after 1 to 2 days (Fagraeus, 1948; Ehrich *et al.*, 1949; Leduc *et al.*, 1955; Nossal, 1959b), and it now seems certain that all the plasma cells arise from the multiplication of these early plasmablasts (Nossal and Mäkelä, 1961a, 1962b; Mäkelä and Nossal, 1962; Baney *et al.*, 1962; Urso and Makinodan, 1961). This leaves us with the critical questions: Where do the plasmablasts themselves come from? Which of the normally occurring cells in lymphoid tissues

are capable of being stimulated by antigen to take on the form and function of a plasmablast? Following a primary response, which of the cells in the resting immunized animal bear the burden of immunological memory and make the intense plasmacytopoiesis of a secondary response possible?

Ever since Sabin (1939) first discussed the role of the monocyte in antibody formation, many studies purporting to show that one cell or another can turn itself into an antibody-former have been reported. Fagraeus (1948), on the basis of her brilliant histological survey, inferred that plasma cells were derived from the reticulo-endothelial system, but it seems that she made little or no distinction between reticulo-endothelial cells and primitive lymphocytes. A macrophage origin of plasma cells has also been proposed (Dixon *et al.*, 1957). Early workers had been convinced that plasma cells came from primitive lymphocytes (Downey, 1911; Maximow, 1928; Michels, 1931), a view which has been recently restated (Leduc *et al.*, 1955; Congdon, 1961). Some have speculated that the small lymphocyte might be the "immunological messenger" (Wissler *et al.*, 1957) and might reverse its life history on appropriate antigenic stimulation by transforming itself into a plasmablast and by thus giving rise to a clone of plasma cells (Burnet, 1959). Finally, it has been argued that antibody-forming cells themselves might be the ancestors of further antibody-forming cells (Wissler *et al.*, 1957). Unfortunately, none of the techniques used by these workers were decisive. Conclusions depended either upon subjective interpretations of a series of complex static pictures (sections or cell smears) or upon studies utilizing transfers of large heterogeneous populations of cells from one animal to another (see article by Dr. Cochrane and Dr. Dixon, this volume). Clearly the important changes are the earliest ones, i.e., the transformation of some cell or cells into plasmablasts; and histological studies will never give more than suggestive evidence of this subtle alteration. In recent experiments, we have adopted an approach to this question which, while not providing the final answer, has given us more precise insight and has clearly eliminated many of the cell types mentioned in the foregoing as the primordial cells in immunity.

The principle behind the experiments (Nossal and Mäkelä, 1961a, 1962b) was a simple one. If one could introduce a set of stable markers into the lymphoid cell population which would differentiate various categories of cells, one might be able to induce an immune response and follow the progress of the markers into the resulting plasma cell population. No ideal markers exist, but we felt that the well-known differences in the capacity of different cell types to become labeled after *in vivo*

exposure to tritiated thymidine could be used to good effect. Tritiated thymidine has several valuable characteristics as a stable marker (Hughes *et al.*, 1958). (1) A single intravenous injection is rapidly removed from the circulation and, for the most part, firmly bound within a few minutes; (2) it is incorporated into deoxyribonucleic acid (DNA) during synthesis by cells as a prelude to mitosis. As DNA is believed to be metabolically stable, any tracer incorporated should mark that cell permanently, subject only to dilution at each mitotic division of the cell; (3) a single pulse allows labeling of some lymphoid cells and not others; and (4) detection of the label is relatively easy using high-resolution autoradiography. In our experiments we have so far examined only a secondary immune response, that occurring in rat popliteal lymph nodes following a second foot-pad injection of a *Salmonella* flagellar antigen. Four weeks after a primary stimulus, the popliteal lymph nodes of such rats are morphologically indistinguishable from unstimulated nodes. If one then gives a single pulse of tritiated thymidine and kills the animal 1 hour later, only a small proportion (about 1%) of the node cells are found to be labeled. Reticular cells and macrophages were rarely labeled, small lymphocytes and mature plasma cells never. Large lymphocytes were labeled in about 80% of the cases. Into this noncommittal category of large lymphocytes we place all cells found in lymph nodes with a nuclear diameter of 11μ or greater, excluding cells obviously not lymphocytes, such as mast cells and macrophages. The category includes cells named by others lymphoblasts, hematogenous stem cells, reticular lymphocytes, reticulum (as opposed to reticular) cells, blasts, and so forth. The reason that we do not attempt to subdivide this category further is that we have never been able to attach any functional significance to the minor morphological variations among these cells. The general features, apart from size, which distinguish these primitive lymphocytes are the light-staining nucleus often with one or more prominent nucleoli, and the obvious, usually complete, rim of cytoplasm exhibiting various degrees of affinity for basic dyes. Medium lymphocytes (nuclear diameter 8–9 μ) were labeled in 13% of the cases. This was in agreement with the findings of other workers in the field (Schooley *et al.*, 1959; Hughes *et al.*, 1958; Cronkite *et al.*, 1959; Yoffey *et al.*, 1958; Schooley and Berman, 1960). We, therefore, gave a group of resting primarily immunized animals sufficient isotope to ensure that the label could still be detected even after nine or ten mitotic divisions. Two hours after the isotope injection, the animals were injected with "cold", i.e., carrier, thymidine, and they also received a secondary stimulus. No exogenous tracer was thus available to the cells at any stage of the secondary response. The prediction

was made that if the "memory cells" or plasmablast precursors were small lymphocytes, reticular cells, macrophages, or plasma cells, few or none of the crop of plasma cells called into being would be labeled; if large lymphocytes were the source, then most of the plasma cells would be labeled; if a mixed ancestry existed, a mixed labeling picture would ensue. The results were surprisingly clear cut. Over 95% of all plasma cells resulting from the secondary stimulation were labeled and must thus

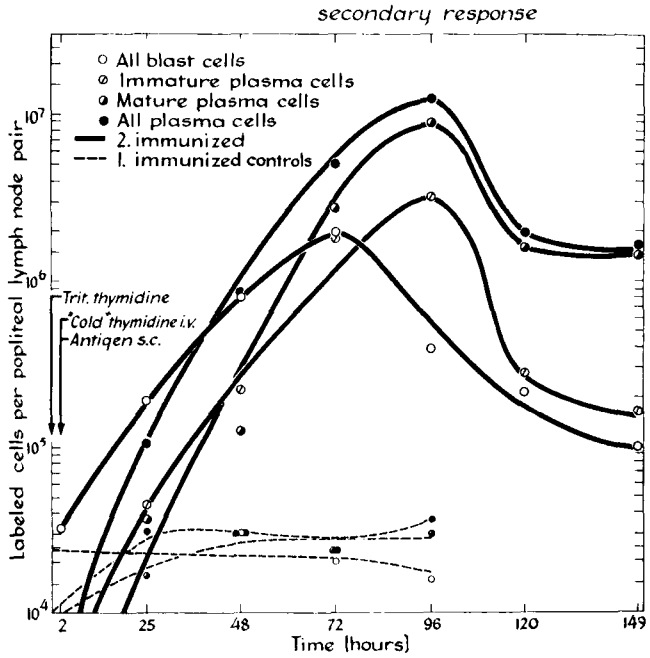

FIG. 1. The appearance of labeled plasma cells in popliteal lymph nodes of rats; tritiated thymidine was pulsed in briefly at time 0 and a secondary antigenic stimulus given at 2 hours. Reproduced from Nossal and Mäkelä (1962a).

have been derivatives of cells synthesizing DNA at the time of tracer injection, i.e., 2 hours *before* the secondary antigenic stimulus (Fig. 1). This strongly suggests that the cell type (in resting, primarily immunized nodes) capable of responding instantly to antigenic stimulation and thus presumably carrying specific immunological memory, is a rapidly proliferating, primitive lymphocyte. We shall have much more to say about the behavior of these lymphocytes on antigenic stimulation when we consider the problem of induction (Section VI).

A further interesting feature of these experiments (Nossal and

Mäkelä, 1962b) was that even 40 weeks after a primary stimulus, a brisk plasma cell response could be obtained on secondary stimulation and again the plasma cell precursors were primitive lymphocytes. Studies on resting nodes suggested that these lymphocytes divided about every 12 hours, as judged by a fall in mean grain count of labeled cells. If one makes the assumption that immunological memory is a cellular phenomenon (Burnet and Fenner, 1950) and not a property of the serum (Jerne, 1955), the findings suggest that immunological memory remains intact in a stem line of primitive lymphocytes for at least 600 generations. This tends to argue against the role of persisting antigen in immunological memory (Haurowitz, 1953; Garvey and Campbell, 1956) though antigen-trapping mechanisms that would preserve at least one template molecule within members of the stem line could no doubt be postulated. It also makes an epinucleic type of inheritance of immunological memory less likely (Monod, 1959; Szilard, 1960) and suggests a nuclear inheritance (Schweet and Owen, 1957; Burnet, 1957b, 1959; Lederberg, 1959) although the evidence is in no sense decisive.

At first sight, the idea that a continuously proliferating, primitive line of cells is the storehouse of immunological memory seems inefficient and wasteful. Would it not have been more reasonable of nature to have made some stable, long-lived cell, such as the small lymphocyte, the resting repository of immunological information? On the other hand, a memory system depending on primitive cells has a remarkable plasticity and necessitates very little inertia when the emergency (infection) strikes. In the *absence* of antigenic stimulation, one daughter of each mitotic division of a large lymphocyte, *on the average,* remains primitive and preserves the *status quo;* the other either dies or differentiates in some direction other than a plasma cell. If it dies, as many primitive lymphocytes do (Cronkite *et al.,* 1959; Nossal and Mäkelä, 1962b), it is phagocytized and its molecular constituents presumably are reutilized; thus, little has been lost. If it differentiates toward a small lymphocyte, presumably some other (unknown) useful function is subserved. On the other hand, if the antigenic signal does come, a sensitive, primitive population exists which is ready to respond instantly by plasmacytopoiesis. The cells do not even have to increase their mitotic rate, although we have some evidence that they might do so. All that is needed for a clonal expansion of the memory cells is that for a few mitotic divisions, *both* daughters remain primitive, but differentiate into plasmablasts and finally into plasma cells. Details will be considered in Section VI. We thus can postulate that the primary antigenic stimulus has called into being [either by "selection" or "instruction" (Lederberg, 1959)], a popu-

lation of primitive lymphocytes that are adapted to respond to further antigenic stimuli by clonal expansion and differentiation to the plasma cell form. The proliferating stem line is preserved indefinitely, subject only to random drift, and, possibly, contraselective pressures if other strong immune reactions result in competition for *Lebensraum* or nutrients. Immunity lasts as long as the stem line is maintained.

If this model correctly explains the plasmacytopoiesis in the secondary response, we still are left with the question of the primary response. Experiments on the primary response are technically more difficult, as plasmacytopoiesis is less intense and thus "noise levels" resulting from normal processes in the node are more confusing. Further, with Gram-negative bacterial antigens, there is always the risk that one is not dealing with a primary response at all. Although concrete evidence is lacking, the author would speculate that the sequence of events in the primary response also involves multiplication and differentiation of primitive lymphocytes.

Since the first draft of this manuscript was written, I have become aware of the work of Gowans (1962) and of Porter (1962). These rather similar but independent studies claim that when small lymphocytes are radioactively labeled by a variety of techniques, and injected into the circulation of homologous animals, a proportion of the small lymphocytes lodge in the recipient spleen and within a day or two assume the form of a primitive cell rather like a plasmablast. It is presumed that these in turn mediate a graft-versus-host type immunological attack. The authors claim that primitive lymphocytes play no part in this process. Taking these findings at their face value, we must assume a basic difference either between a primary and a secondary response or between homograft reactions and humoral antibody formation. Experiments are in progress in our laboratory to attempt to solve this question.

C. The Life Span of Plasma Cells

Many observations suggest that the antibody-forming life of a plasma cell is short. After antigenic stimulation, particularly in a secondary response, plasmacytopoiesis reaches a peak after 4 to 7 days (Fagraeus, 1948; Leduc *et al.*, 1955; Wissler *et al.*, 1957; Nossal, 1959; Nossal and Mäkelä, 1961a, 1962a) and then rather suddenly declines sharply. This would suggest that plasma cells live for a few days only, migrate out of the area under study, or change their morphology (perhaps to that of a small lymphocyte) and are no longer recognizable. It has been suggested that antibody-forming cells shed their cytoplasm, come to look indistinguishable from small lymphocytes and circulate round the body dispers-

ing the immunological message (Wissler *et al.*, 1957). We have already cited the evidence against this attractive concept (Section II, B). To attack the problem of the life span of plasma cells more directly, Schooley (1961) has studied a steady-state situation of plasmacytopoiesis by autoradiographic means. Mice received three injections of dog serum in Freund's adjuvant, and, one day after the last, an intravenous injection of tritiated thymidine. The differential cell count, which did not change materially over the next 25 days, showed that from 12 to 14% of all cells seen belonged to the plasma cell family. Of the mature plasma cells, none were labeled at 1 hour, but 80% were labeled at 8 hours. This suggested that the unlabeled mature plasma cells seen at 1 hour had been almost entirely replaced and, although it could not be determined whether the disappearance of the unlabeled cells was the result of cell death or of migration, the inference could be drawn that the life span of the plasma cell was of the order of 8 to 12 hours.

In recent experiments (Mäkelä and Nossal, 1962), we have obtained some evidence bearing on this point. To gain more precise information, we studied antibody formation by single cells isolated *in vitro* and then performed autoradiography on each antibody-forming cell. One of the questions we wished to answer concerned the age of antibody-forming plasma cells. All rats studied were in the fifth day of a secondary immune response to a *Salmonella* flagellar antigen; from 1 to 48 hours before killing, each had received a single injection (intravenous) of tritiated thymidine followed after 2 hours by an injection of "cold" thymidine where applicable. Single cell suspensions from antibody-forming lymph nodes were prepared, and single plasma cells were studied for antibody formation *in vitro* using a variety of techniques (Mäkelä and Nossal, 1961a,b). Then each active cell was removed from its microdroplet, pipetted onto a marked spot on a clean, dry slide, artificially flattened onto the glass, fixed in acetic acid–methanol, and subjected to autoradiography. Labeling of the cells gave us an indication of the approximate maximum age of each—a cell could only have been labeled if it, or its ancestor, was preparing to divide at the time of isotope injection. These data indicated that labeling of antibody-forming cells was rare at 1 to 4 hours and had increased to 20% at 12 hours, to 50% at 30 hours, and to over 95% at 48 hours. Thus, very few antibody-forming cells at the height of the cellular immune response were more than 48 hours old, and few if any of the plasma cells that had been "born" on the third day were still in the node on the fifth day. Though again we know little about possible migration or morphological change, the inference which might be drawn from the experiments is that it

usually takes from 4 to 12 hours from the most recent division to the assumption of typical plasma cell morphology, and that the complete life span might be about 36 to 48 hours. This figure is longer than that cited by Schooley (1961), but he was dealing with a species (mice) in which metabolic processes are generally quicker and with a more intense (adjuvant-type) immune response.

D. The Mitotic Capacity of Effector Cells

It seems clear that plasma cells are the result of recent division. It would be of considerable interest to know whether cells actually engaged in antibody formation could be capable of further division. Leduc *et al.* (1955) noted the presence of mitotic figures in islands of cells in which specific fluorescence due to antibody could be detected, although resolution was inadequate to decide whether the mitotic figures themselves were labeled. Accordingly, we used the ability of cells to incorporate tritiated thymidine as presumptive evidence of further mitotic activity and studied antibody formation by single cells at various stages of the secondary response (Mäkelä and Nossal, 1962b). Rats were given a secondary stimulus with *Salmonella* flagella and were killed at intervals over the next 7 days. One hour before killing, each rat received an injection of tritiated thymidine. Single, lymph node cell suspensions were prepared, antibody formation by single cells was studied, and autoradiography was performed on all active cells. The results are summarized in Fig. 2. Only occasional active cells were seen in resting, primarily immunized nodes, but they were incapable of incorporating thymidine, i.e., they were not preparing for mitosis. Most looked like old plasma cells and were perhaps the last remnants of the primary response. At 2 days, there were roughly two equal groups, i.e., old plasma cells as in the resting animals and very large immature typical plasmablasts of which about 90% were labeled. From 3 to 5 days, the proportion of plasma cells with detectable antibody-forming activity continued to rise, but labeling fell rather sharply. The shaded area of Fig. 2 demonstrates the extent to which cells simultaneously synthesizing DNA and antibody occurred. There was a limited period during which DNA-synthesizing antibody-formers were common. At the peak of the cellular immune response, i.e., 4 and 5 days after a secondary stimulation, most antibody-forming cells were not capable of synthesizing DNA. Thus, in an antibody-forming clone derived by antigenic stimulation from a given primitive lymphocyte (Section II, B), there is a stage when some members simultaneously perform the highly specialized task of antibody synthesis and prepare for further mitosis. The great bulk of antibody

formation, occurring after the third day, however, is the duty of non-dividing more or less mature plasma cells.

Baney *et al.* (1962) have recently confirmed the salient features of these experiments. In their elegant work, the Coons fluorescent antibody technique was combined with autoradiography. Rabbits were heavily immunized against bovine γ-globulin; on the fourth day of the typical

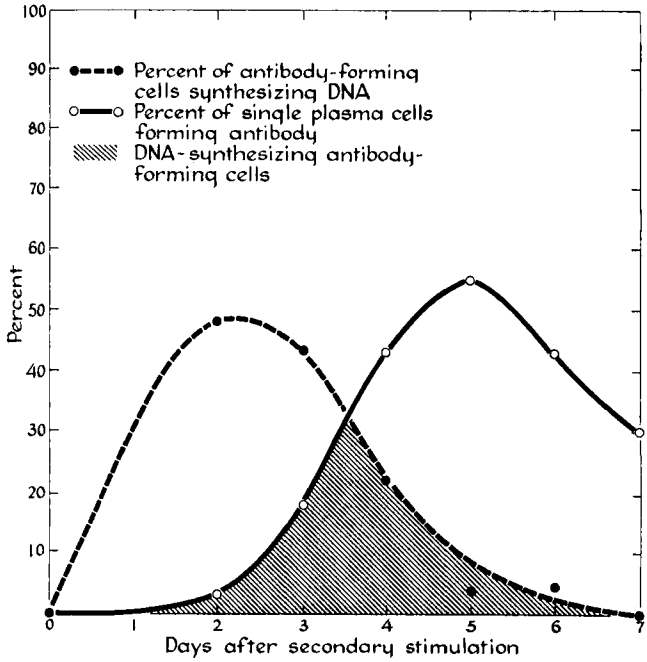

FIG. 2. Deoxyribonucleic acid synthesis among single cells forming detectable antibody. Plasmablasts occasionally formed detectable antibody, but most antibody-forming cells were mature plasma cells, incapable of DNA synthesis. The shading is diagrammatic and has no quantitative meaning. The *product* of the two curves would give a real measure of the frequency of DNA-synthesizing antibody-formers.

anamnestic response, the rabbits were killed, spleen cell suspensions were incubated with tritiated thymidine *in vitro*, and cell smears were prepared. On these smears, active cells containing intracellular antibody, as determined by the fluorescent "sandwich" method (Coons *et al.*, 1955) were carefully located and were then examined for labeling after autoradiography. Since only 4–5% of the antibody-forming cells were labeled, the great majority of them were not preparing to divide at that time.

The lack of DNA synthesis among mature plasma cells is of great

importance to those interested in antibody formation *in vitro*. Obviously our approach would be made much easier if one were in a position to study the clonal behavior of a single primitive cell and perform a detailed pedigree analysis of antibody-forming phenotype. However, when cells from an immunized animal are placed into tissue culture, they gradually stop forming antibody after several days (reviewed by Stavitsky, 1961) and, in general, cannot be restimulated with antigen. Since mature antibody-forming cells seem incapable of further division even in the body, a study of continuing antibody formation in tissue culture depends upon the maintenance of functional, sensitive primitive cells which tend to die out early *in vitro*. Until this problem can be overcome, the prospects of producing an antibody-forming clone *in vitro* appear slight.

E. The Fate of Effector Cells and the Problem of Continuing Immunological Memory

Many of the experiments relevant to this topic have already been cited. We have seen that after a typical secondary immune response, few plasma cells can be detected after 7 to 10 days, and that their fate is obscure. Typical plasma cells do not circulate in the blood, though limited transport in the lymph is a possibility. On the whole it seems not unreasonable to postulate that plasma cells for the most part are born, live a short antibody-producing life, and die in the one place. Since memory between a primary and a secondary response appears to be the duty of a line of primitive lymphocytes, continuing memory after secondary stimulation would appear to depend upon the persistence of this line. Since cells are refractory to further antigenic stimulation for some time after a typical secondary response according to Sercarz and Coons (1960), it would seem that a secondary stimulus fires off nearly all the relevant primitive memory cells into plasmacytopoiesis and that, thereafter, a new population of reactive memory cells has to be built up, perhaps from the few blasts that are not fired off. Several mechanisms suggest themselves to account for the spread of the immunized state around the body. First, antigens themselves move widely around the body, either as soluble molecules or inside phagocytic cells. Second, primitive cells are present in lymph (Schooley *et al.*, 1959; Schooley and Berman, 1960) and blood (Hughes *et al.*, 1958), and lymphocytes apparently circulate around the body to a considerable extent (Gowans, 1959). This would seem to allow for some movement of immunological information. Third, it is not yet proven that small circulating lymphocytes play *no* role in immunological memory. One modification of Wissler's

hypothesis (Wissler *et al.*, 1957) could be that small lymphocytes *do* carry some information and, at intervals, *spontaneously* turn themselves back into blasts—although to account for our autoradiographic results (Section II, B), one would have to assume that antigen in no way influences this process. This situation is tantamount to saying that although plasmablasts and plasma cells come from primitive lymphocytes, the primitive lymphocytes themselves come from a dedifferentiation of small lymphocytes. To us, this view seems clumsy, but no concrete evidence against it can be cited.

With this, admittedly tenuous, information about the nature of our microorganismal population and about some of the important interrelationships, we are now in a position to examine further the actual potentialities of single members and to speculate about how single cells are integrated into the general plan of immunity.

III. Phenotypic Restriction among Members of the Population

One of the striking observations about immune responses is the heterogeneity of cell behavior encountered. When an antigen is injected for the first time, many of the cells in the draining lymph node appear to take it up, but only a very few subsequently produce antibody (Leduc *et al.*, 1955). In a secondary response, far more antibody-forming cells are produced and thus mainly account for the characteristic booster effect (Leduc *et al.*, 1955; Nossal, 1959a). Taken together in the light of the observations already mentioned, this suggests that a greater proportion of the primitive cells are capable of responding during a second stimulation than in the unstimulated animal. In what way are these morphologically indistinguishable cells different from each other? What is the basis of functional heterogeneity? To gain further insights into these and related problems, some years ago we undertook an extensive analysis of the antibody-forming activity of single cells.

A. Single Cell Studies on Multiply Immunized Animals

A considerable amount of information now exists on the antibody-forming capacity of single plasma cells—the "antibody-forming phenotype." We have recently reviewed this field in detail (Nossal and Mäkelä, 1962a) and will confine ourselves to salient points here.

*1. Studies on Anti-*Salmonella *Antibodies in Rats*

We have developed several *in vitro* techniques to study anti-*Salmonella* antibodies made by single plasma cells from the rat, as already

mentioned (Nossal and Lederberg, 1958; Nossal, 1958, 1959a,b, 1960a; Mäkelä and Nossal, 1961a,b, 1962; Nossal and Mäkelä, 1962b,c). Some of these depend on isolating single cells from immunized animals *in vitro* by micromanipulation; others can be readily performed on mass cell suspensions. In the micromanipulation experiments, rats were immunized by injections of *Salmonella* antigens into the hind foot pads; at the desired time, the popliteal lymph nodes were removed, teased with needles in a full tissue culture medium to give a single cell suspension, and washed. A de Fonbrune oil chamber was then set up, containing many large droplets of clean medium (washing droplets) and a pool droplet of the washed cell suspension. In a de Fonbrune chamber, droplets adhere to the under surface of a coverslip and are prevented from evaporating by mineral oil which fills up the chamber. Using a micropipette and manipulator, we then prepared fifty or more pairs of microdroplets of clean medium, each containing about 10^{-6} ml. Single cells believed by virtue of their size to be plasma cells were manipulated out of the pool drop, placed into a washing drop, and moved around in order to give each cell an individual wash. They were then pipetted into one of the microdroplets, together with a minimal quantity of the washing fluid. A similar quantity of washing fluid was pipetted into the paired control drop. After a suitable number of single cell droplets had been prepared, the chamber was placed in a 37°C. incubator for 3 hours. The antibody content of each droplet was then tested. Anti-H antibodies were detected by instilling five or more bacteria of a strain sharing the H (but not the O) antigen with the immunogen, and observing for immobilization. Alternatively, more bacteria could be instilled and typical H agglutination observed. Finally, a reaction depending on the combination of cell-bound antibody with bacterial surface antigen could be scored; in some drops, bacteria adhered to the surface of the antibody-forming cell and actually moved the cell around the microdrop if they retained some motility. This phenomenon, which we called H adherence, was noted in nearly all cells positive for immobilization (provided enough bacteria were instilled to make collision of bacteria and cell likely), and also in a minority of cells demonstrating no detectable immobilization or H agglutination. Anti-O antibodies were also detected in three ways. Thirty or more bacteria sharing the O antigen, but not the H, with the immunizing strain, were instilled into each drop. In positive droplets, characteristic, bizarre-shaped, motile clumps of O agglutination were often formed, and dividing bacteria during the course of 1 or 2 hours at room temperature, by failing to separate, led to the formation of the motile chains. This chaining phenomenon seemed to be a more sensitive test for anti-O

antibody than O agglutination and was sometimes positive when O agglutination was doubtful or negative. Finally, O adherence, similar to H adherence but generally stronger, was noted in most droplets exhibiting O agglutination or chaining and in some other droplets. It is not clear whether bacterial adherence measures the presence of subthreshold amounts of the same antibody as the other tests or detects the presence of a different sort of antibody. There is, however, much evidence to indicate that it is a specific immunological reaction (Mäkelä and Nossal, 1961a).

Bacterial adherence, especially O adherence, can also be used to detect the presence of antibody-forming cells in mass cell suspensions. A quick method for isolating many single antibody-forming cells depends on O adherence (Mäkelä and Nossal, 1962). To a large drop of a cell

TABLE I
INCIDENCE OF DOUBLY ACTIVE CELLS IN ANIMALS IMMUNIZED WITH 2 TO 4 ANTIGENS (SUMMARY)

Cells	Tested	Total active	Singly active	Doubly active[a]	Actually releasing 2 antibodies[a]
No. of cells	7046	2637	2589	48	2
% Tested	—	37.4	36.7	0.7	0.03
% Active	—	—	98.2	1.8	0.08

[a] Most "doubles" released one antibody and manifested the second reactivity by adherence only.

suspension containing anti-O antibody-forming cells on a coverslip in a de Fonbrune oil chamber is added a large number of motile bacteria sharing the O but not the H antigen of the immunizing strain. As the bacteria swim into the drop and encounter and adhere to antibody-forming cells, they confer a very characteristic motility on the cell. These spinning cells can then be removed, one by one, from the large drop, and studied further in any desired fashion (e.g., as in Section II, C).

To understand the genetic control of antibody formation, we must know how many antibodies a single cell is capable of making at one time. This question has been a major concern in our laboratory for over 3 years. All our experience indicates that cells capable of producing more than one sort of antibody are rare. In Table I, four different studies on this point are summarized (Nossal, 1958, 1960a; Mäkelä and Nossal, 1961b; Nossal and Mäkelä, 1962c). Most experiments dealt with two unrelated mixtures of *Salmonella* flagellins which also contained immunogenic amounts of O antigen, but more recent experiments (Mäkelä and Nossal, 1961b; Nossal and Mäkelä, 1962c) have dealt with single

cells which were tested for two different anti-H antibodies and two different anti-O antibodies, making four assays on each microdroplet. In view of the more complete testing in the more recent experiments, the figures in Table I are minimal both for the active cells and for the doubly active cells. Of 7046 cells tested, 48 or 1.8% of 2637 active cells were doubly active. Furthermore, the reactivities of the doubly active cells from our most recent experiments (Table II) are striking. In most cases, the doubly active cells did not actually *release* two antibodies; rather, they released one and manifested the second reactivity by adherence only. This was true both of cells positive for two different anti-H antibodies or for one anti-H and one anti-O antibody. No cell was positive for two types of anti-O antibody. Moreover, the adherence reaction of these

TABLE II
ANALYSIS OF "DOUBLES"

	Number of cells		
	Expected	Observed	Significance
H–H	14.2	16	Not significant
H–O	6.6	5	
O–O	0.2	0	
Release–release	15.2	2	$P < 0.001$
Release–adherence	10.1	22	
Adherence–adherence	1.7	3	
Strong–strong	27.7	9	$P < 0.001$
Strong–weak	11.1	28	
Weak–weak	1.1	3	

doubly active cells was frequently weak and consisted of the attachment of only one or two bacteria. If we arbitrarily classify immobilization and agglutination, and adherence reactions involving three or more bacteria, as evidence of "strong" antibody-forming capacity, then 83% of singly active cells were "strongly positive." Postulating a random pairing of the reactivities among doubly active cells, one would expect that most "doubles" would also be strongly positive against each of two antigens. The data (Table II) showed a paucity of cells strongly active against two bacterial strains; in fact, most were strongly active against one and weakly active against the second.

To explain these unexpected characteristics of doubly active cells, we have proposed the following three alternatives (Nossal and Mäkelä, 1962c): (1) As two adjacent cells, each making a different single antibody species, are forcibly disassociated *in vitro*, a small fragment of one, invisible under the light microscope but containing enough antibody to

give an adherence reaction, may remain attached to its former neighbor. Thus, an *artifact* having the properties of many of our "doubles" could result. If this proves to be the correct explanation for many of our doubles, then the "one cell–one antibody" rule as originally proposed by Burnet (1957b) might really apply in our system. (2) Some cells may form two antibodies, but not at the same time. Thus, release could reflect present, and adherence past, antibody synthesis. (3) Perhaps cells do form two antibodies simultaneously, but can only form one at the usual rapid rate. Adherence, being a very sensitive test, would then be the only detectable manifestation of minor antibody synthesis. Within this formulation, the synthesis of still smaller amounts of a third or fourth species could not be excluded.

TABLE III
EXPERIMENTAL VARIATIONS INTRODUCED TO RAISE INCIDENCE OF DOUBLY ACTIVE CELLS[a]

Parameter	Range
Age at first injection	< 4 hours to 6 months
Duration of immunization	4 to 76 weeks
Number of injections	2 to 25
Antigen dose	0.001 to 6000 μg.
Immunization order	Simultaneous, mixed, sequential

[a] Effect on incidence of "doubles": nil.

It seemed possible that the immunization schedule used could influence the proportion of doubly active cells in the plasma cell population. To study the kinetics of formation of doubly active cells, we varied the immunization schedule within very wide limits (Table III), but were unable to influence the incidence of doubles in any way. The theoretical implications are discussed below.

2. *Studies on Antiphage Antibodies in Rabbits*

Experiments very similar to ours have been independently carried out by another group (Attardi *et al.*, 1959). Their findings on the production of antiphage antibodies in microdroplets containing rabbit lymph node cells differed in three important respects from our own. First, though doubly active cells were in the minority, they were about ten times as frequent as in our experiments. Second, the doubly active cells showed no tendency to be strongly active against one phage and weakly active against the other. Third, far more of their active cells were classified as small lymphocytes. The reasons for these differences are not clear. If, however, phage adhered to very small amounts of cell-bound antibody, the second and third of the differences would become less striking.

3. *Studies by the Fluorescent Antibody Technique*

The "one cell–one antibody" hypothesis has also been tested by the fluorescent antibody technique (Coons, 1958; White, 1958; Vazquez, 1961) which involves saturating accessible antibody sites on a frozen section of tissue with the homologous antigen, and then staining with a fluorescent dye coupled to the homologous antibody, thereby making an antibody-antigen-antibody "sandwich." When two antigens are used, the two homologous antibodies are prepared in different animals and each is combined with a different fluorescent dye, frequently rhodamine (red) and fluorescein (green). In such preparations some cells "light up" red, others green; none have been found exhibiting mixtures of color. Experiments with cells from animals simultaneously immunized with two antigens support the contention that most antibody-forming cells are specialized for the formation of one antibody only; but it is not clear how many doubly active cells might have been present but not detected by the methods used.

B. Theoretical Implications of Phenotypic Restriction

In this review I have attempted to separate, for didactic purposes, topics which are obviously interrelated and difficult to understand without reference to each other. Clearly, we cannot decide why most cells form only one antibody without considering the original source of the immunological information which makes the formation of an antibody possible. However, in line with my desire to allow the data to speak for themselves, I would still like to postpone detailed consideration of current theories of immunological specificity (Section V, A). For now, let us merely say that there are two conflicting groups of hypotheses, the first stating that immunologically competent cells are "instructed" how to pattern the globulin they make by an antigen template or some replica of it; a tacitly acknowledged by-product of these hypotheses being that any immunologically competent cell is potentially capable of forming any antibody; and the second postulating that the information for the synthesis of any possible globulin is already present in the normal, unstimulated adult animal, possibly in a variety of cell lines or clones. Antigen then would act as a "selective" agent, only accelerating a process of which the body is already capable. Models to explain restriction of a cell's antibody-forming capacity could be constructed on either view. For example, on an instructive hypothesis we could claim that the first molecule of antigen which effectively "hits" a cell completely pre-empts its machinery, making simultaneous formation of a second antibody impossi-

ble; in the same way, a cell preoccupied with the formation of one virus may be resistant to infection with a second; or an ovum after penetration by one sperm is impervious to the entry of another. The occasional double producer might be explained by assuming that occasionally, on a chance basis, two different antigen molecules hit a cell simultaneously. This model clearly predicts that increasing the antigen dosage, thereby making simultaneous double hits more likely, should increase the proportion of doubles. Table III shows that this is not so—that the proportion of doubly active cells is not significantly different when a difference of almost 10^7 in the immunizing dose was used. On the clonal hypothesis, singly active cells should be the rule. We could then explain doubly active cells by assuming that each immunologically competent cell has one gene locus determining antibody specificity and that a cell heterozygous at this locus could form two antibodies. As discussed elsewhere (Nossal and Mäkelä, 1962a,c), the model predicts that repeated immunization, particularly with a low dose, should favor the emergence of doubles. Our experiments failed to produce support for this view (Table III).

We are thus in the quixotic position of being able to explain, in a variety of ways, both the preponderance of singly active cells and a low incidence of doubly active cells, but not the fact that doubles appear to form such a constant proportion of the population. The data incline one to the view that one antibody-forming capacity per cell is the rule; and that doubles are either an artifact, or the result of some sharing of cytoplasm or genetic information (RNA or DNA) between singly active plasma cells proliferating simultaneously in intimate contact with each other.

C. Conclusion

A new issue has been raised by these single-cell experiments. Why are most antibody-forming cells restricted, at least phenotypically, to the formation of one antibody? Subject to the qualification that the synthesis of very small amounts of one or more other antibodies might escape detection, the finding has been substantiated by all workers. Doubly active cells, though rare in our system, apparently do occur; they appear to form large amounts of one antibody and much smaller amounts of a second one; and their numbers are not increased by increasing either the number or the intensity of antigenic stimuli. The experiments do not decide between "instructive" and "selective" theories of antibody formation; but they must be considered in any complete formulation of either theory.

IV. Genotypic Restriction among Members of the Population

In contrast to the detailed knowledge we have of the antibody-producing phenotype, i.e., the immunological potential possessed by a given plasma cell at a given time, we know very little about the cell's genotype. Immunological memory appears to reside in primitive lymphocytes (Section II, B and E), and a given memory cell may be genotypically restricted to having information for the formation of only one or two antibodies. In that case, it would respond to the corresponding antigen(s), and to no other, by plasmacytopoiesis during which all of its progeny would form the same antibody. If the inductive antigenic stimulus is not given, it presumably would give rise to a stem line of cells, each one also being restricted in potential. Alternatively, a primitive lymphocyte may be multi- or even totipotent. In that case, different members of the plasma cell clone derived from it might each form a different antibody, restriction then being purely a phenotypic phenomenon. Unfortunately, no techniques are available as yet for *in vitro* cloning of lymphoid cells, and the knowledge that we have about the potential of primitive cells is mainly inferential.

A. Studies on Replicating Cell Populations

1. Serial Passage of Antibody-Forming Cells

Simonsen (1957) has shown that after the intravenous injection of adult homologous lymphoid cells into a chick embryo, extensive replication of donor cells takes place in the host spleen and elsewhere. In recent studies (Nossal, 1960b; McDonald and Nossal, 1963), we have made use of this finding to study the immunological potentials of cells transferred from immunized rats and mice to a series of "neutral" isologous or homologous hosts (Fig. 3). The experiments which gave the best results (McDonald and Nossal, 1963) involved the transfer of adult, immunized spleen cells to a series of isologous mouse hosts, whose own immunological capacity had been temporarily neutralized by a sublethal dose of whole body X-irradiation. Mice were immunized with a single intravenous injection of a mixture of flagella from two unrelated *Salmonella* species, "AD" and "685." After some weeks, their spleens were removed, were stimulated *in vitro* with only AD flagella, and were then transferred intraperitoneally into isologous mice that had received 700 r the day before. After 4 weeks, the recipients were killed, their sera were titrated for antibody, and their spleens were removed and studied. If some of the donor cells had colonized the host spleens, such transferred daughter cells could then be restimulated *in vitro* and transferred to further irradiated isologous hosts, and so forth. The results of such an

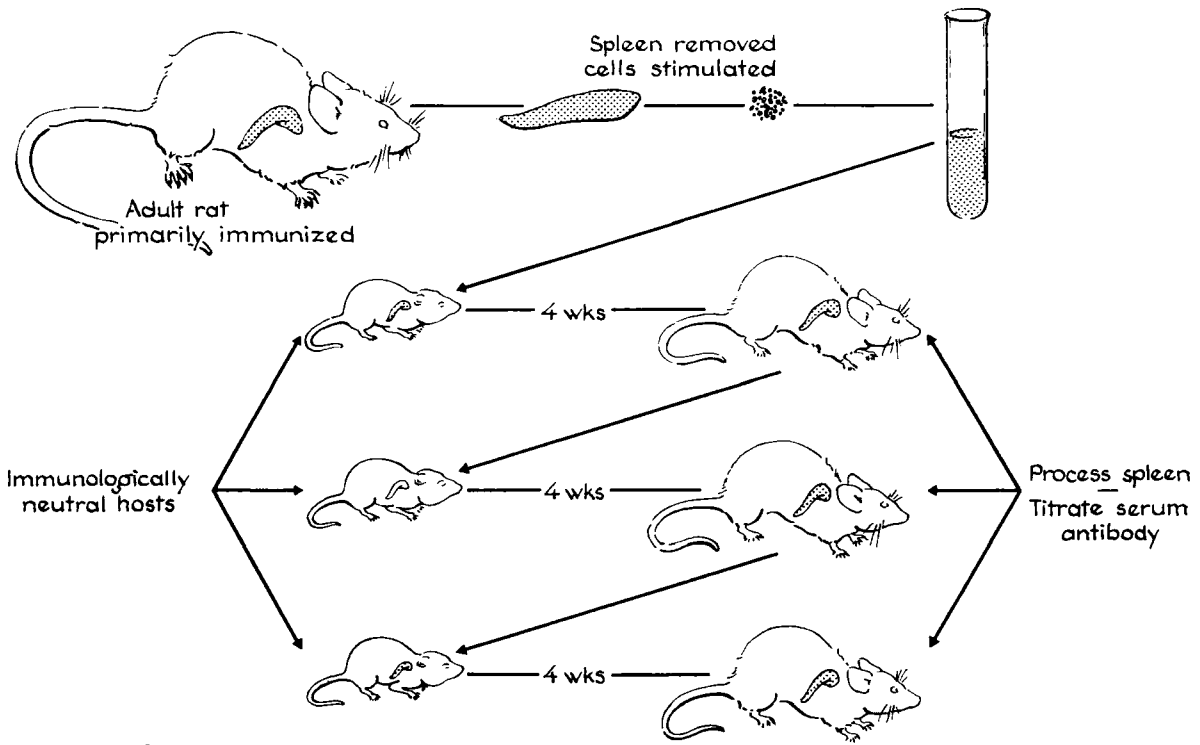

FIG. 3. Scheme for the serial transfer of antibody-forming cells. *In vitro* antigenic stimulation was by a modification of Harris *et al.* (1956) technique. In our best experiments, e.g., Fig. 4, sublethally irradiated isologous mice were used rather than newborn homologous rats.

experiment are given in Fig. 4. On the first passage, the mice made considerable amounts both of anti-AD and of anti-685 antibodies. On the second and third passages with cells stimulated *in vitro* with AD only, the mice formed anti-AD but no anti-685. On the fourth and subsequent passages with cells stimulated *in vitro* with 685 only, the mice formed some anti-685, but it never approached the titer of anti-AD. Thus, contrary to the situation in the whole animal, immunological memory can be maintained through a number of transfers only by repeated stimulation of the cell population with antigen. Of course, to detect antibody-forming potential on repeated transfer, we have to contend with losses resulting from handling the cells, pipetting losses, and probably most important, losses in the host animal owing to the fact that presumably only a few of the cells injected intraperitoneally find their way, settle down, and proliferate in the host spleen. It is, therefore, not surprising that a specific proliferative stimulus is needed at each passage. In the experiment cited, antibody-forming potential was maintained for a series of ten monthly passages. Despite repeated stimulation with 685 after the third passage, the population, while retaining high anti-AD capacity, never developed equal anti-685 capacity. This suggests that the memory population was genotypically restricted. If one postulates that a given large lymphocyte from the original donor spleen was multipotent, carrying memory both for AD and for 685, one could explain the absence of anti-685 at the second and third passage by the lack of an inductive stimulus; but the results of subsequent passages would be difficult to explain. On the other hand, if the original donor contained two different populations of memory cells, each carrying the information for one of the antibodies, one could postulate that only those cells specifically stimulated *in vitro* would replicate to a sufficient extent for their progeny to be detectable subsequently in the host spleen. Thus, if the 685 stimulus were omitted in the first three passages, the 685 memory large lymphocytes would rapidly become diluted out on passage, and antibody formation would be minimal even after subsequent repeated reintroduction of the 685 stimulus.

One important criticism of this formulation was pointed out by Dr. N. A. Mitchison (personal communication). The experiment "works" only when antigen is presented *in vitro* at each transfer (Nossal, 1960b) and does not work when antigen is omitted even once. The cell population may be "so busy" forming one antibody that it will not form the second one even though it possesses the capacity until the pressure to form the first is relaxed. Accordingly we set up an experiment in which the donors were immunized with AD and 685 as before and were then stimulated

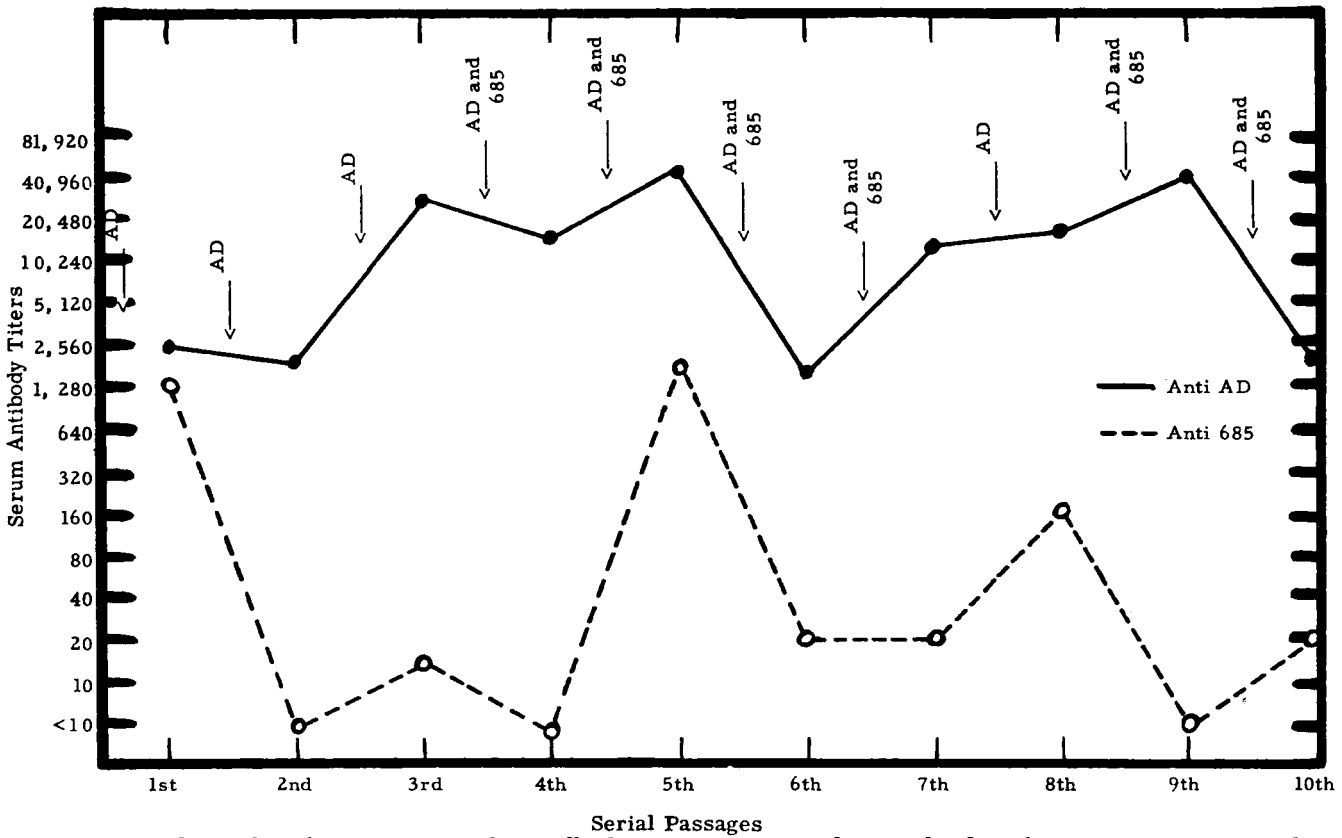

FIG. 4. Serial transfer of C3H mouse spleen cells from animals primarily stimulated with two antigens. Note that following the *in vitro* omission of 685 antigen, the cells continue to form anti-AD antibody only; when 685 stimulation is recommenced, only partial reactivity returns. Various groups of controls were included and demonstrated an absence of host contribution; these are not shown.

in vitro with AD for two passages and with 685 for two subsequent passages. The cells formed no significant antibody in the latter two passages and had evidently lost the anti-685 memory in the early passages. Under these highly artificial circumstances we seem to be maintaining a population committed to the formation of one antibody.

These experiments, involving the transfer of large heterogeneous populations of cells cannot lead to very firm conclusions, particularly as many unknown factors could lead to a negative result. They merely represent the best we can do until more precise methods for analyzing the progeny of immunologically competent cells become available and are consistent with the notion of a genotypic restriction of memory cells.

2. Serial Passage of Cells Mediating Graft-versus-Host Reactions

Simonsen's (1957) original experiments with serial transfer of fowl leucocytes, on which our studies were based, are relevant here. He took peripheral blood from an adult fowl, injected it into a homologous embryo, and some days later noted that the host, now a newly hatched chick, was suffering from severe graft-versus-host reactions. When the blood of the first host was injected into further homologous embryos, the sequence of events was repeated, and so on for nine serial passages. As the population of fowls had not been systematically inbred, it is not clear whether the original population of donor leucocytes was reacting repeatedly to antigens shared between the various hosts but not present in the original donor, or whether it was being induced anew to react to different host antigens at each passage. Isacson (1959) extended the observations to even younger embryos than those used by Simonsen. Following the homologous transfer of adult lymphocytes, he noted the development of discrete foci in host spleens which enlarged for about 5 days and then regressed. The foci appear to consist of a mixture of donor and host cells. Using splenomegaly as an index, the intensity of the reaction in Isacson's hands diminished rapidly after three serial transfers.

Further evidence that the splenic enlargement and hemolytic anemia noted by Simonsen (1957) were manifestations of a graft-versus-host reaction was provided by Cock and Simonsen (1958). They used chickens which had been bred by full-sib matings for nineteen or more generations and were considered to be antigenically pure "for most practical purposes." Parental leucocytes injected into an F_1 hybrid between two strains caused a severe reaction; F_1 cells injected into the parental strain caused little or no reaction. Following the discovery by Boyer (1960) that foci similar to the ones observed by Isacson (1959) could

be produced on the chorioallantoic membrane of chick embryos, Burnet and co-workers have extensively studied the reactivities of fowl leucocytes against homologous antigens (Burnet and Boyer, 1960; Burnet and Burnet, 1960). Using two fairly highly inbred lines of White Leghorn fowls, they showed that homologous transfer of adult leucocytes to the chorioallantoic membrane of 12-day embryos produced about one proliferative (graft-versus-host) focus per 20,000 leucocytes; isologous transfer produced fewer foci. In fact, the degree to which transfers within a line were capable of producing foci could be used as a measure of persisting heterozygosis within that line (Burnet and Burnet, 1960). Of special interest to us were the results of serial transfer of leucocytes (Burnet and Boyer, 1960). When leucocytes were homologously transferred, either via the chorioallantoic or the intravenous route, nodular lesions developed in the spleen and elsewhere, and a cell suspension from such a host spleen when inoculated on the chorioallantoic of further chick embryos produced proliferative foci macroscopically indistinguishable from the ones produced by adult lymphocytes. A remarkable change in the specificity of the proliferative ability of the donor cells had taken place during the first passage, however. Whereas usually adult leucocytes transferred isologously produced few foci, leucocytes that had been passaged once homologously became capable of producing many foci even in embryos of the original donor strain. The authors believed that this loss of specificity represented the first step in a series of changes by which the initially competent cells lose their capacity to produce graft-versus-host reactions and that one of the next steps was apparently a loss of the capacity to grow at all on further serial passage. In contrast to the results originally reported by Simonsen (1957), Burnet and Boyer were unable to maintain the proliferative capacity of homologously transferred spleen cells for more than three to five passages.

While these studies have many interesting implications, the demonstration that a heterogeneity of cell potential exists among fowl leucocytes is most relevant to our discussion. Of every 20,000 cells, only one can react to the presence of homologous antigens by focal proliferation. Terasaki (1959) had previously arrived at a very similar figure. This diversity could reflect genetic heterogeneity of cell potential or it could merely be a physiological difference. It has not yet been reported whether the proliferating cells are primitive lymphocytes or mature (small) lymphocytes. If the cells which initiate the foci are, indeed, large lymphocytes, this first-set homograft reaction would seem to have all the characteristics that we predicted for a primary response to *Salmonella* antigens (Section II, B).

Serial transfer of cell populations capable of mediating homograft reactions in mice has recently been reported by Siskind *et al.* (1960). When adult CBA spleen cells were injected into newborn A/Jax mice, runting resulted. When spleen cells of some of the runts that had survived to 8 weeks were transferred to further newborn A/Jax mice, a higher and earlier incidence of runt disease, reminiscent of a secondary response reaction, occurred. Whether the phenomenon was specific for the A/Jax strain was not reported. Simonsen (1960) noted quite a different sequence of events on serial transfer of spleen cells. When A × B F_1 hybrid adult spleen cells from two isogenic mouse strains, A and B, were transferred to an adult A animal, the host spleen cells developed "productive sensitization" against the B antigen; that is, when the host cells were transferred to infant A × B hybrids, they caused a more profound graft-versus-host reaction than normal A spleen cells. By contrast, when adult A cells were transferred to infant A × B hybrids, the persisting presence of large amounts of B antigens apparently caused "exhaustive sensitization" of the adult A cells. Far from causing a second-set response on further transfer to infant A × B hybrids, they failed to cause any graft-versus-host reaction, though still retaining reactivity against an unrelated antigen, C. The explanation of the differences between these two groups of experiments is not at hand.

3. *Conclusions*

I have considered these homograft reactions in such detail to point out the many parallels between the induction of a homograft reaction and of antibody formation. In both cases: (1) a heterogeneity of inducibility exists within the cell population mediating the reaction; (2) a small proportion of cells undergo clonal expansion; (3) the proliferative burst is self-limited in that it reaches a maximum within some days and then ceases; and (4) an alteration of the reactivity of the population following antigenic stimulation usually involves "productive" but occasionally involves "exhaustive" sensitization. The analogy could be completed did we but know (5) the nature of the effector cell in homograft reactions and (6) its phenotypic and genotypic immunological potentials.

B. The Degree of Cell Commitment

We have seen that plasma cells, the effector cells in antibody formation, are relatively short lived, do not appear to divide, and at any time each usually forms only one antibody. It seems reasonable to postulate that a given plasma cell is committed, at least from the time of the last mitosis in its ancestral line, to the formation of a given antibody. Small

lymphocytes, though longer lived, also seem to be end cells with little likelihood of being able to react to further stimuli. They may be the effector cells of different types of immune responses, such as delayed hypersensitivity and homograft rejection, although it is difficult to speculate about their mode of action. They may also be committed, from an early state, to the performance of a given task. At least a proportion of the primitive lymphocytes in the body appear to be specialized in some way—presumably they carry the memory of an immune response that the animal has undergone at some previous time. Though the evidence is not decisive, I would incline to the view that cells carrying a given memory are also committed in that they can be induced only by the corresponding antigen and not by any others. This would give a plausible explanation for the heterogeneity of the responses of a cell population to a given antigen. Thus, more than 99% of the lymphoid cells present in an animal may be committed as the result of immunological experience. One may well ask, which are the cells capable of responding to new, or apparently new, antigenic stimuli?

The notion of cell commitment forms an important part of Szilard's (1960) theory of antibody formation. Szilard postulated that antigenic stimulation may "lock" a cell in a stationary steady state in which it forms large amounts of antibody but is "insensitive" to other antigens. I would like to extend the concept of cell commitment to cover all circumstances, genetic and environmental, which destine a cell to proceed along an irreversible pathway of differentiation and specialized function. For example, the *absence* of a given antigenic stimulus may well be the circumstance which decides that a cell should become a small lymphocyte (Section II, B).

V. The Ultimate Source of the Population

A. The Source of Immunological Information

We can now no longer postpone considering the source of immunological information. Antibody is a highly specialized protein, made as the result of an interaction between antigen and immunologically competent cells. Although the evidence is fragmentary, especially in mammalian systems, it is generally supposed that proteins are made according to the specifications of the corresponding section of the genetic material, DNA (Crick, 1958).

This "central dogma" is far from proven. Lederberg (1959) has formally dissected the following various ways in which antibody specificity could be generated:

1. If antibody behaves like other proteins that have been extensively studied, e.g., ribonuclease or insulin, then the sequence of amino acids in the globulin polypeptide chain will chiefly or solely determine specificity; the way in which the molecule folds would be dependent on the primary structure, and thus, in the last analysis, on the sequence of nucleotides in the genetic materials, RNA and DNA.

2. The sequence of amino acids may be irrelevant in determining tertiary structure, and combining specificity may be determined solely by different patterns of pairing of half-cysteine residues. Thus, Karush (1961) using a number of plausible assumptions, has calculated that variable pairing could account for some millions of different types of globulin molecules.

If we accept the first view, we must either assume that the information for antibody synthesis is a built-in property of the cell and that antigen merely acts as a "selective" agent or adopt the rather heretical notion that antigenic stimulation creates the formation of a new sort of genetic structure within the cell. If we accept the second alternative, we would adopt the more familiar notion that antigen "instructs" the cell to fold globulin in a special way by some template mechanism (Haurowitz, 1953). Many theories of antibody formation have been proposed in recent years (Pauling, 1940; Mudd, 1932; Haurowitz, 1953; Karush, 1961; Jerne, 1955, 1960; Burnet and Fenner, 1950; Schweet and Owen, 1957; Burnet, 1957a,b, 1959; Talmage, 1957; Lederberg, 1958, 1959; Boyden, 1960; Monod, 1959; Szilard, 1960; and Gorman, 1961).[2] To arbitrate between them would require far more wisdom (and tact!) than the author possesses. Phenotypic and genotypic restriction of cellular antibody-forming capacity and a stem line of rapidly dividing specialized cells as the seat of memory are the key concepts we have developed here. These are readily fitted into the clonal selection model of Burnet (1959). One could postulate that the population of primitive inducible cells is diversified into a large number of types with each cell carrying one or two alleles for antibody specificity and that antigen induces the selective proliferation of cells expressing the corresponding allele. The secondary response need not be different in principle from the primary; it could

[2] Gorman has conceived of a novel modification of Burnet's clonal selection hypothesis, which to my knowledge is still unpublished. It depends on the existence of 2 sorts of clones corresponding to each antigenic determinant, namely immunologically competent and immunologically incompetent; the 2 varieties each respond to antigenic stimulation by proliferation, and a competitive situation is postulated to exist between them. Gorman explains on his model many of the more obscure facets of immunology, such as those considered in our Section VII.

be explained by assuming a larger starting population of reactive primitive cells. The high rate of cell division in the stem line would not endanger the integrity of memory since the antibody-producing capacity of a cell would be genetically determined. The observed phenotypic and genotypic restriction of capacity would be a *sine qua non* of the model. However, subcellular selective models, in which the self-replicating unit is not the cell but some fraction of it (e.g., a ribosome) would also fit the data, at least in part. Thus, every cell could have every gene for the synthesis of any conceivable globulin, provided the number of types is not much larger than 10^4. Heterogeneity of cell response could then be explained by postulating that the chance of a given cell being *effectively* hit by antigen is small (Monod, 1959); phenotypic restriction by some pre-emptive or locking mechanism (Szilard, 1960); apparent genotypic restriction by an epinucleic type of inheritance (Monod, 1959; Szilard, 1960; Lederberg, 1959). Two facets are hard to explain, however: (1) the number of doubly active cells is not increased by increasing the dose of a mixed antigen; and (2) immunological memory appears to be maintained for more generations (>500) than would seem plausible for cytoplasmic inheritance. The genetic phenomena which form the bulk of this review are difficult to explain on Jerne's (1955, 1960) or Boyden's (1960) theories, which do not stress cell heterogeneity; or on the direct template hypotheses, which explain antibody specificity but not the other characteristic features of immune responses. However, Jerne (1960) points out in his scholarly review that "no general theory can possibly account, at present, for all the innumerable complexities of antibody formation." Whether the ultimate source of immunological information is in the genome or is generated by an interaction between cell and antigen has not been clearly established.

B. The Source of Primitive Cells as the Key

If our postulate that all immune responses involve the induction of certain primitive cells, with subsequent clonal expansion and concomitant functional differentiation, is essentially correct, then the origin and life history of primitive cells becomes a most important problem. This is true whether we adopt a clonal selection model, in which *all* cells are considered to be committed, or other models, in which primitive cells could be regarded as multipotent and uncommitted until induced by antigen. A relationship between large lymphocytes proliferating in germinal centers and plasma cells developing in the medullary cords of lymphatic tissues has long been suspected (Rebuck *et al.*, 1958, cite many references). Lymphopoiesis, as well as plasmacytopoiesis, is a part of most

immune responses, and on purely histological grounds, it is difficult to decide whether two independent processes are going on *pari passu,* or whether the germinal centers are actually feeding primitive cells into the medullary cords which then can differentiate morphologically into plasma cells. Immature plasma cells frequently congregate fairly close to lymphoid follicles (Thorbecke and Keuning, 1956), and occasional sections show the center of a secondary nodule in intimate contact with proliferating plasma cells (Thorbecke, 1960). Such findings suggest a "spilling over" of primitive, germinal center lymphocytes into the medullary areas where differentiation into plasma cells occurs. The morphological sequence of events in immunized tissues suggests to Congdon (1961) that the ultimate source of plasma cells is the germinal-center large lymphocyte. This idea which implies that large lymphocytes are bipotent in that they are capable of differentiating either into the plasma cell or into the small lymphocyte, would fit our data very well. In addition, there are probably some primitive cells residing permanently outside the follicles. However, our question is still not fully answered. From where do the primitive, germinal center and other blast cells arise?

Here we must ask two questions of a controversial nature. (1) Are mature cells capable of reversing their life history and turning back into blasts? (2) To what extent do primitive cells, or cells potentially capable of transforming themselves into primitive cells, migrate around the body? Yoffey (1957) has long claimed that the high incidence in bone marrow of cells resembling small lymphocytes indicates that they may change locally into multipotent blasts. I repeat from Section II, E that there is nothing in our data to contradict the notion that the small lymphocyte has the potential of turning itself spontaneously back into a blast. All we can claim (Nossal and Mäkelä, 1962b) is that, if such a conversion takes place, it must be intermittent, spontaneous, and not influenced in any way by secondary antigenic stimulation. We can exclude the *recruitment* of small lymphocytes by antigentic stimulation into plasmacytopoiesis; our data have no relevance to the question of whether immunological memory might be transferred into small lymphocytes and spontaneously resurrected into a usable form by conversion back into a large lymphocyte. All one can say is that this alternative model seems clumsier than the one we have proposed.

On the second question, we can cite more concrete data. Primitive lymphocytes undoubtedly appear in the thoracic duct lymph (Schooley and Berman, 1960) and in peripheral blood (Hughes *et al.,* 1958), and subsequently migrate back into lymphoid tissue and elsewhere (Gowans, 1959; Bond, 1959). In fact, cutting off this back migration soon leads to

considerable lymphopenia (Gowans, 1959). Metcalf and Nakamura (1961; Nakamura and Metcalf, 1961) distinguish between recirculation, the mere flow of lymphoid cells through tissues, and redistribution, an actual tendency for lymphoid cells to be formed by one organ and transferred to another. On the basis of careful study of mitotic indices in various lymphoid tissues, they have speculated that certain organs, notably the thymus, may be chronic exporters of lymphocytes; and others, particularly peripheral lymph nodes, chronic importers. In the latter location, the mitotic rate is insufficient to maintain the *status quo*, and an excess inflow over outflow of cells is needed.

It is well known that little antibody formation takes place in the thymus, and it is tempting to postulate that the thymus is the body's largest factory of immunological potential for producing either many different patterns (Burnet, 1959) of primitive cells or many uncommitted primitive cells. Lymph nodes and lymphoid accumulations in the spleen may then represent areas in which this potential can reach expression by antigenic induction, by cell replication and differentiation, and by the setting up of committed memory stem lines. A further possibility may be a specific chemotactic effect of antigen on cells capable of responding, and net immigration of cells into lymph nodes draining the site of a secondary antigen injection may occur (Nossal and Mäkelä, 1962b).

While the present article was in preparation, the extremely important work of Miller (1961) was published. Miller has shown that thymectomy performed in certain strains of mice within the first 24 hours of life leads to profound lymphoid atrophy and that such thymectomized mice will frequently accept skin grafts from homologous mice or even from rats. These findings provide experimental evidence for the speculations contained in the last section.

C. Studies on Germ-Free Animals

We have argued that a large proportion of an adult animal's lymphoid tissue consists of committed cells, called into existence by the many antigenic insults which each animal suffers in its daily life. It would be most helpful if we could remove all such cells, and study only the skeleton population of cells still awaiting induction. Theoretically, such a situation might pertain in germ-free vertebrates; but, on the whole, studies of the germ-free animal (Reyniers *et al.*, 1959) have been disappointing. It is true that, as compared to normally reared animals, germ-free animals possess, in general, somewhat less lymphatic tissue (Miyakawa, 1959), fewer secondary lymphatic nodules, and less plasmacytopoiesis (Thorbecke, 1959), less "natural antibody" (Wagner, 1955;

Springer *et al.*, 1959) and less γ-globulin (Wostmann, 1959), but these differences are far from "all-or-none" – presumably because even the germ-free animal encounters antigenic molecules in the diet or via dust constituents in the air. The results, however, as far as they go, are consistent with our thesis. The thymus appears to be normally developed, and lymph nodes are atrophic (Gordon, 1959). Of special interest is the fact that germ-free animals respond to antigenic stimulation with normal plasmacytopoiesis and antibody formation (Thorbecke *et al.*, 1957; Wagner, 1955, 1959). This fits in well with our ideas of cell commitment. If immunologically competent cells were multipotent, an animal having little lymphatic tissue should respond less well to antigen than an animal having a lot. If the excess lymphatic tissue of the normal animal mainly represents cells committed to one pathway or another, then the germ-free animal, possessing the same inherent genetic capacity, would respond as well as the normal one. The only case in which the normal animal might have an advantage would be when faced by the investigator with an antigen identical or related to one which it had encountered naturally.

VI. The Problem of Induction

Although I have mentioned the induction of plasmacytopoiesis several times, I think that the topic is of sufficient importance to warrant more detailed consideration. Antibody formation has frequently been likened to adaptive enzyme formation (Burnet, 1957a; Monod, 1959). About the induction of the latter process we know much; of the former, less. In this section I wish to highlight some important differences and attempt to summarize the knowledge we have gained through our autoradiographic studies (Nossal and Mäkelä, 1961a, 1962b; Mäkelä and Nossal, 1962).

I will confine myself to a single concrete model–the secondary response to a bacterial antigen. In Section II, B, we concluded that some primitive cells arising in lymph nodes during primary stimulation are specifically adapted to react to a secondary stimulation. Let us assume that these cells have the information for antibody synthesis without specifying the mechanism. How are they induced to express this potentiality as massive protein synthesis?

1. Do these primitive memory cells synthesize any antibody in the resting state? In our resting, primarily immunized nodes, we found very few cells making detectable antibody, and those few were not blasts (Mäkelä and Nossal, 1962). Of course, failure to detect antibody formation does not mean that none is occurring. However, resting cells obvi-

ously do *not* form antibody at the high rate characteristic of the fully induced plasma cell as evidenced by histological appearance and ultrastructure and proven by microdrop experiments. Therefore, induction cannot solely be explained by cell multiplication and must involve at least accelerated, if not *de novo,* synthesis by the cells involved.

2. What becomes of the memory cells in the absence of induction? Evidently, if no further antigen is presented, they still divide every 12 hours or so, but half the progeny are lost from the memory pool through differentiation or death. In other words, spontaneous phenotypic loss of inducibility seems to occur in a sufficient proportion of the population to ensure a total number of inducible cells which changes little over many months. If inducibility were determined by the presence of a cytoplasmic entity, such as antigen or an antigen permease (Monod, 1959), we would have to postulate uneven distribution of the entities among daughter cells; otherwise memory would be lost after a comparatively small number of generations. If inducibility is genetically determined, its loss by some daughter cells would have to be explained by the development of repressors. Certainly detailed genetic models are difficult to construct, and the galactoside–permease model does not present close parallels.

3. What happens to the memory cells when antigen is again presented? The first important change is a removal of the condition which prevents enlargement of the pool of primitive cells: the total number of blasts increases and, concomitantly, a series of changes in the cell's machinery is induced. A large population of ribosomes and an active endoplasmic reticulum are generated, which are obviously the cytoplasmic factory for massive protein synthesis and export. Finally, the cell, apparently as a specific end result of the process of induction, loses the capacity to divide. Figure 5, which summarizes our results in relation to this question (Nossal and Mäkelä, 1961a, 1962b; Mäkelä and Nossal, 1962), indicates how a single, large lymphocyte gives rise to a clone of some hundreds of plasma cells. The induction of a memory cell may thus be described by a sequence involving self-limited clonal expansion, formation of the antibody-forming factory, and death of most of the progeny.

It is unnecessary to stress the fact that antibody formation represents a more complex problem than that of enzyme induction in bacteria, but one interesting difference should be clarified. Induction of an enzyme such as β-galactosidase in *Escherichia coli* (Cohn, 1957; Cohen and Monod, 1957) involves no change in cell multiplication—bacteria continue in log phase; whereas the induction of antibody formation involves a striking and profound change in the proliferative pattern of the cells being induced. Thus, theories which attempt to explain immune induc-

tion will have to concern themselves not only with changes in RNA, but with altered patterns of DNA synthesis. It will never be enough to delineate the processes occurring within any one cell during one generation. An understanding of the whole process will require knowing what makes clonal expansion start and stop so suddenly.

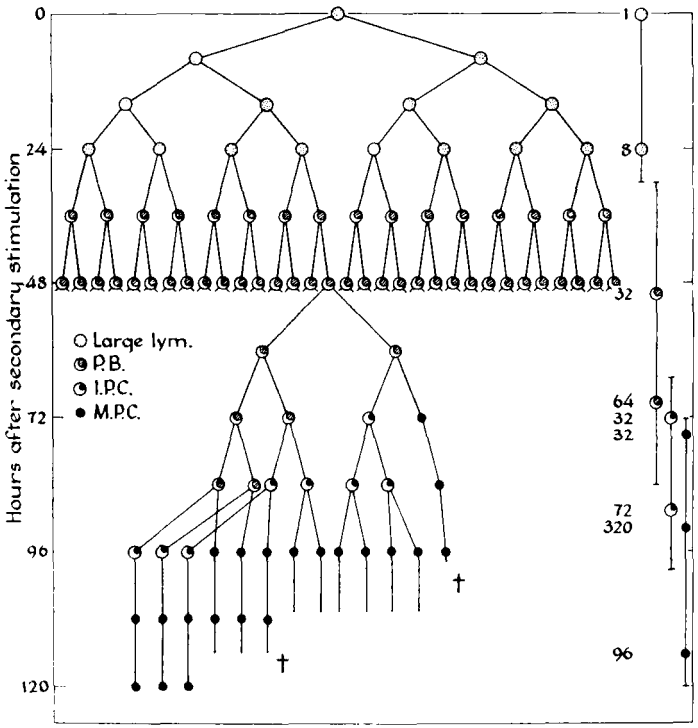

FIG. 5. Schematic representation of the development of an antibody-forming clone. Forks indicate mitoses; continuous lines, persistence without mitosis. The occasional primitive cell, which presumably is responsible for continuing memory, is omitted. PB = plasmablast; IPC = immature plasma cell; MPC = mature plasma cell. Reproduced from Mäkelä and Nossal (1962).

One intriguing feature of the antibody induction process has so far defied detailed interpretation. The process of induction can be largely inhibited by whole-body irradiation of the animal 1 or 2 days before the injection of antigen. If conditions of partial inhibition are carefully chosen, some degree of restoration of the immune response may be achieved by a variety of materials, such as yeast extract, partially depolymerized nucleic acid derivatives, kinetin, 3-indole acetic acid, and ribonuclease (Taliaferro and Jaroslow, 1960). These and related results

suggested to the authors that one of the limiting factors for the induction of antibody formation in X-irradiated (and even in normal) animals is the concentration of certain nucleic acid derivatives in the antibody-forming tissues. It is by no means certain whether the restorative action of such derivatives is owing to the provision of more building blocks for DNA and RNA synthesis or to some more obscure mechanism.

VII. Immunological Tolerance

Although the key facts of immunological tolerance have been recently reviewed in this series (Hašek *et al.*, 1961) and elsewhere (Burnet, 1957a; Burnet, 1959; Brent, 1960; Hašek and Lengerová, 1962), no review dealing with the cellular genetics of immune response could be complete without some reference to the problem.

Acquired immunological tolerance signifies the specific partial or complete loss of responsiveness to a given antigen which is induced by perinatal exposure of an animal to that antigen. The treatment must result in a reduction or loss of cells inducible by that antigen, or a reduced effectiveness of induction in each cell, or both. The clonal model (Burnet, 1959; Lederberg, 1959) states that cells having the genetic capacity to react to an antigen can be eliminated or, more likely, suppressed by perinatal exposure of the immature cell population to that antigen. This results, in Jerne's terms (1960), in a "purged dictionary" of inducible clones. The return of reactivity after the antigen has been metabolized or otherwise eliminated (Smith and Bridges, 1958; Mitchison, 1959; Trentin and Sessions, 1961) could be explained by a depression of clones held in an unresponsive state by persisting antigen or by a mutation of cells from noninducibility to inducibility (see following). Autotolerance, Burnet's axiom of "self-recognition," would result from the elimination or suppression of all clones reactive to idiotopes ("self"-antigenic determinants) and the presence of clones reactive to all possible xenotopes (foreign determinants).

If primitive, noninduced cells are multipotent, it is more difficult to construct tidy models of tolerance. Subcellular selective and instructive hypotheses would require, respectively, specific repressors for, or the actual presence of, *each* idiotope in *each* cell. Both ideas evoke the rather unattractive picture of a cell packed with a lot of inhibitory entities to permit reactivity to xenotopes but not to idiotopes. That the idea seems clumsy to us does not invalidate it. Substantial repression of gene action and other types of blockage of potential synthetic pathways may be an essential part of normal cell function.

It is becoming clear that there is nothing unique or magical about the immunological response of the perinatal animal. Autotolerance and experimental tolerance with continued booster injections of antigen are generally permanent. To Lederberg (1958, 1959), this suggested that, on theoretical grounds, immunological depression must occasionally occur even in adult animals. On the clonal model, it seems likely that mutations in reactivity must take place in adult life, particularly as they would tend to guard against the total loss of immunological information by random drift or contraselection. On nonclonal models, it seems equally likely that some slipup of the mechanism maintaining tolerance must occur at some time in some cell. If the unerring response of such an aberrant cell to an idiotope were that of antibody production, then autotolerance would eventually break down.

Much work backs up this theoretical viewpoint. Felton's (1949) early experiments showed that comparatively small doses of pneumococcal polysaccharide, an antigen which tended to persist for a long time, could produce nonreactivity (paralysis) in adult animals. Dixon and Maurer (1955) noted that temporary suppression of antibody formation could be obtained in adult rabbits by infusing large quantities of bovine serum albumin for long periods. Sercarz and Coons (1959) have presented evidence that a real central depression of cellular reactivity may be concerned in such nonresponsiveness. Simonsen (1960) introduced a new concept which I believe to be of great importance, namely, exhaustive sensitization (Section IV, A). His experiments suggested that under some circumstances, adult cells confronted with unlimited amounts of antigen could mediate an immune response, but if antigenic stimulation persisted, they would enter an exhausted, nonreactive phase. At least three other groups of findings fit in: (1) animals recovered from runt disease soon pick up and look quite healthy; (2) long-lived radiation chimeras appear to exhibit mutual tolerance between donor and host, though both were adult at the time of making the chimera (Cole and Davis, 1961); and (3) adult mice can sometimes be rendered tolerant to H-2 compatible homografts by prolonged parabiosis or repeated massive injections of homologous living spleen cells (Mariani *et al.*, 1960).

The circumstances required for the induction of nonreactivity in adults seem to be a prolonged availability to all immunologically competent cells of a fairly weak antigen. I have had to modify our previous (Nossal and Mäkelä, 1961b) explanation for this in the light of our finding (Nossal and Mäkelä, 1962b) that small lymphocytes seem to play no role in memory and following many fruitful discussions with Dr. N. A. Mitchison. On the genetic model we have developed here, nonreactivity in adults

could occur as follows. Antigen induces all the large lymphocytes capable of reacting; a self-limited burst of plasmacytopoiesis follows, which usually ensures the removal of most of the antigen; in the absence of further antigen, a new, and eventually an enlarged population of inducible cells is built up. However, if antigen is present all over the body in unlimited amounts even after the original induction, the buildup of reactive cells (Sercarz and Coons, 1960) might be inhibited. It is clear that there is a large gap in our knowledge concerning the mechanisms whereby our memory stem line is created originally and recreated following induction. Until it is filled, more detailed speculation seems pointless.

It is often stated that the facts of immunological tolerance provide the main justification for a clonal selection hypothesis. This may be true, but I hope I have shown that there are many other facets of immunity which suggest some degree of clonal individuation, at least of induced cells and their progeny.

VIII. Conclusions

I tentatively conclude the following:

1. There is a heterogeneity of responsiveness to a given antigen among lymphoid cells; this is owing either to genotypic or to phenotypic variation within the population.

2. An immune response is induced by stimulating large lymphocytes, which have not been forming much antibody, to undergo clonal expansion and concomitant differentiation to the plasma cell form.

3. The inducible cells found after primary immunization are members of a stem line and carry immunological information; they divide rapidly even in the absence of antigenic stimulation, but in such a fashion as not to increase in numbers.

4. The burst of plasmacytopoiesis following a single inductive stimulus is self-limited and involves the creation of some hundreds of plasma cells from a single memory cell.

5. These differentiating cells begin to form a little antibody while still dividing, but reach peak productive capacity when no longer capable of mitosis.

6. Antibody-forming cells are usually typical plasma cells; plasma cells usually or always form antibody.

7. Each plasma cell produces antibody for a few days and then either dies or assumes a different morphology and function.

8. Most plasma cells form one antibody at one time.

9. Most memory cells carry information for the synthesis of one antibody only.

10. The great bulk of lymphoid tissue in an adult consists of cells either mediating a current immune response or carrying the memory of a past one, and are, thereby, committed.

I also venture the following frank speculations:

1. Our experiments fit better (a) the selective than the instructive theories of immunity and (b) the cellular rather than the subcellular selective models.

2. Cells simultaneously producing two antibodies will be found to be genetic mosaics, transformants, transductants, or artifacts.

3. The cellular mechanisms involved in perinatal tolerance and in adult paralysis will be found to be identical, and the chief difference in the perinatal animal depends upon the absence of an environment favoring induction.

4. The basic principle that antigen induces clonal proliferation and differentiation in large lymphocytes will describe both the primary and the secondary response, and both antibody formation and homograft phenomena.

5. Study of induction will soon establish a nucleic acid source of immunological memory.

6. Real advances in immunology in the next decade will be made by immunochemists aware of the importance of biological concepts, such as the cellular genetics of immune responses, and by biologists willing to come to grips with the molecular implications of their findings.

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Antibody Production by Transferred Cells[1]

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One of the most useful tools for the study of antibody production has been the technique in which cells that have the capacity to form antibody are transferred from one animal to another. With this technique, immunologically competent lymphoid cells are removed from one animal and their immunologic function studied after their transfer to another animal. Thus, one observes antibody production by an isolated group of cells and presumably excludes participation by cells or tissues other than those transferred since the response of the recipient may be blocked. In this way investigators have been able to study, among other things,

[1] Publication No. 7 of the Division of Experimental Pathology, Scripps Clinic and Research Foundation, La Jolla, California.

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the sources and the types of cells capable of antibody formation; the ability of various antigens to stimulate such cells; the quantity and quality of antibody produced by certain cells; and the temporal aspects of the immune response. Questions concerning cell division during antibody formation and the precursors of antibody-producing cells have been and are being probed; certain situations resulting in a suppression of antibody formation have been analyzed through the use of cell-transfer techniques; and the question of homograft rejection has been approached by these methods. Finally, through the use of transferred cells, information has been collected regarding the actual mechanisms by which antibody is synthesized by cells.

I. Consideration of the Transfer System

Antigens Employed and Methods of Antibody Detection

The earliest workers employing the techniques of transferring cells used bacterial antigens and agglutination techniques (Deutsch, 1899; Luckhardt and Becht, 1911; and Topley, 1930). Advantage was taken of the sensitivity of the bacterial agglutination techniques by S. and T. N. Harris (1951) and S. Harris *et al.* (1954a,b) who stimulated donor rabbits with foot-pad injections of *Shigella paradysenteriae* or *Salmonella typhosa* and after isolating regional lymph nodes and transferring them to recipient rabbits, detected a rapid appearance of agglutinins in the serum. Bacterial antigens have been used extensively by others, e.g., see Sterzl (1958a).

Chase (1951 and 1952) sensitized guinea pigs with simple chemical compounds, such as picryl chloride or *o*-chlorobenzoyl chloride, and found that after transfer their lymph node or spleen cells bestowed anaphylactic sensitivity on recipients.

Heterologous red blood cells were also found to stimulate donor lymphoid cells to produce hemolysins after transfer to recipients (Chase, 1951). Red cell antigens have subsequently enjoyed wide usage because of their strong antigenicity and the sensitivity of antibody detection methods.

The transfer of the capacity to produce antibody against the PR8 strain of the influenza A virus has also been accomplished (S. Harris *et al.*, 1954a). Antibody in the recipients was detected by inhibition of viral agglutination of chicken erythrocytes.

Purified serum protein antigens were employed by J. C. Roberts and Dixon (1955), using previously sensitized donor rabbits, and advantage was taken of two methods to detect the presence of antibody formation

in recipients of the transferred cells. Iodine^{131}-labeled protein antigens were injected intravenously into recipient rabbits immediately after transfer of lymph node cells taken from previously sensitized donors, and the disappearance of the circulating antigen in the recipient was then followed. Recipients supporting antibody synthesis by the transferred cells demonstrated first a sudden rapid elimination of the circulating antigen. Then, after 3 days, the level of free antibody rose to a point where it could frequently be measured by precipitation techniques. This method allowed the measurement of the weight of antibody produced in the recipient and studies of certain qualitative properties of the antibody.

Serum protein and chicken ovalbumin antigens were used by Rosenberg *et al.* (1958) to sensitize guinea pigs, and the lymphoid cells were then transferred intracutaneously into normal recipients. Antibody production was measured by the passive cutaneous anaphylaxis test, i.e., by injecting antigen and T-1824 dye intravenously into the recipient and observing the appearance of bluing at the cutaneous site of transferred cells when antibody had been produced. Similar results were reported by Chase (1952).

The use of diphtheria toxoid to stimulate donor animals (Wager and Chase, 1952; Chase, 1953a,b; and Chase and Wager, 1957) has also allowed studies of the quality and quantity of antibody produced by transferred cells in guinea pigs and rabbits. Antibody was measured by its ability, compared to that of a standard antiserum, to neutralize the necrotizing effect of known amounts of diphtheria toxin in the skin of normal rabbits. Tetanus toxoid has also been satisfactorily employed as antigen in transfer studies (Hale and Stoner, 1953; Stoner and Hale, 1955).

In summary, many types of antigens have been employed with success. Some, such as bacterial or red-cell antigens, have offered advantages in the sensitivity of techniques used to detect antibody. Soluble protein antigens, on the other hand, have enabled fluorescent antibody studies to be carried out on the cells that presumably synthesize antibody and have permitted an investigation of the quantity and certain qualities of the antibody produced in the recipient.

II. Studies of Donor Organs or Tissues Capable of Transferring Antibody Formation

Prior to cell transfer studies, considerable evidence pointed to lymphatic tissue as the main seat of antibody synthesis, and it was to be expected that the earliest successful transfer experiments used lymph nodes and spleen taken from previously immunized animals. The next

steps taken attempted to extend these observations to other lymphoid structures or to concentrations of certain lymphoid cell types, and to vary the region of antigenic stimulation of the donor in order to compare the productive capacity of lymphoid tissues taken from various parts of the body.

S. Harris *et al.* (1954b) compared the ability of lymph nodes draining the site of antigenic stimulus with nodes taken from other sites to respond with antibody after transfer. After injections of *Shigella paradysenteriae* into the hind foot pads of rabbits, subsequent transfer of the draining popliteal nodes, or axillary and mesenteric nodes or spleen into separate animals, revealed that only the draining popliteal nodes produced appreciable amounts of antibody in the recipients. In addition, the transfer of homolateral popliteal nodes after a foot-pad injection with *Shigella* was followed by antibody synthesis whereas transfer of the contralateral node was not—despite large injections of antigen. Under the same circumstances, when S. *paradysenteriae* were injected into the hind foot pads and *Salmonella typhosa* into the front foot pads, the popliteal nodes produced anti-S. *paradysenteriae* only, whereas the axillary nodes produced anti-S. *typhosa* only, and the mesenteric nodes produced neither. In work carried out concurrently, Stavitsky (1954) found that both the draining popliteal node and the spleen shared in the response to a foot-pad injection of soluble diphtheria toxoid, but that only the spleen was important following intravenous stimulation of the donor. This, then, indicated that, for antigens in bacteria, the lymph node draining the site of injection played an important, and at times, the only significant role in the antibody response. Further, such nodes were found to have a capacity for antibody production over and above that evoked by the single bacterial stimulation, for when a number of different bacteria were presented to them simultaneously and transfer was made to recipients, antibody to all the bacteria was formed without depression of synthesis of antibody to S. *paradysenteriae* (S. Harris *et al.*, 1954b).

Whereas S. Harris *et al.* (1954b) reported that intravenous injection of S. *paradysenteriae* failed to sensitize mesenteric lymph nodes, Roberts and Dixon (1955) found that soluble protein antigens, when injected intravenously, sensitized the mesenteric nodes. The explanation for this difference probably rests in the longer duration of the soluble protein antigens in the circulation and their ability to reach the mesenteric nodes in higher concentration. Moreover, T. N. Harris *et al.* (1955) found that mesenteric lymph node cells, sensitized *in vitro*, synthesized antibody on transfer into rabbits, although to a lesser degree than popliteal or axillary nodes.

While lymph node and spleen cells have generally been employed in cell transfer experiments, other organs that contain lymphatic tissue have been studied in an attempt to estimate the potentiality of cells in each site. *Thoracic duct lymph* has been used as donor material in cell transfers (Konda, 1959; Wesselen, 1952). It contained 98% lymphocytes, which provides morphologically the purest population of cells yet utilized, and was found to mediate agglutinin production to typhoid bacilli in normal homologous rabbit recipients. *Circulating leucocytes* after *in vitro* sensitization and transfer produced antibody (T. N. Harris *et al.*, 1956). *Oil-induced peritoneal exudates,* for the most part macrophages, have been assayed for their ability to produce antibody after transfer to a recipient. Dixon *et al.* (1957a,c), using rabbits sensitized with bovine serum albumin (BSA), compared the antibody-forming capacity of such peritoneal exudates containing an average of 71% macrophages (65–77%), 11% lymphocytes (7–16%), and 15% polymorphs with that of a lymph node suspension (popliteal and mesenteric) from the same donors containing an average of 90% lymphocytes (82–95%) and 8% macrophages (2–15%). The two sources of cells produced comparable antibody responses in the recipients. The ability of peritoneal exudate cells to transfer a secondary antibody response has been confirmed in recipient rabbits (Konda, 1959) and also in irradiated isologous mice (Perkins *et al.*, 1961). The peritoneal exudate cells in mice were apparently as effective as spleen cells. A primary response was also obtained in the isologous recipients when larger numbers of exudate cells were used.

Studies of the ability of *thymus* and intestinal *Peyer's patches* to produce tetanus antitoxin have shown that each of these tissues in mice is capable of antibody production (Stoner and Hale, 1955). However, in rabbits and guinea pigs, thymic cells taken from presensitized donors have produced little or no antibody in recipients (Dixon *et al.*, 1957c; Rosenberg *et al.*, 1958) as have similar normal thymic cells sensitized *in vitro* with *Shigella* antigens (T. N. Harris *et al.*, 1955). Tissue culture studies have shown thymus to be inferior to lymph nodes and spleen in its antibody-producing capacity (Stavitsky, 1961). Marshall and White (1961) have found that antigenic stimulation of the thymus occurs only when antigen is injected directly into the gland, presumably because of a blood-tissue barrier. After direct antigen injection, antibody-containing plasma cells were found in the thymus by fluorescence microscopy. Although such observations suggest a thymic origin for the antibody-containing cells, they cannot rule out the migration of immunologically competent cells from elsewhere in the body into the thymus as a result of the injection of antigen.

The transfer of bone marrow cells from mice shortly after primary sensitization to sheep red-blood cells resulted in no antibody formation. A similar study in repeatedly injected rabbits during a primary immunization revealed antibody formation (Taliaferro and Taliaferro, 1962), and, after preimmunization, transferred marrow cells in the recipient successfully mediated a secondary response (Makinodan, 1956; Makinodan *et al.*, 1956, 1957, 1958, 1962; Gengozian and Makinodan, 1956; Taliaferro and Taliaferro, 1957, 1962; Stoloff, 1960; Perkins *et al.*, 1961; Gengozian *et al.*, 1961a). It is possible that, with preimmunization of the donor, enough primitive cells of the bone marrow might differentiate to form detectable antibody or that cells capable of antibody synthesis could enter the marrow space from other organs and thus lead to positive results following transfer. *In vitro* studies (review by Stavitsky, 1961) have also indicated that marrow from hyperimmunized animals conveys antibody synthesis after being transferred into tissue culture. More recent data, however, by Doria *et al.* (1962), have shown that if transferred, unstimulated homologous bone marrow cells were left in the irradiated recipient for a prolonged time, they would produce a primary response to antigenic stimulation. It was demonstrated that the transferred cells and not the host's cells were responsible for the antibody that appeared.

Many other sites have been tested by cell-transfer techniques for their ability to accumulate antibody-producing cells after presensitization: cornea (Oakley *et al.*, 1955), omentum (K. B. Roberts, 1955a,b), and fat pads of rabbits (Oakley *et al.*, 1954).

Although antibody production by individual tissues or organs has been stressed in the preceding paragraphs, it must be re-emphasized that each tissue is made up of numerous cell types, some of which may be more capable than others of responding to an antigen. The number of these more capable cells probably varies from tissue to tissue within a single animal. Since these cells, and not the whole organ or tissue, are probably the common denominator of antibody production, studies of the comparative ability of organs or tissues to produce antibody may lead to irregular results. Unfortunately, the particular cells which produce antibody have not been identified and isolated at present.

III. Studies of the Recipient

A. Routes of Injection of Cells into Recipients

Different routes of injection of the donor cells into the recipients have been utilized, and each has offered certain advantages. The intravenous route of injection has been eminently satisfactory in offering a conducive

climate for the cells to produce antibody. Some difficulty with the intravenous route in mice has been encountered because of the high incidence of fatalities following injection, presumably resulting from embolization of clumps of cells in the lungs. In addition, the intravenous route disperses the transferred cells so that morphologic studies are impractical. This disadvantage has been in great part overcome by injecting washed suspensions of cells into the *abdominal musculature* and *subcutaneous tissues* of the recipient (J. C. Roberts and Dixon, 1955). As will be discussed later, this method of transfer has allowed the addition of a morphologic dimension, both by routine and by fluorescent antibody studies of the transferred cells. Furthermore, the use of *Millipore chambers* as containers into which the transferred cells are placed has permitted, along with general cytologic observations, a more accurate study of cellular division and function by eliminating the contaminating host cells from the field of observation (Makinodan, 1962; Holub, 1958; Holub and Riha, 1960). The *intraperitoneal route* of injection of a suspension of donor cells had produced varying results (K. B. Roberts, 1955a,b; S. Harris *et al.*, 1954a; J. C. Roberts and Dixon, 1955; Chase and Wager, 1957; Sterzl and Trnka, 1959; Oakley *et al.*, 1954; Gengozian *et al.*, 1961b; Perkins *et al.*, 1961). It was noted by J. C. Roberts and Dixon (1955) that, although antibody was produced by cells transferred into the abdominal wall, when cells were introduced by mistake into the peritoneal cavity, less antibody was detected in the recipients. S. Harris *et al.* (1954a) also noted less satisfactory titers after intraperitoneal injection as compared to the intravenous route. On the other hand, Chase and Wager (1957) have reported the production of antidiphtheria toxin by cells transferred to the peritoneal cavity of guinea pigs and rabbits, and others (e.g., Perkins *et al.*, 1961; Gengozian *et al.*, 1961b; Sterzl and Trnka, 1959) have utilized this route consistently with satisfactory results. The *intraocular space* (Hale and Stoner, 1953; Stoner and Hale, 1955) and the chorioallantoic membrane of chicks (Trnka and Říha, 1959; Sibal and Olson, 1958) have been used as transplantation sites and offer some protection from the host's homograft response.

B. The Possible Role of the Recipient of Transferred Cells in the Synthesis of Antibody

Probably the greatest concern in experiments utilizing cell transfer techniques is with the possibility that the recipient might contribute at least some, if not all, of the antibody formed after transfer. To study this possibility several different approaches have been taken. It was found in the earliest experiments that when the cells were damaged or killed

before transfer, significant amounts of antibody could not be detected in the circulation of the recipient. The cells could be treated by mechanical disruption (Chase, 1951; Wager and Chase, 1952), by freezing and thawing (S. and T. N. Harris, 1951; S. Harris *et al.*, 1954a; Taliaferro and Taliaferro, 1962), by lysis in distilled water (S. Harris *et al.*, 1954a; Taliaferro and Taliaferro, 1962), and by X-ray (S. Harris *et al.*, 1954a; Dixon *et al.*, 1957c) and ultraviolet light (S. Harris *et al.*, 1954a). Another type of physical damage to the transferred cells that has resulted in an abrogation of the antibody response is the homotransplant reaction by the recipient against the transferred cells. T. N. Harris (1957), T. N. Harris *et al.* (1958a), and S. Harris *et al.* (1958) showed that presensitization of the recipient with the donor's leucocytes caused rapid rejection of the subsequently transferred cells and prevented antibody formation. This again indicated that the donor cells and not the recipient are responsible for the antibody production.

The possibility existed that the transferred cells or fractions of these cells might be capable of inducing the host's cells to carry out the antibody synthesis. However, S. Harris *et al.* (1954a), Chase (1946), Chase and Wager (1957) and Taliaferro and Taliaferro (1962) showed that any antibody response to antigen by the host in normal, nonirradiated recipients came at a time distinctly later than that by transferred cells. That the first rise in the recipient's antibody level was, indeed, produced by the transferred cells was evident in the experiments of S. Harris *et al.* (1954a) in which the first response was nullified by physical damage to the cells before transfer (ultraviolet, freezing-thawing, etc.) but the second response was still apparent. In addition, in another experiment, it was found that by irradiating the recipient animal before transfer, the second or host response could be abrogated or markedly reduced, allowing only the early response on the part of the transferred cells to be detected (S. Harris *et al.*, 1954a). Hence, not only a clear-cut chronological separation of the two responses—first the passive and then the active—in the recipient was possible, but also one or the other of the responses could be nullified specifically.

Other evidence has indicated that the antibody emanating from the transferred cells is different in quality from that which may be produced by the recipient animal. Chase (1953a,b) employed a technique that differentiated guinea-pig antiserums according to their ability to neutralize toxin antigen under adverse conditions.[3] This was presumably a test for

[3] For an index of antibody "avidity," guinea-pig antidiphtheria toxin was tested under optimal and suboptimal conditions for neutralization of diphtheria toxin (see

the avidity of the antibody, which increased with time after stimulation of the guinea pigs. It was found that when guinea pigs were presensitized twice or even just once with diphtheria toxoid and their cells were transferred into normal recipients, a double peak of antibody was detected, as was discussed in the preceding paragraph, one occurring in the first few days after transfer and one appearing later. The earlier peak of antibody was found to have a high avidity which was the same as that of the donor animals before sacrifice. The second peak had a lower avidity, typical of a first response antibody. Upon further immunization with toxoid, the avidity of the recipient's antibody rose.

Roberts and Dixon (1955) found it possible to separate the response of the transferred cells from that of the recipient using quality of the antibody as a measure. By using prestimulated donors, they found that, after transfer of the cells into irradiated recipients, an accelerated rate of antigen elimination occurred, typical of a secondary response, which only the donor cells were capable of producing since the recipients had never had contact with the antigen before and, in addition, were irradiated. Moreover, the antibody that appeared in the circulation after complete elimination of the antigen had an initial combining ratio (Roberts and Dixon, 1955) with its antigen and an avidity [as determined by the ammonium sulfate technique of Farr (1958)] typical of the secondary type antibody.

Grey (1962), in studying the antigen–antibody dissociation rates[4] of

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Jerne, 1951). Optimal conditions consisted of mixing antitoxin with toxin at high concentrations for several hours, then diluting for the performance of skin tests in rabbits. Suboptimal conditions consisted of combining the antitoxin with the toxin, already diluted to 1 Lf./liter, for a period of only 5 minutes before test injection into the skin of rabbits. Under these conditions the serums showing about a fourfold difference in the amount of antibody needed for neutralization were considered to have high avidity whereas those antiserums exhibiting differences as great as 32- or 64-fold between optimal and suboptimal conditions were considered to be of low avidity.

[4] An expression of the quality of the particular antiserum was given by its dissociation rate from antigen. This was accomplished by use of the method of Farr (1958) in which the dissociation of I^{131}-BSA from the antibody is measured after the addition of an enormous excess of unlabeled BSA. The I^{131}-labeled BSA then would dissociate from the antibody at a rate that would probably reflect the avidity of bonds of the population of antibody molecules. At any time the amount of I^{131}-BSA remaining bound to antibody was measured by precipitating the antibody globulin at 50% saturated ammonium sulfate and counting the amount of activity in the precipitate. A population of avid antibody molecules would show relatively slow dissociation whereas nonavid antibody would show more rapid dissociation from the antigen.

antibody taken from donor rabbits primarily and secondarily stimulated with BSA, found that the dissociation rate of antibody taken from secondarily stimulated rabbits was distinctly slower than that taken from primarily stimulated animals as had been noted previously by Farr (1958). When cells from such secondarily stimulated donors were transferred to normal or irradiated recipients, the antibody formed in the recipients was of the secondary type, i.e., it exhibited a dissociation rate far slower than that of primary antibody produced actively by the recipient. This again leads to the conclusion that the recipient's cells do not themselves synthesize a significant part of the detected antibody molecules.

Morphologic observations have supported the role of the transferred cells in the formation of antibody in the recipient. Histologic and immunohistochemical studies of the cells after transfer into the recipient by Neil and Dixon (1959) have shown that a definite morphologic sequence of events takes place in the transfer site leading to the appearance of antibody within what were presumed to be the transferred cells. These studies were complicated by the fact that donors' and recipients' cells were intermixed, making separation difficult or impossible. By transferring cells in Millipore chambers, Makinodan (1962) has avoided this mixing of donor and recipient cells. In this situation antibody-containing cells developed in the chambers containing only the transferred cells—thus giving morphologic support to the thesis that the donor cells are responsible for the antibody response.

The possibility that the donated cells carry over significant amounts of preformed antibody with them into the recipient has been ruled out by the frequently used control in which, after homogenizing the transferred cells, little or no antibody is found. Other studies of the time in which labeled amino acids are incorporated into antibody (Taliaferro and Talmage, 1955; Stavitsky, 1958) have shown that the incorporation of labeled amino acids into antibody occurred only in the recipient during the rapid synthesis of antibody, several days after antigenic stimulation, and not in the donor prior to or within the first hours or days after stimulation.

All these results, based on different approaches, experimental animals, antigens, and methods of antibody detection, taken together show, quite definitely that neither the transferred cells nor the antigen carried with them cause the recipient, irradiated or not, to form the specific antibody, but that it is the donors' cells, existing in a foreign milieu, that are carrying out the task.

C. Increased Synthesizing Ability of Transferred Cells in Irradiated Recipients

It was observed by S. and T. N. Harris (1954a) and by T. N. Harris *et al.* (1954a), in comparing normal and irradiated recipients of stimulated homologous lymphoid cells, that higher titers appeared in the recipients irradiated 24 hours prior to transfer. The advantage enjoyed by cells in the irradiated recipient over those in the normal recipient was apparently not because of a longer half-life of antibody or hemoconcentration after irradiation of the animal but, possibly, because of a decreased homograft reaction against the transferred cells. Another possibility that has received recent support is that there might have been an increased stimulus-to-lymphoid proliferation in the radiated recipient as a result of the postradiation depletion of the host's own lymphoid tissue. In support of this, it has been observed by Makinodan (1962) and by Mark (1962) that in the transfer of antibody-producing cells to isologous recipients, where the homograft rejection is avoided, irradiation of the recipient before the transfer heightens the response. While more than one theoretical explanation of these phenomena are apparent, the possibility should be considered that the rapidly dividing donor cells that have received stimulation to produce antibody could be occupying the partially emptied lymphoid beds to a greater extent than the unstimulated cells. Other observations may be added here in support of such a hypothesis. In transferring increasing numbers of stimulated lymphoid cells to isologous recipients, Perkins *et al.* (1961) observed that the resulting antibody titers increased to a point beyond which no rise was noted. This suggests an internal autoregulatory mechanism of lymphoid tissue. Moreover, it was found that the transfer of 12×10^6 presensitized nucleated spleen cells, the equivalent of 8% of a normal mouse spleen's nucleated cells or 2% of the total animal's "spleen type" lymphoid tissue, was capable of producing in the isologous irradiated recipient an amount of antibody equal to that produced by an intact 25-gm. mouse similarly stimulated (Gengozian *et al.*, 1961a; Makinodan, 1960; Makinodan *et al.*, 1962). In summary, evidence has accumulated suggesting an internal regulation of the total amount of lymphoid tissue and, therefore, antibody produced in either an intact animal or a recipient of transferred cells.

D. Inhibitory Effects of Irradiation

The inhibitory effect of ionizing irradiation on the antibody response is well known, but the actual mechanism involved remains clouded. In the intact animal it has not been possible to determine whether the effect

is wholly caused by direct injury to the cells [i.e., to an area of 0.4 μ diameter of cell, Makinodan *et al.* (1962)] by the irradiation or whether secondary humoral factors might also play a role. Evidence to support the latter possibility was found by using presensitized donor rabbit cells that were X-rayed *in vitro* and by comparing the amount of antibody produced by them after transfer to irradiated or normal recipients (Dixon *et al.*, 1957b). A large majority of irradiated recipients failed to support antibody synthesis by cells given 400r or more whereas normal recipients of irradiated cells formed antibody. In addition, although no measurable primary production of antibody to bovine γ-globulin occurred following transfer of normal lymphoid cells to irradiated recipients, Weigle and Dixon (1959) found that by transferring the normal cells to recipients previously made tolerant to bovine γ-globulin, a primary response could be obtained. Hence, it would seem that the irradiated recipient either harbors some sort of inhibitor or is in some way deficient, and, therefore, at least in part the effect of irradiation on antibody formation may be brought about by more than just the direct ionizing injury of the lymphoid cells.

Thus, a recipient with all the favorable qualities of an irradiated animal, such as the possible decreased ability to reject a homograft and depleted "lymphoid tissue," but without the deleterious indirect effects of an irradiated recipient might well support synthesis of antibody to a greater extent than heretofore realized. In this regard, the lethally irradiated isologous recipient used frequently by Makinodan *et al.* (1960) probably approaches as close to the optimum as has been presently attained.

E. Antibody Formation by Transferred Cells in Neonates

For many years it has been generally accepted that neonatal animals of many species are less capable of producing circulating antibody than their adult counterparts. In recent years attempts have been made to determine the exact nature of this inadequacy, i.e., whether the fault lay in the cells of the neonatal animal or whether there was some deficiency or some inhibitor in the internal environment of the neonate that could be held responsible for this inability to form protein molecules bearing antibody specificity. In search of an answer to this problem, the transfer of lymphoid cells from adults to neonatal animals has been undertaken to find if the antibody formed by these adult cells in the neonate was comparable in quantity to that produced in other adult recipients. In this way, the milieu of the neonate could be tested for its ability to support antibody synthesis. The results obtained from different laboratories have

been conflicting, however, and have led to controversy. Sterzl (1954) reported that neonatal rabbits would support antibody production by adult lymphoid cells. However, in studies comparing the relative ability of adults and neonatal rabbits to support antibody synthesis by the transferred adult cells, Dixon and Weigle (1957) found the neonatal recipient to be far inferior to the irradiated adult. Subsequent work has substantiated the finding that under similar conditions the neonatal rabbit does not appear to provide as sufficient an environment as its adult counterpart for the synthesis of antibody by adult cells (Dixon and Weigle, 1959). In the recent work of T. N. Harris *et al.* (1962), neonatal rabbits were found to support antibody synthesis by transferred adult cells, but to a lesser degree than adult recipients, and Sterzl (1960a) has found nearly equal responses in adult and neonatal rabbit recipients. However, despite this possible inadequacy of the neonatal rabbit recipient, the neonates of other species of animals (see following) have been found to offer a satisfactory environment for transferred adult cells. It would appear, nevertheless, from the available data that at least two possible explanations of this phenomenon in rabbits exist: that the neonatal rabbit is able to reject the transferred cells before they have fully accomplished their task of antibody synthesis, i.e., either by an accelerated homograft rejection or because of a delay in the antibody response of the transferred cells, or, perhaps, that in the neonatal animal more antigen may be required than in the adult to produce an effective stimulus.

1. Rejection of Transferred Cells by the Neonate

Despite the tendency to consider neonates as having a generally immature immunologic constitution, there is ample evidence that neonatal animals are capable of rejecting homologous cells. Sterzl (1958a, 1959) suggested that neonates might well be capable of rejecting the transferred cells before the latter had made an antibody response on the basis of experiments showing that the stimulation of adult cells with antigens within the first few days after transfer to neonatal rabbits was followed by antibody formation whereas stimulation later than the third day failed to elicit a response. Similar delays in antigenic stimulation of cells transferred to adult rabbits gave similar results, indicating comparable rates of rejection of transferred cells in neonates and adults (Dixon *et al.*, 1959). In 2-day chicks, time of responsiveness by transferred cells was found to be even shorter (Sterzl and Trnka, 1959).

In other experiments indicating a rejection phenomenon in neonatal animals, Trnka and Říha (1959) found that when adult chicken spleen cells were mixed with *Brucella* bacteria or BSA antigen and then trans-

ferred to the chorioallantoic membrane of 18-day chick embryos, a primary response was measurable in both cases. However, when such cells were transferred to 2-day chicks, far less antibody was detected subsequently to *Brucella* and no hemagglutinin titer was found to BSA. While the site of transfer is by necessity different in the two cases, the possibility of a homograft rejection of transferred cells by the more mature chick is evident.

Further evidence of the ability of the homograft reaction to prevent the significant formation of antibody in the neonate may be seen in the experiments of Nossal (1959) and Mark (1962) in which the transfer of stimulated cells to neonatal *isologous* mice resulted in antibody formation whereas the transfer to neonatal *homologous* recipients failed to yield detectable antibody. In addition, Holub and Říha (1960) found that by protecting transferred adult cells from a cellular rejection mechanism by the neonate through the use of Millipore chambers, a primary response could be obtained both to bacterial and to serum protein antigens. Antibody to the latter antigen appeared after the seventh day and was found in significant amounts only in the chamber fluid.

That the homograft reaction can take place in neonatal rabbits has been indicated by the work of Sterzl (1958a,b) and Sterzl and Rychlikova (1958), previously referred to. In addition, Egdahl (1957) reported the ability of rabbit fetuses to reject homografts 1–3 days before birth and T. N. Harris *et al.* (1962) showed that neonatal rabbits could be sensitized by injections of prospective donors' leucocytes. Then, at a later time, cells capable of antibody synthesis from the same donor were transferred to them these cells failed to produce antibody. The neonates also supported greater antibody synthesis if irradiated prior to the transfer of sensitized cells (T. N. Harris *et al.*, 1962; and Barnett and Grey, 1962). However, in order to explain the lesser degree of antibody synthesis by the adult cells in the neonate on the basis of a homograft rejection, it must be presumed that the neonate is capable of rejecting the cells more rapidly than the adult or perhaps that a longer latent period for antibody formation is required in the neonatal environment. In this regard, recent observations of Najarian and Dixon (1962) have shown that the time required for the rejection of skin homografts in neonatal rabbits was slightly less than in adult recipients. The mean time of rejection of adult skin grafts on adult rabbits was found to be between 8 and 9 days whereas similar skin on neonates was rejected between 6 and 7 days. Microscopic evidence of early damage to the graft could be found around the fourth day in the neonate recipients. This, then, indicates that a neonatal rabbit can reject homologous tissue as rapidly,

if not more rapidly, than an adult, assuming that anatomic differences in the graft beds do not account for the shorter rejection period in the neonate. Therefore, the time needed for rejection of homologous cells appears to be close to the time needed to achieve maximum antibody synthesis by transferred cells. If the neonate is able to reject the transferred cells slightly more rapidly than the adult, it should form significantly less antibody. Such an effect would be even more pronounced if there were any delay in the antibody response of cells transferred to a neonate.

Another possible factor in the production of antibody by transferred cells in a neonatal animal may be the quantity of antigen needed for stimulation. Observations derived from studies in intact neonates has indicated that an antibody response could be obtained at an earlier age when large quantities of antigen were used for immunization than when small quantities were used (Sterzl and Trnka, 1957; Sterzl, 1959; S. Harris *et al.*, 1962). Although in experiments utilizing the transfer of cells to neonatal rabbits it has been pointed out that adequate amounts of stimulating antigen must be used (Sterzl and Trnka, 1959; Trnka and Sterzl, 1960), studies comparing neonatal and adult rabbit recipients at various stimulating dosages of antigen are lacking.

In regard to the mechanisms of action of the augmented quantity of antigen, if such is indeed required, Sterzl and Trnka (1959) postulated that extra antigen might be needed to sensitize further generations of transferred cells in the recipient. Recently, Hrubesova (1961b) found that bacterial antigens or labeled, denatured, serum protein antigens were removed from the circulation more rapidly by neonates than adults. It is possible that such a rapid removal of the antigen by the neonate could account for lowered antibody synthesizing capacity of adult cells transferred to neonates when these antigens are used.

Despite the question of adequacy of the neonate recipient, many studies have been carried out in which the neonate has been satisfactorily utilized as a recipient of cells. Significant amounts of antibody have been synthesized to a greater or lesser degree by adult cells in neonatal recipients in the rabbit (Sterzl, 1954, 1957, 1958a; Holub, 1958; Holub and Riha, 1960; Sterzl and Rychlikova, 1958; Sterzl and Trnka, 1959; Sparck, 1959; S. Harris *et al.*, 1962; Dixon and Weigle, 1957, 1959; Barnett and Grey, 1962) and in the chicken (Simonsen, 1957; Trnka, 1958; Sibal and Olson, 1958; Trnka and Říha, 1959; Papermaster *et al.*, 1959), the duck (Sterzl, 1959), the mouse (Nossal, 1959; Mark, 1962), and the rat (Nossal, 1960).

With the possibility in mind that the neonatal rabbit might not afford

a sufficient milieu for antibody synthesis by competent adult cells, Dixon and Weigle (1959) attempted to find if the neonate's cells would be able to carry out synthesis if removed from its own environment and transferred into an adult. These studies showed that substantial synthesis of antibody by neonatal cells occurred in about half the adult recipients but not in neonatal recipients, although the amount of antibody was less than that produced by adult donors. This again favored the interpretation that the neonatal animal's immunologic immaturity lay in part in an environmental inadequacy. These studies have been confirmed by Nossal (1959) in neonatal rats from 1 to 5 days of age but not in rats less than 1 day old or in transfers of embryonic rabbit liver to lethally irradiated rats (LaVia *et al.*, 1958). However, S. Harris *et al.* (1962) have recently reported a failure of cells of donor rabbits under 1 month of age to produce detectable antibody when transferred into the adult milieu. There were few differences other than rabbit strains and the fact that spleen cells were used by Dixon and Weigle (1959) and lymph node cells by S. Harris *et al.* (1962) to explain the conflicting results. Sterzl (1962) also has found cells from neonatal rabbits incapable of synthesizing antibody when transferred to irradiated adults. Recently, Makinodan and Peterson (1962) have shown that cells from 1-week old mice produced only 1% of the optimal amount of antibody that could be obtained from isologous adult mice. In summary, the cellular inadequacy that appears in neonatal mice seems not as marked as in rats. The degree of such an inadequacy in rabbits must await further evaluation.

IV. Studies of the Transferred Cells

A. Morphologic Changes of Transferred Cells in the Recipient

Studies of the morphologic appearance of transferred cells have revealed certain basic information on the sequence of events that accompany the appearance of antibody in the circulation of the recipient. J. C. Roberts *et al.* (1957) studied sequentially the histological appearance of the transfer site in the abdominal wall in irradiated recipient rabbits after transfer of cells from donors that had been previously sensitized with serum protein antigen and simultaneous stimulation of the recipients by intravenously injected antigen labeled with I^{131}. The cellular changes were as follows: numerous well-preserved lymphocytes and a few macrophages and polymorphs were seen the first day after injection; 2 days later the relative number of small lymphocytes decreased and the relative number of larger lymphoid cells with pyroninophilic cytoplasm increased, especially in areas surrounding nerves and vessels. These were

thought to be "transitional" and "preplasma" cells as described by Fagraeus (1948). In a subsequent paper using identical methods, Neil and Dixon (1959) found that these transitional cells on the third day after transfer and antigenic stimulation contained small amounts of specific antibody for the first time, as detected by the fluorescent anti-

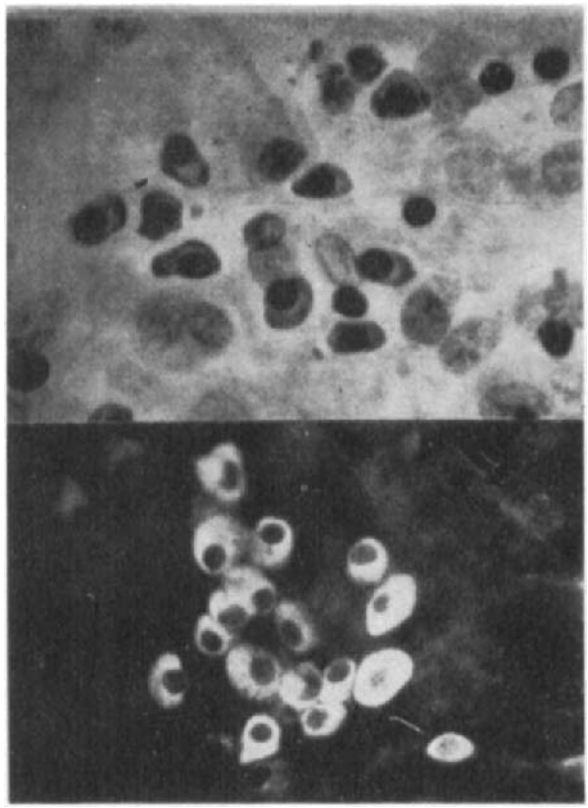

FIG. 1. Plasma cells taken from subcutaneous transfer site in rabbits 5 days after transfer. Upper photograph stained with Giemsa to show plasma cell morphology; bottom fluorescence photograph stained to show presence of anti-BSA in the cytoplasm of similar plasma cells. (From Zlotnick and Vazquez, 1962.)

body technique. This was at a time prior to the immune elimination of antigen from the recipient and before the appearance of free circulating antibody. From 5 to 9 days after transfer, these transitional cells were replaced by mature plasma cells containing cytoplasmic antibody as seen in Fig. 1, although after the fifth day there were fewer antibody-containing cells, and by the fourteenth day such cells were only rarely

found. By using as controls the transfer of killed cells or living cells with no simultaneous antigen stimulation, J. C. Roberts *et al.* (1957) failed to find these sequential morphologic changes.

In addition to the lymph node cells, transfers of suspensions of mineral-oil-induced peritoneal exudates, in large part macrophages, of rabbits were performed in the same manner as noted in the preceding paragraphs, to study the transfer site (J. C. Roberts *et al.*, 1957). The sequence of morphologic changes was similar to those of the lymph node cell transfers. Instead of a predominance of lymphocytes in the first few days there were oil-containing macrophages and, as in the case of lymph node cells, the predominant cell type gave way to transitional and preplasma cells in the next few days, and finally to mature plasma cells. Good antibody responses again correlated with the formation of the plasma cell series.

In all these experiments the cell transfer sites were studied using H&E stained sections and insignificant numbers of dividing cells were identified. Therefore, it appeared that the major cell types transferred, i.e., lymphocytes from lymph nodes and macrophages from peritoneal exudates, had changed directly without division into plasma-cell precursors (J. C. Roberts *et al.*, 1957; Dixon *et al.*, 1957a).

More recent work by Urso and Makinodan (1962) has indicated that significant cell division can occur in the antibody-producing line of cells after transfer. Mice that had received 950r shortly before transfer were used as recipients of lymph node cells from rabbit donors previously immunized with BSA. The donor cells were transferred into Millipore chambers of 0.1 μ pore size to avoid admixture with the host's cells, and antigen was administered simultaneously to provoke primary antibody synthesis. At various times thereafter, tritiated thymidine was given so that all cells synthesizing deoxyribonucleic acid prior to mitotic division during a 24-hour period would incorporate the radioactive label into their nuclei and could be easily detected at a later time by autoradiography. Then, since serum protein antigen had been employed, it was possible to ascertain by fluorescent antibody techniques which of the cells contained antibody (and presumably had synthesized it). By making autoradiographs of the slide that had been studied with the fluorescent technique and by counting the antibody-containing cells that had also previously incorporated tritiated thymidine, the extent of mitosis could be ascertained. The results of these studies showed that 95% of all antibody-containing cells had divided within the 7 days after transfer and simultaneous antigenic stimulation. Further studies of the mitotic rates

of cells after antigenic stimulation were performed by Capalbo *et al.* (1962) and Capalbo and Makinodan (1962) by transferring spleen cells to irradiated isologous mouse recipients either into diffusion chambers or intravenously. The cells were then stimulated with antigen, and at various times tritiated thymidine was administered to tag cells undergoing division. The total number of cells labeled with the tritiated thymidine was counted (radioautograph), and also the total number of grains per cell was recorded at various times following administration of the radioactive marker. The results showed that whereas nonstimulated cells divided at a rate of once per 24 hours on the average, or had a generation time of 24 hours, that antigenically stimulated cells undergoing a secondary response had a reduced generation time of 10 to 12 hours. After primary stimulation, on the other hand, the average generation time remained at 24 hours for 2 days before dropping to 10 to 12 hours. In other studies measuring the amount of antibody produced by increasing the number of transferred cells, Makinodan *et al.* (1962) demonstrated that what was true of cells in a secondary response in terms of division and maturation was also true of the primary response. The role played by antigenic stimulation in cellular proliferation was underscored in work by Nossal (1960) in which detectable antibody production by transferred cells occurred extensively in serial passages of the cells to homologous neonatal recipients only when antigenic stimulation of the cells took place at each passage time. More recently, counts of mitotic figures in histological sections of abdominal wall transfer sites have also revealed the presence of mitotic figures as shown by Sainte Marie and Coons (1962) and Zlotnick and Vazquez (1962). In these studies, numbers of mitoses in transfer sites comparable to those seen in lymphoid tissue of intact antibody-forming animals have been observed.

In view of the observations revealing significant mitotic activity in transferred cells, it is impossible to be certain which cell types transferred in a mixed population give rise to the antibody-forming cells. Since transferred cells destined to give rise to antibody-forming cells appear to divide repeatedly during the immune response it is possible that all of the antibody-containing cells could be derived from any cell type capable of division even if it comprised only a few per cent of the cells transferred. Thus, a minor cell type such as a primitive blast cell which is present in teased lymph node cells, peritoneal exudates, or any other lymphoid tissue, could, through mitoses, give rise to a sufficient number of cells to account for a large number of antibody-containing cells within 4 to 5 days after transfer.

B. The Homograft Reaction against Transferred Cells

After transfer of the cells, as has been discussed in the previous section, marked changes take place with many cells dying in the first few days and surviving cells assuming the responsibility for synthesizing antibody.

The life of these latter cells, however, appears under certain conditions to be limited. From a serologic standpoint, the transferred cells have been found to respond to antigenic stimulation only up to 3–4 days after transfer in a homologous recipient, whereas they may be stimulated for a longer time in an isologous mouse recipient (Makinodan, 1962; Mark, 1962). Sterzl (1958a) using neonatal rabbits as recipients found that transferred adult cells were capable of producing antibody against *Brucella suis* when the antigenic stimulation was given at the time of transfer, but not when given 4 days after transfer. Similarly, using homologous donor and adult X-rayed recipient rabbits, Dixon *et al.* (1959) found that the transferred cells produced antibody when antigen was injected at the time of transfer, but not when injected 3 days after transfer. These studies indicated that the cells were no longer susceptible to stimulation by the antigen from 3 to 4 days after transfer and/or that the cells, even if stimulated, were no longer capable of detectable antibody synthesis. This latter possibility could conceivably be explained by several mechanisms but among the foremost is that of the homograft rejection: the host animal, irradiated or not, rejects the donor's cells before they are capable of synthesizing enough antibody to be detected. Thus, in the experiments with delayed injections of antigen (Sterzl, 1958a; Dixon *et al.*, 1959), the authors considered that the cells might well have been rejected by the homologous recipient before detectable amounts of antibody had been released into the circulation.

Evidence of homograft rejection of the transferred cells has already accumulated. Mitchison (1956), T. N. Harris (1957), T. N. Harris *et al.* (1958a, 1962) and S. Harris *et al.* (1958) found that preimmunization of the recipients with peripheral leucocytes obtained from pooled donors decreased or eliminated the antibody response after subsequent transfer of sensitized homologous lymph node cells. Transfer of individual rabbit's leucocytes resulted in variable decreases in the subsequently transferred antibody response, whereas cells pooled from several rabbits were more uniformly effective. Polymorph- or mononuclear-rich peritoneal exudates worked equally well in rabbits (T. N. Harris *et al.*, 1958a), and preimmunization of recipients with as few as 10^6 nucleated cells was effective in diminishing the antibody response by subsequently transferred

lymphoid cells. Rabbit red blood cells did not bring about the effect; nor did leucocytes obtained from several other species with the exception of human leucocytes that apparently were partially effective. Regarding the importance of time, injection of the preimmunizing leucocytes from 2 to 3 days before transfer of antigen-sensitized lymph node cells resulted in some diminution of eventual antibody titers, whereas injection from 5 to 6 days before transfer resulted in no significant antibody titers and indicated full inhibition. A diminished antibody response occurred up to 107 days between the preimmunization with pooled leucocytes and the transfer of sensitized cells. This effect was partially blocked by irradiation of the recipient before preimmunization (S. Harris *et al.*, 1958).

All these experiments indicated that the recipient was presensitized to the eventual donor's cells and that before the transferred cells could produce enough antibody to be detected, they were destroyed by some immunologic rejecting mechanism of the host. The type of rejection appeared similar to, if not identical with, the usual second set homograft rejection mechanism as it occurs in skin transplantation (T. N. Harris *et al.*, 1958a). However, it was soon discovered that the rejection of the transferred suspension of antibody-producing cells could be mediated by serum taken from rabbits preimmunized with leucocytes, that the rejecting factor was carried in the γ-globulin fraction of the serum, that it was absorbed by white cells, and that it was not destroyed at 56°C. In short, the factor in the serum responsible for the rejection phenomenon had the characteristics of circulating antibody. When such "antiserum" obtained from rabbits injected with leucocytes was incubated with stimulated lymph node cells just prior to transfer of the lymph node cells, a reduction in the recipient's antibody was found (T. N. Harris *et al.*, 1961). Moreover, when the "antiserum" was given to a nonimmunized recipient within the first few days after cell transfer, a marked suppression in detectable antibody was noted, as may be seen in Fig. 2. The effect of the "antiserum" was nearly complete when injected the first few days after transfer. Similarly, Siskind *et al.* (1960) found that the injection of "antiserum" against a donor strain of mice into newborn recipient mice suppressed or prevented the development of runt disease in the newborns when donor strain spleen cells were later administered. These, then, represent without much doubt examples of homograft rejection by circulating antibodies. Peculiar to models is the type of rejected tissue, a suspension of cells that is injected intravenously into the recipient, which is different from the usual solid tissue or organ graft. The latter is transferred as a cohesive unit and has not, at least to date, in a comparable situation

been found to be rejectable by circulating antibody. Suspended cells, on the other hand, after coming to rest in the small vessels of the recipient rabbit could well be far more susceptible to damage by circulating antibody than solid tissue grafts. In addition, the indicator here, i.e., the synthesis of antibody, may be a more sensitive one than the visible survival of grafted tissues and, therefore, more susceptible to host reactions.

Other information regarding the rejection of transferred cells has been provided by the studies of Makinodan (1962) in which isologous, homol-

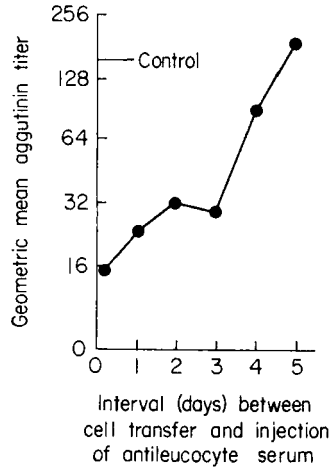

FIG. 2. The effect of passively administered antileucocyte serum on the antibody produced in recipient rabbits by transferred cells. The cells had been stimulated *in vitro* with trypsin-treated *Shigella* antigen. When the antiserum was administered within the first 3 days, a marked or, since the prints represent an average value, complete inhibition of the antibody response was obtained; 152 recipients were used. The control value given is the geometric mean titer of recipients not treated with antileucocyte serum. (From T. N. Harris *et al.*, 1961.)

ogous, and heterologous systems were compared. Spleen cells from mice transferred in Millipore chambers into the peritoneal cavities of irradiated *isologous* recipients survived and, after stimulation, produced antibody in the host for periods greater than 20 days. On the other hand, when such cells were transferred into irradiated *homologous* recipients, the antibody levels were maintained for a shorter time, between 9 and 20 days, whereas when *heterologous* rabbit cells were transferred in chambers to irradiated mice the levels of antibody dropped prior to the ninth day after transfer. Since host cells could not traverse the 0.1μ pore size of the chamber, a system of rejection other than that involving cells must

be implicated. Then, assuming a free flow of plasma nutrients across the chamber membrane, some humoral factor that inhibited antibody formation, perhaps through death of the cells, was probably functioning. Without Millipore chambers, evidence for the rejection of homologous antibody-producing cells within 9 days has been obtained by Mitchison (1957a,b) who found that antibody titers in such recipients fell rapidly. Corroborative data have been reported by Stavitsky (1959), Stoloff (1960), and Dineen and Perry (1960).

The Millipore chamber studies indicate a humoral mechanism in the rejection of transferred homologous and heterologous lymphoid cells. This humoral mechanism may well operate in addition to the well-established cellular mechanisms of graft rejection. That this latter means of rejection may function could be implied from the numerous studies not involving cell-proof chambers in which the transfer of heterologous antibody-producing cells have shown almost uniformly negative results. That is, in the absence of protective chambers, the host's white cells with free access to the transferred cells could reject them rapidly enough to prevent significant antibody production, whereas with the protective Millipore chambers, as used by Makinodan, the transferred cells survived long enough to produce detectable antibody. Transfer of heterologous antibody-synthesizing cells in which such a rapid demise of the transferred cells appears to be operative include rabbit to guinea-pig transfers (Chase and Wager, 1957; S. Harris *et al.*, 1954a), guinea-pig and horse cells to rabbit (Oakley *et al.*, 1954), rabbit to chick (Trnka, 1958), rabbit to duckling and chicken to neonatal rabbit (Sterzl and Trnka, 1959), and adult guinea-pig to neonatal rabbit (Sterzl, 1958a). Exceptions to the above results include rabbit to guinea-pig transfers (Wesselen, 1952) and rabbit or rat to lethally irradiated rats or mice, respectively (LaVia *et al.*, 1958; Chin and Silverman, 1960).

Further evidence showing the response to the host against the transferred cells has been collected from studies of radiation chimeras. Gengozian *et al.* (1961b) found that lethally irradiated mice when injected with rat bone-marrow cells did not in themselves contain detectable antibodies against the rat red cells, but that chimerous spleens in a lethally irradiated isologous mouse produced detectable, antirat, red-cell antibodies. To find if rat antibodies to mouse red cells were produced in the environment of a rat, the same chimerous spleens were transferred to irradiated homologous rats, but failed to show production of antimouse red blood cells. Other studies have indicated that antibody produced by the lethally irradiated host against certain constituents of either a homologous or heterologous protective bone-marrow graft was associated with

the elimination of the protective graft and the demise of the host (Makinodan, 1956, 1957; Gengozian and Makinodan, 1956). Under the circumstances of these studies, therefore, circulating antibody may be produced by the host cells against the antigens in the graft despite lethal irradiation of the recipient.

An interesting exception to the phenomenon of host rejection of the graft in conditions other than the isologous transfer was presented by Hale and Stoner (1953). They found that, from 6 to 12 days after transferring homologous spleen or lymph node cells to the anterior chamber of the eye of Swiss mice, antigenic stimulation was still followed by an antibody response on the part of the transferred cells. Since this was past the time when a homograft rejection would normally have taken place in other sites of the mouse, and assuming that the Swiss mice were not isologous (they came from a colony that had been inbred, but no mention of brother-sister matings or graft testing were reported), a special protective function must be attributed to the anterior chamber. This is by no means a new concept and the anterior chamber has been frequently used for transplantation experiments for this reason. The brain also offers a certain protection from the recipient's rejection mechanisms, but so far has not been used as a site for the transfer of antibody-producing cells.

The rapid homograft rejection of transferred antibody-forming cells by preimmunized recipients has been used by Celada and Makinodan (1961) as the basis of a test for homotransplantation antigens. In their system, recipient mice were given a priming or presensitizing injection of homologous cells, cell fractions, or other material to be tested and, after 10 days, were irradiated and were given donor spleen cells (homologous transplant) and sheep red blood cells. The homologous transferred cells produced antibody to the sheep red blood cells in a predicted manner unless antigenic material had been present in the original priming injection and immunized the recipient against them. Within this model, the constituents of the priming injection could be altered or treated in any manner and the antigenicity or lack thereof tested. In preliminary results, the effect of a presensitizing injection of viable nucleated bone-marrow cells was greater than the same cells killed. Further, these cells had greater effect than liver cells which, in turn, were more effective than testicular cells in presensitization of the homologous recipient. Red cells were ineffective and hence contained insufficient antigenic material to cause detectable rejection of the antibody-producing transplant.

C. Antibody Formation by Transferred Cells against Constituents of the Host

There are several studies showing the production of antibody by the transferred cells against constituents of the host. Simonsen (1957) found that adult chicken spleen cells when transferred to chick embryos produced antibody against the recipient's red cells as detected by the Coombs antiglobulin test. In homologous radiation chimeras, Doria *et al.* (1962) found that the spleen cells responsible for antibody production were of the donor cell type. Additional evidence of a graft vs. host reaction was noted when spleen cells were injected into irradiated homologous recipient mice, i.e., C57BL cells into irradiated C3H recipients, and the recipients' spleens and nodes were removed after 12 days and injected back into a C57BL mouse. Agglutinins were found against the C3H red-cell antigens (Feldman and Yaffe, 1958). Further studies by Porter (1960), Piomelli and Brooke (1960), and Doria (1962a,b), have indicated clearly that cells transferred to an irradiated recipient produce an immune response against the host. These studies, then, involving the production of circulating antibody by the transferred cells against constituents of the host, complement the many other studies that have been performed on the graft vs. host reaction, e.g., runt disease, but in which the presence of a circulating antibody against the host has not been demonstrated.

V. Estimation of the Quantity of Antibody Produced by Cells in the Transfer System

It has been possible, through the use of a known number of cells that are taken from one animal and transferred to another, to obtain an estimate of the amount of antibody synthesized by that group of cells and their progeny in a secondary response. J. C. Roberts and Dixon (1955) transferred 0.5×10^9 to 9.7×10^9 lymph node cells from previously sensitized rabbits to irradiated recipients and found that anywhere from 0 to 159 μg. anti-BSA N with an approximated average of 33 μg./ml. serum (3.3 mg. in the total intravascular pool) appeared in the irradiated recipients 3 days following complete elimination of antigen from the circulation.

The quantitation of diphtheria antitoxin synthesis by transferred cells was studied by Chase and Wager (1957) both in guinea pigs and in rabbits. These authors transferred an unspecified number of splenic or lymph node cells taken from 3 to 4 days after restimulation of donors with diphtheria toxoid and measured the production of antibody. They found that 100 μg. of antibody nitrogen were found in the total intra-

vascular serum pool in several of the guinea-pig recipients. In a few recipients, larger amounts (up to eight times more) were detected. The high values are probably more meaningful since, as mentioned by the authors, they might well reflect a more compatible environment. Similar quantities were found in the case of rabbit to rabbit transfer of lymphoid cells taken from donors restimulated from 3 to 4 days previously with toxoid.

In the mouse, Gengozian *et al.* (1961a) and Makinodan *et al.* (1962) reported that in transferring 12×10^6 cells (about 8% of a spleen), both primarily and secondarily sensitized, to irradiated isologous recipients, antibody responses could be obtained comparable to those in intact mice. In addition, it was calculated that the antibody-forming capacity of spleen cells increased 182-fold (Makinodan *et al.*, 1962) during a secondary as compared to a primary immunization.

VI. Studies of the Qualities of the Antibody Produced by Transferred Cells

As has been noted in Section III, B, the avidity of the antibody produced by a donor's cells after transfer into a recipient is the same as the avidity of the antibody in the donor just prior to cell transfer. Chase (1953a,b) selected donor guinea pigs that showed a range of individual "avidity" values, and their lymph node cells were transferred to individual recipients (see footnote 3). The avidity of the resulting antibody in the recipient was tested. A striking similarity was found to exist between the avidity value of the donor and recipient antibody. These data strongly indicate that the cells continue to produce the same quality of antibody that they, or their predecessors, had previously produced in their original (donor) environment.

Grey (1962) compared the quality of antibody produced in hyperimmunized donor rabbits with that found in recipients of cells of individual donors (see footnote 4). Donors were bled 10 days after a secondary antigenic stimulus and again about 2 weeks later, at the time of transfer. Although dissociation curves of the antibody of donor rabbits exhibited differences, the curves obtained from any individual rabbit at various times after secondary stimulation displayed little difference. Popliteal and mesenteric nodes from a single donor were then pooled and transferred to between one and three recipients along with a stimulating injection of BSA. Each recipient was bled 10 days following transfer and the serums were tested for the dissociation rate from the antigen. As is shown in Fig. 3, the dissociation curves obtained from the serum of each of the recipients paralleled the dissociation curves obtained from the donor rabbit's serum tested simultaneously.

These data indicated not only that the donor's cells were responsible for the production of antibody in the recipient, as noted previously, but, of special importance, strongly suggested that certain qualities of the antibody were determined by the transferred cell and were not affected by the foreign milieu. It would seem likely that each recipient supplied essential building blocks to produce the machinery necessary for the fabrication of antibody molecules as well as to form the antibody mole-

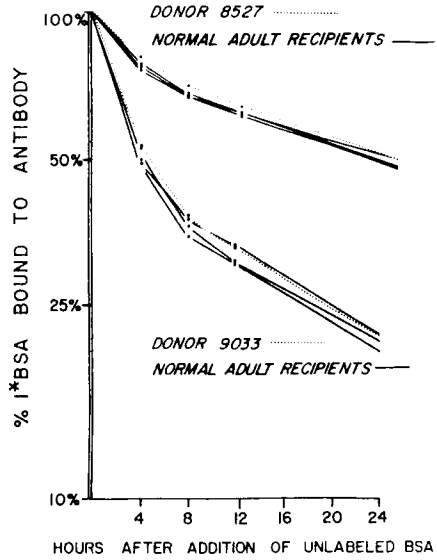

FIG. 3. The dissociation rate of I^{131}-labeled BSA antigen from antibody in the presence of an excess of unlabeled BSA. Antibody was obtained from two secondarily stimulated donor rabbits and their respective recipients (for method see footnote 4 in text). The dissociation rate of the antibody produced by the transferred cells in the recipients is similar to that produced in the donors shortly before transfer. (From Grey, 1962.)

cules themselves. However, these studies and others mentioned previously (J. C. Roberts and Dixon, 1955) indicate that the antibody produced from and in the foreign host resembles in several ways the molecules found in the original donor environment. These results suggest that the major controlling influence in the formation of certain qualities of the antibody molecules rests neither in the raw materials provided for the synthesis of the antibody nor in the antigen necessary for its stimulation, but rather in the constitution of the antibody-forming cell and involves a certain predisposition and functional autonomy of the cell.

VII. The Latent Period of the Antibody Response

A. Studies on the Times Required for Various Phases of the Latent Period

The same lag or latent period between the injection of antigen and the appearance of detectable antibody which exists in the intact animal was also found in cell transfer situations. In a series of thorough studies involving the transfer of antibody-producing cells, the Harrises (S. and T. N. Harris, 1954a, 1958; T. N. Harris *et al.*, 1954b, 1955, 1958b, 1959; S. Harris *et al.*, 1956) noted that a period of between 3 and 4 days was required after antigenic stimulation before antibody could be detected. This period was unchanged regardless of whether partially degraded or intact bacteria were used as antigen. Stimulation of donors more than 4 days prior to transfer resulted in diminished titers in the recipients indicating probably that a significant part of the formation of antibody had already taken place by that time (S. and T. N. Harris, 1954a). Moreover, cells taken from donors injected with antigen 2 days or 1 day or even 10 minutes before transfer formed antibody in the recipient that was detectable, respectively, 2, 3, or 4 days after the time of transfer, i.e., a latent period of between 3 and 4 days was maintained. As a further step, when normal donor rabbit cells were incubated *in vitro* with the *Shigella* organisms (S. and T. N. Harris, 1954b; T. N. Harris *et al.*, 1954b) and subsequently transferred to X-rayed recipients, a similar latent period was required before antibody could be detected. Similar observations have been made in different laboratories using different antigens and differing circumstances, the actual period of latency depending more or less on variables such as type of antigen and method of detection of antibody. That the length of the latent period may be influenced by the activity of the response is demonstrated by work of the Taliaferros (1962). Thus, the latent period was practically reduced to zero in recipients of spleen, lymph node, or bone-marrow cells transferred at the height of hemolysin formation during the primary or secondary response in repeatedly injected donors.

Whether the latent period reflects the interval occupied by catabolism of the antigen into active particles or the formation of the antibody-synthesizing mechanism or a delay in the secretion of antibody from those cells forming it or all of these together cannot be determined. It could even be that antibody is formed and secreted at an ever increasing rate from the time of stimulation but that the amount synthesized during the first few days is too low for detection. In many instances, at least a portion of the latter part of the latent period is required for the newly

formed antibody to saturate the persisting antigen before it can appear free in the circulation.

The Harrises (T. N. Harris *et al.*, 1955, 1958b; S. Harris and T. N. Harris, 1958; S. Harris *et al.*, 1956) studied the role that catabolism of antigen played in determining the length of the latent period. They found that the latent period was not appreciably reduced even when they used highly stimulating antigenic fragments of about 10,000 mol. wt. or less obtained by degradation of *Shigella* with trypsin. However, the antigen after degradation with trypsin might not be the final stimulating material and could well require further catabolic modification by the donor cells.

In a study of the actual time that the formation of antibody molecules begins, the incorporation of S^{35}-labeled amino acids into antibody was used by Taliaferro and Talmage (1955). They injected S^{35}-labeled amino acids at various times into either secondarily stimulated donors or into normal recipient rabbits and found that labeled amino acids were not incorporated into antibody in detectable amounts during the latent period (3 days) but only during the time when the level of precipitating antibody was rising in the recipient. The authors thus suggested that neither peptide precursors of antibody were formed during the latent period nor was antibody synthesized and retained during this time. Rather, other activities of importance might have occurred during the latent period such as the formation or induction of enzymes important in the synthesis of antibody. Stavitsky (1957, 1958) found similarly that S^{35}-labeled donor cells or fractions of these cells transferred before the time antibody made its appearance failed to introduce the S^{35}-label into the antibody molecules produced in the recipient. Again this indicated that by far the greatest synthesis of antibody starts after the latent period. Furthermore, the work of Taliaferro and Taliaferro (1957), involving the incorporation of S^{35}-labeled amino acids into circulating antibody during its rapid rise in rabbits, showed that the transit time from individual amino acids to circulating antibody occurred in 40 minutes. It was pointed out that this short transit time of amino acids indicated that antibody molecules are released from the cells shortly after their fabrication. This result, together with the previously mentioned data of Taliaferro and Talmage (1955) on the latent period, suggest that peptide precursors of the antibody are not formed in measurable amount in the early stages of the latent period and that probably the true precursors of antibody are individual amino acids.

B. Studies of the Latent Period Using the Subcellular Material for Transfer

Attempts have been carried out over the past several years to determine if subcellular units of "induced cells," taken from a presensitized animal, could transfer antibody formation. Sterzl and Hrubesova (1956) in an initial study and Friedman (1959) in a later one reported that nucleoproteins isolated from spleen cells of adult rabbits injected 48 hours previously with *Salmonella paratyphi* B and transferred to neonatal rabbits brought about a small but measurable production of antibody. Such antibodies were not detected in the transferred ribonucleoprotein fraction. Subsequently, however, an attempt was made to see if such antibody was fabricated *de novo* in the recipient by administering C^{14}-labeled amino acid at a time when maximal synthesis was supposed to occur. These results were negative (Hrubesova *et al.*, 1959) although antibody titers in the recipients were low. Again it was found that no detectable antibody existed in the transferred ribonucleoprotein (RNP), but it was suggested that antibody complexed with antigen in the RNP fraction could exist undetected. Furthermore, using *Brucella suis* antigen, to which the animals were far less likely to be exposed naturally, no transfer of antibody-synthesizing capacity by RNP could be measured. This finding suggested that the antibody which had been detected (Sterzl and Hrubesova, 1956) in the *Salmonella paratyphi* B experiments was probably formed in the donor as the result of a secondary response and had been carried to the recipient with the RNP, perhaps bound to antigen. Furthermore, negative results using *Brucella suis* antigen and human globulin were found in rabbits and chicks (Hrubesova, 1961a; Hrubesova and Trnka, 1961; Janković *et al.*, 1959) in which transfers of RNP, ribonucleic acid, and 2,4-dinitrophenol were performed. Thus, the production of antibody by transferred subcellular fractions obtained from adults primarily stimulated with antigen (and containing no "natural" antibody) has not yet been convincingly demonstrated.

C. Studies of the Latent Period by the Use of Metabolic Inhibitors

Use has been made recently of metabolic inhibitors to find if an alteration of certain cellular functions might affect the mechanism which is eventually responsible for the synthesis of antibody molecules. Sterzl (1960b) injected the purine antagonist 6-mercaptopurine into neonatal rabbit recipients of normal adult spleen cells that had been incubated *in vitro* with *Brucella suis* antigen. He found that injection of the 6-

mercaptopurine throughout the first 5 days after transfer completely abolished the antibody response. However, presensitized adult cells elicited an antibody response in spite of injections of the chemical into the recipient. These results were interpreted to indicate that steps involving synthesis of nucleoprotein are carried out during an inductive phase in primary but not secondary sensitization. To determine the part of the inductive phase that was sensitive to 6-mercaptopurine, the antimetabolite was introduced into the neonatal recipient at various times after transfer of the primarily sensitized cells. It was found that inhibition or elimination of the antibody response was achieved by injections at 24 and 72 hours after transfer. In further studies, Sterzl (1961) found that while purine derivatives were effective, pyrimidine antagonists in general were not. It was concluded that these experiments demonstrated a direct interference in the adaptive metabolic processes during the induction of cells. The incorporation of amino acids into antibody protein was apparently not affected by treatment with 6-mercaptopurine (Sterzl, 1960b).

The theory of clonal selection of antibody formation was tested experimentally by Sterzl (1961) in other studies using inhibitors. It was reasoned that if mitotic proliferation of the transferred cells was required for the establishment of clones of antibody-forming cells, the inhibition of mitoses in the recipient should reduce antibody formation. However, using the highest doses of colchicine, actinomycin C and 5-fluorouracil tolerated by recipients for a period of 5 days after transfer, no suppression of the synthesis of antibody was found. Hence, these data suggest that a maximal or near maximal response to a primary antigenic stimulation, at least in the experimental situation used by Sterzl (1961) is not dependent upon mitotic proliferation of cells such as would lead to clone formation. These conclusions appear to be at variance with the findings of Makinodan (1962) and of Nossal (this volume) that antibody-forming cells have all, or nearly all, arisen from cells which have incorporated thymidine into their nuclei and have, presumably, divided shortly beforehand. It would be desirable to be certain that the concentration of mitotic inhibitors used by Sterzl was sufficient to prevent mitosis in the precursors of antibody-forming cells, for the conclusions to be drawn are of the greatest theoretical importance.

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Phagocytosis

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I. Introduction

In writing this chapter I have attempted to keep in mind the desirability that it should be readable and for this reason I have struggled against the inclination to turn out a review of all the current literature pertaining to phagocytosis. In the selection of material, I have tried to be discriminating and in doing this I have been unfair to some of my colleagues in the world of science. For this I apologize and in mitigation I plead that the account of phagocytosis which is presented here is only intended to represent my current impressions of a field with which I have been closely connected for the past several years. This account is by no means exhaustive and deals mainly with work published during the past 10 years. An earlier review to which the reader is directed is that by Berry and Spies (1949).

Since the beginnings of immunology, at the turn of the century, the engulfment of bacteria and other particulate materials by various cells in the animal body has been recognized to be an important mechanism by which animals remove toxic and foreign materials. For this process to occur efficiently it has also been established that a coating of antibodies or opsonins on the bacteria is desirable. Because our knowledge of the details of this process has not greatly advanced since that time, the present account will probably pose more questions than it will provide answers.

By the term phagocytosis we mean, of course, the whole act by which cells swallow other particulate material. Some of the information on phagocytosis has accumulated from studies of whole animals in which the cell system involved is referred to as the reticulo-endothelial system. Other data have been achieved by work with isolated populations of phagocytic cells *in vitro*. Both these sources will be considered in detail because they largely complement each other. For the sake of simplicity they will first be examined separately and an attempt will be made later to synthesize the two parts into one functional whole.

II. The Reticulo-endothelial System

Since we are concerned here with phagocytosis, we will adhere to Payling Wright's (1953) description of the reticulo-endothelial system (RES) as being a collection of mesenchymal cells characterized by the readiness with which they ingest small particles and colloids.

This definition will include collectively the following groups of cells:

1. Those stationary cells lying in close apposition to the blood stream in the liver (Kupffer cells), the spleen (splenic macrophages), bone marrow, and adrenals
2. The macrophages of the lymph glands which mainly filter the lymph
3. Those wandering macrophages that are to be found in the serous cavities such as the peritoneum
4. The blood monocytes
5. The histiocytes of the connective tissue
6. The microglia of the central nervous system.

The interrelations of these various groups of cells have been briefly described by Howard (1961), and it is clear that there is no general agreement on this. It seems quite possible that each cell type may have some ability to change into cells belonging to one or more of the other groups, and it has certainly been established from studies in a rabbit ear chamber (Ebert and Florey, 1939) that blood monocytes may change into macrophages which are much more phagocytic than the monocytes. The origins of these groups of reticulo-endothelial (RE) cells are obscure and little is known of their turnover. It has recently been shown by Nicol and Bilbey (1958) that macrophages may be shed from the liver and spleen into the venous return to the heart and that they are then picked up in the lungs and may be demonstrated lying free in the alveoli. Although this follow-up of marked macrophages was necessarily done in

animals under conditions of hyperactive RE function, it suggests that the same course of events may occur to a lesser extent normally and may be a pathway in the disposal of effete phagocytic cells which may thus be finally swept up or coughed up from the lungs and swallowed.

A. The Phagocytic Function of the RES

The earlier work on the functional activity of the RES has established the extraordinary efficiency of the system in removing inert particles such as carmine and Thorotrast from the circulation. A similar efficiency was noted in clearing some bacteria. Bull (1915), for example, observed a reduction in blood count of *Salmonella typhi* in rabbits from 10^7 per milliliter, 1 minute after injection, to 40 per milliliter after only 15 minutes. On the other hand, the greater part of an initially injected dose of *Streptococcus pneumoniae* was still circulating in rabbits after half an hour (Manwaring and Coe, 1916). These early studies were somewhat handicapped by a lack of quantitative precision and the clearance of inert colloids was usually followed by observing the accumulation of the material by microscopic methods. This deficiency has been well remedied by the development of a quantitative technique for measuring the rate of clearance of India ink from the circulation of mice and other small animals (Halpern *et al.*, 1953). By this method, a dose of suitably prepared colloidal carbon is injected intravenously into mice, and samples of venous blood withdrawn at carefully timed intervals from the retro-orbital venous plexus of the mice. The measured volume of this blood sample is delivered into sodium carbonate solution to lyse the red cells, and the quantity of colloidal carbon still in suspension in the blood is measured in a nephelometer. This method has been further elaborated in the numerous studies of Benacerraf *et al.* (1957) in which the rate of clearance of particles is expressed as an exponential function. Thus, a phagocytic index (K) is obtained as follows:

$$K = \frac{\log \text{concentration } a \ - \log \text{concentration } b}{t_b - t_a}$$

where t_b and t_a are the times at which concentrations b and a occurred. By this means, the kinetics of clearance of particles by the RES have been put on a reasonably quantitative basis and the changes in functional activity which may follow other procedures have been securely established. Halpern, Benacerraf, and their colleagues have demonstrated that the phagocytic index for inert colloids decreases in the series consisting of mouse, rat, guinea pig, and rabbit, but it seems that this is the

result of the relative sizes of liver and spleen in these animals and that the phagocytic activity per unit weight of RES is the same in all the animals measured. An important point revealed by these workers (Halpern *et al.*, 1957) is that the larger the particle the faster its rate of removal. This has been confirmed by Jancsó (1955) using colloidal gold and by Dobson (1957) using chromium phosphate.

B. Factors Changing the Phagocytic Activity of the RES

Following an injection of colloidal material into the blood stream, the clearing efficiency for some time afterward may be measured by injecting subsequent doses of the same or similar particulate material. In this way it is commonly found that there is a depression of the clearing function for a period of a few hours after the initial assault and that this is followed several hours later by an increase in phagocytic activity usually well above the normal levels. This hyperactive state may last for approximately 1 week. If the deposition of particulate material in the RES is followed in the course of this hyperactive period, the number of cells participating in the uptake of colloid is seen to be greatly increased and, in addition, the individual activity of each phagocytic cell appears to be greater as they may each contain a larger amount of colloid.

A question of some importance not yet settled is the origin of the apparently greater number of, say, Kupffer cells in the liver following stimulation of the RES. Do these arise from existing Kupffer cells by division or are they merely transformed endothelial cells, or is it possible that a Kupffer cell is an endothelial cell which has taken up some particulate material and that the numbers of endothelial cells which transform depend on the numbers of particles available and suitably prepared for phagocytosis? There have been many attempts to answer this question but most of them suffer from the uncertainty that the demonstration of the reticulo-endothelial cells has involved using their phagocytic function which we know alters rapidly the very parameter in question. One possible approach seems to be to follow the incorporation of some nucleic acid precursor into the RE cells during the phase of stimulation. Kelly (1958) found a 2- to 10-fold increase in the specific activity of P^{32} in the deoxyribonucleic acid (DNA) isolated from the livers of mice during the phase of stimulation. This result implies that the cells in the livers of mice having a stimulated RES were synthesizing new DNA and were, therefore, dividing, but, unfortunately, no information was presented as to the particular cells involved. The origin of the Kupffer cells and other phagocytes will be referred to later.

The method of quantitative study developed with carbon has more recently been applied using bacteria and antigens as the agents to be cleared by the RES. Benacerraf *et al.* (1959b) found that the clearance of P^{32}-labeled, living *Escherichia coli* and *Staphylococcus aureus* from the circulation of mice could only be expressed exponentially for the first few minutes when approximately 60% of the initial amount circulating was cleared. The distribution of the bacteria was similar to that of other colloids and, in general, the results paralleled those using inert materials, with one difference. The rates of clearance in different animal species correlated with the level of circulating antibody against the organism cleared. More rapid clearance was also noted if the bacteria were sensitized with antibody before injection.

C. Correlations between RES Activity and Immunity

One of the few undisputed observations in immunology is the fact that possession by an animal of antibody specifically reacting with a certain bacterial or viral parasite may serve to protect that animal against future disease from the particular microorganism. The mechanisms by which the antibody ensures this immunity are often obscure but probably its opsonic effect on bacteria, which permits phagocytosis to occur, is an important one. There appears to be a close correlation between the antibody content of the blood and the rate of clearance of the corresponding antigen. Indeed, Patterson *et al.* (1960) have suggested that this is the most sensitive test for antibody.

Support for a causative relationship between rate of clearance by the RES and immunity is provided by the finding that injection of various colloids into animals, such as to cause a blocking and later a stimulation of RES function, will often result in a concurrent depression followed by an elevation in the animals' resistance to a variety of infectious agents, and these changes follow a quite closely parallel time course. These changes in nonspecific immunity follow injections of bacterial lipopolysaccharides (Rowley, 1956) as well as injections of the lipid A component of lipopolysaccharide (Howard *et al.*, 1957), Thorotrast (Howard *et al.*, 1958), colloidal sulfur (Kiser *et al.*, 1956), and heat-denatured albumin–globulin aggregates (Benacerraf *et al.*, 1955).

Soon after the reopening of research on this phenomenon of nonspecific immunity (Rowley, 1955), it was suggested by Landy and Pillemer (1956) that the concurrent rise in the level of properdin was an important factor in its causation. They were thinking in terms of the direct bactericidal action which properdin and the complement system exert on some Gram-negative bacteria, but this explanation, involving

a direct action, was later abandoned when it was shown that the immunity extended to bacteria resistant to the properdin system (Howard *et al.*, 1958). It now seems possible that an increase in humoral serum factors may be decisive in controlling the over-all clearing activity of the RES, since Rowley (1960) and Jenkin and Benacerraf (1960) have shown that serums from animals with hyperactive RES possess increased opsonic activity. It may well be that the increased properdin level contributes to a nonspecific increase in RES clearing function (see Howard and Wardlaw, 1958). It must, of course, be appreciated that an increased rate of clearance does not necessarily lead to an increased immunity because clearance is not always followed by intracellular killing. An added complication is that stimulation of the clearing capacity of the RES to an equal degree by two different colloids, such as Thorotrast and lipopolysaccharide, will not necessarily be accompanied by increases in resistance to infection. In fact, Howard (1959) has shown that opposite effects may occur—the lipopolysaccharide leading to increased and the Thorotrast to decreased immunity. It would be interesting to know if pretreatment with these two colloids, both of which increase the rate of clearance of lipopolysaccharide, would also lead to increased clearance of the bacterial challenge strain.

D. Opsonic Factors in RES Clearance

Bacterial clearance differs from that of such "inert" materials as Thorotrast and carbon in possessing an element of specificity. Different strains of bacteria, although of the same species and the same size, may be removed at different rates (Wardlaw and Howard, 1959). Such differences may be related to the virulence of the bacterial strains for the animal being studied (Jenkin and Rowley, 1959). It seems fallacious, however, to regard any particle as inert, as was recognized long ago by Fenn (1921). He showed that phagocytosis of carbon by polymorphs was promoted by contact with serum.

New light has been shed on the phenomenon of RE blockade by the demonstration during this phase of suppressed activity that serum opsonin fractions were limiting the over-all process and that when these opsonins were provided the blockaded RES cleared particles in more or less normal fashion (Jenkin and Rowley, 1961). It seems probable that the phagocytosis of all particles, whether inert or living and specific, is enhanced by the presence of serum fractions, and it is possible that the different abilities of colloids to produce RE blockade may be attributable to their adsorptive properties for suitable serum proteins.

When using whole animals it is difficult to design experiments which

will enable the nature of these opsonins to be discovered and for this purpose several workers have followed the uptake of bacteria and of particles by the perfused liver *in situ*. Manwaring and Coe (1916) and Manwaring and Fritschen (1923) perfused suspensions of *Streptococcus pneumoniae* and *Klebsiella pneumoniae* through the livers of dogs and rabbits and measured the rates at which the organisms disappeared from the perfusion fluid. In spite of many technical difficulties, they demonstrated that high dilutions of immune serum were effective in promoting clearance by the liver RES. The same sort of study with rats enabled Howard and Wardlaw (1958) to define the components of normal rat serum that were necessary for opsonic activity. They found that if the bacteria were suspended in Locke-Ringer's solution for perfusion, little removal took place on passage through the liver, but addition of normal rat serum to the suspension enabled up to 40% of the bacteria to be removed in one cycle. Pretreatment of the rat serum by the established methods for removal of complement, etc., showed that the exhibition of full opsonic activity by normal sera required all the components of complement, specific antibody and another heat-labile component, which was possibly properdin.

The vital and rate-determining role of humoral opsonic factors in clearance by the RES is further supported by work on the radiosensitivity of this function. The general finding has been that large doses of the order of 800 rads, which do not affect the clearing ability of RES, greatly affect the potentiality for change in this system. The depression in phagocytic activity produced by carbon blockade in irradiated animals lasted for longer periods than in normal controls (Benacerraf *et al.*, 1959a). Moreover, the RES in irradiated animals could not be stimulated by the injection of the yeast-cell wall material, zymosan. On the other hand, irradiation at these dose levels greatly increases the susceptibility of animals to experimental infection as well as to invasion and infection by their own normal intestinal organisms. If there is an explanation for this increased susceptibility in the face of the normal clearance by the RES, it is likely to be found in the diminished capacity for change which this system shows after irradiation. The finding that blockade may be caused by a limitation in the supply of serum opsonins (Jenkin and Rowley, 1961), coupled with Taliaferro and Janssen's (1952) demonstration that the antibody response is radiosensitive when the antigen is a particulate red cell, suggests that the action of irradiation on antibody and opsonin synthesis may only be apparent when RES function requires a rapid replacement of these humoral factors. During infection, of course, the RES is constantly in such a dynamic state.

III. *In Vitro* Studies on Phagocytosis

In attempting to understand the various ramifications and interrelationships of the humoral and cellular components which lead to resistance of animals to infection, we are forced to try to take the systems apart to simplify the many complexities.

In order to assess our ignorance of the phagocytic process, we should recall the various stages involved about which we need information. These are:

1. The nature of opsonins
2. The combination of these with bacteria
3. The nature of the bacterial component for this combination
4. The collision between bacteria and phagocyte—is this a matter of chance only?
5. The actual process and manner of engulfment
6. Postengulfment factors, leading to death or survival of bacteria, the enzymes, or other substances which cause this
7. Any effect of the particle on the host phagocytic cell
8. Reasons why this over-all process possesses considerable specificity
9. The origin of the phagocytic cells.

Data on some of the these points have been provided by work necessarily involving isolated populations of phagocytic cells, but it is advisable to remember that in supposedly simplifying events in this way we may, in fact, be adding other variables which so complicate the issue that little advance is made or false conclusions are reached.

A. Cellular Immunity

In most of the work reported so far, the importance of antibody or opsonin both in protecting animals against infection and in promoting RES clearance has been a persuasive influence toward acceptance of a causal relationship between RES function and immunity. In one important instance where the RES still appears to be implicated in immunity, there has never been a satisfactory demonstration of circulating antibody—this is in tuberculosis. For this reason, the work of Lurie (1942) has considerable importance. He injected tubercle bacilli into normal rabbits and into rabbits immunized with BCG and later removed the local lymph nodes, parts of which were used to assess the bacterial count both by microscopic and by culture techniques. The remainder of the lymph node material was minced up and injected into the anterior chambers of rabbits' eyes—one eye for bacilli in "immune" cells, the other eye for the normal cells. After about 10 days, the animals were killed and the numbers of acid-fast bacilli in the two anterior chambers were as-

sessed. He found that bacteria that had been phagocytized by immune cells were inhibited in their growth or killed even when transplanted into normal animals. Since this result could not be attributed to the presence of immune serum, Lurie concluded that immune cells were more bacteriostatic than normal ones. In other words, he postulated the development of *cellular immunity.*

These findings, which seem to indicate that monocytes in an immune animal may be made bactericidal by previous contact with acid-fast organisms, have gone unchallenged. Mackaness (1954b) has pointed out, however, that the transfer of cells from immune animals may have caused the transfer of hypersensitivity and other local tissue reactions that affected the viability of the monocytes and indirectly of the contained acid-fast organisms.

Nevertheless these findings by Lurie stimulated others to work with isolated populations of monocytes in tissue culture systems and to infect them with mycobacteria. The commonly used technique established by Mackaness (1952) was to obtain monocytes from the peritoneum of guinea pigs or rabbits previously injected with insoluble materials such as starch, glycogen, or paraffin oil to stimulate a peritoneal monocytosis. These cells were washed by centrifuging in balanced salt solution, were brought into contact with acid-fast bacilli and, by one device or another, were stuck to a glass surface in order that the intracellular bacteria could be counted. The media in which the infected cells were maintained during observation usually contained antibiotics to prevent extracellular growth of the bacteria. By this general method, using an ingenious tissue culture cell, Mackaness (1954a) found that strains of *Mycobacterium tuberculosis* always survived inside rabbit monocytes and that engulfed virulent strains grew faster than engulfed attenuated or avirulent strains. Monocytes from normal or BCG vaccinated rabbits behaved in an identical fashion when infected with mycobacteria *in vitro.* In both instances there was a steady and similar rate of intracellular growth of the bacteria. Similar studies by Suter (1953), using guinea pig and rabbit mononuclear cells from glycogen-induced peritoneal exudates, pointed to the same main conclusion, i.e., that monocytes were unable to reduce the numbers of intracellular mycobacteria even though the bacteria were from attenuated strains. The major difference between these two workers was that Suter found macrophages from BCG immunized animals to be more capable of suppressing growth of intracellular bacteria than those from normal animals, in which growth was rapid. Suter's results were more in accord with Lurie's demonstration of cellular immunity, although this was in the form of decreased intracellular growth of the bacteria rather

than a clear bactericidal effect. If one has to try to criticize these excellent experiments of Mackaness and Suter, it would seem that both suffer from the necessity of obtaining their phagocytic cells from stimulated animals. Thus, the cells would already have been involved in phagocytosis in the peritoneum before they were handled *in vitro*. Moreover, their monocytes were washed free of oil or glycogen, etc., by centrifugation. In our hands, this treatment has been found to reduce greatly the phagocytic and bactericidal properties of mouse peritoneal macrophages. This type of experiment, furthermore, is unfortunately unable to tell us whether a proportion of the phagocytic cells is able to kill bacteria while the rest permit intracellular growth. In other words, the macrophage population under study must be treated as homogenous and the results averaged out per cell. It seems more likely that the population of phagocytes is in a dynamic state, and that individual ones vary so markedly that in the course of this life cycle they may vary in their phagocytic and intracellular capabilities.

The existence of a type of cellular immunity in phagocytic cells has been supported by the studies of Elberg (1960) and colleagues. They noted a decrease in the rate of degeneration of monocytes from BCG vaccinated animals after these cells had been infected *in vitro*. Within 10 hours of infection with *M. tuberculosis* H37RV, up to 35% of the monocytes derived from normal rabbits showed signs of breakdown, whereas none of the infected, immune cells showed this. A similar protection against the toxic effects of intracellular mycobacteria was found in monocytes from animals immunized with unrelated antigens such as *Salmonella* vaccine or ovalbumin. Part of the cellular protection was provided by the serum in which the cells were suspended and part was a property of the immune cells themselves. The best survival was obtained when cells from animals immunized with BCG were suspended in serum from similarly immunized animals. This *in vitro* protection of cells against degeneration, if related to immunity, appears then to be of a nonspecific character and could possibly be the result of the increased intracellular metabolic rate which phagocytosis itself seems to induce in leucocytes. Elberg has suggested that this effect may be somewhat analogous to the immunity from phage attack which a bacterium possesses when it is already infected with a related lysogenic phage. A strict analogy here would require that all the monocytes from the vaccinated animals had been in direct contact with and still carried antigenic material from the vaccine.

The correlation between bacterial virulence and survival within macrophages has been found by numerous workers. Vischer and Suter

(1954) found that *Toxoplasma gondii* would grow well in some macrophages and that the rate of intracellular growth was correlated with the susceptibility of the species to infection with *Toxoplasma*. Macrophages *in vitro* which had been derived from immunized animals had a definite inhibiting effect, particularly in the presence of immune serum. As a further example of this correlation, Furness (1958) infected mouse peritoneal macrophages with a virulent and an avirulent strain of *Salmonella typhimurium*. He found in contrast to most workers that both strains were phagocytized equally well. Both were killed at the same rate for the first 3 hours after ingestion, but the virulent organisms subsequently multiplied while the avirulent ones were eliminated. He ignored the initial killing found with both strains and concluded that virulence in *S. typhimurium* is dependent on the capacity to survive intracellularly. A further paper by Furness and Ferreira (1959) dealt with rat and guinea pig macrophages and their interactions with virulent and avirulent strains of *S. typhimurium* and *S. paratyphi* B. Once again the figures presented show that 3 hours after infection (the first reading) the macrophages from all three animals had reduced the number of the intracellular organisms. After this time there was a sharp divergence. The surviving virulent organisms began to multiply rapidly while the count of avirulent organisms remained low. The conclusion reached by the authors, that different susceptibility to *Salmonella* infections between animal species depends on the relative abilities of the phagocytes to kill the ingested organisms, is not well supported by the results presented. Moreover, their finding that virulent organisms are taken up by cells just as effectively as avirulent ones has been strongly challenged by a number of workers (Rowley and Whitby, 1959; Cohn and Morse, 1959; Jenkin and Benacerraf, 1960; Mackaness, 1954b).

Jenkin and Benacerraf (1960), using the same two strains of *S. typhimurium* which Furness employed, found little phagocytosis of the virulent strain at a time when 50% of the avirulent organisms had been ingested. They also showed that once phagocytosis had been achieved by using an opsonic antiserum the virulent strain was killed at about the same rate as the avirulent one. Another important point arises from this work which has a bearing on the question of cellular immunity. Macrophages from animals immunized with BCG were much more effective at ingesting and killing several strains of *Salmonella* than were normal ones. The possibility is suggested here that the cellular effect may be quite nonspecific and different from the precise and specific antibody-type of immunity.

In spite of considerable evidence that the humoral and cellular com-

ponents of immunity are mutually interdependent, work is constantly being reported supporting one or another aspect without adequately separating or estimating the effect of the interdependent components. For example, a recent paper favors strongly the existence of cellular immunity (Miya *et al.*, 1961). In these experiments, macrophages were obtained from the peritoneum of normal or immunized mice or rabbits following stimulation with glycogen. These cells were washed three times by slow speed centrifugation, were resuspended in Krebs-Ringer phosphate buffer containing 30% of normal rabbit serum, were mixed in a Warburg vessel with a threefold excess of bacteria, and their respiration rate followed. Controls were set up with organisms and cells alone. The rate of oxygen uptake was greatest with bacteria alone, and least with cells alone; normal cells plus bacteria gave a rate approaching that of the bacteria alone, whereas immune cells plus bacteria respired at the low rate given by cells alone. From these results, the authors concluded that immune cells were killing the bacteria whereas normal cells were not. In fact this type of experiment indicates that in the presence of normal cells bacteria still respire and this could be owing to a decreased amount of phagocytosis by the "normal" as opposed to the immune cells. The results obtained could be explained entirely on the basis of different phagocytic rates in the two systems. If, for example, the normal cells had only ingested 50% of the bacteria, the remaining 50% of extracellular ones could respire at such a rate as to obscure completely any diminished rate of respiration by the phagocytized bacteria. In addition, the different phagocytic abilities of the two cell systems could be accounted for by the presence of traces of antibody in the immune cells. It is unlikely that antibody could be removed completely from *within* macrophages without damaging the cells themselves. A further criticism of this paper is the lack of data on the specificity of the reported effect. In other words, did phagocytic cells from animals immunized against *Klebsiella pneumoniae* behave like normal macrophages toward some other unrelated bacterial strain?

For the moment the case for cellular immunity against infection, in the absence of antibody, is not proven and will be particularly difficult to establish since the phagocytic cells may themselves be able to produce antibody.

The foregoing references are only a few examples pointing to the existence of cellular immunity. All of them have some unsatisfactory features like most experiments. Those of them dealing with isolated populations of cells usually involved one or more of the following disadvantageous features:

(a) *The tissue culture medium contained antibiotics.* This was, of course, used to keep down the multiplication of extracellular unphagocytized bacteria, which otherwise would have overgrown the whole culture and killed the phagocytic cells. It has been assumed, though without proof, that the concentration used did not penetrate the monocytes sufficiently to affect the growth of the intracellular bacteria.

(b) *The cells used were obtained by stimulation with glycogen and oil.* This convenient method to obtain large quantities of cells of necessity harvests cells that are no longer normal and recently such cells which have engulfed particles have been found to possess more active enzymatic systems than do normal cells (Cohn and Morse, 1959). This obstacle could be overcome by using normal cells. Thus, the normal mouse peritoneum contains approximately 10^7 cells of which 90% or so are mononuclear. These can be washed out and cultured *in vitro* (Rowley, 1958). The normal lung is another good source of phagocytic cells, as shown by Myrvik *et al.* (1961).

(c) *Once obtained the phagocytic cells were repeatedly washed by centrifuging.* This procedure has usually been resorted to with the object of removing all humoral factors from the system. Even if this aim could be achieved, it is usually at the expense of the vitality of the cells. In order to maintain these cells at their *in vivo* metabolic rate, they must be carefully handled and not subjected to any sudden changes of pH or temperature.

(d) *The infected cells were cultivated for several days and sampled daily.* The longer the time after removal of the macrophages from the body, the more artificial the conditions become There is the added danger that more rapid changes will be missed by daily sampling. Several workers have ignored the events occurring during the first day of *in vitro* growth of their infected cells and have drawn conclusions from the trend after several days of cultivation. Mitsuhashi *et al.* (1961) describe experiments in which mouse monocytes, obtained by glycogen stimulation, were infected at a rate of 1 monocyte to 10 virulent or avirulent organisms of *Salmonella enteritidis.* Their experiments show that during the first day of *in vitro* cultivation in Hank's solution with antibiotics both virulent and avirulent strains multiply rapidly intracellularly at equal rates. After this time, however, while the virulent strains continued to grow, the avirulent bacteria were gradually killed. Considered at the cellular level, their data would mean that a cell, which originally ingested 6 bacteria and which in the first 24 hours of cultivation had allowed these to multiply to 12, suddenly acquired, in artificial culture, the capacity to kill this large number of intracellular organisms. It seems

more likely that such late effects would be due to the penetration of antibiotics into damaged cells.

B. Quantitative Kinetic Studies

Attempts have been made to design experiments which would circumvent some of the foregoing sources of error. One of the earliest and most elegant of these was the direct observation under the microscope by Wilson *et al.* (1957) that phagocytosis of group A streptococci by mouse or human neutrophils resulted in death of the bacteria with a half-life of 7 to 8 minutes. This same technique has been used recently by Melly *et al.* (1960) to compare the fate of virulent *Staphylococcus aureus* with avirulent *S. albus* within human polymorphs. Both strains were destroyed, the *S. albus* strain the quickest, though even the *S. aureus* strain had a half-life of only 25 minutes. Multiple ingestions increased the over-all rate of destruction. This modified the impression given earlier by Rogers and Tompsett (1952) that *S. aureus* strains survived in cells better than *S. albus* strains.

Another attempt at giving accurate data has been reported by Rowley (1958). Peritoneal macrophages, after isolation from normal mice, i.e., not previously stimulated, were placed without washing into a Porter flask, in a medium without antibiotics, containing a "flying coverslip" to which some of the macrophages attached themselves. To this flask were added a known number of bacteria. At intervals, thereafter, the number of viable bacteria in the extra- and intracellular phases could be estimated by direct plating. Using this simple method, it appeared that opsonized *E. coli* were rapidly killed intracellularly, whereas unopsonized intracellular bacteria were able to multiply. In an extension of this, Whitby and Rowley (1959) found that a variety of organisms, both virulent and avirulent for mice, were rapidly taken up in the presence of antibody and were destroyed by mouse peritoneal macrophages both *in vitro* and *in vivo*. Under these conditions, bacterial strains had an average half-life inside the cells of approximately 8 minutes, except for the strain of *S. aureus*. It had an intracellular half-life of approximately 20 minutes. In the absence of added antibody, little phagocytosis occurred and consequently no killing was measurable. Further *in vitro* experiments lead these authors to the conclusion that once a population of virulent or avirulent organisms has been phagocytized to an equal extent the rates of killing of the two types will not show much difference (Rowley and Whitby, 1959).

The controlling and rate-limiting effect of opsonins on the interaction

between phagocytes and bacteria has also been found by Cohn and Morse (1959) in studies with rabbit polymorphonuclear leucocytes. Using mixtures of bacteria and polymorphs in suspension in siliconed tubes, they determined the number of viable bacteria in the extra- and intracellular phases by centrifugation and disintegration of the cellular pellet in a high-speed homogenizer. The failure of polymorphs to kill strains of *S. aureus* was seen to be because of the lack of phagocytosis. Once this was ensured by adding immune serum, the virulent strains were killed at the same rate as the avirulent ones. Moreover, washed cells from immune animals behaved as did cells from normal animals provided that phagocytosis of the bacteria had occurred to an equal extent as a result of the incorporation of immune serum into the system. The same conclusions were reached by Mackaness (1960) with rabbit macrophages isolated in the same way as in his original work with acid-fast bacteria. He found that a strain of *S. aureus* resisted phagocytosis unless immune serum was added. *Staphylococcus albus,* on the other hand, was rapidly taken up by the cells. Once phagocytosis of either strain had occurred, the bacteria were inactivated at approximately the same rate, with a half-life of approximately 30 minutes, which was somewhat slower than inside polymorphs. Since the rate at which the bacteria died in their intracellular situation decreased with time, Mackaness made the interesting suggestion that the decreasing rate could be accounted for by variations in the macrophage population since bacteria might survive for abnormally long periods in old cells that had lost their antibacterial action.

Further evidence for the importance of opsonins was provided by Hirsch and Church (1960) who used a system involving phagocytosis and killing of group A streptococci by rabbit and human polymorphs. Thus, human polymorphs effectively destroyed the bacteria because they possessed anti-M substances and antihyaluronic acid substances. Rabbit polymorphs will not phagocytize the organisms even in the presence of M antibody, because of the lack of antihyaluronic acid substance. In the presence of hyaluronidase, rabbit cells would take up and kill these bacteria just as well as human cells.

1. Factors Affecting the Rate of Phagocytosis

It will be obvious from much of the foregoing work that the coating of bacteria or other particles with opsonin, either in the form of specific antibody or some other less well-defined serum protein, is usually if not always, a necessary preliminary for efficient phagocytosis. This condition applies to polymorphs and macrophages as well as to the *in vivo* RES. Factors which have a direct effect on this vital, preliminary and often

rate-determining step, may, therefore, affect the whole antibacterial efficiency of the phagocytic system.

The fact that phagocytosis is an energy-dependent process has been known for some time. Stähelin *et al.* (1956) showed that monocytes normally use more oxygen and produce more lactic acid than do polymorphs and that oxygen consumption doubled during the uptake of bacteria. In keeping with these results, they showed that the respiratory activity of leucocytes isolated from tuberculous guinea pigs was greater than that from normal animals. During the uptake of bacteria, the respiration of polymorphs, as measured by the rate of production of $C^{14}O_2$ from labeled glucose, increased fivefold (Stähelin *et al.*, 1957).

Other isolated observations bearing on this subject have appeared. Thus, Crabbé (1956) reported that small doses of cortisone increased the amount of phagocytosis which occurred when staphylococci were mixed with rabbit pleural macrophages. Estrogens were found to increase the rate of uptake by the RES (Biozzi *et al.*, 1957), and certain fats and simple fatty acid esters also have an effect. This appeared to have some chemical specificity since ethyl oleate somewhat depressed the phagocytic index to 0.018 from a control value of 0.034, whereas glyceryl monooleate left it unchanged at 0.036 and glyceryl trioleate elevated the clearance to 0.098. With these changes in RES function, the authors found no increase in liver and spleen weights. They thus concluded that cell multiplication in the RES was unlikely to be the cause of the hyperactivity and suggested the interesting possibility that the fatty chylomicrons circulating in the blood may play a part in controlling RES activity. Unfortunately no data were presented about the effect of such treatments on susceptibility to experimental infections (Stuart *et al.*, 1960).

Studies with isolated cell populations have led to the general finding that phagocytosis of particulate or colloidal materials leads to an increase in the subsequent phagocytic and metabolic ability of the system. Treatment of macrophages both *in vivo* or *in vitro* with bacterial lipopolysaccharides promoted a greater phagocytic efficiency against unrelated particles (Rowley, 1960). Strauss and Stetson (1960) showed that the addition of bacterial endotoxin, soluble antigen–antibody complexes or polystyrene particles to heparinized blood caused great increases in respiratory activity.

A composite synthesis of these various points has been achieved by the careful and systematic studies of Cohn and Morse (1960). From these it is clear that phagocytosis is most efficient in the presence of opsonins and glucose, the process is energy-requiring, and glycolysis is

the normal source for this. Inhibitors which block glycolysis, such as iodoacetate or arsenite, inhibit uptake of particles. Rabbit polymorphs, which ingested heat-killed *Staphylococcus albus,* showed increases in oxygen and glucose utilization and in lactic acid production. A wide range of concentrations of bacterial lipopolysaccharide was found to increase the rate of phagocytosis and killing because of a direct effect on the leucocytes. It seems likely from the work of Cohn and Morse that the lipopolysaccharide itself increases the utilization of glucose and the production of lactic acid in an unspecific fashion, owing to, in some obscure way, an over-all increase in metabolic rate of the phagocytes.

2. *Intracellular Postengulfment Events*

Numerous reports indicate not only that the phagocytic function of cells is increased by phagocytosis but that practically every other metabolic function increases as well. An example of this is the great increase in acid phosphatase in macrophages from tuberculous lesions (Grogg and Pearse, 1952) and which Weiss and Fawcett (1953) observed in tissue cultures of chicken macrophages to be correlated with phagocytic ability. Howard (1959) found that stimulation of the RES with lipopolysaccharides was accompanied by a great increase in the acid phosphatase in the Kupffer cells of the liver, and more recently Auzins and Rowley (1961) have noted that mouse peritoneal macrophages from animals given lipopolysaccharides, bacteria, or other particles contained up to three times the normal acid phosphatase activity.

Once again we are indebted to Cohn and Hirsch (1960a) for a clarification. These authors found that the cytoplasmic granules isolated in a purified state from rabbit polymorphs had many enzymatic activities associated with them, particularly acid and alkaline phosphatases, ribonuclease, and β-glucuronidase. Moreover, they contained a major part of the antibacterial principle, phagocytin, which could be liberated from them with dilute acid. In a further paper, Hirsch and Cohn (1960) reported that phagocytosis caused a loss of granules from rabbit polymorphs and, concurrently, the proportion of granule-associated enzymes decreased in the general cytoplasm. It seems that macrophages may undergo similar granule changes since Old and Benacerraf (1961) have demonstrated acid phosphatase activity confined to granules which may be clearly seen within macrophages. The other enzymes which Cohn and Hirsch found in their polymorph granules and the number of granules appears to correlate roughly with the phagocytic ability of these cells. Thus Auzins and Rowley (1961) found that lipopolysaccharide, when injected into mice, is followed within an hour or so by a decrease

in the number of granules containing acid phosphatase and in the total acid phosphatase activity of the lysed cells. However, 24 hours after the lipopolysaccharide injection, the number of granules appeared greater than in control cells and the total acid phosphatase activity was greatly increased. From this and Cohn and Hirsch's work, one can construct a working hypothesis that ingestion of particles by phagocytic cells causes an immediate increase in metabolic rate leading to an accumulation of lactic acid; and if the local pH becomes sufficiently acid, the enzymatic granules lyse and liberate their enzymes into the vacuoles in which the particulate material has been segregated. If the cell is still viable, the lost granules are replaced by some mechanism and overcompensation may occur.

A pleasing preliminary explanation for the increased metabolic function during phagocytosis has been offered by Evans and Karnovsky (1961). They suggest that decrease in intracellular pH is the first event, that it leads to the stimulation of reduced diphosphopyridine nucleotide (DPNH) oxidase activity of the granules and simultaneously to a reduced triphosphopyridine nucleotide (TPNH) linked conversion of pyruvate to lactate and that triphosphopyridine nucleotide is rapidly regenerated and stimulates the direct oxidative pathways involving the conversion of glucose-6-phosphate to carbon dioxide.

These various demonstrations that phagocytic cells behave at a higher metabolic rate when stimulated by the act of phagocytosis supports the criticism of using cells from stimulated hosts which was mentioned earlier in this chapter. If the cells are already stimulated by having engulfed oil, it is less likely that any differences between cells from immune or normal animals will be seen. This point is well illustrated in some recent work by Berk and Nelson (1961). Macrophages, when obtained from normal mice 16 hours after the intraperitoneal injection of paraffin oil, failed to exhibit any stimulatory effects of lipopolysaccharides on the succinoxidase or glycolytic activity, in contrast to the findings of Cohn and Morse (1960). The level of acid phosphatase activity in such oil-induced macrophages was 3 times greater than normal (Auzins and Rowley, 1961). It seems likely that such an increase might be expected for other enzymatic systems. In other words, this type of experiment is unphysiological.

C. Intracellular Bactericidal Mechanisms

For many years endeavors have been made to isolate from leucocytes bactericidal principles that might account for the extraordinary power of the leucocytes to kill many species of bacteria. Two major possibili-

ties appear as explanations for the antibacterial effects of phagocytic cells. The first is that the cells might contain specific antibacterial substances or antibiotics, each with its own antibacterial spectrum. The second one is that certain normally occurring enzymes or surface active materials exert more pronounced effects on the contained bacteria because of special physicochemical conditions within the leucocyte. Both of these alternatives have been supported in the literature and they may both contribute toward the end result.

1. Intracellular Killing by Specific Chemical Factors

Under this heading would be included factors whose action could still be demonstrated by cell lysates. Such antibacterial principles have been reported, but the evidence, in general, lacks chemical precision.

The most promising work along these lines has been that of Hirsch (1956). He isolated an antibacterial principle from rabbit polymorphonuclear leucocytes by disruption and extraction with salt solutions (1956) or with 0.01 *M* citric acid (Hirsch, 1960). Phagocytin, as this substance is called, appears to be an acid-soluble cytoplasmic protein occurring exclusively in granulocytes of various animals. It has not been demonstrated in extracts of any other cell types, including mononuclear phagocytes. Phagocytin exerts an antibacterial effect over a wide range of organisms, both Gram-negative and Gram-positive, and its lethal effect is increased at the acid pH that occurs inside phagocytic cells. The more recent demonstration by Cohn and Hirsch (1960b) that most of the intracellular phagocytin is associated with granules which can be lysed in the presence of dilute acid and which spontaneously lyse following phagocytosis of bacteria, makes it likely that phagocytin plays some part in the killing of bacteria by polymorphs. It may not be the only mechanism, but, since it is highly effective under the conditions found in infected polymorphs, it seems probable that it operates to some extent. It would be interesting to assess its importance by studying the effect of leucocytes on phagocytin-resistant mutants.

Another antibacterial principle has been isolated from rat peritoneal leucocytes by Fishman and Silverman (1957). The cells were disrupted by ultrasonic waves, and the resulting homogenate was bactericidal at pH 7 against many organisms, particularly beta hemolytic streptococci, *Staphylococcus aureus, Streptococcus pneumoniae* and *Shigella dysenteriae.* Chemical purification, which was carried out by Fishman *et al.* (1957), showed that the activity could be precipitated by 30% saturated ammonium sulfate, was reduced by trypsin and abolished by digestion

with lipase. The authors suggest that the activity may lie in a lipoprotein substance which exerts its effect through surface activity.

2. *Intracellular Killing Owing to the Physicochemical Environment of the Phagocyte*

It has been amply demonstrated that phagocytic cells have the capacity to create acidic conditions within vacuoles in their cytoplasm. This can be seen by following the color changes of ingested particles which have previously been stained with indicator dyes. In this way it is clear that pH 4.5 may commonly be reached (Sprick, 1956). At this pH and in the presence of lactic acid, some normally occurring enzymes, such as lysozyme, may have a pronounced antibacterial effect. Although lysozyme has a limited antibacterial spectrum under normal pH conditions, it has a wider power at more acid pH and in the presence of chelating agents, such as Versene or lactic acid (Repaske, 1956).

It is also possible that the intracellular acid conditions alone might kill the bacteria because most of the strains of pathogenic organisms, except mycobacteria, which have been used in experiments in this field, are killed rapidly between pH 3–3.5 (Looke and Rowley, 1961). More information is required about the physicochemical conditions including local pH inside the various types of phagocytic cells, particularly while they are involved in the act of phagocytosis.

Most of the studies on intracellular bactericidal mechanisms have used polymorphonuclear cells, but monocytes possess a highly effective antibacterial action, which has successfully evaded any explanation. Thus, monocytes do not normally possess significant amounts of lysozyme or phagocytin, and homogenates from them do not readily exhibit an antibacterial activity (Rowley, personal observation, 1959).

3. *Possible Effect of Opsonins on Intracellular Events*

Another difficulty in following the interactions of bacteria and phagocytic cells springs from the possibility that the opsonic proteins which aid the process of phagocytosis may also affect the subsequent intracellular events. Suggestive evidence for this was the finding referred to earlier that *E.coli,* when opsonized with horse serum, was rapidly taken up and killed by mouse peritoneal macrophages, whereas the same bacterial strain unopsonized was phagocytized to a very minor extent and multiplied inside the macrophages (Rowley, 1958). Similar results were reported by Jenkin and Benacerraf (1960) who concluded that opsonization affected the survival of bacteria within the cell. This very important point has resisted attempts to design unambiguous experiments.

Very recently, Jenkin and Rogers (1961) have contributed to the solution of this dilemma using a most elegant technique. They had the idea of promoting bacterial phagocytosis by allowing phage to be adsorbed onto a phage-resistant organism and opsonizing the phage–bacterium complex with serum which had no effect on the bacterial strain alone. Bacteria which had been engulfed as a result of acting as phage carriers were not killed within the macrophages. It is difficult to see how this result can be interpreted other than to indicate that specific opsonins not only promote phagocytosis but also the subsequent event leading to bacterial killing.

D. The Nature of Opsonins

Although it is certain that specific antibodies are effective opsonins, the activity of normal animal serums, although no less real, is not so well characterized. The participation of complement in this activity has been mentioned, and the remaining opsonic fraction can be removed from normal serum by absorption with the bacterial strain. In the case of Gram-negative bacteria, the opsonins in normal serums are not identical with antibody against the O-somatic antigens (Jenkin and Rowley, 1959). Nevertheless, by cross absorption tests, they show a fair degree of specificity (Jenkin, 1961). Part of the stimulation of immunity by lipopolysaccharides, as discussed in the foregoing appears to be the result of greater opsonic activity in the serum. Yet inasmuch as this immunity is nonspecific, we would expect either that there exist nonspecific opsonins or that lipopolysaccharide stimulates the production of many specific opsonins. Moreover, opsonins take part in the uptake of inert particles, such as carbon, starch (Nelson and Lebrun, 1956), and bentonite (Potter and Stollerman, 1961). It is possible that opsonins are specific proteins having a combining capacity for specific antigens as well as having a general affinity or adsorptive tendency toward the inert particles just mentioned. More information is needed about the nature of the natural antibodies or opsonins formed in normal serums. Advantage may be taken of the recently developed methods of protein fractionation which have so successfully been applied to specific acquired antibodies.

IV. Conclusions

In reviewing some of the factors bearing on the phenomena of phagocytosis, one is impressed by the many similarities in the behavior of the intact RES and the populations of phagocytic cells which have usually been studied *in vitro*. Both systems require the participation of opsonins,

even for inert particles, and their bactericidal property can be correlated to some extent with antibacterial immunity.

There is a large area of ignorance, however, which has not yet been mentioned. This area centers in the fact that organisms often re-emerge into the blood stream in spite of rapid phagocytosis and death in the RES and in other phagocytes. For example, *S. typhimurium*, after injection into mice, may disappear completely from the blood stream within a few hours and can only be detected for a few days in the reticulo-endothelial organs. Yet, many days later, perhaps, bacteremia occurs at a time when the organisms are more effectively removed by the RES than at first. From this one can deduce that very large numbers of bacteria are being seeded into the circulation in order to maintain this level of bacteremia. This means that, concurrently, some RE cells are engulfing organisms while other cells (and presumably RE cells) are liberating equally large numbers. Attempts to incriminate the spleen for this reseeding by following the effects of splenectomy on the level of bacteremia have been unsuccessful (Jenkin, personal communication, 1960). In any case, it looks as though effective and defective RE cells, in terms of antibacterial action, may coexist in the same organ. This emphasizes the necessity of following phagocytic cells of various kinds through their life cycles and to ascertain, for example, whether they are more capable of dealing with bacteria at a certain stage in maturation and whether they have equal potentialities in the various RE organs.

One can only conclude, in spite of much effort in the past 60 years devoted to solving the problems associated with phagocytic cells, that there are still enough remaining to keep many interested researchers happy for some considerable time to come!

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Antigen-Antibody Reactions in Helminth Infections

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I. Introduction

Work on the antigen–antibody reactions in helminth infections has involved a wide range of mammalian species as hosts and also has included almost the complete range of parasitic helminths. In the human field, work has been restricted largely to diagnostic tests for various infections such as trichinosis, hydatid disease, schistosomiasis, and filariasis. On the other hand, in the animal field, particularly with respect to economic animals, since diagnostic tests have not been as applicable, work has been concerned with the mechanisms whereby the animal becomes immune to reinfection, in other words, where the antigens and antibodies operate in a "functional" capacity. It is intended to deal with the various helminth species according to their classification and within this frame-

work to deal, first, with the various phenomena on the fundamental side of antigen–antibody reactions; second, with naturally occurring immunity in parasitized animals; and, third, with the mechanisms which lead to "functional" immunity.

There is no evidence in helminth infections that antibodies associated with the immune response differ markedly in their physical or chemical characteristics from any other antibody which is stimulated by an infectious agent. Thus, helminth parasites provide a valuable means of studying the effect of immunity on an infectious organism. Helminths are large enough to allow direct observations to be carried out on reactions which occur between them and various immune substances, they are frequently localized in an organ of the body, and they undergo growth and development but not multiplication in the host.

However, information regarding the intrinsic nature of the mechanism of immunity against helminths is scarce, partly because of the small number of workers in the field and partly because of the failure, until recently, to culture by *in vitro* methods the parasitic larval stages which contribute so much to the immune response. Despite the wide range of organisms studied, from the schistosomes of man to the nematode parasites of mice, it is becoming clear that the immunological responses to any given group of helminths show many similarities and that the effects of immunity on the parasites follow a common trend. In many nematode infections, for example, certain well-defined phases in the life cycle of the parasite are responsible for initiating "functional" immunity and these are also the phases when the immune mechanisms markedly affect the parasitic population.

At this point it is pertinent to recall that a helminth parasite is a metazoan organism which during its life cycle in the host undergoes a marked degree of change and growth. In the class Nematoda, infection may be by skin penetration or by ingestion of an infective larval stage which metamorphoses through at least three further larval stages to become an adult male or female worm, the latter of which lays a large number of eggs. The larval stages are separated by periods of reorganization, designated "moulting periods," during which important antigenic materials are released that stimulate "functional" immunity and during which mechanisms of immunity begin to operate in a manner most disadvantageous to the parasite (Soulsby, 1959). During this period of prepatency, the worms grow and develop, and various organs, especially those responsible for oogenesis, are particularly prominent. Such marked changes obviously involve a wide variety of physiological and biochemical aspects. The response of the host similarly changes in a qualitative

and a quantitative manner. In addition, some parasites undergo a migration in the host. Thus, one larval stage may be found in a certain organ or tissue, whereas the next and subsequent larval stages and probably the adult stage may be found in a completely different site. Thus, the hookworm larva commences its parasitic life cycle as a skin penetrator and ultimately, having migrated via the lungs and trachea, is to be found attached to the intestinal lumen sucking blood. Nevertheless, despite at times their large size and multifarious migrations, the helminths generally stimulate satisfactory immune responses which can be detected by various serological tests and which can also be of such a nature that reinfection does not occur.

II. Natural Resistance

The normal mechanisms of the body which operate against helminth parasites have been studied but little. Standen (1952) showed that cercariae of many species of trematodes were destroyed when exposed to the normal serum of a wide range of animals. Many animals possessing such a normal serum action against a particular trematode cercaria fail to become infected by the cercaria (Culbertson, 1936). This property is not restricted to mammals. The lethal action of the mucus of certain fish on the cercariae of various trematodes is directly proportional to the resistance of the fish to the trematodes (Nigrelli, 1935). The lethal action of normal serum in sheep against the miracidia of the fluke, *Fasciola hepatica,* is increased on infection (Soulsby, 1957a).

Lewert *et al.* (1959) demonstrated that normal serum from several species contained an inhibitory substance active against the skin-penetrating enzymes of schistosome cercariae. This substance was increased in amount in human infections, but the authors were unable to provide unequivocal proof that the inhibitor specifically functioned in immunity. Rather they were of the opinion that the cercarial enzyme inhibitor was a nonspecific substance whose serum level is elevated during active infection. These normal serum reactions against helminth parasites may well be akin to that of normal human serum which destroys almost all the pathogenic trypanosomes with the exception of those infecting man (see Laveran, 1903; Culbertson, 1935). Almost no information is available regarding the relationship of these naturally occurring factors to such entities as the complement–properdin complex, lysozyme, and the β-lysins. Further investigation of these reactions may well lead to a clearer understanding of the mechanisms responsible for the destruction of the parasite.

III. Class Nematoda

Study of this class has mainly involved *Ascaris* spp., *Nippostrongylus muris, Strongyloides* spp., *Filaria* spp., *Trichinella spiralis,* and the parasites of domestic animals, such as *Haemonchus contortus* and *Dictyocaulus viviparus.* The work on *Trichinella* has recently been reviewed by Kagan (1960).

A. Serological Reactions

1. Cross Reactions

From the serological standpoint, cross reactions are prevalent between helminths of given families and even between certain orders. Frequently, these cross reactions are utilized in diagnostic tests. For example, the dog

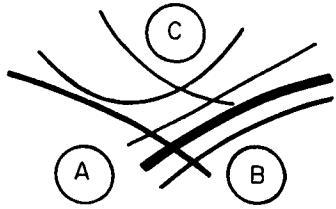

Fig. 1. Diagrammatic representation of the precipitin lines in agar gel (Ouchterlony technique) (A) serum from a sheep immune to *Trichostrongylus* spp., (B) serum from a sheep immune to *Haemonchus contortus* and (C) *H. contortus* third larval stage antigen. Only one precipitin line is shared by these two antisera. Similar reactions are obtained with an antigen prepared from *Trichostrongylus* spp. larvae.

heart worm, *Dirofilaria immitis,* gives a satisfactory skin test in human filarial infections (Bozicevich and Hutter, 1944). Complement fixation and hemagglutination tests also demonstrate a wide range of cross reactions. Precipitation in agar gels not only show cross reactions, however, but also finer distinctions. Thus, although the immune sera from sheep, infected with either *H. contortus* or *Trichostrongylus* spp., react with antigens prepared from either parasite, the pattern of precipitation lines in agar gel demonstrates that the two reactions are essentially different (Fig. 1).

Cross reactions on a serological basis between various nematode species do not indicate a functional immunity or protection against subsequent infection. Thus, sheep, immune either to *Trichostrongylus* spp. or to *H. contortus,* react serologically with antigens from *H. contortus* and *Trichostrongylus* spp., but the functional immunity to *Trichostrongylus* spp. is distinct from that to *H. contortus* (Stewart, 1950a). Such a situa-

tion raises the question of the relationship of antigens which give serological cross reactions to the antigens which are responsible for functional immunity. If the antigens responsible for cross serological reactions were responsible for the production of protective, functional, immunity, it would be anticipated that infection with one parasite would lead to resistance to another parasite which cross reacts serologically. Since this does not occur, a parasite must contain a multiplicity of antigens many of which play no essential part in immunization from the functional point of view, whereas others are essentially functional and specific in nature. The "nonfunctional" antigens may be those which are used in diagnostic tests, such as skin tests, and a purification and characterization of them would be extremely valuable for diagnostic purposes. This is illustrated by the serological relationship between the ascarids. *Toxocara canis* of dogs, which may be responsible for causing visceral larva migrans in children, cross reacts with *Ascaris lumbricoides* (Kagan, 1957, 1958a). Consequently, this infection in children cannot at present be diagnosed by serological tests with certainty.

The recent work of Kent (1960) has demonstrated that the proteins contained in the water extract of fresh adult females of *A. lumbricoides* are separable by paper and agar gel electrophoresis into five major components, of which at least four are active and are precipitated by corresponding antibodies. In most cases, these components produced a single precipitating band in agar diffusion and were possibly specific for *A. lumbricoides* since, of sixteen sera from cases of suspected visceral larva migrans, only two gave positive precipitin reactions. These antigenic preparations did not cross react with sera from other helminth infections, such as echinococcosis, trichinosis, or schistosomiasis. Chemical analysis of the antigens indicated that most of the water soluble proteins of *A. lumbricoides* were glycoprotein complexes.

The possibility that cross serological reactions between various species of helminths might be due to host proteins within the parasite has received little attention, with the exception of work by Goodchild and Kagan (1961). Savel (1955) reported the presence of proteins possessing the same electrophoretic mobility as serum albumin and globulin in the perienteric fluid of *Ascaris*. Whether the proteins were host proteins remains to be demonstrated, but some of the antibodies produced by injecting helminth antigens may be directed against host rather than helminth material, especially when the antigen preparations include the gut of the helminth.

With the lack of specificity of many antigens in diagnostic tests, attention has been centered on the use of the products of the living parasite,

namely the metabolic products. These products from *Trichinella spiralis*, harvested under suitable conditions, can function satisfactorily as antigens in diagnostic tests (Chipman, 1957).

2. *Heterophile Antigens*

In addition to the essentially specific helminth antigens in the bodies of various nematodes, other antigens possess unusual specificities. For example, the Forssman antigen has been demonstrated in *T. spiralis* (Mauss, 1941b), and infection of animals with *A. lumbricoides* leads to a marked elevation of heterophile antibodies (Soulsby, 1958a,b). A high level of heterophile antibody in an animal, however, does not appear to alter its susceptibility to the parasite. Nevertheless, the Forssman antigen has been demonstrated to be of value in the study of immunity to helminths insofar as it can be used as a natural marker. Using it, Soulsby (1958c) was able to show that the moulting period in an *Ascaris* infection initiated for the first time the release of large amounts of antigen. It is worthy of note that heterophile antibody levels are elevated in many helminth infections and cognizance should be taken of this fact when tests such as hemagglutination tests are employed as diagnostic measures.

3. *Blood Group Substances*

Other unusual antigens associated with helminth infections are related to the blood group substances and they have been demonstrated in *T. spiralis, A. lumbricoides*, and other helminths. Soulsby and Coombs (1960) showed that infection of pigs with *Ascaris suum* resulted in a marked elevation of the A antibodies and that materials from *A. suum* neutralized both human anti-A and human anti-B isoantibodies, but had no effect on the anti-D Rh agglutinin. Similarly, Oliver-González (1946) stimulated high levels of A antibodies in rabbits infected with *A. lumbricoides*. Oliver-González (1953, 1960) also showed that polysaccharide materials of *A. lumbricoides* and other parasites can be absorbed onto erythrocytes. He suggests that such new erythrocyte–polysaccharide complexes could induce antibody production which would, in fact, be directed against the host's own erythrocytes. This suggestion indicates that the causes of anemia associated with some parasitic infections may upon further examination be related to the production of isoantibodies.

Oliver-González and Koppisch (1958) further investigated the immunological and pathological phenomena related to the substances from the tissues of *A. lumbricoides*. They demonstrated that the injection of extracts of various organs of ascaris produced in dogs a rapid anaphylactoid reaction resulting in death and in guinea pigs, a marked congestion

of the liver. During these reactions agglutination of erythrocytes was seen, and blood plasma of the inoculated dogs agglutinated the animal's own erythrocytes. The authors suggest that among other mechanisms autoagglutination of erythrocytes may have an important role in the reactions of animals inoculated with ascaris tissue extracts and that the autoagglutinins normally present in animal sera react with the injected antigens. They also postulated that the antigen when injected adheres to erythrocytes. Moreover, since the anaphylactoid reaction was not evident in guinea pigs infected with embryonated eggs prior to inoculation with tissue extracts, it may not be related to previous sensitization with migrating ascaris larvae. On the other hand, Soulsby and Coombs (1960) were able to demonstrate that the metabolic products of *A. suum* larvae contained the A substance. The role of these blood group substances in the pathogenesis of parasitic helminth disease has not been assessed.

Seasonal variations in anti-A levels seen in pigs (Goodwin and Coombs, 1956) may be caused by ascaris infection, and the seasonal variations in the anti-J levels in cattle (Stone, 1956) may be due to parasitic infections, especially since the A-substance exists in *F. hepatica* (Oliver-González and Torregroso, 1944), a parasite which commonly affects cattle. Recently, Tucker (1961) has shown that a seasonal variation in the anti-R isoantibodies occurs in sheep on pasture, but not in sheep which are housed. The effects of parasitic enteritis on the longevity of the red blood cell may be pertinent here because Baker and Douglas (1957) suggested that the phenomenon of autoimmunization may well play a part. However, at present it is not known whether a certain blood group in an animal is advantageous or otherwise with respect to any parasitic infection it may possess. It could be disadvantageous to the host due to the stimulation of autoantibodies by the parasite or it could be used in the wider context as a genetic marker of an inherent susceptibility or resistance to parasitism.

B. Precipitating and Nonprecipitating Antibodies

Various types of antibodies are associated with nematode infections. Particular attention has been paid to precipitating antibodies since Sarles and Taliaferro (1936) reported the presence of precipitates in the gut of and around *Nippostrongylus muris* during *in vivo* immune reactions. These observations led to the demonstration by Sarles (1937) that larvae when placed in homologous immune serum developed precipitates around various orifices such as the mouth, the excretory pore, and the anus. Subsequently, similar findings were reported for hookworm, ascaris, and *Trichinella*. Mauss (1941a) demonstrated that the infectivity of

Trichinella larvae, placed in immune serum prior to their infection of an animal, was reduced by 60%. Recently, Jackson (1960) demonstrated by fluorescent antibody techniques that these precipitates visible at the orifices of various worms, are composed of antigen derived from within the parasite and antibody in the serum.

Precipitating antibodies, not necessarily related to those which cause precipitates at the various orifices of the larvae, are demonstrable by a variety of other techniques and in particular by the Ouchterlony technique. Soulsby and Stewart (1960) have shown that serum from sheep infected with *H. contortus* exhibited a definite pattern of precipitation lines in agar gels, which showed a marked degree of qualitative and quantitative variation depending upon the degree and stage of infection (Soulsby, 1960c, 1961).

In addition to precipitating antibodies, skin-sensitizing antibodies have been extensively used as diagnostic tests in helminth infections.

Nonprecipitating antibodies have also been demonstrated in Nematode infections by Soulsby (1960b) using a chemically linked red cell system. In addition, hemagglutination tests have been used by Kagan (1958a) and Soulsby (1957a and 1960a) both as diagnostic tests and as serological tests to study the response of animals experimentally infected with parasites. In parasitic gastroenteritis in sheep, Soulsby (1960a) demonstrated that the hemagglutination titer was not related to the complement fixation titer in sheep immune to *H. contortus*.

Despite the extensive work which has been done on various serological manifestations of nematode infections, no convincing evidence exists to date to show that antibodies detected by numerous serological tests play any functional part in immunity. The serological response of an animal to infection, although it may be a guide to the immunological status of that animal in respect to its nematode infection, may often be no more than an epiphenomenon.

C. Passive Transfer of Immunity

Experiments using passive transfer of immune serum have been carried out in infections such as *T. spiralis*, *N. muris*, and *Dictyocaulus viviparus* (Sarles and Taliaferro, 1936; Chandler, 1938; Hendricks, 1953; Culbertson, 1942; Thorson, 1954; Jarrett *et al.*, 1955). However, with the exception of the latter parasite, passive transfer of immunity did not induce a marked degree of immunity. In the work of Jarrett *et al.*, it was necessary to transfer to each recipient 10-week-old calf, the equivalent of 4½ liters of donor serum. Even though such passively transferred serum possesses high levels of circulating antibodies, the protective capacity

may not necessarily be related to the antibody detected by the serological test. Thus, infected ewes may possess antibodies to nematodes in their colostrum which, though present in the lambs they suckle (Stewart, 1959), does not afford the lambs protection (Filmer and McClure, 1951). Also, in *H. contortus* infections in sheep, neither the height nor the duration of the antibody response, as measured by the complement fixation or the hemagglutination test, are indicative of resistance to infective larvae (see Soulsby and Stewart, 1960). Nevertheless, failure to produce antibody may be associated with failure to resist infection (Stewart, 1950b). In addition, circulating antibody may at times be functional in immunity since the protective capacity of immune serum was reduced if serum was absorbed with the excretions and secretions of *N. muris* larvae, as reported by Thorson (1954).

Since the white cells of the body are always present at the site of an immune response to helminths and respond in a striking manner, their role in producing antibody at, or transporting it to the local site should be investigated further. Taliaferro (1958) has noted that precipitins may play a lesser role in helminth immunity than was previously thought; he suggests that nonprecipitating antibody and, in particular, skin-sensitizing antibodies should receive further consideration.

D Avidity

Avidity should also be studied in helminth infections, particularly with regard to mechanisms inhibiting development or egg production. Barr (1951) has stressed the necessity for the proper preparation of an animal to be able to produce an antibody of high avidity. The value of minimal infections of helminths to establish a good immunity in sheep is shown by the fact that excessive infections at an early age have a deleterious effect on the acquisition of immunity (Gibson, 1952). It might be possible to ascribe this to an undue forcing of an incompletely developed antibody-producing mechanism with the production of a poorly combining antibody. Other factors may be involved, however, such as lowered immune response due to immunological unresponsiveness following continuous antigenic stimulation. In epidemiological studies of the behavior of parasites in a flock of sheep in Britain, the older members of the flock pass through an immunizing period which is associated with the termination of the spring rise phenomenon and which renders them immune to excessive infection during the rest of the year (Soulsby, 1957b). On the other hand, lambs reflect the challenge of infective larvae present in the pasture when the older sheep are immune by suffering from clinical outbreaks of parasitism. This situation arises

despite the fact that the lambs have been exposed to parasites from an early period in their lives during which they have not acquired a sufficient degree of immunity to protect them. The quality of the early response in such animals may be of great importance.

E. Functional Antigens and Antibodies

It has been mentioned that functional immunity appears to be specific, at least at the generic level, e.g., with *H. contortus* and *Trichostrongylus* spp. (Stewart, 1950a). Whether this phenomenon extends to the species level within various genera is not known. However, since functional antigens are thought to be specific, they are probably not somatic antigens because vaccinations with nonviable antigens, i.e., with essentially somatic antigens, produce only a low degree of protective immunity and show a wide range of cross reactions. A protective property in somatic antigens would lead from an immunity toward one parasite to an immunity toward another parasite which shared common antigens. An illustration of this is seen in *Schistosoma mansoni* and *T. spiralis,* which produce immunologically related substances (Oliver-González, 1949; Senterfit, 1958), but immunization of mice with *T. spiralis* does not induce resistance to *S. mansoni* — in fact, it may make the animals more susceptible (Weinmann, 1960). Functional antigens may, however, be shared by helminths at the oogenesis level (Soulsby, unpublished). Thus, sheep immunized against *Cooperia* spp. were unable to resist a challenge infection with *Trichostrongylus* spp. but the latter, when mature, were markedly retarded in oogenesis. It may be that cross reactions occur in mechanisms responsible for the inhibition of reproduction as distinct from the apparent specific nature of the immunity induced by the larval stages.

Immune mechanisms responsible for the inhibition of oogenesis in helminths may be analagous to the reproduction-inhibiting antibodies (ablastins) of *Trypanosoma lewisi* and *Trypanosoma duttoni,* which show cross relationships (cf. Taliaferro and Pizzi, 1960). Chandler (1953) has suggested that common antigens exist in these two parasites which are involved in nuclear and cytoplasmic division.

Whatever the functional antigens are in helminth immunity, they are associated with the living parasite because the living parasite, either in an unaltered or in an attenuated form, must live in the host to produce a satisfactory functional immunity or, alternatively, the products of a living parasite must be given to the host. When immunity is produced by these methods a variety of manifestations of immunity can occur. These range from the complete elimination of an infection by means of

the spectacular self-cure phenomenon through retarded larval development to a retardation or a cessation of egg production. In order to produce the various effects on the parasitic helminths, the immunological mechanisms need to be active continuously. Thus, parasites which are retarded in their development by immunological processes or female worms which are inhibited in egg production can proceed to develop when the immunity falls below a critical level. Inhibited larval stages of *N. muris*, for example, when transferred from an immune animal to a normal animal, can resume development (Chandler, 1936). Taffs (1960) induced a marked fall in egg production in ascarids in pigs by giving a large number of infective eggs; this was accompanied by a rise in antibody titer. If the worms were not eliminated from the pig by this reaction, the egg count gradually returned to its former high level. At first, the eggs were infertile. Only when full egg production was resumed were fully fertile eggs produced. Obviously, the effect of immunity on oogenesis was temporary and was not irrevocable.

Frequently, a study of how immune mechanisms affect a parasite gives valuable clues about the origin of the functional antigens and with this in mind it is pertinent to consider the self-cure mechanism.

F. Self-cure

The self-cure mechanism, as originally described by Stoll in 1929, involved the elimination by sheep of infections of *H. contortus* in a rather dramatic manner. Subsequently, such sheep were resistant to infection by infective larvae. Further work by Stewart (1953, 1955) demonstrated that the self-cure reaction could be induced in a suitably infected and sensitized sheep by a challenge dose of *H. contortus* larvae. The self-cure reaction so induced was accompanied by a marked rise in circulating antibodies as measured by the complement fixation test. Since, however, the rise in antibody titer occurred after self-cure had taken place, circulating antibody was considered to play no part in self-cure. Further work showed that the self-cure reaction was accompanied by a significant rise in blood histamine which could be eliminated by the administration of antihistamine drugs although the antibody rise persisted. On examination of the mucosa of the abomasum of a sheep undergoing self-cure, Stewart demonstrated that a marked edema occurred and it is likely that infective larvae stimulate an antigen–antibody reaction in the mucosa of the abomasum which leads later to an increase in circulating antibody but, more immediately, to the liberation of histamine or histaminelike substances that adversely alter the mucosa of the abomasum for the worms. It is considered that this alteration in environment leads to a

loss of worms. The timing of the histamine rise is of some importance because it indicates when the antigen stimulates the reaction. The histamine rise, since it occurred between the second and fourth day after infection, indicated that the mere entry of larvae into the body of the animal was not responsible for the initiation of the response, but that some development of the infective larvae was necessary before the antigens were available to initiate the reaction.

Further work on the self-cure phenomenon by Soulsby *et al.* (1959) and Soulsby and Stewart (1960) showed that the moulting of *H. contortus* larvae from the third to the fourth larval stage was the essential stimulus to the self-cure mechanism. These authors were able to show by serological tests that serum taken at the time of self-cure in sheep showed a marked reaction with a substance obtained from larvae which is associated with the ecdysis of larvae, i.e., the exsheathing fluid produced by infective larvae when they exsheath (Sommerville, 1957). Its production is stimulated by suitable low oxygen tensions and by a dialyzable factor present in the digestive tract of the host. At present no direct evidence exists to show that any material comparable to exsheathing fluid is liberated at the time of the first parasitic moult in *H. contortus* infection, but circumstantial evidence strongly favors this idea. Further evidence that the moulting of larvae stimulated the self-cure reaction was obtained by infecting suitably sensitized and infected sheep with larvae to bring about self-cure. Such sheep were slaughtered at intervals after the challenge infection. It was found that, until the third moult (first parasitic moult) of the challenge dose of larvae, the existing population of adult *H. contortus* remained intact. But at the time of self-cure, when much of the population of adults was in a state of disintegration in the intestine, many fourth stage larvae as well as third stage ones were present in the abomasum. Since a period of immunity or protection follows the self-cure reaction, any antigen responsible for the stimulation of the self-cure may also be concerned in the period of protection thereafter.

Soulsby (1960c), studying the variations in the serological response in sheep undergoing self-cure and protection in *H. contortus* infection, showed that the loss of immunity was associated with a disappearance of lines in the agar gel precipitin test which react markedly with exsheathing fluid. In the self-cure reaction, larvae of the challenging dose and not the adult worms were responsible for the initiation of the self-cure mechanism. Many other helminth infections are probably terminated by a comparable reaction. In infections of *D. viviparus* in cattle, the self-cure reaction may lead to an adverse effect on the host itself in

that the host may die because of a marked allergic reaction in the lungs (Michel, 1954).

The self-cure mechanism is not solely an experimental entity; it is also an important mechanism for terminating naturally occurring parasitism. Thus, the spring rise in helminth populations in sheep is terminated by a group self-cure mechanism (Soulsby, 1957b). Similarly, under Australian conditions, natural burdens of *H. contortus* in grazing sheep are eliminated by a self-cure mechanism, which occurs particularly after rain, when infective larvae become numerous (Stewart, 1950b). The reaction is essentially a violent hypersensitive reaction on the part of the host during which the parasites are eliminated almost mechanically.

Although the self-cure reaction in *H. contortus* infection is clearly initiated by the moulting of the challenge dose of larvae from the third to the fourth larval stage, Michel (1952a,b) has suggested that self-cure is initiated when the biomass of helminth material reaches a critical level. Using *Trichostrongylus retortaeformis,* Michel showed that a certain massive reinfecting dose of worm material must be present to evoke the self-cure reaction: light and moderate reinfecting doses had no effect until the parasites of such infections reached maturity. Michel also demonstrated that the self-cure reaction in *T. retortaeformis* infection did not affect inhibited larval stages in the mucosa of a resistant animal. On the contrary, when the immune status of the animal declined, such larvae resumed their development until at maturity the self-cure mechanism was evoked. There is no indication in the above work as to the extent of development of the parasites when the reaction took place.

The self-cure reaction is probably not limited to helminth infections. McCullough and Eisele (1951) observed that carriers of *Salmonella* ceased excreting organisms when reinfection occurred.

Though the self-cure reaction has been studied with particular reference to *H. contortus,* an abomasal parasite of sheep, the reaction is not specific because *H. contortus* larvae will also cause elimination of other abomasal parasites, such as *Trichostrongylus axei* and *Ostertagia circumcincta,* and will stimulate self-cure of the intestinal *Trichostrongylus* spp. *Trichostrongylus* spp. larvae, though inducing self-cure of adults of that species will not affect an infection of *H. contortus* (Stewart, 1953). This result may be accounted for by antigenic differences in the two species or, as suggested by Stewart, by the easy passage of antigenic material of *H. contortus* from the abomasum to the small intestine, but not of similar material of *Trichostrongylus* spp. from the small intestine to the abomasum. It is interesting to recall that the protective immunity of the two species is quite distinct.

Many nematode infections can be rapidly terminated by an immune response. This is illustrated by *N. muris* and *T. spiralis* infections in experimental animals and is associated with a marked accumulation of white cells in the mucosa of the bowel and the presence of precipitates at the various natural orifices of the parasites in the case of *N. muris* (Taliaferro and Sarles, 1939). Such a curative mechanism on the part of the host does not resemble the violent hypersensitive reaction associated with *H. contortus* infection. The reaction appears to involve the parasite as well as the host. In infections of *N. muris*, inflammation is initiated in the lamina propria of the intestine, and precipitates are later noticeable in the worms. As inflammation with an increase in cellular elements becomes more intense and extends into the submucosa, the infection is largely terminated (Taliaferro and Sarles, 1939). By using cortisone, the cellular reactions both in *N. muris* and in *T. spiralis* infections may be retarded and the active infection prolonged (Weinstein, 1955; Coker, 1955).

G. Protective Immunity

Protective immunity, as distinct from self-cure, has various manifestations. The latter vary from a complete inability of helminths in the infective stages to infect an animal, through retarded development at various stages in the life cycle, to suppression of egg production by female worms. They all probably act by retarding development of the worms. Whether the complete inability of infective stages to infect an animal is a widespread aspect of immunity or not is difficult to assess. It seems to occur, however, in some instances. Thus, Stoll (1929) reported that challenge doses of infective *H. contortus* larvae were eliminated in the feces of immune sheep, and Michel (1953) found that infective larvae of *T. retortaeformis* were unable to establish themselves in an immune animal. T. B. Stewart (1958) reported too that exsheathment of infective larvae was inhibited in immune animals. This occurrence would obviously lead to the elimination of the larvae. In other studies, however, a challenge infection does enter a highly immune animal and may persist and even grow for a short while. In the experimental infection with ascaris (Soulsby, 1961), the challenge infection was only adversely affected by the immunity of the host as it approached a moulting period as shown in Fig. 2. This effect was first manifested by a decrease in the growth rate and later by a marked decrease in the number of larvae—ultimately, the challenge infection was overcome. In sheep highly resistant to either *H. contortus* or *Trichostrongylus* spp., a challenge dose of infective larvae after penetration into the mucosa, persisted for a short time, but was

eliminated when the parasites approached the moulting period (Soulsby, unpublished).

No evidence exists at present to indicate that antibodies to helminths are present at mucous surfaces. In fact, Soulsby (1960b) was unable to demonstrate any antibodies in intestinal mucus. Thus, the infective larvae evidently must invade the tissues before they are inhibited or

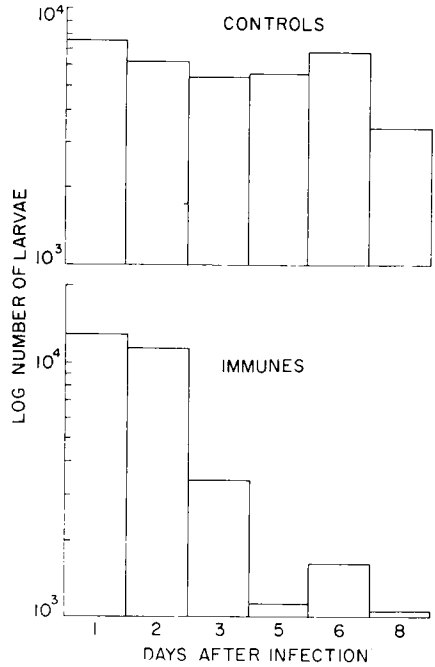

FIG. 2. Number of *Ascaris suum* larvae in the lungs and livers of guinea pigs daily, after a challenge dose of 100,000 infective eggs. The immune group of guinea pigs received three immunizing doses of infective eggs subcutaneously. The figure illustrates that large numbers of larvae are able to invade an immune animal: only when they have developed for a certain time (more than 2 days) is their number greatly reduced. Compare the marked decline in the number of larvae between the third and fifth day (moulting period) in the immune group with the relatively constant number in the controls.

destroyed. Certainly the skin-penetrating larvae, such as hookworms, *N. muris* and *Strongyloides* spp., must penetrate the body integument before being inhibited by immune mechanisms. Once they have entered the body, they may be affected adversely at various stages, particularly at moulting. Such inhibited larval stages, as mentioned previously, when transferred from the immune environment, can develop normally. If development

occurs up to the adult stage, the immune mechanism is often manifested initially by a diminution or cessation of egg production. Thus, a retardation or absence of egg production occurs in infections of *Trichostrongylus* spp. (Stewart and Gordon, 1953), and the elimination of an *N. muris* infection in the rat is heralded by a cessation of egg production.

The role of the adult worm in the production of an immune response is difficult to assess. At times, the presence of a few adult worms appears to influence the development of various dormant larval stages. For example, in infections of *Trichonema* spp. in the horse, Gibson (1953) was able to show that a new crop of larval stages developed to maturity and commenced egg laying after anthelmintic treatment had eliminated the adult worms. This process, when repeated, allowed a further batch of larvae to develop. A comparable situation seems to have been reported by Michel (1952b). He showed that batches of larvae, following self-cure of an adult infection of *T. retortaeformis,* emerged from the mucosa of immune rabbits and developed to adult worms. Roberts (1956) also reported that a few adult *Haemonchus placei* could inhibit a large burden of larval stages in the mucosa of the abomasum of cattle but that, when eliminated by appropriate anthelmintic treatment, such large numbers of larval stages sometimes developed that the animal died. It is ironical that the giving of an anthelmintic, designed to eliminate the worms from the animal, should result in increased rather than in decreased parasitism.

Stewart (1950c) showed that material from the adult *H. contortus* was less satisfactory as an antigen in serological tests than material from larvae, and Jarrett *et al.* (1959) showed that irradiated larvae need not mature completely to produce immunity. It will, however, probably be found that all stages of development of a parasite can stimulate immunity. Recently, in this laboratory, transplanted adult *N. muris* have induced a good immunity to reinfection which is accompanied by precipitating antibodies in the serum. Though all stages of a helminth infection can apparently induce an immune response, it appears unnecessary for the life cycle to be completed to produce a good response. This situation indicates the importance of carrying out more work on various larval stages.

Jarrett *et al.* (1958, 1959) and Dow *et al.* (1958) reported that larvae "attenuated" by ionizing radiation induce a satisfactory immunity to at least three nematodes by undergoing a limited development. Unless some development takes place, no protection occurs. Somewhat similarly, Gould *et al.* (1957) showed that increasing doses of ionizing irradiation diminished the ability of *T. spiralis* larvae to migrate and develop; and that

immunity was not produced if irradiation stopped development of the larvae. The work of Jarrett *et al.* on *D. viviparus* and *H. contortus* indicated a similar situation.

Soulsby (1959) has stressed the importance of the moulting period in nematode infections as an immunizing phase and was able to show, using the Forssman antibody as a natural marker (Soulsby, 1958c), that the moulting period in ascaris larvae was the time when a marked release of antigen occurred; larval stages prior to this did not contribute much antigen. This result could explain why infected larvae, even in a highly immune animal, are able to invade and persist and even grow for a while.

H. Metabolic Products and Their Nature

Little is known about the nature of the antigens which are produced by the various larval stages either during their growth or at the moulting period, but many of them are probably enzymes. Such antigens have been designated as metabolic products in the present review. In studying infections of *N. muris* in the rat, Sarles and Taliaferro (1936) found precipitates in immune rats around and in the gut of the worms in the skin and lungs. In addition, the worms were immobilized, retarded in development, and surrounded by inflammatory cells which acted as scavengers if the worm died (Taliaferro and Sarles, 1939). The immunity to *N. muris* was thus considered essentially a local immunity in strategically placed organs, i.e., in the skin, lungs, and intestines, which was largely dependent upon humoral factors. Precipitates comparable to those observed by Sarles and Taliaferro (1936) and Sarles (1937) have also been seen under certain conditions with hookworm, ascaris, and trichostrongyle larvae (Otto, 1939; Soulsby, 1957c; Hawkins and Cole, 1945). Soulsby demonstrated such precipitates around third stage larvae of ascaris, i.e., larvae that had undergone some degree of development in the body of the host, but not around infective second stage larvae. Such results offer some explanation of why infective larvae, even in highly immune animals, can penetrate, migrate, and grow without being affected by the immune defenses. Hawkins and Cole (1945) were also able to demonstrate precipitates on third stage infective larvae of *H. contortus* in sheep, but Silverman and Patterson (1960) could find them only on the fourth stage larvae. Using *in vitro* culture techniques, Douvres (1960) showed that when larvae of the cattle nodular worm, *Oesophagostomum radiatum*, were placed in media containing either immune serum or extracts of tissue from an immune animal, precipitates occurred at the various natural orifices of larvae which had developed in culture to a stage com-

parable with the moulting period of these larvae. No reaction was demonstrable with larvae at the infective stage.

The conclusion from this evidence appears to be that, before the immune mechanisms of the body can affect a nematode infection, the infection must have progressed to a stage of development at which it is capable of being affected by the immune mechanisms. It may be that functional antigens are not produced early in development or that they exist but are masked at first and only become active later. Or immune substances may only penetrate to the interior of the parasite during the transitional moulting period when the cuticle, as in insects, may be more permeable than at other times.

If metabolic products from the various natural orifices of the larvae are of outstanding importance in immunity to helminths, vaccination of animals with them should result in a high degree of immunity. Such a vaccination, however, has only produced a lower degree of immunity than is produced by a natural infection. The metabolic products, however, are difficult to collect in large amounts and may rapidly deteriorate since they probably contain a large variety of enzymes.

The chemical nature of these so-called secretions and excretions is not known at present although the amino acid composition of some of them has been studied (Haskins and Weinstein, 1957). Data on this subject would probably appear faster if the origin of these materials were not so difficult to determine. The available information regarding their nature is as follows: Thorson (1956) demonstrated that materials produced in the esophageal glands of hookworms were capable of vaccinating dogs against the infection and that they contained a lipolytic enzyme which was inhibited by serum from an immune dog. He (1953) also showed that the infective larvae of *N. muris* possessed a lipase which he considered important in invasion. Similarly, Lewert and S. Mandlowitz (see Lewert, 1958) demonstrated a lipase in extracts of the infective larvae of *N. muris* and *Strongyloides* spp. which was active on tripalmitin, a normal skin constituent.

Whether such enzymes are shared by all stages of the parasite life cycle is unknown and, indeed, they need not be significant in functional immunity. The possession, however, of enzymes common to all stages of the life cycle may explain why adult parasites at times exert such a profound influence on larval stages. Chandler (1953) favored the view that antiparasite immunity is connected with antienzymes but in the absence of much information about the enzyme systems within the body of the helminth or in the metabolic products, it is difficult to predict what kind of antienzymatic immunity might be expected.

The antienzyme hypothesis is attractive since it could explain to some extent why immunity induced by the use of dead and disintegrated helminths is inferior to that induced by active infections, on the ground that the residue of enzymatic protein in dead material is small. It is fairly certain that a vast complex of enzymes must exist in living helminths which might be arbitrarily divided into "internal" and "external" enzymes. The internal enzymes would be those responsible for the metabolism of the parasite, such as carbohydrate metabolism in which the Embden-Meyerhof sequence has been shown to exist (von Brand, 1952). The external ones might be concerned with the preparation or invasion of, and migration through, tissues. In respect to the former idea, Taliaferro and Sarles (1939) suggested that the excretions of the dorsal and subventral esophageal glands play an important part in feeding, and Chitwood and Chitwood (1938) suggested that material from these glands may either be ejected through the mouth for extracorporeal digestion or passed to the intestine for intracorporeal digestion.

Lewert and Lee (1954) studied the passage of helminth larvae through host tissues and found that saline extracts of various larval homogenates exhibited a general proteolytic activity and, in particular, were active against glycoproteins. A collagenase type of activity was limited to those helminths which alter glycoprotein-containing materials in the skin of the host and was absent in tissue invaders, such as *N. muris* and *T. spiralis.*

A spreading factor, possibly related to hyaluronidase was also obtained from the larvae of various helminths: thus, the streptococcal decapsulation test was positive for extracts of larvae of *Strongyloides ratti, N. muris,* and *Ancylostoma caninum,* as reported by Lee and Lewert (1957). These authors showed further that enzymes derived from various infective larvae differ in enzymatic activity not only among themselves but also from comparable bacterial enzymes. Hence it is not surprising that protective immunity is specific for the species of helminth under consideration.

It could well be envisaged that enzymes responsible for skin penetration act as functional antigens and that their inhibition in an immune host might result in an inactivation of the larvae. The immobilization of skin-invading parasites may lead to clinical manifestations such as creeping eruption in *Strongyloides* infection in humans. Lee and Lewert (1957) have described a serum factor responsible for the inhibition of the collagenase-like penetration enzymes of *S. mansoni* and *S. ratti.* This serum factor, however, did not reach a level higher than normal in rats immunized by repeated exposure to *S. ratti,* and may not be an antibody even though it reached a higher level in humans infected with *S. mansoni.*

Most of the foregoing studies have been carried out on the infective stages of various helminths, and little or no information is available concerning the enzymes of the parasitic larval stages of various nematodes. This situation may be accounted for by the fact that parasitic larval stages are difficult to collect in sufficient quantities. With the advent of *in vitro* cultivation, it should now be possible to obtain a much more detailed picture of the enzymes that are produced by the various larval stages, particularly at such important points as the moulting period.

In addition to considering the type of antibody and the source of protective antigens in immunity to helminths, it is necessary to consider how the antibody or other active materials enter a parasite to produce their inhibiting effects. It has been assumed that the precipitates demonstrable at the various orifices of larvae cause mechanical interference with feeding, excretion, and secretion. For example, Culbertson (1941) has suggested that the depression of egg production in female worms might be explained by the occlusion of the genital pore with precipitates. Rogers and Sommerville (1960) showed that a precipitate occurred at the excretory pore of exsheathing larvae when placed in an antiserum against exsheathing fluid. A similar reaction might inhibit the moulting of larvae.

Since antibody appears to produce an intrinsic effect on the parasite, it seems unlikely that the immune mechanisms act merely by affecting the outside environment of the parasite: it seems more likely that the active materials penetrate within the parasite to affect the functional tissues and organs. Some support for this idea is provided by Schwabe (1957). He showed that the oxygen consumption of *N. muris* larvae, when placed in immune serum, was significantly reduced well before the appearance of precipitates at the natural orifices. Schwabe suggests that the mechanism operates by inactivating the enzyme system responsible for oxygen utilization. The inhibition of egg production also appears to be the result of an antibody effect upon oogenesis as such, rather than of any mechanical interference. This effect, it should be recalled, is reversible and it may be directed toward mitotic division, protein synthesis, or even nucleic acid synthesis. It may thus be comparable to the antibody, ablastin, which develops during infections of *T. lewisi* and which has recently been shown by Taliaferro and Pizzi (1960) to be an inhibitor of protein and nucleic acid synthesis.

I. The Cuticle

If antibody acts intrinsically, it must be able to enter the body of the helminth. Taliaferro (1943) considered the cuticle of a nematode to be highly antigenic, but doubted that in the *living* nematode it was a func-

tional antigen. This idea was based on the absence of visible precipitates on the cuticle when nematodes of several species were placed in contact with antibodies *in vitro* or *in vivo* unless there was good presumptive evidence that the precipitate had originally been formed at the orifices of the worm or that a damaged cuticle allowed large amounts of antigen to reach the exterior. Oliver-González (1943) also found that precipitating and larvicidal activities were not produced by immunization with ascaris cuticle and were not lost by absorption with ascaris cuticle. Consequently, the only route into the helminth was via the mouth, the excretory pore, or the anus.

Jackson (1960), using fluorescent antibody techniques, demonstrated not only that the precipitates at the oral, excretory, anal, and reproductive pores contain antibody but also that antiserum specifically stains the digestive tract of infective larvae and adult *N. muris*. Whether antibody in the digestive tract can enter the perienteric tissues of the helminth is not known. In such a situation, the antibody may itself be destroyed by protein-splitting enzymes of the helminth (Rogers, 1941).

Recent work (Soulsby, unpublished) has shown that the cuticle of certain nematodes is antigenic and that antibody and complement can be fixed to the cuticle. Furthermore, under the action of antibody and white cells which become adherent to the cuticle, the cuticle becomes increasingly permeable, at least to low molecular weight substances such as eosin and methylene blue. Further work may show that the cuticle is an additional route for the entry of antibody into the body of the helminth.

J. White Cells

The role of white cells in immunity to helminths is not clear. Silverman and Patterson (1960) reported that the inhibition of the larval stages of *H. contortus* in sheep appeared to be mediated solely by humoral agencies, cellular elements not being in evidence. On the other hand, infections both of *Nippostrongylus* (Taliaferro and Sarles, 1939, 1942) and of *Trichinella* (Larsh and Race, 1954) are accompanied by marked cellular infiltrations. Local lesions associated with the immune reactions to helminths are also characterized by enormous accumulations of white cells, including many eosinophiles. Soulsby (1961) has shown that ascaris larvae, when placed in the peritoneal cavity of an immune rabbit, rapidly become covered with white cells, the majority of which are eosinophiles. This reaction is particularly noticeable with third stage larvae of *A. suum* and is not evident with either second or third stage larvae in normal rabbits (Fig. 3). Comparable reactions with nematode larvae in the digestive tract of immune animals have also been observed; in this

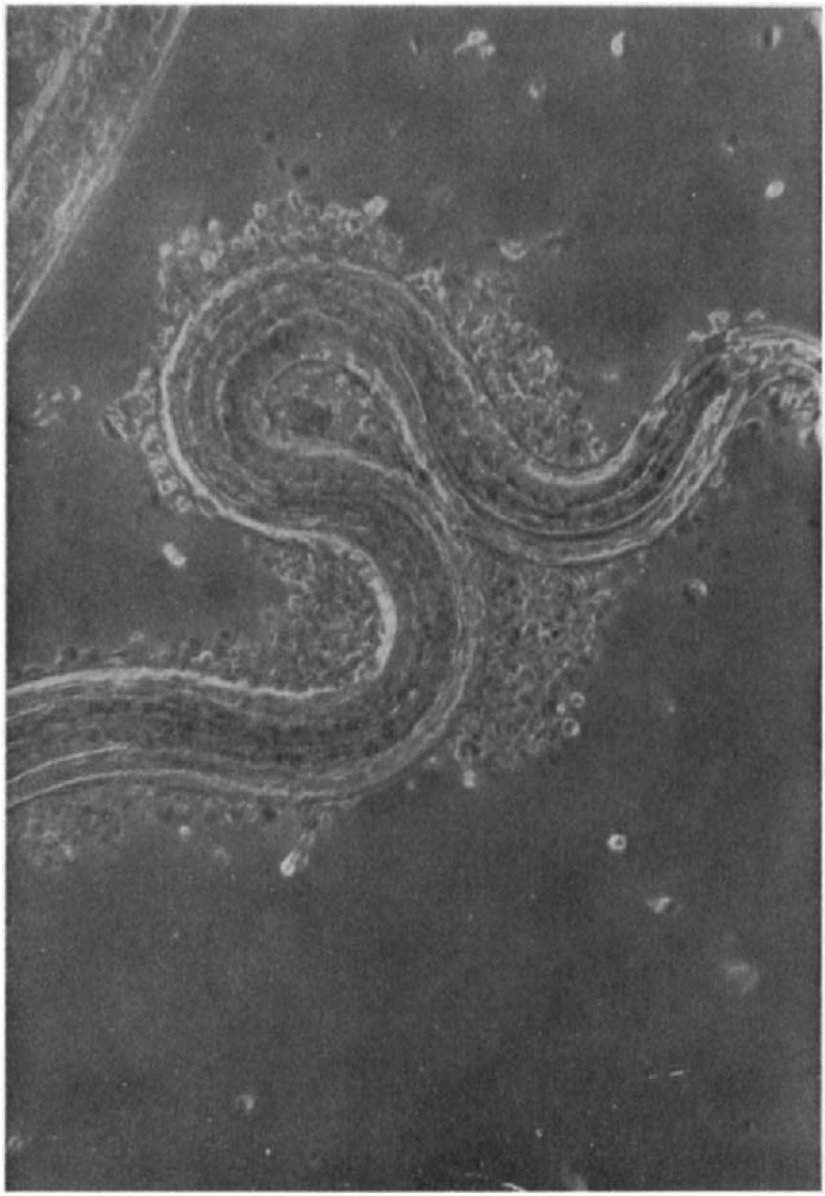

FIG. 3. Third stage *Ascaris suum* larva after 4 hours in the peritoneal cavity of a rabbit immune to *A. suum*. The cells, majority of which are eosinophiles, are firmly adherent to the cuticle. Normal rabbits do not produce this reaction, and second stage infective larvae are not affected in an immune animal. (Phase contrast.) Magnification: ×300.

case, cells actually migrate into the lumen of the crypt to attack the larval stage (Soulsby, unpublished observation, 1961). In addition, Oliver-González (1960) reported that a highly purified polysaccharide from the muscle of ascaris, when injected intraperitoneally into mice caused infiltrations into the liver of cells resembling primitive hematopoietic elements of the erythrocytic and leucocytic series.

The presence of eosinophiles in parasitic invasions is well known, but opinions vary as to their function. Spiers and his associates (Spiers and Wenck, 1955; Spiers, 1958; Spiers *et al.*, 1961) consider that the eosinophile is an important component of the immune mechanism and is involved in the formation of antibody inasmuch as phagocytosis of eosinophiles that are sensitized by antigen causes macrophages to transform into plasma cells. Such a hypothesis could explain how antibody-producing cells are brought into contact with the parasite.

IV. Class Trematoda: *Schistosoma*

A large part of the immunological work carried out on parasites belonging to the Trematoda has been concerned with the schistosomes and in particular those affecting humans, namely *Schistosoma japonicum, Schistosoma haematobium* and *S. mansoni*. The presence of antibodies in infected animals or humans or in animals injected with schistosome antigens has been known for a long time. For example, Hayami and Tanaka (1910) reported positive complement fixation tests with the sera of calves infected with schistosomes, and Yoshimoto (1910) reported similar results with the sera of infected human beings. Since then, many serological reactions have been demonstrated in animals infected with schistosomes, including cercaria immobilization, cercaricidal tests, the "Cercarienhüllenreaktion" (CHR), and the cercarial agglutination and circumoval tests, in addition to the more conventional precipitin, complement fixation, agglutination, and skin tests. These tests have been applied to various stages in the life cycle of schistosomes and are to some extent related to the stage and degree of infection. Some are of value in assessing the efficiency of drug therapy whereas others give an early indication of the status of the infection.

It is generally accepted that man becomes resistant to a subsequent attack of schistosomiasis (Fisher, 1934; Schwetz, 1956), and numerous studies on experimental animals have indicated that immunity is acquired as a result of infection (see Kagan, 1958b). The variety of diagnostic tests that can be applied in schistosomiasis facilitate a closer study of the immune responses of the host and their relationship to the developmental cycle of the parasite than can be obtained in many other infections.

A. Natural Resistance

Natural or innate immune mechanisms can be related to serum factors which endow an animal with a greater ability to resist infection. One of these is the cercaricidal factor, originally described by Culbertson and Talbot (1935) and later investigated by Standen (1952), Stirewalt and Evans (1955), and Kagan and Levine (1956). It has been found in general that hosts, whose serum is cercaricidal, possess a high degree of natural resistance to schistosomes. The cercaricidal factor of normal serum has been connected with complement since it is destroyed by heating for 30 minutes at 56°C., and Kagan and Levine (1956) showed that it was correlated with the properdin level of serum. The cercaricidal activity of serum sometimes masks an agglutination activity for cercariae (Standen, 1952; Kagan and Levine, 1956) because some cercaricidal sera, after heating, agglutinate cercariae. The agglutinins found in normal serum are nonspecific and are not related to any of the immune agglutinins. For example, normal serum which agglutinates living cercariae will not agglutinate sheep cells coated with schistosome antigens by the tannic acid hemagglutination technique. Fractionation studies by Evans *et al.* (1955) showed that the cercaricidal factor was contained in the γ- and β-globulin fractions, whereas a further activity of normal serum, that of inhibiting locomotion of cercariae, was present in the α-globulin fraction. These reactions are probably due to factors involving complement and properdin and can produce different effects depending on the organism tested and on the type of test applied. Since anticercarial factors appear to be more active in the sera of species which are naturally resistant to schistosomes, it is tempting to conclude that they are one of the defense mechanisms of the normal nonsusceptible animal which prevent the establishment of cercariae in the body.

B. Serological Reactions

The serology and immunodiagnosis of schistosomiasis has occupied numerous research workers for a number of years. Acute schistosome infections are relatively easy to diagnose by detecting eggs in the urine or feces, but chronic or minimal infections where eggs are difficult to find are more satisfactorily diagnosed by various laboratory tests, especially in view of the world-wide interest in schistosomiasis. Furthermore, immunodiagnostic tests are sometimes useful for evaluating therapy. One of the greatest problems in carrying out the conventional serological tests has been an adequate supply of a satisfactory antigen. Various alcoholic and saline extracts have been made from adult worms or from cercariae or from infected snail livers (Fairley, 1919). Despite the difficulty in

obtaining satisfactory supplies of antigen, Lurie *et al.* (1952) were of the opinion that the complement fixation test was the most sensitive method for the diagnosis of early schistosomiasis.

1. *Circumoval Precipitins*

Oliver-González (1954) described an interesting test, i.e., the circumoval precipitin test. In this test, precipitates occur around living schistosome eggs when placed in serums from infected humans or monkeys. The precipitates are aggregates resulting from a reaction between specific antibodies in the serum and secretions produced by the living miracidium that have diffused through the egg membrane (Kagan, 1958b). The antibodies responsible for the circumoval precipitates are specific for the egg, in that they are absorbed only by living eggs and not by either adult or cercarial antigens (Oliver-González *et al.*, 1955a). In addition, Oliver-González *et al.* reported that circumoval precipitates were species-specific to the extent that precipitates appear around eggs only when placed in homologous serum. The circumoval precipitin test is stronger in chronic schistosomiasis than in early stages of the disease. It seems likely that the antigens which stimulate the antibodies responsible for this test are derived from eggs deposited in the liver or in the venules of the bowel as the serum of an animal exposed to male worms is negative for circumoval antibodies. The test is also of value in assessing the efficacy of therapy (Oliver-González *et al.*, 1955c) because it gradually becomes weaker during therapy and becomes negative 120–180 days after a successful therapeutic outcome.

The work of Lewert and Lee (1954) is of interest in considering such circumoval precipitins. They showed, using the periodic acid-Schiff method, which detects polysaccharide in protein, that the glands of miracidia in normal eggs passing through host tissues are filled with a stainable material. This substance is not only present within the egg shell but appears to pass through it and to form an exterior coating on the shell, which may assist the egg to move through the tissues. Eggs which are dead or which have been derived from unisexual infections are devoid of this glycoprotein secretion. It is tempting to consider that the circumoval precipitins are directed against a glycoprotein present on the exterior surface of the eggs and derived from the miracidia contained therein.

2. *Miracidial Immobilization*

Another serum reaction with miracidia, i.e., the miracidial immobilization test, has been described by Senterfit (1953). This test is positive with all developmental stages, and Kagan (1955) found it by far the

most sensitive test for detecting antibodies in schistosome infections. Immobilization antibodies appeared between 40 to 50 days after infection with S. *mansoni* and reached a high level by the seventy-fifth day. Some degree of cross reaction occurred between the miracidia of various schistosomes, but reactions were strongest with homologous miracidia. The immobilization antibody occurred in the γ-globulin fraction of serum, and its activity generally paralleled that of the cercarial agglutination titer.

Both the circumoval reaction and the immobilization test depend on the living miracidia, but they appear to be differentially sensitive. Thus the immobilization antibody reacts with all stages of development, is absorbed by all of them, and is generally low in level or completely absent during chronic infection (Senterfit, 1958), whereas the circumoval antibody reacts with secretions of miracidia which diffuse through the egg shell and is stronger during chronic then during early infections.

The antibodies responsible for immobilization are probably directed against somatic antigens and, consequently, might be expected to cross react with other stages of the life cycle. Senterfit (1958) showed that *T. spiralis* infection in rabbits caused an elevation of immobilization antibody titers. This finding can be correlated with the observation of Oliver-González (1949) that S. *mansoni* and *T. spiralis* possess related antigens. Senterfit also noted that immobilization antibodies were not related to protection. This fact further indicates that the antigen concerned is somatic in character. Weinmann (1960), in addition, failed to increase resistance to S. *mansoni* by immunizing mice with *T. spiralis*.

3. *Cercarial Precipitins*

The formation of precipitates around the cercaria of S. *mansoni* when incubated in fresh sera of monkeys infected with schistosomes was reported by Papirmeister and Bang (1948). This reaction was abolished by heating the serum at 56°C. for 30 minutes and could be restored by the addition of complement. Stirewalt and Evans (1955) were unable to relate the cercarial precipitin reaction to schistosome infection. They agree with Vogel and Minning (1949b) that the test of Papirmeister and Bang is not the result of an antigen–antibody reaction, but is rather dependent on the cercaricidal activity of the guinea pig serum used in the reaction. Wilkerhauser (1961) described a somewhat similar reaction, which, however, is apparently a function of immunity. It consists of the formation of precipitates around the excysted cercariae of *F. hepatica* when they are placed in immune serum.

4. *"Cercarienhüllenreaktion"*

A reaction which has attracted particular attention is the CHR of Vogel and Minning (1949a,b). It is characterized by the formation of a membrane around the body and tail of living cercariae when they are placed in immune serum and is enhanced by adding guinea pig complement (Stirewalt and Evans, 1955). It can be used as an index of the efficacy of therapy since it appears in serum 40–47 days after experimental infection and is absent 5–7 months after successful therapeutic treatment. Vogel and Minning found the reaction fairly specific as no reaction was obtained with non-schistosome cercariae. These results were confirmed by Kagan (1958b) who studied the reaction in sera from animals naturally infected with different species of cercariae as well as artificially immunized with various cercarial extracts (see also Kagan and Levine, 1956). Kagan also showed that all stages of *Schistosomatiom douthitti* were capable of inducing the CHR reaction and that the activity could be absorbed from serum with large concentrations of cercariae. The nature of the CHR reaction was clarified by Kruidenier and Stirewalt (1955). These authors observed with the electron microscope that it was formed from a film of excretory substance present on cercariae as they emerged from snails. This substance was derived from the penetration gland complex of the cercaria, the initial discharge being from the glands of immature cercariae prior to their emergence from the sporocyst. Material discharged after emergence from snail tissues does not appear to contribute to the CHR reaction. Consequently, periodic acid-Schiff positive material observed by Lewert and Lee (1954), which is discharged from the lateral penetration glands of cercariae, would appear not to be concerned in the CHR phenomenon. The cercaricidal factor of normal serum prevents the formation of the CHR envelope, and elimination of this factor by heating allows the envelope to form (Stirewalt and Evans, 1955). Kagan (1958b) is of the opinion that the CHR reaction is limited as a diagnostic test since it lacks sensitivity and may, for example, cross react with bird schistosome antibodies induced during schistosome dermatitis (Hendricks and Cort, 1956).

5. *Cercarial Agglutinins*

In addition to the foregoing reactions, cercarial agglutinins have been demonstrated in the serum of acute infections of *S. mansoni*. They are distinct from the normal agglutinins found in various sera. Frequently, a normal cercaricidal factor masks an agglutinin, but can be removed by heat (Kagan and Levine, 1956). The specific cercarial agglutinin can be

induced by vaccination, reaches peak titer in the early phase of infection, and is present in unisexual infections (Oliver-González *et al.*, 1955a). However, Stirewalt and Evans (1955) are of the opinion that cercarial agglutination is a manifestation of the CHR reaction.

It is difficult to assess the import of the multitude of reactions which occur with the various larval stages of schistosomes. Some, such as the circumoval precipitin test, appear to be quite specific for certain stages whereas others, such as the miracidial agglutination test, are stimulated by any antigenic material from any phase of the infection. In fact, it may well be that many of the reactions, particularly those involving cercariae, are phases in a sequence of events and depend on the amount of antibody in the serum under test.

C. Distribution of Various Properties in Serum

Elegant work was conducted by Lee and Lewert (1960) on the distribution of the various properties of sera from schistosome-infected humans. Fractionation by starch block electrophoresis showed that a cercarial immobilizing factor was present in the most rapidly migrating albumin fraction and extended into the prealbumin area. A miracidial immobilization activity was present in the α-globulins and its peak corresponded with the serum inhibitor of cercarial protease. These activities were present in many sera but were highest in immune sera. Complement-fixing antibodies were found in the β- and γ-globulin fractions with peak activity in the γ_2-region. It was thought that cercarial agglutinins and cercarial precipitins were caused by the same antibody, whose distribution was the same as that for the complement-fixing antibody. Similarly, it was considered that miracidial agglutinins and circumoval precipitins were due to the same antibody, which occurred in the γ_1- and γ_2-fractions. The electrophoretic mobilities of antibodies reacting with miracidia, however, were distinct from those reacting with cercariae. Similar work by Evans *et al.* (1955) with moving boundary electrophoresis showed that the γ-globulin contained factors responsible both for cercaricidal and for CHR effects and partly contradict the findings of Lee and Lewert. Evans *et al.* also described a cercaricidal inhibitor in the α-globulin fraction which was necessary for the development of the CHR reaction.

D. Intradermal Reactions

The skin test has been widely used in diagnosing schistosomiasis. Practically every stage in the life cycle of the parasite can be used as antigen. Several other trematodes also serve as antigens. For use on a

global scale in epidemiological surveys, the antigen requires standardization. Thus, the reaction needs to be assessed objectively; a standard reference antigen is needed and should be standardized on some other basis than dilution; and fractionation studies are required. It may be noted that Kagan *et al.* (1961) found no significant differences with antigens from various sources if the nitrogen content of the antigen was adjusted to the same levels before injection.

The skin test is of the immediate type and the optimum time for reading it is 15 minutes after injection of the antigen (Pellegrino and Macedo, 1956). The reaction becomes evident about 4–8 weeks after infection and persists for many years, even after suitable treatment (Mayer and Pifano, 1945; Pifano and Ron, 1957). It is stronger in adults than in children and in boys and men than in girls and women (Kagan *et al.*, 1961).

As a diagnostic test, the intradermal test, which is relatively simple, can be used for epidemiological surveys to screen large numbers of persons. Other serological tests, however, such as the complement fixation test, the slide flocculation test using cholesterol–lecithin crystals and cercarial antigen (Anderson, 1960), and a fluorescent antibody technique (Sadun *et al.*, 1960) offer greater sensitivity.

The skin test, when egg antigen is used, in addition to being a diagnostic tool, may give some indication of the success of therapy. Oliver-González *et al.* (1955a, 1955b) found with the egg antigen that patients passing viable eggs gave negative skin tests, but that patients previously treated for schistosomiasis were positive.

Lurie *et al.* (1953) confirmed the absence of a skin reaction in children infected with *S. haematobium* and stated that the skin test became positive approximately 6 months after cure. This length of time is too long to evaluate therapeutic measures, but may be shortened by the development of more specific antigens. Nevertheless, a negative circumoval test and a positive skin test with the egg antigen would tend to indicate the termination of an infection (Oliver-González *et al.*, 1955c).

The Prausnitz Küstner (PK) reaction, based on the passive transfer of sensitivity to the skin of normal individuals, is positive in schistosome infections (Taliaferro and Taliaferro, 1931). This reaction appears to be specific for schistosomiasis since *F. hepatica* antigen does not produce a positive response (Guerra *et al.*, 1945). It is possible that the antibody transferred in the PK reaction is a nonprecipitating skin-sensitizing antibody because it is not absorbed with relatively large amounts of antigen. Coker and Oliver-González (1956) were able to induce a positive PK reaction in normal human recipients, using an antigen prepared from schistosome eggs, but were unable to demonstrate it in infected recipi-

ents. No information exists to show how soon after cure the PK reaction becomes positive with egg antigen. The findings of Coker and Oliver-González, however, might suggest that circulating *egg* antigen is present in active cases of schistosomiasis. Consequently, the PK reaction, using egg antigen, may provide a useful means of assessing the efficacy of therapy. This is based on the following considerations. A circulating antigen, if present during active infection, might neutralize passively transferred serum (containing antiegg antibody) from active cases of schistosomiasis, and the subsequent challenge dose of egg antigen would then produce no skin reaction. In the absence of circulating antigen, either in a normal recipient or in a recipient cured of schistosomiasis, such a neutralization would not occur, the antibody would be fixed to the site and would react with the challenge dose of antigen. Some suggestion that circulating antigen may be present in active infections is given by the work of Okabe and Tanaka (1958) and Tanaka (1960) who demonstrated a substance in the urine of persons infected with *S. japonicum* which precipitated with antiserum against *S. japonicum* prepared in rabbits.

E. Protective Immunity

The role played by materials responsible for the various serological reactions in protective immunity is uncertain. Vogel and Minning (1953) demonstrated a similarity in behavior of the disease in man and monkeys and produced experimental evidence for the acquisition of a high grade of resistance to superinfection in monkeys. A similar resistance may thus be acquired in man. These authors studied the reaction in rhesus monkeys exposed either to a single infection with several hundred cercariae or to repeated infections with about twenty-five cercariae of *S. japonicum* at monthly intervals. There was a partial resistance to challenge infections after 10 or more months and complete resistance after 14 or more months. Complete resistance was assumed to be present when massive and ordinarily fatal superinfections failed to increase the low number of eggs passed as a result of the previous infection. These authors were unable to produce active immunity by the injection of dead worms or by passively transferring immune serum from resistant animals, but showed that resistance could develop in the absence of eggs and could outlast the presence of living worms in the body. In addition, complement fixation tests were always positive for a certain period during the infection but titers could not be correlated with egg counts or with an increased resistance.

Kagan (1953) suggested that resistance to schistosomiasis may begin as early as the second week after exposure because challenge doses of

cercariae did not reach the lungs after rhesus monkeys were exposed to between 2000 and 45,000 S. *douthitti* larvae. There was no cross protection against S. *mansoni* as a result of previous infection with S. *douthitti*, but one sexual phase induced protection against the other. Thus, Kagan (1952) showed that infection with male worms caused stunting of female worms and vice versa. In the mouse infected with S. *douthitti*, the egg appears to be an important antigenic stimulus for immunity in contrast to the situation in infections with S. *japonicum* in the monkey (Vogel and Minning, 1953). The egg of S. *douthitti* is very resistant to chemotherapy and survives in the tissues for 3 to 4 weeks after the death of the worms. Mice infected with S. *douthitti* and cured by chemotherapy lose their immunity approximately 3 weeks after the death of the worms at a time when the eggs disappear from tissues. Kagan and Lee (1953) suggest that the living embryo within the egg secretes or excretes substances that are responsible for stimulating protective immunity. Whether these postulated substances derived from the egg react with immune serum to form circumoval precipitates is not known. Though Vogel and Minning (1953) were unsuccessful in immunizing monkeys against S. *japonicum* by the injection of dead parasite material, other workers, notably Ozawa (1930) and Lin *et al.* (1954), showed that injections of saline suspensions of whole worms induced some degree of protection. Though Watts (1949) reported that mice injected with cercarial antigen of S. *mansoni* were more resistant to infection than uninjected mice, both Thomson (1954) and Kagan (1958b) were unable to verify these results. Using the so-called "metabolic product" antigens derived from a large number of cercariae, Levine and Kagan (1960) have been able to increase the survival of mice infected with S. *mansoni*.

The passive transfer of serum antibodies does not, in general, result in an adequate degree of protection against schistomiasis. Thus, although immunity in dogs and rabbits infected with S. *japonicum* was reported to have been passively transferred by Kawamura (1932), this result was not confirmed by Vogel and Minning (1953), by Stirewalt and Evans (1953), and by Levine and Kagan (1960). On the other hand, Sadun and Lin (1959) produced resistance in mice with worm extracts, with worm metabolites, and even with immune serum from rabbits. The serum adversely affected growth and development of S. *japonicum* to a certain degree. Such a result lends support to the idea that acquired resistance to S. *japonicum* is at least partly mediated by circulating antibody.

The immune responses to schistosomiasis, particularly in the higher primates, appears to be low grade. Indeed Schwetz (1956) considers that humans infected with schistosomes are in a state of premunition, viz.,

immunity persists only as long as the active infection persists, that clinical cure of an individual in an endemic area of schistosomiasis would be disadvantageous, and that treatment should be administered not to cure but to relieve chronic symptoms. Kagan (1958b), however, points out that, although conditions in the mouse support the concept of Schwetz, therapeutic cure should be pursued in man and monkeys in whom resistance to reinfection may persist for some time after termination of the infection (see Vogel and Minning, 1953).

F. Other Trematodes: *Fasciola hepatica*

Other serological studies of trematodes have been concerned mainly with the liver fluke, *F. hepatica,* in man and in domestic animals. In this infection clear-cut evidence of immunity to reinfection is lacking. No immunity is evident against the adult parasite in the bile ducts as parasites can exist there up to 11 years (Durbin, 1952). Some immunity, however, has been produced by vaccinating animals with extracts of *F. hepatica,* as reported by Kerr and Petkovich (1935) and Urquhart *et al.* (1954). This protection may be manifested by a decreased number of parasites and by smaller parasites. Healy (1955) reported only minor differences between controls and rabbits injected with extracts of *F. hepatica* upon challenge. These differences consisted of a smaller number of abnormal eggs produced by some of the trematodes in the vaccinated rabbits and a slight retardation of some worms of the challenge dose, especially before they entered the bile duct.

In *F. hepatica* infections, conventional serological tests, such as complement fixation and precipitation, have demonstrated circulating antibodies and have been used diagnostically in man although there is considerable confusion in interpreting the results of the tests.

The allergic response to *F. hepatica* has been studied in a variety of animals including human beings, and the immediate skin test shows a good correlation with infection in cattle, according to Soulsby (1954). In addition, Soulsby demonstrated the passive transfer of skin sensitivity by means of the PK reaction, and it is possible that univalent or cell-bound antibodies that sensitize the skin and possibly other organs may be found in this infection as well as in schistosomiasis.

Recently, Wilkerhauser (1961) demonstrated the formation of precipitates round the cercariae of *F. hepatica* when they were placed in serum from infected animals. These precipitates were most noticeable at the extremities of the cercariae. The active serum factor, presumably an antibody, was stable at 56°C., could be absorbed by the "metabolic products" of adult *F. hepatica,* disappeared from serum at about 150 to

180 days after treatment, and caused a slightly shorter survival time of cercariae than normal serum and a slight loss in infectivity.

V. Class Cestoda

A. Adult Cestodes

Evidence concerning the production of antibodies to adult tapeworm infections in humans and in animals is conflicting. A variety of serological tests have been used. Precipitins were demonstrated in humans infected with the broad fish tapeworm, *Diphyllobothrium latum,* as early as 1904 by Isaacs and van der Velden. Ghedini (1906) reported positive results with a complement fixation test in individuals infected with *Taenia solium.* Similarly, Kolmer *et al.* (1916) and Ravetta (1937) reported positive complement fixation tests with the sera of dogs infected with tapeworms. However, a number of other authors including Busson (1911), Lebas (1924), and Deschiens and Renaudet (1941) could only occasionally detect complement-fixing antibody in the serums of humans and animals infected with tapeworms. For many years the human tapeworms, *Taenia saginata* and *Taenia solium,* were thought to occur singly. In fact, *T. solium* is popularly called the solitary tapeworm and the name "solium" was considered to mean sole although it is now thought to be derived from the Arabic *silsilla* meaning chain. Nevertheless, multiple infection with *T. saginata* is by no means rare in endemic foci, and individuals can be infected with from 2 up to 25 scolices (Du Cazal, 1891; Palais, 1937; Altman and Bubis, 1959). The adult tapeworms of man and the larger animals reside in the lumen of the bowel where little absorption of antigen would occur unless the bowel mucosa were damaged.

1. Hymenolepis *in Mice*

The dwarf tapeworm, *Hymenolepis nana* var. *fraterna,* in mice may undergo a developmental, cysticercoid, phase in the villi of the final host. In this infection, Hearin (1941) and Larsh (1942, 1943) showed that a high level of immunity was induced by infection. This immunity was humoral in character, could be passively transferred to normal animals, was possessed by young born to and nursed by infected mothers, and was demonstrable in the serums of mice by means of precipitation, complement fixation, and agglutination tests (Larsh, 1943). The antibody responses were produced by infection with cysticercoid stages which migrate into the villus of the intestine, but partial protection was artificially afforded by adult tapeworm materials (Larsh, 1943). It is noteworthy that a marked antibody response was not produced by a closely

related worm, *Hymenolepis diminuta,* which does not migrate into the villi of the intestine (Chandler, 1940). In dwarf tapeworm infections in mice, the migrating cysticercoid phase is responsible for immunization. Eggs fed to mice normally undergo migration into the villus with the formation of a cysticercoid. However, eggs of the tapeworm fed to beetles develop into cysticercoids which, when the beetles are fed to mice, lead to the direct development of an adult tapeworm without any cysticercoid stage in the villus—in this case no immunity is produced (Hunninen, 1935).

2. Echinococcus granulosus

Successful artificial immunity against the dog tapeworm, *Echinococcus granulosus,* was claimed by Turner *et al.* (1933, 1936) who immunized dogs with intermediate hydatid cysts prior to infection with hydatid scolices. Gemmel (1960) was able to induce a low degree of immunity against *E. granulosus* by vaccinating dogs with adult tapeworm materials. Though infection was not completely suppressed, development was retarded, particularly at the onset of oogenesis.

Hypersensitivity to adult tapeworm infections was noted by Ramsdell (1927) who stated that it persisted for as long as 6 years after removal of the tapeworm. Brunner (1928) reported specific skin reactions of the immediate type in persons infected with *T. solium* or *D. latum.* A few early studies on antigen–antibody reactions in adult tapeworm infections have been followed by little recent work.

B. Larval Cestodes

1. Echinococcus

Work on larval stages of cestodes has largely been concerned with diagnostic tests for echinococcus (hydatid) disease. Serological studies on the immune response to hydatid infections was initiated by Ghedini in 1906 who described a complement fixation reaction in individuals infected with the cysts. The early work of Weinberg and Parvu (1908) was concerned with the specificity of the test and the duration of antibodies in the infected human after surgical removal. Weinberg and Boidin (1909) found that the level of complement-fixing antibodies was not correlated with the size or the site of the cyst or with eosinophilia, but was related to the site and the degree of fibrosis of the adventitial wall of the cyst.

Since this early work, many serological methods have been used to diagnose hydatid infections in humans and in animals, but the results vary with respect to the value of the tests. In general, the presence of an

active cyst is associated with the production of antibodies. The rupture of the cyst frequently produced a marked rise in the amount of circulating antibody and often led to acute anaphylactic symptoms in the patient. Removal of cysts by surgical operation resulted in the loss of antibody.

The intradermal skin test for hydatid infection was introduced by Casoni (1911) and is still used today as a method of diagnosis.

More recent serological tests for hydatid disease include the hemagglutination test (Kagan *et al.*, 1959) and a bentonite flocculation test (Norman *et al.*, 1959). For example, serum from patients with the active disease show hemagglutinin titers up to 1:120,000. However, certain sera negative in other tests tend to be positive when tested with fluid from the hydatid cyst. Such reactions may be traced to the fact that autoantibodies that are prevalent in the sera of patients ill with various hepatic diseases (Gajdusek, 1958) may cross react with host material in the tissues and fluids of the hydatid parasite. The presence of host protein in the tissues and fluids of parasites is not unexpected because studies with labeled radioactive tracers have shown an active exchange of proteins between parasite and host (Stoner and Hankes, 1955). Kagan *et al.* (1960) showed by agar diffusion methods that preparations both from *E. granulosus* and from *Echinococcus multilocularis* have antigens in common with host liver.

Recently, Goodchild and Kagan (1961) have compared, by means of electrophoresis on paper and starch, hydatid fluid from cysts of *E. granulosus* collected from a variety of animals including the moose, pigs, cattle, and man, cyst fluid of *E. multilocularis* obtained from experimental infections of cotton rats and sera from uninfected hosts. The electrophoretic patterns revealed striking similarities in all these materials. From this work the authors presume that serum albumins and globulins occur in hydatid fluid but at a much lower concentration than in serum. Undoubtedly greater specificity of hydatid antigens must await the production of materials free of host proteins, as, for example, in *in vitro* cultures.

Antigens other than helminth antigens have been demonstrated in hydatid cysts. Thus, Graña (1949) showed that a non-Forssman heterophile antibody which agglutinates sheep cells, markedly increased when hydatid fluid was injected into man and into animals. Similarly, Cameron and Staveley (1957) reported the existence of P substance in the germinative membrane of hydatid cysts. Later these authors (Staveley and Cameron, 1958) found that hydatid fluid could inhibit anti-Tj^{a} and anti-P sera. It is uncertain whether this P substance is elaborated by the germinative membrane of the hydatid cyst or is selectively absorbed by the cyst when in the animal.

2. Cysticercus fasciolaris *and* Cysticercus pisiformis

The larval stages of cestodes infecting various animals usually evoke a marked antibody response and nonviable materials can be used to stimulate protective immunity. Such materials in nematodes, it should be recalled, are generally of no value in producing functional immunity. The larval stages of the cat and dog tapeworm occurring in rats, mice, and rabbits produce very satisfactory immunity. Thus, rats and rabbits infected with *Cysticercus fasciolaris* and *Cysticercus pisiformis*, respectively, evoke antibodies which protect control animals against infection by passive transfer. Such antibodies are transmitted from mother to offspring and can be produced by the vaccination of animals with adult tapeworm materials (Miller, 1931, 1934; Miller and Gardiner, 1934; Miller and Massie, 1932).

Campbell (1938a,b,c) reported the occurrence of two forms of antibody in animals infected with the somatic stages of the dog or cat cestodes. He described an "early antibody" which was protective in character, was demonstrable within 7 days of infection, and could be produced by artificial immunization with adult or larval forms of the cestode. The naturally and artificially produced early antibody could be absorbed *in vitro* by adult and larval tissues and was responsible for the protection of the host against the migration of the early larval stage (hexacanth embryo) from the egg. A "late antibody" appeared several weeks after infection. It was also protective in character but could not be stimulated by adult or larval forms of the cestode and could not be absorbed by adult or larval tissues. The late antibody was thought to be stimulated by antigenic materials synthesized during the parasitic cyst's metabolism and was considered to be responsible for the destruction of the cyst once it had become established.

3. Cysticercus bovis

Early work on the immunological response to the intermediate stages of the human tapeworms, *T. saginata* and *T. solium*, was chiefly concerned with diagnostic tests. For example, Trawinski (1936, 1947) reported the presence of precipitating antibodies in humans and pigs infected with *Cysticercus cellulosae*. Recent work, however, has shown that animals infected with the intermediate stage of the human tapeworm, *T. saginata (Cysticercus bovis)*, show a marked antibody response which has two main peaks of production, lasting over a period of 7 to 9 months (Soulsby, unpublished). In animals infected at 4 to 6 months of age (i.e., immunologically competent), a marked antibody response is evident, but animals infected at birth develop little or no response despite

the fact that the parasite lives in the animal for several months. Early work by Penfold and Penfold (1937) showed that if cattle were infected with eggs of *T. saginata* the resulting *C. bovis* infection was destroyed within a period of 9 months and that the cattle were then immune to subsequent infection. These results have been confirmed (Soulsby, unpublished) although calves infected at birth may still harbor viable cysts after 10 months and are capable of being reinfected. It is possible that the early antibody against the migrating larval stages of *C. fasciolaris,* as described by Campbell, also appears in *C. bovis* infection. It can be envisaged that, when infection takes place at birth, no antibody is produced against the early larval phase, and reinfection occurs because there is no secondary response of the early antibody. Some evidence exists for an early antibody since Silverman (1955) has demonstrated the occurrence of precipitates at the penetration gland complex of hexacanth embryos of *T. saginata* when they are placed in immune serum.

That some calves, when infected at birth with *C. bovis,* cannot produce a satisfactory antibody response and can become reinfected may suggest that some degree of immunological tolerance has developed to the infection. The persistence of cysts in animals infected at birth has great pertinence to the epidemiological situation which exists in tropical countries, particularly in Africa, where viable *C. bovis* cysts can be found in animals up to 4 or 5 years of age despite the fact that these animals are solidly immune to reinfection. Since the incidence of *C. bovis* in cattle in these areas is high, it can be strongly suspected that the animals were infected at birth and developed a degree of immunological tolerance to the cysts (Urquhart, 1958).

Some degree of immunity to *C. bovis* infection in cattle can be induced by vaccinating animals subcutaneously with viable eggs (Soulsby, unpublished). This can be compared to the production of immunity to ascaris infection by the subcutaneous administration of viable eggs (Soulsby, 1957d). Both techniques introduce a living parasite into a site in a host in which normal migration cannot occur.

VI. General Résumé

In the study of antigen–antibody reactions in helminth infections, the worker must deal with metazoan parasites composed of different tissues and organs with vastly different functions and probably composed of many antigens. Some of them are large; others are small and quite embryonic in their early infective stages. In addition, following infection of a host with the infective larval stage, there is a complicated cycle of de-

velopment involving not only a change in size and physiology but frequently a change in environment. Specific materials may be produced by different larval stages which facilitate penetration of the skin or parasitism in various organs. With this marked change in environment, physiology, and structure, it can be anticipated that responses of the host must also vary markedly. Up to the present, there has been little work done to characterize individual antigens in helminths. Experiments have largely been concerned either with diagnostic tests or with the ability of antigens to function in inducing protective immunity.

For diagnostic serological or skin tests, attempts have been made to fractionate and purify specific test antigens. Bearing in mind that the test antigens are usually prepared from parasites that have lived in the host, it is possible that the antigen preparations contain host proteins which may be responsible for many of the cross reactions that occur. With the advent of the *in vitro* cultivation of helminths, it may be anticipated that host materials can be eliminated from the test antigens.

Essentially, protective immunity to helminths depends upon the presence of the living parasite or of metabolites of the living parasite within the host. With the exception of the intermediate stages of cestodes, immunization with essentially nonviable materials of helminth origin has generally been found to produce little or no protective immunity. Protective immunity does not, however, depend upon the occurrence of the complete life cycle of the parasite in the host. It can be established by partial development and the migration of larval stages. Thus, in nematode infections, at least, development which includes at least one moulting phase, is associated with the marked release of immunizing antigens. Some evidence indicates that these antigens may be related to materials involved in the exsheathment of the larvae, although exsheathing or moulting fluid has not been shown to be the sole material produced by larvae at the time of moulting. Such a fluid may merely accompany antigens which can be readily detected by various serological tests. The source of the functional antigens in helminth infections is unknown. There is still no evidence available to show which organs or tissues produce the protective or functional antigens. However, it would appear that actively metabolizing organisms are more antigenic than those which are not metabolizing, and it is likely in the future that these functional antigens will be found to be associated with the synthesis of protein or even of nucleic acids. Undoubtedly numerous enzymes exist within the tissues of helminths that could act as antigens, but they may not become available to the host in antigenic form during a normal infection with a helminth.

There is little evidence in nematode and trematode infections to show that circulating antibody, as detected by various serological tests, is concerned in protective immunity. Thus, serums from such infected animals when passively transferred have usually resulted in only a low level of immunity as contrasted to the high level conferred by serums from cestode infections. In this connection, it should be mentioned that antibodies other than precipitating antibodies, particularly the skin-sensitizing antibodies, should be investigated with respect to their functional role in helminth immunity.

Little information exists with respect to the importance of white cells in the antibody response. That they occur in large numbers, however, at the site of an immunological reaction between the host and the parasite is probably not a fortuitous finding.

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Embryological Development of Antigens

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[1] The author wishes to dedicate this paper to the late Professor A. M. Schechtman, Department of Zoology, University of California at Los Angeles. His outstanding achievements in the fields of Developmental Biology and Immunoembryology have earned the sincere respect of research workers everywhere. The author salutes Professor Schechtman for his fundamental contributions and expresses the sorrow that is shared by his many friends at his passing.

I. Introduction

Most native proteins are antigens and therefore any discussion of antigens in developing embryos becomes, for the most part, a treatment of proteins of the embryo. It is believed that proteins may play a major causal role in the various phases of development, and they have received a proportionate amount of attention. One of the most sensitive tools that the developmental biologist has utilized for investigation of the proteins of the embryo has been the immunochemical method. Antigens are defined as substances which can provoke the formation of specific antibodies, and it has been repeatedly demonstrated that embryos possess numerous proteins with antigenic properties. It is to be stressed that antisera are produced in response to various specific determinant groups on antigens and that an apparent identity of two proteins by an immunological test may be caused by a similarity of some of their determinant groups. In this paper the term antigen will be employed with this reservation in mind.

It is also necessary to emphasize that the determinant groups of an antigen may be different from the sites on a protein which possess enzymatic activity. The immunological method is only one way of investigating macromolecules, and the use of other methods as well will result in a more complete characterization of the macromolecules in question.

For the most part this review will be limited to immunological investigations of development, with only infrequent departures to other methods of analyzing the numerous important roles that proteins play in developmental processes. This does not imply that the immunological approach is necessarily the best approach to these problems, but that this reviewer has limited himself primarily to those instances in which the proteins in question have been demonstrated to be antigenic. Needless to say, many of the proteins involved in the economy of the embryo that have not yet been studied immunologically are also antigenic.

This paper will be concerned primarily with certain aspects of the work dealing with the maternal synthesis and transfer of antigens to the egg; the description, distribution, and time of appearance of specific antigens, with special attention given to the muscle protein, myosin, and to the lens proteins; to immunological studies related to the embryonic processes of competence, induction, determination, differentiation and specific growth stimulation or inhibition; and to the biological effects of specific antisera upon developing embryos. No attempt will be made to refer to all of the published work in this field, and the author's main aim will be to present results which afford insight into the mechan-

ism of embryonic development. There have been a number of recent reviews concerning immunology and development: Ebert (1955, 1958a,b, 1959), Edds (1958), Nace (1955), Schechtman (1955, 1956), Tyler (1955, 1957), and Woerdeman (1955). Several papers have dealt primarily with immunological methods which relate to the study of development: Feldman and Yaffe (1957), Croisille (1960), Clayton (1960), and Wyttenbach (1960). Agar-plate diffusion reactions (Ouchterlony, 1948; Oudin, 1952; Wilson and Pringle, 1955) have been widely utilized in developmental studies.

II. The Significance of Antigens in Development

Since differentiation and growth are processes which seem to be intimately linked with the synthesis of proteins, it appears that immunological studies, by virtue of their specificity and sensitivity, afford a critical means of investigating the role of proteins in development. The objective of this reviewer will be to draw attention to instances where antigens may play a causal role in development; and he will try to avoid, as often as possible, the mere presentation of an inventory of the antigens of the embryo. In this regard the following questions will be considered: Does the time of appearance of a tissue-specific antigen always coincide with the morphological and functional differentiation of that tissue, or does it correspond to the time when the tissue is determined, i.e., when the future fate of the tissue is set, although there is no morphological nor functional differentiation? Does the antigen appear at the time the tissue attains the potential to form the tissue (embryonic competence) and disappear when the tissue loses this competence? Is the distribution of a tissue-specific antigen the same as the area of the organism that is competent to form that tissue? Is there critical evidence that supports the notion of organ-specific stimulation, or inhibition, of differentiation and/or growth? Are specific macromolecules passed between cells during embryonic induction, and, if so, what is their function? How do proteins upon cell surfaces act in promoting or inhibiting cell movement, selective reaggregation of cells, and cellular adhesion? Are antigens end products of differentiation or are they part of the causal mechanisms of development? Finally, what are the responses of embryonic cells to exposure to antigens or antisera? The answers obtained to the last-mentioned question are undoubtedly critical because they may allow the embryo itself to answer some of the previous questions that are being considered.

III. Maternal Synthesis of Proteins and Their Transfer to the Egg

A. Serological Comparison of Chicken Vitellin, Livetin, and Serum

In discussing the embryological development of antigens, it is necessary to emphasize that not all of these antigens are synthesized in the egg and embryo itself. The evidence that macromolecules may be synthesized by the maternal organism and transferred to the ovarian eggs and to embryos by way of the blood stream is reviewed in recent papers by Brambell *et al.* (1951), Brambell and Hemmings (1954), and Schechtman (1955, 1956). Tyler (1955) has emphasized that there is no critical evidence to indicate that the growing oocyte itself synthesizes most of its macromolecular constituents, but that they are apparently supplied to the oocyte by the maternal organism. However, the embryo itself does synthesize macromolecules, particularly after gastrulation. This review will be concerned only with maternal transfer of the homologous or natural proteins of the embryo rather than the passage of antibodies and heterologous proteins, although this latter type of transfer does, of course, afford convincing evidence for the phenomenon of maternal transfer of macromolecules (see Knight and Schechtman, 1954).

The production of the serum proteins of the embryo and their relation to the egg yolk has been the subject of a number of investigations. Roepke and Bushnell (1936) developed an antiserum to vitellin, the water-insoluble phosphoprotein fraction of hens' eggs, and found that it reacted strongly in precipitin reactions with the serum of the laying hen but not at all, or weakly, with the sera of male birds or nonlaying hens. Schechtman (1947) showed a serological similarity between the water-soluble yolk protein of hen's yolk (livetin) and serum of the adult, and he suggested that this pseudoglobulin fraction (livetin) was the main constituent of the serum of the embryo during the first few days of development. In his work antisera to whole serum and the euglobulin fraction gave positive ring tests against embryos (primitive streak to 15–17 somite stage), but after absorption with yolk the antisera did not react with the embryo. However, they still react with adult serum and the blood of 15-day chicks, a fact indicating that certain serum proteins of the adult have determinant groups different from those in yolk and appear some time between the 15–17 somite stage and the fifteenth day of development.

Immunological studies by Tanabe *et al.* (1961) of ultracentrifugal fractions of laying hen sera and of egg yolk demonstrated a low-density protein with no bound phosphoprotein phosphorus and also a phospho-

protein, or phosphoprotein complex, both in the sera and in the yolk. These two components were not found in the sera of cocks. The specific antibodies to either one of these antigenic fractions could not be exhausted by absorption with the other preparation. The low-density phosphorus-free protein in the serum of laying hens, and the phosphorus-free protein in the ultracentrifugal supernatant solution of egg yolk, gave one band in agar-diffusion reactions which showed the reaction of identity. The phosphoprotein of laying hen serum was soluble in isotonic saline whereas the phosphoprotein of egg yolk was not, and these fractions also differed in their serological properties. This evidence, suggesting antigenic relationships between the serum and the yolk, implies that some of the serum proteins are derived from the yolk. There is also evidence that nonyolk proteins of the hen's egg are similar to serum proteins. Marshall and Deutsch (1951) used quantitative precipitin tests to show that the ovalbumin of egg white is similar to yolk albumin and to adult serum albumin. The conalbumin of egg white is similar to proteins of yolk and adult serum.

1. Isotopic Evidence for Maternal Transfer of Chicken Yolk Phosphoprotein

The idea for a maternal synthesis of yolk proteins is strongly supported by the isotopic evidence of Flickinger and Rounds (1956). These authors injected two laying hens and two nonlaying hens with $Na_2HP^{32}O_4$ and killed one layer and one nonlayer 6 hours after injection and the other two 12 hours after injection. Phosphoprotein fractions were obtained from trichloroacetic acid insoluble fractions of liver, blood, and egg yolks of the four hens, and phosphoprotein phosphorus was hydrolyzed in boiling 2% NaOH. The samples were neutralized and inorganic phosphate was precipitated with 10% CaCl saturated with $Ca(OH)_2$ (pH 8.8). No detectable amount of phosphoprotein phosphate was obtained from the liver and blood of nonlaying hens. The laying hen samples were counted with a thin-window counter, and the data are presented in Table I. The isotopic activity of the phosphoprotein fraction of the liver is greater than that of the blood, which in turn is higher than that of the yolk. Although the amount of phosphoprotein is accumulating rapidly in the oocyte during this time, the higher isotopic activity of the phosphoprotein fraction of the liver suggests that the liver is the site of phosphoprotein synthesis. The lower activity of the liver at 12 hours and the higher activity of the blood and egg yolk indicates that the phosphoprotein (vitellin) is transported to the yolk by way of the blood stream.

Chargaff (1942) injected radioactive phosphate into laying hens and found the rate of yolk phosphoprotein formation was similar to that of the maternally synthesized phosphatides (Hevesy and Hahn, 1938), a fact suggesting that vitellin was synthesized by the hen.

TABLE I

ISOTOPE LEVEL OF PHOSPHOPROTEIN PHOSPHATE OF LIVER, BLOOD, AND EGG YOLK FROM LAYING HENS KILLED 6 AND 12 HOURS AFTER INJECTION OF 100 μC. OF P^{32}

Organ	Activity (c.p.m./phosphate) at: 6 hours	12 hours
Liver	164.0	101.0
Blood	38.3	45.4
Egg yolk	0.62	4.1

2. *Appearance of Nonvitelloid Serum Proteins in the Chick Embryo*

Nace and Schechtman (1948) found that an antiserum to adult chicken serum reacted with the serum of the 3- to 4-day embryo and that this reaction was removed if the antiserum was absorbed with yolk; however, they detected serum proteins with determinant groups characteristic of adult proteins by the fifth to sixth day of incubation. The appearance of these nonvitelloid antigens in the blood corresponded to the time of disappearance of the primitive blood cells and the occurrence of many disintegrating cells in the blood stream. Schechtman and Hoffman (1952) noted that rabbit antisera against the α-β-globulin fraction of adult chicken serum reacted against whole blood of 3-day chick embryos and the serum of 6-day embryos. These authors postulated that the high rate of blood cell disintegration at 4 to 5 days of development is a source of embryonic serum proteins, in this case, α-β-globulin. This finding supports Sabin's (1917) hypothesis that embryonic serum comes from disintegrating blood cells.

Nace (1953) obtained antisera against serum albumin and γ-globulin of adult chicken serum obtained by cold ethanol fractionation. After yolk absorption of these antisera, antigenic determinants similar to serum albumin were detected in the serum of the embryo on the fifth day of incubation, those similar to α-β-globulin on the sixth day, and those resembling γ-globulin between the ninth and twelfth day. Just before the appearance of these antigens, the unabsorbed antisera detected the presence of yolklike antigens in the serum of the embryo which resembled each of the nonyolk antigens. Nace suggested that yolk proteins are a source of the early serum proteins, that they are derived intact from the maternal organism, and, further, that the transferred antigens would

"seed" the reaction of the embryo's own mechanisms for serum protein synthesis.

B. Serumlike Antigens in Frog Eggs and Embryos

The evidence presented in the foregoing for serological similarity of yolk proteins and adult serum proteins is also supported by work with the amphibians. Cooper (1946, 1948, 1950) detected serumlike antigens in the saline-soluble fraction of eggs and embryos, and Cooper's (1950) agar-diffusion experiments demonstrated that this relationship between adult and embryo could be referred to a multiple number of antigens. Flickinger and Nace (1952) used an antiserum to adult frog serum and found that it reacted with the centrifugal supernate fraction of oocytes and embryos as well as with the vitellin and livetin fractions of yolk.

1. Similarities between Female Serum Proteins and Frog Yolk Proteins

Flickinger (1956, 1957, 1960) has obtained evidence that frog vitellin is a water-insoluble complex of two components; one is a protein low in phosphorus, which is present in high concentration, and the other is a phosphoprotein present in a low concentration. Dephosphorylation of the small protein of the insoluble complex by phosphoprotein phosphatase releases the large protein from the complex in a water-soluble state. This view is supported by the electrophoretic and ultracentrifugal evidence for the identity of the phosphorus-free protein component of the water-insoluble complex (vitellin) and the water-soluble phosphorus-free protein of the livetin fraction (Schjeide *et al.*, 1955). In this regard Schechtman (1955) mentioned that his chicken antivitellin sera reacted as strongly with livetin as with vitellin itself. Flickinger and Rounds (1956) found that an antiserum to the livetin fraction of frog yolk produced five lines of identity in Ouchterlony plates between livetin (water-soluble yolk fraction low in protein phosphorus) and vitellin (water-insoluble yolk fraction high in protein phosphorus) (Fig. 1). The antigens, antiserum, and agar were made up with a 10% concentration of NaCl in these tests in order to solubilize the vitellin fraction. The similarity in reactivity is attributed to the common phosphorus-free protein in the two fractions. The antilivetin serum showed reactions of identity of at least three of these antigens with frog serum (Fig. 2) (Flickinger and Rounds, 1956). The antilivetin serum gave five lines in reactions with female frog serum, but only two when reacted against serum of the male frog (Fig. 3). These two antigens of male serum were identical to those in the female serum. The anomalous reactivity of female serum was also noted in another investigation (Flickinger, 1958a) in which antisera to

adult male frog brain were absorbed to completion with male frog serum, but the antisera still reacted with female frog serum in precipitin tests. Since the antisera were directed against a male frog organ, this finding is difficult to understand unless one postulates that female serum con-

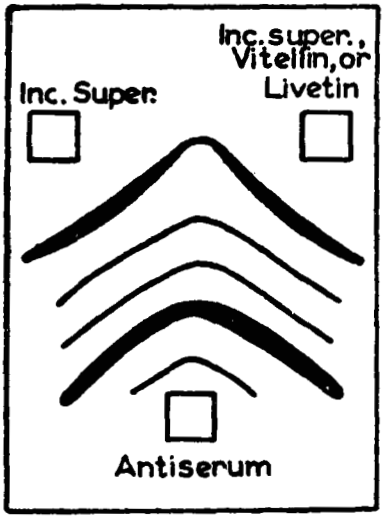


FIG. 1. An antiserum against the water-soluble livetin fraction of frog yolk was tested against livetin (here called incubation supernate) and vitellin in Ouchterlony agar-diffusion reactions.

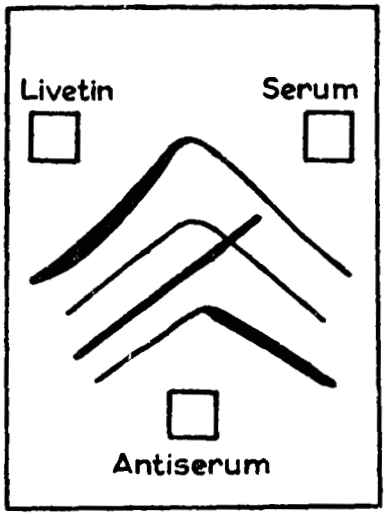


FIG. 2. Diffusion-patterns of antilivetin tested against livetin and frog serum.

tains antigens with combining groups also shared by brain but not by various proteins of male serum. These possibly might be the three antigens detected by agar-plate diffusion in the female serum which are not present in the male serum (Flickinger and Rounds, 1956).

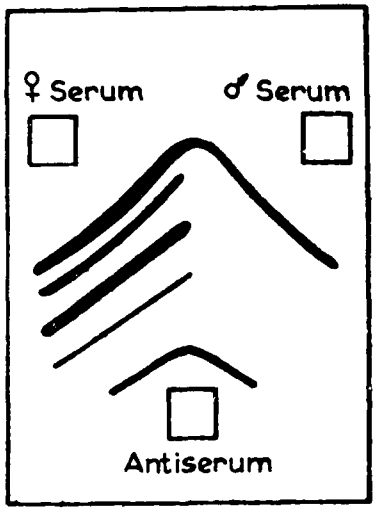


FIG. 3. The reaction of female and male frog serum with the antiserum to the livetin fraction of frog yolk.

2. *The Demonstration by Fluorescent Antibody of Serumlike Antigens in Frog and Mouse Eggs*

Glass (1959) used fluorescent-conjugated γ-globulin fractions of antisera directed against female frog serum and observed fluorescence in the preyolk oocytes and around the periphery of the egg at early stages of yolk formation. At later stages of vitellogenesis there was bright fluorescence around the nucleus and the yolk platelets. The fluorescent staining of the follicle-thecal cells increased during the period of yolk formation. She suggested that her data support the idea of maternal synthesis of some proteins and their subsequent transfer to the oocyte by way of the maternal blood and the follicle-thecal cells. Similar use of the fluorescent antibody technique with the mouse embryos allowed the detection of serumlike antigens in the cytoplasm of ovarian oocytes (Glass, 1961).

In the work of Nace *et al.* (1960), the localization of fluorescein-labeled γ-globulin fractions of antisera against centrifugal supernates of mature frog oocytes was examined. It had previously been shown that

such antisera cross-react strongly with adult frog serum (Flickinger and Nace, 1952). Nace *et al.* found fluorescence in the cytoplasm of young oocytes and in the surrounding follicle cells, a weak fluorescence in the cortex of mature eggs, and none in the follicle cells. They also found a strong fluorescence in the ciliated epithelial cells of some levels of the oviduct, but none in the ostial end of the oviduct, the egg jelly, or the jelly-secreting glands. The antigen that accounted for this fluorescence was observed to be extruded from the egg into the perivitelline space following fertilization. After ovulation the oviduct epithelium had a diminished fluorescence. The authors suggested that a specific antigen is synthesized in the follicle cells and oviduct and is transferred to the oocyte in which it plays a developmental role.

In the same paper, these authors described Ouchterlony agar-diffusion experiments and immunoelectrophoresis experiments in which they employed a stain for lactate dehydrogenase. They identified four antigens with different electrophoretic and serological characters in the centrifugal supernate of mature oocytes which had lactate-dehydrogenase activity. They also outlined a technique in which they cut a given fluorescent antibody–antigen precipitate band from an agar-diffusion or immunoelectrophoresis pattern and placed this agar upon a fixed tissue section. The antigen–antibody complex was dissociated at pH 10.5 or 3.5 and the specific fluorescent antibody diffused into the tissues. The pH was adjusted to neutrality and the sections were examined for antibody localization after washing. This technique indicated that lactate-dehydrogenase antigen 1 was localized on the yolk platelets of the egg and connective tissue of the oviduct, whereas lactate-dehydrogenase antigen 3 was present in egg cytoplasm, in certain cells of the oviduct epithelium, and in the jelly-secreting glands of the oviduct.

C. Maternal Antigen Transfer in the Cecropia Silkworm

In the Cecropia silkworm, Telfer and Williams (1953) and Telfer (1954) demonstrated an antigen in the blood of females whose concentration is about a thousand times greater than in the male. This antigen was immunologically similar to one in the yolk of unfertilized eggs. The concentration of this antigen was decreased significantly after egg formation had been completed, but was not decreased by ovariectomy, which, of course, prevents egg formation. When ovaries were transferred to the hemocoel of males where egg formation continued, this antigen was only incorporated into the yolk when it could be detected in the blood of the males. If females of another species were transfused with Cecropia blood, this antigen was transferred into the yolk of the ovarian eggs of the

other species. Laufer (1960) has examined agar-gel diffusion plates of blood from female Cecropia pupae by direct histochemical staining of the agar plates for esterase activity. Two of these antigens show esterase activity even though they are in combination with antibody. His results suggest that several of the antigens described in the work of Telfer (1954) are enzymes.

Telfer (1961) has used fluorescein-labeled antibodies to demonstrate that blood proteins are present between the follicle cells, at a brush border at the surface of the oocyte and in the yolk spheres within the oocyte. He proposes that blood proteins are transformed into yolk spheres by a process similar to pinocytosis.

D. Developmental Significance of Maternally Synthesized Antigens

Data at hand suggest that certain frog, chicken, and silkworm proteins synthesized by the mother are transferred to the egg and further that some proteins of the early chicken embryo itself are derived from the livetin fraction of the yolk.

Schechtman (1955) has speculated that the serum and fluids of the adult body supply factors necessary for the differentiation which follows the gastrula stage, but he also states that these heterosynthesized molecules may not be necessary after the organ rudiments have been established. He compared the differentiation capacities of amphibian, bird, and mammalian gastrula tissues *in vitro* and in transplantation experiments and suggested that the superior differentiation in the transplantation experiments is due to the supply of heterosynthesized macromolecules.

Flickinger (1956, 1957, 1958, 1960) and Rounds and Flickinger (1958) have emphasized the importance of solubilization of protein (including ribonucleoprotein) of the yolk platelets in relation to the process of embryonic induction in frog embryos. Temporary acidity produced by CO_2 promoted the differentiation of dorsal tissues such as the neural tube, notochord, and nephric tubules from the prospective ventral areas of the frog gastrula, and similar treatment of isolated yolk platelets resulted in solubilization of yolk protein. It is thought that differentiation is triggered when soluble yolk proteins are hydrolyzed to amino acids and rebuilt into specific kinds of cytoplasmic proteins which characterize the cells as differentiated. The data of Rounds and Flickinger (1958) and of Flickinger (1960) show that solubilization of yolk proteins (and ribonucleoproteins) begins first during gastrulation in the area of the primary organizer (chorda mesoderm) and that proteins

(and nucleoproteins) diffuse to the overlying ectoderm. This finding supports the suggestion that the induction of the neural plate of the frog embryo by the underlying mesoderm may consist of a stimulus to protein synthesis in the overlying reacting tissue by provision of the raw materials for protein synthesis from solubilized yolk ribonucleoproteins of the inductor tissue.

Regarding the questions which were posed in the section dealing with the significance of antigens, the demonstration of maternally synthesized serumlike antigens in eggs and embryos, before the appearance of the plasma itself, would suggest that tissue-specific antigens may appear before the differentiation of the tissue itself. However, the evidence indicates that nonvitelloid antigens, with determinate groups similar to the adult chicken serum proteins, do arise in an epigenetic manner during development. Maternal synthesis and transfer seem to be a fact, and the work of Brambell (reviewed by Brambell *et al.*, 1951) and of Schechtman (1956) indicate that heterologous molecules can be transferred from mother to embryo with little or no apparent alteration. The critical question seems to be: Do the serumlike yolk antigens have anything to do with the formation of the nonvitelloid serumlike antigens? The question of the causal role of the vitelloid and nonvitelloid serumlike antigens in the differentiation of the fluid phase of the blood must await further evidence which will elucidate other characteristics of these proteins, such as their amino acid sequence. In this way the investigator will have other standards of judgment to ascertain whether the presence of serumlike immunological determinant groups in vitelloid molecules is a sign that they are precursors of the definitive serum proteins of the organism.

IV. Description, Distribution, and Time of Appearance of Antigens in the Embryo

A. The Use of Antisera Directed against Saline-Soluble Antigens of Frog and Sea Urchin Eggs and Embryos

In presenting an inventory of some of the antigens found in the embryo, their biological role should be listed, but, unfortunately, it is not at present known for most antigens. Most of the descriptive immunological work with embryos to date has dealt with the time of appearance and location of tissue and organ-specific antigens.

Variations in the saline-soluble antigens of blastulae and gastrulae of *Rana pipiens* have been considered by Clayton (1953) and Spar (1953). Flickinger and Nace (1952) have demonstrated two new antigens in the centrifugal supernate fraction between the immature and mature stages

of the oocyte and between fertilization and the stage 19 larva, respectively. Perlmann (1953) demonstrated that the saline-soluble antigens in the developing sea urchin are remarkably similar beginning with the unfertilized stage up to the pluteus larva. However, the 48-hour plutei and gastrulae have at least three saline-soluble antigens not detectable in extracts of unfertilized eggs. The recent work of Couffer-Kaltenbach and Perlmann (1961) also emphasized the similarity of antigens during development. By using single diffusion in agar, at various dilutions of extracts and of antisera, they identified six identical antigens in eggs and embryonic stages.

B. The Mitotic Apparatus of the Sea Urchin Egg

Went and Mazia (1959) employed a cytological structure with a known function as an antigen. This antigen, consisting of mitotic apparatuses isolated from cleaving sea urchin eggs by the digitonin method and dissolved in water at pH 10.5, was used to obtain antisera. These materials gave a single line in agar-diffusion reactions, and the antisera showed a reaction of identity with an extract of unfertilized eggs although an extra minor band appeared with the latter. Apparently the mitotic apparatus contains a single major antigen and probably a second minor antigen. Since the major antigen is present in cells before the time of cell division, it is probably incorporated ready-made into the mitotic apparatus at the time of cell division. This antigen is also present in the ovaries of the homologous species, but not in testes, muscles, gut, or sperm flagellae. The authors believe that the lack of common antigens between the mitotic apparatus and the muscle and sperm flagella argues against the concept of a common molecular basis for these "contractile structures" (see also Finck and Holtzer, 1961; Laufer, 1959). This concept may be at fault, but certainly more evidence is necessary since not all chemically similar proteins show serological cross reactions, and recently Mazia *et al.* (1961) demonstrated a highly specific adenosine triphosphatase in the mitotic apparatus. Furthermore, antisera to eggs of *Strongylocentrotus purpuratus* reacted against extracts of unfertilized eggs of *Strongylocentrotus franciscanus* and *Lytechinus anamesus*, but the mitotic apparatus precursor was shared only by *S. purpuratus* and *S. franciscanus*.

C. Paternal Antigens in Hybrid Sea Urchin Embryos

Another interesting use of embryonic material as an injection antigen is the work of Harding *et al.* (1954). Antisera were obtained to lyophilized whole extracts of three species of sea urchin embryos and one

group of hybrid embryos at the 48-hour stage of development (early pluteus stage). The species utilized were *Arbacia lixula, Paracentrotus lividus,* and *Psammechinus microtuberculatus.* The hybrids were *Psammechinus* ♀ × *Paracentrotus* ♂. These antisera were tested in Ouchterlony reactions with the species mentioned as well as with two other types of hybrids (*Paracentrotus* ♀ × *Psammechinus* ♂ and *Paracentrotus* ♀ × *Arbacia* ♂) at various stages of development. Paternal antigens were detected in hybrids by the late blastula stage (24 hours of development) as well as at later stages. Control experiments indicated these results were not caused by contamination with sperm. The appearance of paternal antigens in these hybrids before morphological differentiation was ascribed to nuclear activity since the fertilizing sperm contributed primarily nuclear material.

D. The Use of Antisera Directed against Saline-Soluble Antigens of Adult Organs

A number of investigators have searched for adult antigens in the egg and embryo. It is to be expected that a tissue or organ-specific antigen would appear, or increase in amount, at the time of differentiation and growth of that particular tissue or organ. The aim has been to find organ-specific antigens before differentiation and to ascertain if they appear at the time when the fate of the tissue is established (embryonic determination) and if their localization corresponds to the areas of the embryo capable of forming that organ (embryonic competence). Of course the ultimate aim is to discover causal relationships between antigens and development. It will be seen that this goal has been attained in only a few cases.

Schechtman (1948) obtained an antiserum to saline extracts of perfused brains of 19- to 20-day chick embryos and carried out precipitin tests with it against heart, muscle, liver, and blood from 19- to 20-day embryos, as well as against the primitive streak, neural plate, and 4–5 somite stages. The brain antisera were absorbed with blood which also removed the activity toward yolk. These absorbed antisera still reacted with the primitive streak and neurula stages of the embryo and the other organ extracts. It should be pointed out that no organ differentiation has occurred in the primitive streak stages. These antigens in the embryo are apparently general antigens and are not organ-specific as absorption of the antibrain serum with any of the heterologous organ antigen preparations could completely remove precipitin activity against the homologous antigen (brain) and against the embryo. It is probably not the

Forssman antigen that is present in chick blood since the antisera had been absorbed with blood.

Ebert (1950) found that antisera to adult chicken heart, brain, and spleen react with the primitive streak stage but that when these antisera were made specific to each of the adult organs by absorption with the heterologous organs, they did not then react with the embryo. Absorption with heterologous antigens did not remove activity for the homologous adult antigen. Ebert states that the early embryo has an organ antigen which is of a more general nature than adult tissue-specific antigens. Ebert (1951) has also studied the development of adult chicken spleen antigens and finds one group arising at 9 days of development and another group after the fourteenth day.

Using the immunoelectrophoresis method of Grabar and Williams (1953, 1955), Croisille (1960) has studied the development of adult chicken liver antigens in the chick embryo. He identified fourteen constituents in adult chicken liver, and one of these was detected in the 5-day embryo, six were seen in the 6½-day embryo, seven antigens were present in the 8- to 9-day embryo, nine in the 17- to 18-day embryo, and eleven constituents were found in the chick liver 5 days after hatching.

E. Hemoglobin

The use of adult tissue-specific proteins as immunizing antigens sometimes allows a more extended biological interpretation of the results than the work with complex organ extracts. One such tissue-specific protein that has been utilized is hemoglobin.

D'Amelio and Salvo (1959) found that 36-hour chick embryos contain antigens serologically similar, but not identical, to hemoglobin. Complete similarity of the embryonic antigen with adult hemoglobin was attained at 136 hours of development. The use of crystalline hemoglobin as an immunizing antigen allowed Beard (1959) to detect a protein at the primitive streak stage which would react with the antihemoglobin serum in a precipitin test. This antiserum did not cross-react with saline extracts of egg white, yolk proteins, or serum globulins. Not all the hemoglobin could be removed from preparations of serum albumin which did cross-react. Agar-diffusion reactions with the purified adult hemoglobin showed four bands, but only one band is found in the reaction with the primitive streak stage. Three of the four bands of the homologous reaction could be removed by absorption with purified globin. The hemoglobin preparation showed only two bands in starch gel electrophoresis which spectrally were both hemoglobins.

F. Myosin and Actin

1. *Appearance of Myosin and Actin in Frog and Chick Embryos*

In searching for an antigen which is tissue specific and also constitutes a major portion of the protein population of the tissue, attention was naturally drawn to the muscle proteins, myosin and actin. Myosin makes up more than half of the dry weight of muscle, and it has been shown that myosin and actin are the major components of this contractile system.

Antigens produced against myosin and actin of adult muscle have been found to react with extracts of early amphibian and chick embryos before the differentiation of muscle itself. Ranzi and Citterio (1955) extracted late gastrula (yolk plug) stages of frog embryos with Weber and Edsall solution and discovered that the extract reacted with both antimyosin and antiactin sera. Ogawa (1958), with serological methods, detected muscle actin at stage 19 in developing *Triturus pyrrhogaster* embryos, whereas myosin was detected at stage 24. By using antisera to actin and actomyosin of skeletal muscle of adult chickens, Ogawa *et al.* (1958) obtained positive precipitin reactions with saline extracts of 72-hour embryos for actin and of 96-hour embryos for actomyosin.

Ebert (1953) and Ebert *et al.* (1955) obtained antisera to adult chicken cardiac myosin and actin and rendered these antisera organ specific by absorbing them with skeletal myosin and actin. When the precipitin method was employed, myosin was found in all parts of the epiblast of the primitive streak blastoderm. Actin was detected in the head process stage and was localized in only two areas to either side of Hensen's node, although traces were detected in the region of the primitive streak. Rawles (1943) showed previously that only these areas form heart when grafts from this stage embryo are made to the chorioallantoic membrane. At this same time myosin becomes restricted to the heart-forming areas. It seems most likely that the disappearance of myosin from a large part of the embryo may be due to the loss of a particular synthetic pathway, but it may be ascribed to cell movements in the mesodermal layer which would segregate the cells capable of producing myosin. Although the very anterior and posterior parts of the blastoderm lost their reactivity for antimyosin when myosin localization in the heart-forming areas of the intact blastoderm occurred, the capacity of the anterior and posterior region to react was retained if the blastoderm was cut into three regions (anterior, middle, and posterior) and the parts cultured singly *in vitro* (Ebert, 1959). Aside from retaining traces of cardiac myosin for 36 hours, the posterior region also had the capacity

to differentiate into a heart, but the anterior piece reacted with antimyosin for 12 hours and then the reaction disappeared. If the isolated middle piece which contains the heart-forming areas was cut so that it did not contain Hensen's node, antimyosin reactivity disappeared. The localization of cardiac actin and myosin in prospective heart tissue of the early chick embryo some time before morphological or functional specialization of cardiac tissue is one of the best examples of the location of a protein with organ-specific determinant groups in areas of the embryo that are capable of forming that specific organ.

2. *Localization of Myosin in Developing Chick Myoblasts*

Fluorescein-labeled antisera against adult chicken skeletal muscle have been used by Holtzer *et al.* (1957) to study the development of trunk myoblasts in the chick embryo. The antimyosin sera were specific in that they stained only differentiating myoblasts. An example of this staining is illustrated in Fig. 4. The sensitivity of this method was illustrated by the fact that the fluorescent antisera could detect myofibrils shortly before cross striations were seen at stages 16–17 of Hamburger

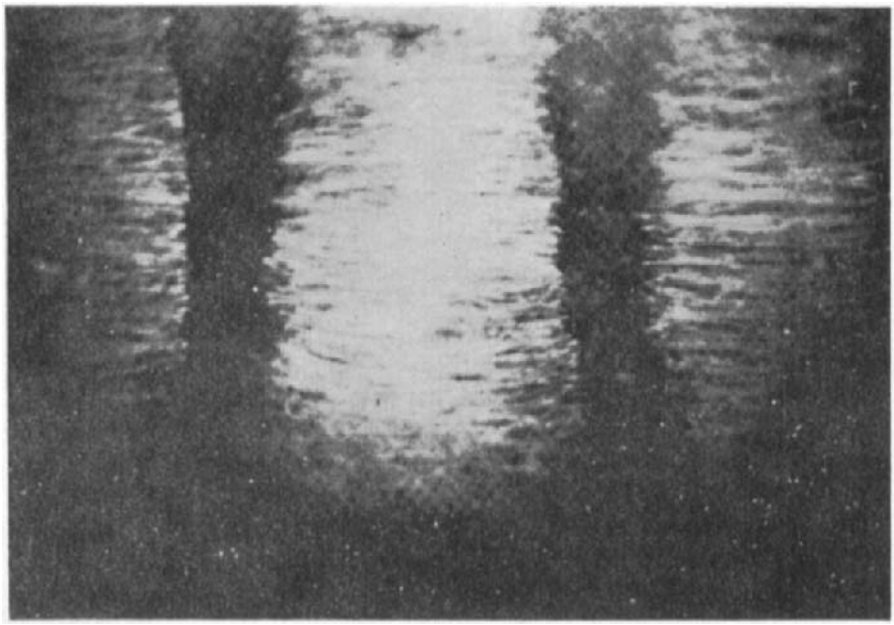


FIG. 4. The staining of myotomal plates of three successive somites by a fluorescein-conjugated antimyosin serum. Only the myofibrils fluoresce (Holtzer *et al.*, 1957).

and Hamilton (51–64 hours of incubation), but only at stages 18–19 (65–72 hours of incubation) if conventional staining methods were used. At stage 10 (33–38 hours of incubation) the myotomes were not fluorescent, whereas at stage 13 (48–52 hours of incubation) the anterior cervical myotomes reacted. Even at later stages the anterior myotomes stained more strongly than the posterior ones. If myoblasts of stage 21 (3½ days) or of older stages were glycerinated and adenosine triphosphate added, contraction occurred. Interestingly, prior exposure to fluorescein-labeled antimyosin prevented this effect. In 2–4 day myoblasts, the first fibrils appeared to adhere to the sarcolemma. The authors point out that many investigators have seen this peripheral location of the early forming fibrils and suggest that the fibrils may form from the cell surface. The individual sarcomere of a 3-day myofibril has bands resembling those of the adult, and myosin is localized only in the A bands. Engel and Horvath (1961) have obtained comparable results.

One fundamental difference between the work of Ebert *et al.* (1955), using the precipitin method, and that of Holtzer *et al.* (1957), using fluorescent antisera, is that the former work showed the presence of myosin in advance of myofibril formation, whereas the latter did not. This difference may be accounted for by the fact that Ebert *et al.* were using antiserum to cardiac myosin, whereas Holtzer *et al.* employed an antiserum to skeletal myosin. Another possible explanation for the apparent difference in results may be ascribed to the fact that the two groups of investigators prepared myosin by different methods.

Holtzer *et al.* (1959) used fluorescein-labeled antimyosin to demonstrate cross-striated myofibrils in myoblasts of the chick heart at 26–40 hours of development, i.e., before the heart starts to beat. The antiserum reacts with myofibrils both of cardiac and of skeletal myoblasts, just as do myofibrils of mature cardiac and skeletal muscle. Apparently cardiac and skeletal myosin have common antigenic sites. The insoluble fibrous protein of glycerol-extracted mitotic figures did not react with the fluorescein-labeled antimyosin. Fibroblasts and presumptive myoblasts did not react with the antibody.

3. *Myosin and Actomyosin in Regenerating Limbs*

Myosin antisera have also been used to investigate the process of limb regeneration. DeHaan (1956) prepared antisera to actomyosin of larval salamanders *(Amblystoma)* and detected the appearance of myosin at the time myofibrils are forming in the regenerating limbs of larval salamanders. Laufer (1959) prepared antisera against myosin and actomyosin in order to follow changes in these proteins during limb regen-

eration in the adult salamander *(Triturus)*. The antimyosin sera and myosin test antigen showed one band in Ouchterlony plates. Unabsorbed antiactomyosin gave three bands in agar-diffusion reactions, attributable to myosin, actomyosin, and a general tissue nucleoprotein antigen present in all of the *Triturus* tissues tested. The nucleoprotein antibody activity could be removed by absorption with blood. Precipitin and agar-diffusion reactions using extracts of various stages of the regenerates with Weber's solution showed myosin and actomyosin first occurred in the regenerating limbs at the time of hand formation (palette stage), i.e., about the time of appearance of the myofibrils and 21 days after amputation of the limbs. It has been suggested by some investigators that some of the blastema cells of the regenerating limb come from dedifferentiated muscle cells. This, as Laufer points out, would involve an extensive chemical dedifferentiation since his evidence shows that muscle proteins disappear during the early stages of limb regeneration. One would expect a relative loss of muscle proteins with a loss of muscle cells, but a total disappearance of muscle antigens in the blastema would seem to represent a chemical dedifferentiation.

G. Lens Antigens

The lens of the vertebrate eye is admirably suited for use in immunoembryological research because its antigens are more quantitatively organ specific than most organs and because studies of its embryological formation have yielded a wealth of information concerning competence, determination, induction, differentiation, and regeneration of the lens.

In 1950 Ten Cate and Van Doorenmaalen utilized antisera to adult lenses of the chick and frog in precipitin reactions with extracts of parts of the embryos in order to ascertain the time of development of adult lens antigens. The latter could be detected at 60 hours of development in the chick embryo when the lens vesicle is still open to the surface, and at Shumway stages 19–20 in frog embryos when only the lens bud is present. It would seem that proteins with determinant groups characteristic of adult lens antigens are present in the extracts of the young lens vesicles before actual morphological differentiation of the lens. These results were confirmed by Flickinger *et al.* (1955).

1. Increase in Lens Antigens in the Developing Chick Embryo

Langman (1959a) employed saline extracts of embryos and antisera directed against 10% saline extracts of adult chicken lenses in Ouchterlony tests and noted an increasing number of lens antigens in the embryo fractions during development. Although presumptive lens ectoderm and

the eye cups did not give a reaction, one band was formed at the lens placode stage (50 hours) by an extract from the lens areas of one hundred embryos; three bands after 60 hours of development when the lens vesicle invaginates; four bands by 72 hours when nuclear lens fibers are forming; five bands at 96 hours when lens fibers are seen in the marginal zone; six bands in 10-day embryos; and seven bands in the lens of newly hatched chicks and adult chickens. Two globulins (α- and β-crystalline) and an albuminlike protein (γ-crystalline) form the bulk of the water-soluble lens proteins (François *et al.*, 1955, 1956).

Maisel and Langman (1961) have used chemically and electrophoretically isolated lens fractions, together with embryo supernates, in agar diffusion reactions with antisera against 10% saline extracts of adult chicken lenses. They found that α-crystalline was in the first lens fraction which appeared at the time the lens placode forms; β-crystalline, characterized by four antigenic subfractions, appeared at the time the lens fibers formed; and the final fraction of lens antigens that appeared contained γ-crystalline. Langman (1961) states that α-crystalline plays the "key role" in lens development in the embryo and during regeneration.

A similar type of study on the development of adult lens antigens in the chick embryo has been carried out by Beloff (1959) using an antiserum to a low-speed centrifugal supernate of adult chicken lenses. She described how in lens formation the posterior wall of the lens vesicle thickens by elongation of the epithelial cells and, up to the eighth day of development, primary lens fibers continue to fill the central part of the lens. She observed one antigen at this time, but at 8 days a second antigen appeared at the time that concentric secondary lens fibers surrounded the nucleus of the lens. Oudin agar tube diffusion methods showed that four antigens had appeared by the adult stage.

Perlmann and De Vincentiis (1961) used an anticattle lens serum to detect lens antigen on the microsomes isolated from 44- to 46-hour chick embryos. The lens antigen was not detected in the microsome-free supernate of chick embryos until 72 hours of incubation.

2. *Retinal Lens Antigens*

Antigens with combining groups immunologically similar to those of the adult lens were detected in the pigmented retina of the chick embryo by noting the biological effect of antilens sera in the work of Burke *et al.* (1944). Clayton (1954) used fluorescein-labeled antilens serum, and Clayton and Feldman (1955) used I^{131}-labeled antilens serum to detect lens antigens in the retina of the mouse embryo. Van Doorenmaalen (1958) revealed lens antigens in chick embryo iris extracts with pre-

cipitin tests. Langman and Prescott (1959) found that their antiadult chicken lens sera showed precipitin reactions not only with the pigment layer of the retina and the dorsal and ventral parts of the iris but also with the aqueous humor and extracts of the cornea and vitreous body. These antigen preparations were all made from eyes in which the lenses had been removed 6–7 weeks before the preparations were made. Anti-cattle and antifrog lens sera were used in precipitin tests to show lens antigens in the retinas of adult frogs and salamanders (Flickinger and Stone, 1960).

In the agar diffusion tests of both Langman and Prescott (1959) and Flickinger and Stone (1960), certain retinal antigens were shown to be identical to some antigens in extracts of adult lenses. Precipitin reactions were always negative with extraocular tissues of the adult in both of these investigations. It is well known that lens regeneration can occur from the dorsal rim of the iris in larvae and adults of the genus *Triturus* (work reviewed by Reyer, 1954). In lentectomized eyes, the iris cells depigment and form a vesicle, and later lens fibers form in this new lens. The presence of lens antigens in the pigmented retina and iris led Langman and Prescott (1959) to suggest a relation between the ability to regenerate lenses and the presence of lens antigens. Flickinger and Stone (1960) detected lens antigen not only in the pigmented retina of adult salamanders (*Triturus torosus*) but also in adult frog retinas, using antisera to frog and cattle lenses. Since adult frogs cannot regenerate a lens after lentectomy, it appears that the relationship between lens antigen and the developmental capacity to form lens is complex and further data are necessary to clarify the question.

3. *Distribution of Lens Antigens in Early Chick and Mouse Embryos*

A fluorescein-conjugated antiserum to adult chicken lenses was used by Van Doorenmaalen (1958) to stain sections of chick embryos which had been prepared in acetone at 0°C. In embryos of 5 to 16 days of development, only the lens was stained with the labeled antiserum. The lens epithelium of the marginal zone, the lens fibers, and the nucleus of the lens showed marked fluorescence. It is interesting to note that Van Doorenmaalen's fluorescein-conjugated antiserum did not stain the iris when applied to the sections, although it did react with iris extracts in precipitin reactions. It would appear that under the conditions he used, tests with the same antiserum demonstrate a greater sensitivity of the precipitin reaction than of the fluorescent-antibody technique.

Van Doorenmaalen (1958) found that the entire embryo before 5 days, including the eye and head, showed a weak fluorescence. An anti-

serum against adult cattle lens, which gave one line in agar diffusion reactions with adult chicken lens supernates, detected microsomal lens antigen both in the anterior and in the posterior halves of 60- to 90-hour chick embryos (Perlmann and De Vincentiis, 1961).

Clayton (1954) noted that her fluorescein-conjugated antiadult mouse lens sera gave a diffuse reaction with the brain, eye cup, and epidermis of the early mouse embryo, although later only the lens, ciliary process, retina, and perhaps the epithelium of the cornea reacted. I^{131}-labeled antimouse lens sera, used by Clayton and Feldman (1955) with frozen-dried and sectioned mouse embryos, demonstrated lens antigens in lenses, retinas, and corneas.

4. Distribution of Lens Antigens in the Early Frog Embryo

The globulin fractions of antisera against *Rana pipiens* tailbud supernatants (stages 19–20) were conjugated with fluorescein isocyanate in the work of Nace and Clark (1958). Localization was observed in the optic cup and lens of stages 19 and 20 embryos, but fluorescent antibodies could not be detected at stage 17 or 21, and, hence, there apparently may be transitory antigens which characterize this particular stage of development.

Recently Flickinger and Stone (1960) used the precipitin method with antifrog lens and anticattle lens sera to detect antigens with lens-determinant groups in immature frog oocytes, neurulae, the pigmented retina-iris, and the aqueous humor of adult frog eyes. The antifrog lens serum was absorbed to remove cross-reacting antibodies to frog brain and serum. The anticattle lens serum showed a very weak cross reaction with brain which was removed by absorption. The antifrog lens and anticattle lens antisera could detect 1.25 and 1.6 μg. of frog lens protein per milliliter, respectively. Both the frog and cattle lens antisera, after having been absorbed with brain centrifugal supernate, gave a positive precipitin reaction with a centrifugal supernate of immature frog oocytes which contained 560 μ protein per milliliter. The use of the anticattle lens serum to demonstrate lens antigens in immature ovarian eggs of the frog is particularly significant since the reactions undoubtedly result from combining groups of lens proteins and not merely from combining groups of frog proteins in general. The strong reactivity of the supernate of the immature frog oocytes with other antisera, as noted earlier (Flickinger and Nace, 1952), may possibly be due to the presence of soluble proteins and the absence of yolk platelets at this stage. These results are further illustrated by agar plate diffusion reactions (Figs. 5 and 6) with unabsorbed antifrog lens serum. Of 8 antigens found in

the frog lens, one was found in immature oocytes, the tails of late-stage feeding larvae, and the aqueous humor, a second one, similar to brain antigens was found in the immature oocytes, and a third one was detected in immature oocytes and in neurulae. The pigmented iris-retina

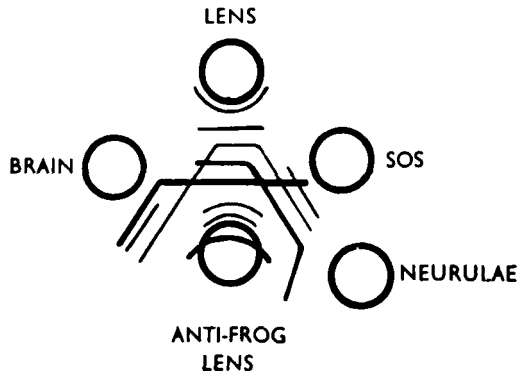


FIG. 5. Agar-diffusion patterns of the reaction of unabsorbed antifrog lens serum with saline extracts of brain, lens, immature oocytes (SOS), and neurulae. Note the identity reaction of lens antigen 5 (numbering from the antiserum well toward the lens antigen well) with the immature oocytes and neurulae.

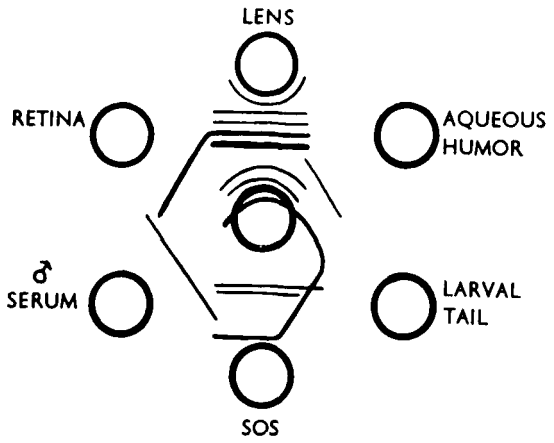


FIG. 6. Reaction of the unabsorbed antifrog lens serum with a number of fractions. Note that lens antigen 5 gave a reaction of identity with the retinal antigen.

had antigens in common with the lens. The use of antisera to the centrifugal supernate fraction of immature oocytes and adult testes (Figs. 7 and 8), together with the antilens serum, showed that the three antigens of immature oocytes that reacted with the antilens serum are different

from the three antigens of this fraction that reacted with the antiserum to immature oocytes and the three antigens which reacted with the anti-testes serum. The two antigens of aqueous humor which reacted with

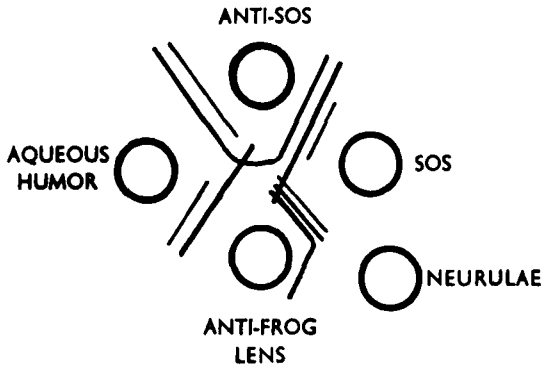


FIG. 7. The use of antisera to the immature oocyte supernate (anti-SOS) and to frog lenses indicates that the antigens in aqueous humor and the immature oocytes that react with the anti-SOS are different from those that formed precipitate lines with the antifrog lens serum.

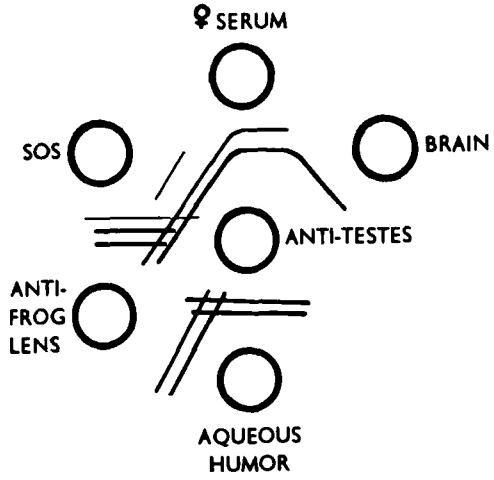


FIG. 8. A similar use of an antifrog testes serum, together with antifrog lens serum, indicates the dissimilarity of the antigens of aqueous humor and the immature oocytes that react with the two antisera.

the lens antiserum are dissimilar to the two antigens of aqueous humor that reacted with the antiserum to immature oocytes and also dissimilar to those that reacted with the antitestes serum.

Positive precipitin tests were elicited by centrifugal supernates of the optic cup, otic vesicle, and oral sucker areas of stage 16 early tailbud embryos, after absorption with antifrog lens serum. The tests were negative with weak dilutions of the antiserum. It would seem that lens antigens are more dispersed than the actual prospective lens area at this stage and that there is no quantitative specificity. The results confirm and extend the idea of Langman and Prescott (1959) and suggest that the presence of antigens with lens determinant groups in immature oocytes, neurulae, and pigmented retina-iris is a reflection of latent lens-forming capacity and that this antigen is not present in tissues which have lost the competence to form lens. It is realized that the ability for lens regeneration from the retina has never been demonstrated in the frog as it has for the salamander. However, it was once thought that adult frogs could not regenerate limbs, but now limb regeneration has been induced experimentally by Rose (1945) and Singer (1954) in adult frogs. Perhaps the frog retina would regenerate a lens if the proper conditions were provided. This does not imply that all of the eight frog lens antigens might be indicators of the ability to form lenses since it has never been demonstrated that the brain, aqueous humor, and larval tail can form lenses. However, frog lens antigen 5 (numbering from the antiserum well outward) reacted only with the lens, immature oocyte supernate, neurulae, and pigmented iris-retina. It may be that this antigen is an indicator of the presence of the synthetic machinery for the production of lens proteins since lenses can develop from all the tissues just listed.

H. Developmental Significance of the Antigens of the Embryo

The results in this section partially answer several questions raised earlier with regard to the significance of antigens. Both Schechtman (1948) and Ebert (1950) have demonstrated that the early chick embryo (primitive streak stage) contains organ antigens of a general rather than of a tissue-specific nature. In addition, several groups have been detected in early embryos before differentiation occurs. In the frog embryo this includes antigens with determinant groups of myosin and actin (Ranzi and Citterio, 1955) and of lens antigens (Flickinger and Stone, 1960). The early chick embryo contains antigens which react to antisera against myosin and actin (Ebert, 1953; Ebert *et al.*, 1955) and hemoglobin (Beard, 1959). These results indicate that antigens with tissue-specific determinant groups may arise before morphological differentiation of the homologous tissue. The demonstration of the major antigen of the mitotic apparatus in the unfertilized sea urchin egg by Went and

Mazia (1959) is another example of the same principle. The lens antigens of the frog embryo also appear before embryonic determination of that structure (Flickinger and Stone, 1960).

The question whether the distribution of antigens with tissue-specific groups is similar to the parts of the embryo which possess the competence or potency to form that particular tissue finds a partial answer in the work with muscle and lens antigens. Thus, cardiac actin and myosin are restricted to the prospective heart-forming areas of the head-process stage chick embryo (Ebert *et al.*, 1955) and the lens antigen is localized in the iris and pigmented retina of chicks and salamanders, which can regenerate the lens (Langman and Prescott, 1959; Flickinger and Stone, 1960). The presence of lens antigen in the egg and various parts of the early embryo itself (Flickinger, 1958a; Flickinger and Stone, 1960) would seem to reflect the future lens-forming capacity of the embryo. The widespread distribution of lens antigens in the early chick embryo, before optic vesicle and lens placode formation, is seen in the work of Van Doorenmaalen (1958) with fluorescein-labeled antibodies, in the results of the precipitin reactions of Flickinger *et al.* (1955) and Perlmann and De Vincentiis (1961), and in the results of Langman *et al.* (1957) in which lens antisera cause cytoxic changes in flank ectoderm and optic vesicles as well as in the head ectoderm. Clayton (1954) found that a fluorescein-labeled antiadult mouse lens serum reacted with brain, optic cup, and epidermis of the early mouse embryo. Later stage mouse embryos only showed reactions of the lens, retina, and cornea.

In the experiments just cited it would seem that the localization of antigens with tissue-specific combining groups is restricted to tissues potentially capable of forming the particular organ. The detection of traces of such antigens may be indicative of the presence of synthetic mechanisms with future potentialities of action. Although these examples suggest some sort of relation between the distribution of specific antigens and embryonic competence, more experimental data are needed.

V. Immunological Investigations of Tissue Interactions in the Embryo

A. Graft-versus-Host Reaction

Cellular and tissue differentiation are often ascribed to interactions between embryonic tissues, and various immunological methods have been utilized in work on this problem. This review will not concern itself in detail with the graft-versus-host immunological reaction which has recently been reviewed by Billingham (1959).

Simonsen (1953) and Dempster (1953) were among the first to suggest that grafted tissues could react immunologically against the host organism, and Billingham and Brent (1956, 1957) added confirming evidence. The enlargement of the spleen of the chick embryo, following the injection of adult chicken blood, was ascribed to proliferation of donor blood cells in the spleen reacting immunologically against the host cells (Simonsen, 1957; Biggs and Payne, 1959, 1961a,b). The latter authors injected cockerel blood intravenously in order to detect the presence of donor cells in the spleen of female host embryos. This was possible since the fifth largest chromosome is paired in the male and single in the female. In spleens that were enlarged five to twenty times they found about half of the mitotic figures to be of donor origin and half of host origin.

Cock and Simonsen (1958) showed that the host spleen is only slightly enlarged when the injected blood and host embryos are from the same inbred line of chickens. This slight enlargement is ascribed to antigenic diversity in the strain. Recent work of Payne and Jaffe (1961), using two inbred lines of chickens, suggests that one strain carries an antigen capable of stimulating splenic enlargement in the inbred line that lacks this antigen. Gene dosage may be involved since embryos homozygous for a given gene seem to give a stronger stimulus to the injected blood cells of the other line with a subsequent larger spleen.

The principle of the graft-versus-host reaction has been utilized by Tyler (1960) in a stimulating theoretical treatment of the cancer problem. Tyler speculates that a malignant tumor develops from a single cell in which a single gene has been lost or inactivated. If the alleles of this gene determine the compatibility antigens of the host, the precancer cells will lack these antigens. These cells then regard the histocompatibility antigens of normal host cells as foreign and the precancer cells proliferate in response to the "foreign" antigen just as normal antibody cells would respond to a foreign antigen.

The graft-versus-host reaction seems to alter the antigenic nature of the host in a quantitative sense. Van Alten (1959) followed the appearance of adult duodenal antigens in the 11- to 21-day chick duodenum using the Ouchterlony technique and found an increased number of antigens when grafting adult duodenum upon the chorioallantoic membrane. There was an increase of three antigens in the host duodenum and an increase of one antigen in the spleen. Grafts of adult spleen did not affect the number of host spleen antigens, but produced an increase of one antigen in the host duodenum.

B. Organ-Specific Growth Stimulation

Some experimental results apparently cannot be explained on the basis of the graft-versus-host reaction and may possibly represent a sort of organ-specific growth stimulation. Van Haeften (1958) placed cell-free homogenates of adult spleen upon the chorioallantoic membrane of a 9-day chick and noted hypertrophy of the host spleen. These spleens appeared histologically normal and therefore the enlargement was evidently not produced by the graft-versus-host reaction. However, Wilt and Stolz (1962) could not repeat these experiments. Croisille (1958) injected cell-free extracts of adult chicken spleen intravenously into the chick embryo starting at 3 days of development and recovered host spleens near the time of hatching. These spleens were 25% larger than normal host spleens of an equivalent age. Croisille also reported that liver extracts injected *in ovo* stimulated liver growth, but he could not demonstrate a similar effect in cultures of embryonic liver. The injection of chick embryo liver or kidney homogenates into chick embryos results in a quantitatively organ-specific stimulation of mitosis in the homologous organ 12 hours after injection (Andres, 1955). Ebert and DeLanney (1960) found a quantitative tissue specificity of host localization when isotopically labeled tissues were injected into embryonic blood vessels. Sulfur-35 methionine-labeled, adult, chicken spleen homogenates when injected into 9-day embryos gave a higher level of S^{35} in the host spleen than in the host kidney, but isotopically labeled kidney homogenate gave a higher level of radioactivity in the host kidney than in the spleen. Centrifugal isolation of nuclei, mitochondria, microsomes, and supernate demonstrated that only the microsomes and supernate showed a selective localization. The same authors found that the injection of 0.1 to 0.15 cc. of spleen microsomes on the ninth or eleventh day caused a significant increase in weight of the host spleen. In adult rats, Teir and Ravanti (1953) reported that the injection of liver homogenates stimulates mitosis in the livers of the host rats, whereas Stich and Florian (1958) found an inhibition of mitosis in similar experiments.

1. Developmental Significance of Organ-Specific Growth Stimulation

The results of various investigations suggest that organ-specific growth stimulation may possibly exist, but a final evaluation awaits further evidence with a larger number of different organs. In order to insure that supposed instances of organ-specific growth stimulation are not actually instances of the graft-versus-host reaction, it will be necessary to ensure that no viable immunologically competent cells are present in the homogenates. The question of organ-specific growth stimulation or inhibition

will rest upon a much stronger experimental basis when biological tests are made with purified materials rather than with crude extracts and homogenates. The subject of growth stimulation and control has been reviewed by Paschkis (1958) and Swann (1958).

C. Organ-Specific Inhibition of Differentiation and Growth

One school of thought emphasizes the view that organ-specific inhibitors may act upon embryonic differentiation and growth. Rose (1952, 1957a,b, 1958) stresses the unspecific nature of embryonic induction and states that cellular differentiation depends in the first place on metabolic differences between different regions of the embryo. Once such differences are established, the sequence of differentiation results automatically from the expression of those inherent properties of the cells that are favored by the locally prevailing environment. The specific products of differentiating regions next act upon other areas and suppress like differentiation there, so that these specifically inhibited regions are reduced to a lower order of differentiation. Inhibition of the expression of single gene-controlled activities of the cells can then occur seriatim, as the result of the accumulation of inhibitory concentrations of specific metabolites. This process is reflected by the progressive loss of potencies in a differentiating system.

Weiss (1947, 1955) and Weiss and Kavanau (1957) have developed a theory of specific regulation of organ growth that involves the interaction of intracellular templates with intra- and intercellular antitemplate molecules of a complementary configuration. According to this theory, growth is stimulated by the presence of free organ-specific template molecules in the cell, and an increased population of intracellular antitemplates renders more of the templates inactive and causes a decline in the growth rate.

The theories of organ-specific feedback effects in differentiation and growth are indeed challenging. Unhappily, the experimental data are as yet insufficient to support these theories. Rose (1955) reported that bloodless, heartless, and nerveless tadpoles have been produced by culturing frog embryos with living adult blood cells, pieces of heart, or pieces of brain in the medium. However, other investigators have found these experiments difficult to repeat. Tucker (1959) claimed that inhibitory information contained in supernates of the heads of nemertean worms can inhibit the formation of the regeneration head blastema on the anterior cut surface of sections from any level of the body. Lender (1956) reported that extracts of planarian heads can inhibit brain regeneration in that animal. Other evidence which supports the idea of organ-specific

inhibition comes from the work of Braverman (1958), R. B. Clarke and McCallion (1959a,b), Lenicque (1959), Rose (1957a), Saetren (1956), and Tardent (1960). Glinos (1958) has proposed that the level of plasma proteins acts as a regulator for the growth of the liver during development and regeneration. Rat liver regeneration, after partial hepatectomy, is accelerated by depletion of the plasma protein level by plasmapheresis and, on the other hand, increasing the relative concentration of plasma proteins by fluid restriction retards liver regeneration.

Feedback controls have been convincingly demonstrated in the biosynthetic pathway leading to the synthesis of ornithine. Vogel (1958) has shown that arginine in *Escherichia coli* is a specific repressor of the synthesis of acetylornithinase, and Gorini and Maas (1958) discovered that arginine can also repress the synthesis of ornithine transcarbamylase. The inhibition of the synthesis of an enzyme by a specific enzyme product would seem to offer encouragement to investigators who wish to demonstrate specific inhibition of differentiation or growth at the tissue or organ level.

1. *Developmental Significance of Organ-Specific Inhibition of Differentiation and Growth*

In attempting to interpret embryonic differentiation and growth in the light of the theories of organ-specific inhibition or stimulation, it appears that the inhibition concept has greater utility. In embryonic induction one sees the interaction of unlike tissues resulting in a regular pattern of differentiation and growth. If the concept of like tissues stimulating like tissues were to operate, then it becomes more difficult to explain the limitation of growth of a given tissue and the growth of other tissues, whereas the inhibitor idea would seem to explain more easily the observed course of embryonic development.

The use of defined chemical compounds, instead of homogenates or centrifugal supernates, in these inhibition experiments will certainly allow a critical answer to this question. An example of the possible inhibition of differentiation by a well-defined compound comes from experiments illustrating the control of sex differentiation in lower vertebrates with low concentrations of sex hormones. Burns (1955) has suggested that the hormones act as repressors. The male sex hormone (testosterone) is thought to inhibit the cortical ovarian field of the gonad of a genetic female embryo at a time when the cortex is competent to form ovary, whereas the female sex hormones are thought to block the medullary portion of the prospective male gonad which usually differentiates into the testis. In these experiments, the unrepressed portion of

the gonad then differentiates. The cortex forms an ovary and the medulla differentiates into a testis.

Feedback effects upon the formation of a functional product and growth of the organ that synthesizes that product have been established for a number of endocrine glands which are under the direction of trophic hormones of the anterior lobe of the pituitary (see Furth, 1953). It is hoped that the question of organ-specific inhibition, of the kind described by Rose (1952, 1957a) and by Weiss and Kavanau (1957), will be answered by experiments as convincing as those provided for feedback effects in the endocrine system.

D. Embryonic Induction

1. Use of Antisera to Heterologous Inductor Tissues

In relation to the problem of embryonic induction, Vainio (1958) has found that a rabbit antiserum to guinea pig bone marrow inhibited the inductive action of this tissue upon amphibian ectoderm, and that ethanol-fixed guinea pig liver, which ordinarily induces mostly neural tissue from gastrula ectoderm, tended to induce more mesodermal tissues after it had been treated with the homologous antiserum. Vainio *et al.* (1960) have demonstrated the passage of antigens from alcohol-fixed guinea pig liver and bone marrow to salamander gastrula ectoderm. Clayton and Romanovsky (1959) obtained antisera against alcohol-treated guinea pig bone marrow and liver and conjugated the γ-globulin fractions with fluorescent dyes. Explants of salamander gastrula ectoderm, fused with the alcohol-fixed guinea pig inductor tissues, were fixed after 12, 24, and 48 hours of culture and treated with the labeled anti-guinea-pig sera. Some of the individual cells of the salamander ectoderm which were near the guinea pig explants showed fluorescence, and in two cultures the entire ectoderm was stained. Sections, which were exposed to an unlabeled rabbit anti-guinea-pig serum and then to fluorescein-labeled sheep antirabbit globulin, also indicated a small level of antigen passage from inductor to at least some of the embryonic cells bordering the inductor.

2. Localization of Neural and Lens Antigens in Frog Embryos

In regard to embryonic induction, it is of some importance to localize antigens that may characterize an induced differentiated tissue. For example, in the case of the induction of the medullary plate by the underlying chorda mesoderm: What is the distribution of neural antigens just before induction occurs? The presence of a greater quantity of neural antigens in the inductor (chorda mesoderm) would suggest the possibil-

ity of passage of these organ-specific proteins to the reacting tissue during induction. On the other hand, more neural antigens in the reacting tissue (gastrula ectoderm) would suggest that organ-specific macromolecules are not transferred during embryonic induction and that the critical action may be the promotion of synthesis of more neural protein in the reacting tissue. In the work of Flickinger (1958a), antisera were developed to brains and lenses of adult male frogs and the antibrain sera were absorbed with serum, heart, and kidney until they were specific for brain. The antilens sera were absorbed with serum and brain until they were organ specific. An antiserum to cattle lenses was also used and was absorbed with frog brain in order to remove a weak cross reaction. The absorbed antilens sera did not cross-react when tested against a large number of antigen preparations of various adult frog organs, but did react with frog lens. The specific antifrog brain sera reacted positively in precipitin tests with four regions of the early frog gastrula (ectoderm, dorsal mesoderm, ventral mesoderm, and endoderm) and also with three regions of the hatched frog larva (head, trunk, and gut). It is apparent that antigens with combining groups characteristic of adult frog brain are located throughout the embryo at these stages of development.

The absorbed antifrog lens and anticattle lens sera were found to react strongly both with the head and with the trunk fractions of the hatched frog larvae. Proteins with lens determinant groups apparently are localized in areas other than the lens-forming regions at this stage. The positive reactions obtained with the organ-specific absorbed anticattle lens sera are particularly significant since the proteins of the embryo that are reacting with the anticattle lens sera are reactive not because of their froglike determinant groups, but undoubtedly because of their lenslike combining groups. As mentioned previously, subsequent work with another group of anticattle lens sera has resulted in the detection of antigens at the neurula stage and in immature ovarian oocytes with combining groups characteristic of the lens (Flickinger and Stone, 1960).

Woerdeman (1953) has reported that saline extracts of presumptive lens ectoderm of salamander neurulae and the young eye cups did not react with an antiserum to adult lenses. However, if he mixed these ectoderm and eye cup extracts and incubated the mixture at 37°C. for 24 hours, then the incubation supernate gave a positive precipitin reaction with the antilens sera. He interpreted this finding as the production of specific lens proteins during the incubation. It seems to this author that, since lens antigens have now been detected throughout the embryo during the early periods of development, increased amounts of

soluble antigen produced during incubation may have reacted with the antisera in the precipitin tests.

3. *Passage of Frog Embryo Antigens in Chimeric Frog–Salamander Explants*

Rounds and Flickinger (1958) and Flickinger *et al.* (1959) have provided isotopic and serological evidence for the movement of proteins

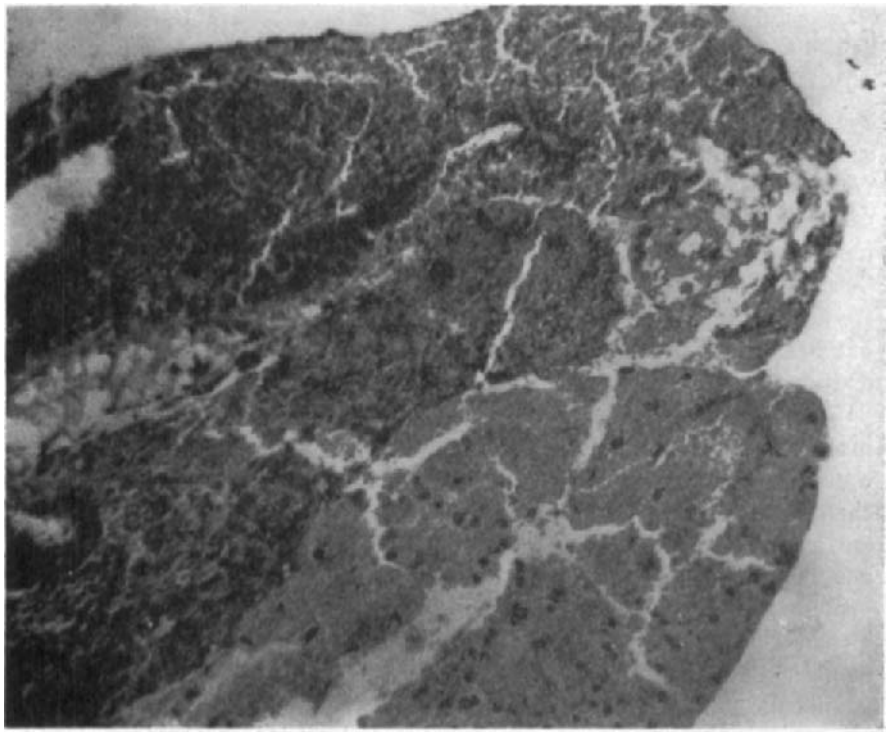


FIG. 9. A chimeric explant in which the chorda mesoderm of a frog gastrula (darker tissue with smaller nuclei) was fused with the gastrula ectoderm of a salamander embryo. After 3 to 4 days the salamander ectoderm was separated and tested for the presence of antigens that reacted against an antifrog gastrula serum.

between embryonic tissues in chimeric salamander–frog explants. Explants of frog embryo (*Rana pipiens*) chorda mesoderm were fused with salamander embryo (*Taricha torosa*) gastrula ectoderm (Fig. 9), and cultured for 3 to 4 days, and were then separated from the *Rana* tissues. Centrifugal supernates were prepared from the *Taricha* tissues, as well as from control explants of *Taricha* ectoderm which had been cultured

alone. These test antigens, at equivalent protein concentrations, were tested against two anti-*Rana* gastrula sera. One antiserum was directed against a total frog homogenate and the other was obtained by injecting a high speed (13,000 × *g*) centrifugal supernate of frog gastrulae which contained microsomes and soluble proteins. Similar results were obtained with the two antisera. The *Taricha* preparations from ectoderm that had been in contact with *Rana* chorda mesoderm gave positive precipitin reactions at antiserum dilutions of 1/64, whereas the control *Taricha* tissues usually did not react at all, although in one case a cross reaction at a ¼ antiserum dilution was observed. This finding strongly suggests the passage of frog antigens into the salamander explants.

Although these frog antigens may only have localized outside the salamander cells, it seems quite possible that they entered the cells since Haurowitz and Crampton (1952) have shown that iodinated protein can pass into certain types of cells. Cytological examination of the chimeric explants of salamander gastrula ectoderm and frog chorda mesoderm or larval frog brain (denuded of epidermis) did not reveal any passage of frog cells into the salamander tissue. The nuclei of the salamander cells are distinctly larger and the two cell types can thus be distinguished.

a. Maintenance of Frog Antigens in Salamander Tissue. In numerous instances the *Taricha* tissues were separated after having been cultured together with *Rana* tissues for 3 to 4 days and the *Taricha* tissues were then cultured for an additional period (2 or 4 days) by themselves. The antifrog gastrula sera always gave a higher titer with *Taricha* test antigens obtained immediately after separation than with *Taricha* tissues grown with *Rana* tissue for 3 to 4 days and then cultured alone for 2 to 4 more days. The decrease of activity with the anti-*Rana* gastrula serum of test antigens from this type of explant implied that frog proteins cannot increase in amount in the isolated salamander tissue once the frog explant is removed. The frog antigens in the isolated *Taricha* explants either diminish in amount or are diluted by an increased production of *Taricha* cytoplasmic proteins.

4. *Developmental Significance of Passage of Specific Macromolecules during Embryonic Induction*

The results of the following investigations demonstrate that some proteins can to a limited extent pass between an inducing and a reacting tissue. Although the salamander tissues in our experiments did not form neural tissue, it is known that frog chorda mesoderm can induce neural tissue from salamander gastrula ectoderm. However, it is this author's

opinion that such molecular passage is not a means of exchanging specific information between cells, but merely a nutrient or metabolic stimulus to the reacting cells. The demonstration of the widespread localization of neural and lens antigens in the gastrula and tailbud stages of frog embryos would suggest that no new specific macromolecular information need be introduced into the reacting cells in an embryonic induction. The stimulation of a complex level of differentiation of amphibian embryo tissues by solutions of low and high pH (Holtfreter, 1944; Flickinger, 1958b; Yamada, 1950) indicates that experimentally one can omit the embryonic inductor. This result serves to emphasize that the reacting tissues in an induction system possess the specific mechanisms needed for differentiation. One would not expect that new genetic information need be introduced into the reacting cells by the inductor since all the cells of the early embryo are thought to be genetically similar. The demonstration of nuclear equivalence of frog blastula nuclei by Briggs and King (1952) and the widespread distribution of certain antigens with tissue-specific determinant groups may be indicators of this biological totipotency. In this regard it has been suggested that embryonic induction is a stimulus to protein synthesis in the reacting tissues (Flickinger, 1958a). The specific type of proteins synthesized depends on the particular state of competence of the reacting tissue, or to put it another way: the type of differentiation depends on the kind of proteins synthesized, which, in turn, depends on the genes that are active at that time. In the author's opinion the functional and structural differentiation of embryonic cells within a whole group of cells, which usually occurs at the same time as changes involving groups of cells, is due to the interaction of two variables. One of these is sequential gene action during development, whereby the various genes accounting for the synthesis of specific proteins act in a given temporal order (embryonic competence), and the other is the provision of the materials and energy needed for protein synthesis (embryonic induction).

As an example of this concept, assuming that four genes (A, B, C, D) act in serial order, but that an adequate supply of amino acids, or a threshold level of energy-yielding metabolism, is attained only by the time that gene C is active, then the specific "C" protein would be formed. The sequence of gene action may depend primarily on nucleic acid anabolism, which would vary in different parts of the embryo, so that all cells would not necessarily be at the same stage of potential genetic activity. However, it is known that cells are much more restricted temporally in their ability to respond to an inductor than in their ability to act as an inductor. It seems that competence (sequential gene action)

is temporally more restricted than embryonic induction (stimulus to protein synthesis). This idea is explored in some detail by the author in an article in the *International Review of Cytology* (1962).

VI. Biological Effects of Antisera upon Developing Embryos

Perhaps the role that antigens play in normal embryonic development is best revealed by experiments in which developing embryos are exposed to specific antisera. In this way the response of the embryo reveals the role the antigens have in the various embryonic processes such as activation of the egg, cleavage, tissue and cell movements, cellular differentiation, cellular and tissue affinities, and growth. A review by Nace (1955) was concerned with the topic of development in the presence of antibodies.

A. Effects of Normal Rabbit Serum

Before considering the effect of specific antisera, it should be noted that normal rabbit sera are not without effect upon embryos. Witebsky and Neter (1935) described a severe toxic action of normal rabbit serum upon chick embryos, and they discovered that heating the sera at 56°C. for ½ hour removes this toxic activity. Recently, Mun (1958) confirmed their findings. He could not restore the toxicity to normal rabbit serum by adding fresh guinea pig serum containing complement. Antisera to saline extracts of 72-hour chick embryos had a toxic effect upon the embryos and there was a weak effect even after the antisera were heated. The toxic action of the heated antisera was stimulated by adding fresh guinea pig serum alone, or guinea pig serum plus fresh rat serum. The latter is thought to be rich in properdin (Pillemer *et al.*, 1954). Mun suggests that complement and properdin (or a properdinlike factor) may be needed in the interaction between antiserum and the embryo.

B. Cell Reaggregation

1. The Action of Antisera upon Sponge Cell Reaggregation

Antisera have been used by Spiegel (1954a, 1955) to study the reaggregation of dissociated sponge cells, and similar work has been done with dissociated cells of the amphibian embryo (Spiegel, 1954b). If a sponge is squeezed through a fine-mesh bolting silk, most of the cells are pulled apart. Then a process of reaggregation begins by which numerous small clumps of sponge cells appear in the medium. Spiegel obtained rabbit antisera to suspensions of *Microciona* cells, *Cliona* cells, and to a mixture of cells from both of these species of sponges. The homologous

but not the heterologous antisera prevented sponge cell reaggregation of the cells of that species. The antiserum added to a mixture of cells from the two species allowed the formation of large aggregates which contained both types of cells. Ordinarily the cells of two different species exclude the other type in a mixed reaggregation situation. Thus, a mixture of anti-*Microciona* and anti-*Cliona* sera did not allow the formation of mixed aggregates. According to Spiegel, the injection of a mixture of cells may cause the formation of antibodies with one or more groups that react with *Microciona* antigens and one or more groups that may react with *Cliona* antigens. Such heteroligating antibodies might account for the formation of the mixed aggregates.

In considering the mechanism by which homologous antibodies inhibit reaggregation, the possibility that antisera act by combining with an intercellular cementing material is ruled out by Spiegel since it could not account for the fact that reaggregation of mixed aggregates occurs in the presence of antiserum to both kinds of cells. That there are antigens on the surface of cells, which might react with each other by calcium bridges, seems unlikely since chelating agents did not block reaggregation, and he does not believe that antibody in excess can saturate the cell surfaces before the cells can make contact.

Spiegel gives more weight to the possibility that combining sites of homologous antibodies contact two or more receptor sites on the surface of the same cell which can thus not combine with receptor sites on other cells. A direct bonding between two molecules, which have complementary reactant groups, must mean that a single cell would have both reactant molecules since similar cell types can unite with each other. This in turn implies that the reactant molecules are arranged on the cell surface in such a manner that they can combine with their complementary molecule on another cell, but not with their complementary molecule on the same cell. Spiegel states that the inhibition of cellular reaggregation by antisera is not necessarily attributable to antibodies combining with the molecules that function in cell aggregation and adhesion. It is possible that the antibodies attached to antigens on the surface of the cells may block the interaction of other molecular groups which function in cell adhesion, by virtue of steric hindrance.

2. *Other Examples of Selective Cellular Affinity*

The problem of the causal forces which operate in directing the selective cellular aggregation of dissociated cells has been emphasized by the recent work of Townes and Holtfreter (1955) and by Moscona (1957, 1960) in which it has been established that various types of verte-

brate embryonic cells, when dissociated and mixed, can segregate and re-establish the tissue types which were first present. Moscona (1957) dissociated cartilage of chick and mouse embryos and, by using nuclear size for the identification of chick and mouse cells, discovered that the re-formed cartilage was composed both of chick and of mouse embryo cartilage cells. When chick chondrogenic cells were mixed with mouse nephrogenic cells, the cells became associated according to cell type and formed separate masses of cartilage and kidney. From these and other examples, Moscona (1957) concludes that the organ specificity apparently is more important than class specificity in the reaggregation of cells.

The forces involved in the social behavior of cells in tissue culture has been extensively studied by Abercrombie and Heaysman (1954) and by Weiss (1958). Abercrombie and Heaysman (1954) have demonstrated that when fibroblasts in tissue culture make contact with each other, the movement of the cell in the direction of contact is prevented. They have termed this "contact inhibition." However, chick heart fibroblasts are unable to inhibit the movement of mouse sarcoma cells, and this may mean that malignant cells have lost the "contact inhibition" response (Abercrombie *et al.*, 1957).

3. *Theories of Cellular Affinity*

There have been several general theories which attempt to devise mechanisms that will account for the selective aggregation and affinities of vertebrate cells. Steinberg (1958) has proposed that cell surfaces possess a highly ordered tangential lattice arrangement of certain ionized acidic groups. Animal cell membranes are usually negatively charged, and this negativity is most probably attributable to the ionized phosphoric acid groups of phospholipids and to the carboxyl groups of proteins. He believes that the cations, calcium and magnesium, would associate with the surface anions and that cells adhere because of a large number of calcium or magnesium bridges between acidic groups on two different cell surfaces. To account for selective cell affinity, these lattice spacings of ionized groups are specific both for cell type and for cell stage, and, further, the spacings are far enough apart to prevent the formation of calcium or magnesium salt bridges between two acidic groups upon the same cell membrane.

A different view is put forth by Curtis (1960). He emphasizes that electron-microscopic evidence shows that cells may be separated from each other by distances of 100 to 200 Å with no cementing material between them (Robertson, 1959). The direct interaction of chemical

groups of surface molecules takes place at a range of only a few angstrom units, whereas calcium bridges would necessitate that cell surfaces be closer than 10 Å. Curtis states that van der Waals-London forces would provide attraction between two parallel cell surfaces at the greater distances of 100 to 200 Å. His reasoning is based upon the Verwey-Overbeek theory (1948). The point of stable adhesion is reached when the repelling force between two similarly negatively charged cell surfaces is balanced by the attracting van der Waals-London forces.

4. *Developmental Significance of Surface Antigens in Cell Movement and Adhesion*

No matter at what range or by what mechanism cells may selectively adhere, it seems that the proteins that make up the cell surface play some role. Gregg (1956, 1960) has demonstrated that new antigens appear at the beginning of the aggregation stage of the slime molds when the individual amebae move together to form a pseudoplasmodium. In certain mutants of the slime mold, *Dictyostelium discoideum,* which do not aggregate, the surface antigens are lost or altered (Gregg and Trygstad, 1958). Antisera against the wild type can agglutinate wild-type amebae, but not the amebae of several of the nonaggregating variants.

The problems of cell movement and cell contact have been considered in a recent symposium (*Exptl. Cell Research, Suppl.* 8, 1961).

C. Sea Urchin Embryo Development

1. *Effects of Antisera upon Unfertilized Eggs*

Perlmann (1956) found that placing unfertilized sea urchin eggs in a homologous rabbit antiserum caused a precipitation on the jelly layer surrounding the eggs in some cases and an invariable wrinkling of the egg surface. This wrinkling could be reversed by placing the eggs back into sea water. His most interesting observation was that some of the jelly-free eggs showed a partial parthenogenetic activation by the antisera; he attributed this to the antigen–antibody reaction. There was elevation of the fertilization membranes and nuclear activation, i.e., migration of the nucleus to the center of the egg and a disappearance of the nuclear membrane, but activation was then terminated. Further work by Perlmann (1957) and Perlmann and Perlmann (1957a,b) demonstrated that the antigen that induced jelly precipitation is located in the jelly coat, whereas an antigen accounting for activation of the eggs is primarily located in the egg, although it may also be present in the jelly layer. He assumes this antigen is located in the egg surface. Homol-

ogous species antisera also depress the rate of fertilization and damage the cortex of the eggs. This action is ascribed to antibodies directed against antigens of the egg rather than to those of the jelly. Perlmann stated that jelly precipitation and partial egg activation was only obtained with a small number of eggs in a large volume of antiserum and that activation only occurred with the eggs of certain sea urchins.

Tyler (1959), in attempting to repeat the experiments of Perlmann, exposed unfertilized demembranated eggs to homologous rabbit antisera, but he never observed any sign of parthenogenesis—only cytolysis. Thus, the claim of artificial parthenogenesis by Perlmann needs corroborative evidence.

2. *Cleavage Blockage by Fertilizin Antisera*

Antisera against extracts of sea urchin eggs and against fertilizin (the glycoprotein of the jelly coat) can block cell division of eggs which have had their own jelly coats and fertilization membranes removed (Tyler and Brookbank, 1956a,b; Tyler, 1957, 1959). The fertilizin antisera are much more effective than the antisera against egg extracts, and antisera against sperm, blood, and epidermal tissue of the adult were without action. If hatched blastulae or gastrulae were placed in an antiserum to fertilizin, their cilia were immobilized and development was blocked. Neither nuclear nor cellular division occurred and cytolysis later ensued. As short a treatment as 15–30 minutes with a strong antiserum to fertilizin was sufficient to inhibit cell division irreversibly. Inactivation of complement did not alter the action of the antisera. If the antisera against fertilizin were absorbed with gastrulae, then about 90% of the cleavage-blocking antibodies were removed. When unfertilized eggs were treated with the antisera to fertilizin they were no longer fertilizable. The action was not species specific since antisera against fertilizin preparations of S. *purpuratus* could block cleavage of *Lytechinus pictus* eggs, as well as those of the homologous species. The antisera-treated eggs showed a temporary increase in respiration as compared to controls (Tyler and Brookbank, 1956b; Brookbank, 1959a). The level increased four to five times during 20–40 minutes and then decreased in the next 40 minutes to the control level. After 4–5 hours of exposure to the antisera, cytolysis occurred.

Tyler (1959) emphasizes that fertilizin is the specific receptor molecule for complementary molecules (antifertilizin) on the surface of the sperm and that the reaction has a causal role in the fertilization process. He obtained fertilizin extracts by the mild procedure of merely extracting suspensions of unfertilized, demembranated sea urchin eggs with slightly

acidified sea water. This preparation appeared as one component during ultracentrifugation and electrophoresis and reacted with the antisera to fertilizin. It seems that antigenic groups are present on the surface of the egg and their reactions with the antifertilizin sera accounts for the inhibition of cell division. The blockage of development of later stage embryos implies that these surface constituents of the egg persist at more advanced stages of the embryo. Brookbank (1959b) has adsorbed antisera against fertilizin with adenosine triphosphatase-bearing granules of unfertilized sea urchin eggs and thus removed their cleavage-blocking ability.

Tyler believes the interaction of fertilizin of the jelly and surface of the egg with a protein with complementary reacting groups (antifertilizin) on the surface of the sperm during fertilization is only one example of specific macromolecular interaction in development. His autoantibody concept of growth and differentiation (1947) states "that each of the various macromolecular substances of which cells are constructed bears the same sort of relation to another of these substances as do antigen and antibody, and they are formed by processes analogous to antibody formation." He considers the formation of immune antibodies as a variation of a process which normally occurs without the presence of a foreign antigen.

D. Effects of Antisera upon Chick Embryo Development

There are numerous instances in which antisera affect the development of chick embryos. The work of Mun (1958), in which antisera to 72-hour chick embryos caused cytotoxic effects in chick embryos, has already been mentioned. Nettleship (1953) injected hamsters with homogenates of 1-, 2-, and 6-day chick embryos and observed blockage of development when these antisera were placed on developing chick blastoderms *in ovo*.

1. Antiheart Sera

More specific effects have been obtained by Ebert (1950) with the inclusion of complement-inactivated antisera to adult chicken organs into the saline-agar-albumin media upon which primitive streak stages were cultured. Antibrain serum (1/30 dilution) primarily affected developing nervous tissues, whereas antiheart and antispleen sera (1/80 dilution) affected mesodermal tissues. The latter embryos lacked somites and some of their lateral plate mesoderm. The most specific effect noted was that embryos cultured in antiheart sera usually lacked hearts or showed very retarded heart development. At lower concentrations these

antisera affected growth and at still lower concentrations they caused cell clumping. Similar results have been obtained by Johnson and Leone (1955) who exposed embryos *in ovo* and *in vitro* to antisera to actomyosin and observed the failure of heart formation or the production of abnormal hearts. Some nonspecific effects were noted *in ovo*, and somite formation was inhibited in the *in vitro* tests.

2. *Antimyosin Sera*

Holtzer's (1959) experiments lead him to a different point of view. Suspensions of cells were obtained from dissociated muscle fibers of 9- to 14-day chick embryos. These cells were cultured for 3 to 7 days and were then incubated with antimyosin serum, which had been heated to inactivate complement, conjugated with fluorescein, and dialyzed against Tyrode solution. The only muscle fibers to take up the antiserum were those that were damaged by trypsin during the dissociation of cells. No visible incorporation of antibodies occurred in the healthy living fibers and no cytotoxic effects were noted. Holtzer emphasized that embryonic cells are no more likely to incorporate proteins by pinocytosis than are mature cells and should be no more susceptible to antibodies than mature cells in spite of the numerous papers reporting cytotoxic effects of antisera upon embryonic cells. He urges caution in interpreting the experiments in which antisera appear to affect embryos.

3. *Antilens Sera*

Langman *et al.* (1957) cultured explants of chick embryos of 5 to 20 somite stages in antisera to adult chicken lenses in a fluid medium for periods of 48 to 72 hours. The culture medium consisted of equal parts of rabbit serum (antiserum or normal rabbit serum), embryo extract, and Tyrode solution. The antilens sera precipitated extracts of lens, retina, cornea, iris, and the vitreous body of the eye, whereas an anti-α-crystalline serum reacted with lens, iris, and retina. After 12 to 24 hours, necrosis was noted not only of the presumptive lens ectoderm but also of the surrounding ectoderm and, in some cases, of the entire ectoderm. With higher levels of antilens serum, the optic vesicle was affected. The damage to the optic vesicles by antisera to the α-crystalline fraction of lenses would also seem to be an effect of antibodies to lens proteins. In antilens sera, lenses did not form in explants of embryos of 5–13 somites, during 48 hours of culture, but did form in 14–20 somite stages. In control, normal, rabbit sera, lenses formed normally. In antimyosin sera, lenses also formed normally, but cells decreased in number or were necrotic. Langman (1959b) repeated these experiments with similar re-

sults. In addition, in lens ectoderm cultured alone, he found normal epithelial outgrowth in antilens sera up to the 11 somite stage but degeneration in later stages. He thus postulated that lens antigen first arises at the 11 somite stage, i.e., shortly after the optic cup contacts the ectoderm. The experiments of Langman *et al.* (1957) demonstrate that antigens with lens determinant groups are localized in areas other than the lens-forming regions at early stages of development. These results confirm the results with the precipitin reactions by Flickinger *et al.* (1955) and Flickinger and Stone (1960) as well as the work with the fluorescein-conjugated antilens sera by Van Doorenmaalen (1958) and Clayton (1954). The failure of Flickinger *et al.* (1955) to observe any effects of antilens sera upon cultured chick embryos may possibly be attributed to the fact that the embryos were cultured upon agar media instead of a fluid medium. Ehrlich and Halbert (1961) demonstrated that heated duck antisera against rabbit lens and rabbit heart have cytotoxic effects upon corneal epithelium *in vitro*.

If demembranated frog gastrulae or the isolated heads of neurulae were cultured for a week in the heated antifrog lens serum, which had been dialyzed against a saline isotonic for frog embryos, normal development of lenses with fibers occurred. However, there was evidence of cytotoxic effects upon some of the lenses and the brains and retinas (Flickinger, unpublished data). Apparently the antibodies affect the antigens which characterize the differentiated tissues, and do not selectively inhibit lens development. Neither was there extensive damage to the early embryos as was true in the experiments of Langman (1959a) with the chick embryo.

Effect on the Inducing Ability of the Optic Cup. A different approach to the question of lens induction was made by W. M. Clark and Fowler (1960). They removed the ectoderm from chick embryo optic vesicles and cultured the optic vesicle for 18 hours in a 1/20 dilution of γ-globulin from antiadult chicken lens sera.The optic cups were then put inside competent ectoderm and cultured for 3 to 4 days in media which did not contain antiserum. Lens induction occurred in 3% of the cultures, as compared to 53% when γ-globulin from normal rabbit serum was used in the optic vesicle cultures before they were enclosed in ectoderm. Similar results were obtained if the antisera and normal rabbit sera treated optic cups were implanted beneath ectoderm of a 4–7 somite embryo. Another control, in addition to normal rabbit serum, consisted of culturing hindbrain and overlying ectoderm in a 1/20 dilution of γ-globulin from antilens serum. Normal induction and development of the inner ear occurred. Clark and Fowler stained sections of

embryos with fluorescent lens antisera and observed a reaction with antigens in the optic vesicle just before induction. When lens induction was almost complete, the optic cup and ectoderm showed an equal amount of fluorescence. These investigators suggest that antigens in the optic vesicle, which react with the antilens sera, are essential for lens induction. They do not take into account the fact that lens determination can occur even if the optic cup is removed in *Rana esculenta* and that free lenses can be induced from isolated ectoderm by dead mammalian tissues (reviewed by Holtfreter and Hamburger, 1955).

E. Mammalian Development

1. Kidney

Passive and active maternal immunization have been utilized to expose developing mammalian embryos to antibodies. Rabbit antirat kidney sera (0.2–1.0 cc.), injected intravenously into pregnant rats on the eighth day of gestation, caused a variety of severe malformations in fetuses of 14 to 31 litters recovered on the twenty-first day of gestation (Brent *et al.*, 1961). Fused kidneys or the absence of kidneys was one type of abnormality observed, and there were numerous nonspecific effects. The injection of normal rabbit plasma and antisera to rat plasma or rat blood cells caused anemia in the mother, but the fetuses were not affected. I^{131}-labeled γ-globulin of antikidney antiserum, after injection into pregnant and nonpregnant rats, localized in the kidneys, adrenals, and spleens, as well as in the placentas of the pregnant rats, as ascertained by I^{131} counts in a scintillation well counter.

2. Lens

Miller (1958) attempted to repeat some older work of Guyer and Smith (1918) by actively and passively immunizing pregnant rabbits with lens antigens and antisera. Only 4 of 460 such newborn rabbits showed eye defects. This result, although alleged to confirm the work of Guyer, is not convincingly corroborative. Huxley and Carr-Saunders (1923), Finlay (1923), and Flickinger *et al.* (1955) were unable to obtain lens defects by either active or passive immunization of rabbits or rats.

3. Brain

Brain emulsions in Freund's adjuvant have been injected into female mice, and abnormalities were observed in the nervous systems of 8 to 9% of the embryos born to these mice (Gluecksohn-Waelsch, 1957). Microcephaly, a suppression of normal neural differentiation, a thinning of the

walls of the neural tube, and an abnormal neural fold closure were observed. Mice injected with heart emulsions did not produce embryos with abnormalities of the nervous system. Precipitating antibodies against brain were demonstrated in seven of ten injected mothers who produced one to three abnormal embryos, whereas they were not produced in three mothers who only produced one abnormal embryo in their litters.

4. *Aspermatogenesis Induced by Active Immunization with Testis Emulsions*

Perhaps the most successful example of active immunization affecting a differentiation system is the work of Freund *et al.* (1953), who injected guinea pigs with emulsions of homologous testes in Freund's adjuvant and severely damaged the spermatogenic tissue in the testes. This work has been confirmed and extended by Katsch and Bishop (1958) who found that heterologous testes and homologous brain tissue are also effective in depleting the spermatogenic tissue although to a lesser extent than homologous tissue. Rooster testis, monkey testis, and human ejaculate were active in this respect. Saline injections of the homologous testes by any route was ineffective. The active factor seemed to be located in the spermatogenic tissue itself since the rest of the testis was not damaged and preparations made from previously injected animals that had been rendered aspermatogenic did not render animals aspermatogenic. Typical damage could be noticed 40–45 days after one injection, and more severe damage occurred at 64–169 days after three to five injections (Fig. 10). An undamaged testis is shown in Fig. 11. Mild aspermatogenesis has also been induced in the rat, but no positive results have yet been obtained with mice and rabbits.

The fact that brain is active suggests an autoimmune mechanism as the basis of these results since brain and testes have a common antigen. Also the sera of a testis-injected guinea pig, containing complement-fixing antibodies, immobilized 50% of fresh sperm in a ½ hour. Katsch (1958) sensitized guinea pigs with homologous testes or sperm in Freund's adjuvant and, using their ilea 60 days later in Schultz-Dale tests, showed maximal contraction in the presence of homologous but not of heterologous sperm. Ilea from animals with severe testicular damage contracted very strongly in the presence of the sperm antigen: those from animals with slight testis damage showed minimal contractions. Circulating antibody, however, does not seem to be directly correlated with the induction of aspermatogenesis since aspermatogenesis is only induced with difficulty in rabbits showing a high level of such antibodies. This fact could be explained by a spatial isolation of the spermatocytes

from the antibodies by some barrier. Katsch (1959) suggested the following mechanism for this response. The antigen, bacteria, and oil are injected beneath the skin where the oil extracts a lipopolysaccharide from the bacteria; this substance after combining with the antigen to form an antigen–haptene complex, is transported to antibody-forming sites

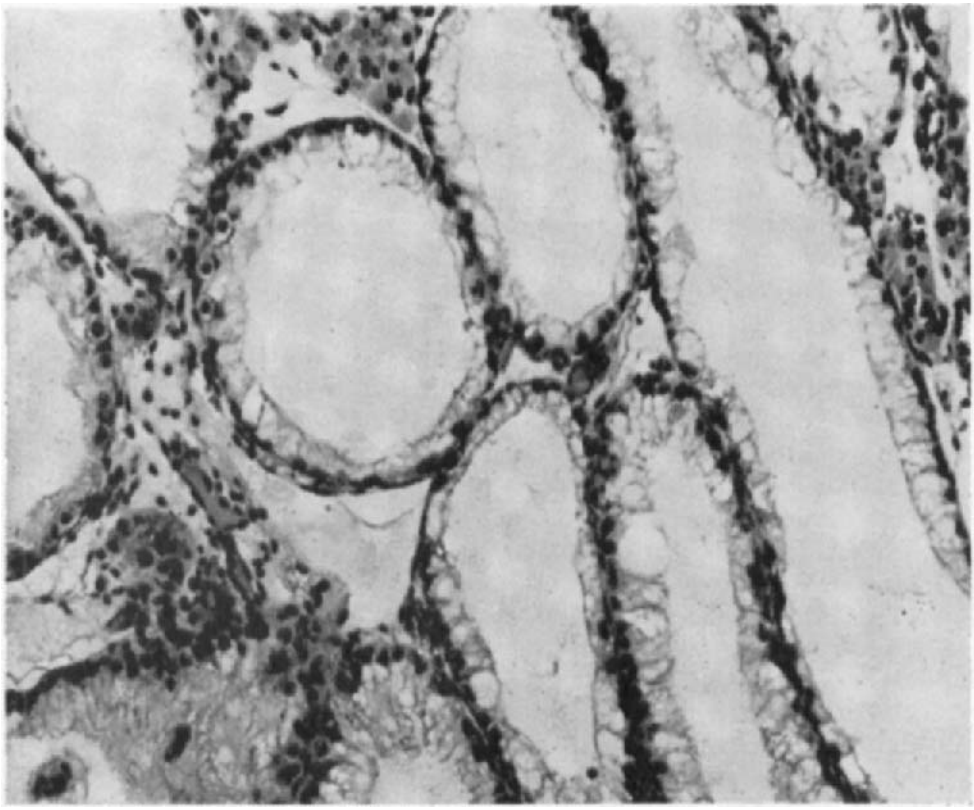


FIG. 10. Severe damage to the testis of a guinea pig 91 days after the first of five injections with an emulsion of guinea pig testes in Freund's adjuvant (Katsch and Bishop, 1958).

by macrophages. Antibodies are formed against this complex, but also against the native antigen of the organ. Sensitizing antibody is formed, as has been demonstrated for the ilea; in the testes the antigen reacts with sensitized lymphocytes there and in the draining lymph nodes. Katsch believes that the reaction of the sensitized lymphocytes with spermatogenic cells releases more antigen, which sensitizes more lymphocytes, and hence the spermatogenic tissue may be completely destroyed.

Examples of several other autoimmune responses are seen in the work of Freund *et al.* (1947), in which brain emulsified in Freund's adjuvant caused encephalomyelitis in guinea pigs, and in the work of N. R. Rose and Witebsky (1956), in which homologous thyroid damage in rabbits occurred after active immunization with rabbit thyroid extracts.

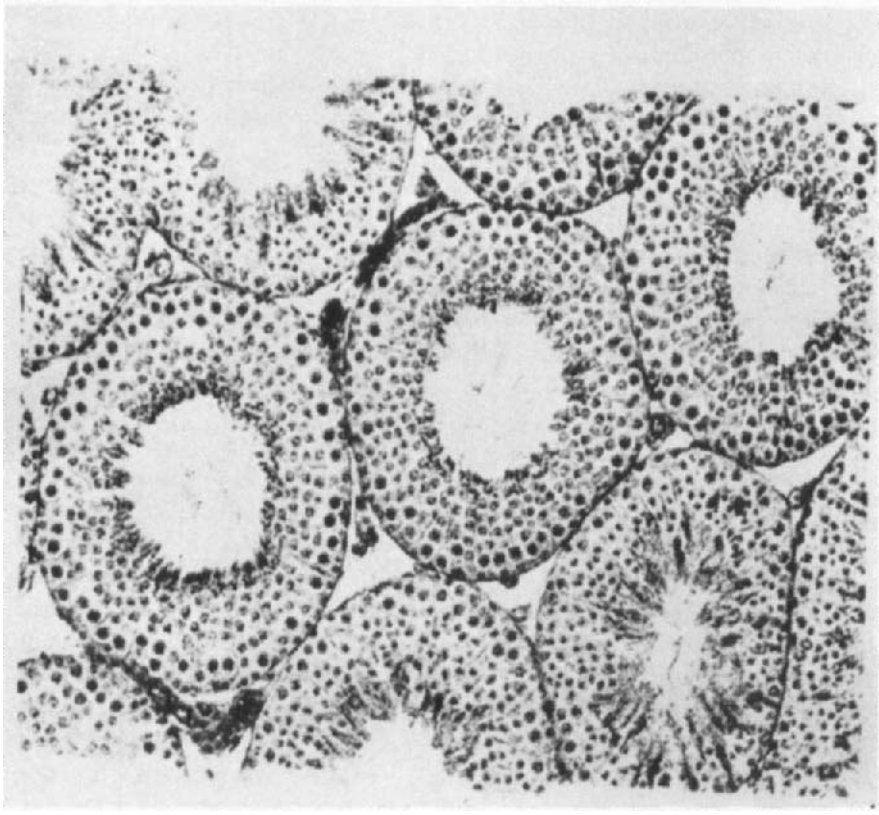


FIG. 11. An undamaged guinea pig testis of a comparable stage (Katsch and Bishop, 1958).

Recently, Bishop (1961) has induced aspermatogenesis in adult guinea pigs by repeated intracutaneous injections of homologous testicular antigen without adjuvant. Injections of 0.1 to 0.05 ml. three or six times weekly resulted in severe lesions of germinal cells after 100 days. Separate site injections of adjuvant and antigen on the same side of the mid-line gave results similar to those obtained by injecting emulsions of antigen and adjuvant. Injections of adjuvant and antigen in separate

sites on different sides of the mid-line did not affect spermatogenesis. Bishop suggests that these results imply the necessity for a common lymphatic drainage system of sites which cause aspermatogenesis when injected separately.

In more recent experiments, Katsch (1960) could not elicit aspermatogenesis in guinea pigs by injecting homologous testicular tissue containing spermatogonia and primary spermatocytes. Homogenates containing secondary spermatocytes and derived cell types induced aspermatogenesis in adult male guinea pigs. Katsch speculates that the antispermatogenic factor is localized in the idiosomic-acrosomal apparatus which first appears in the secondary spermatocytes.

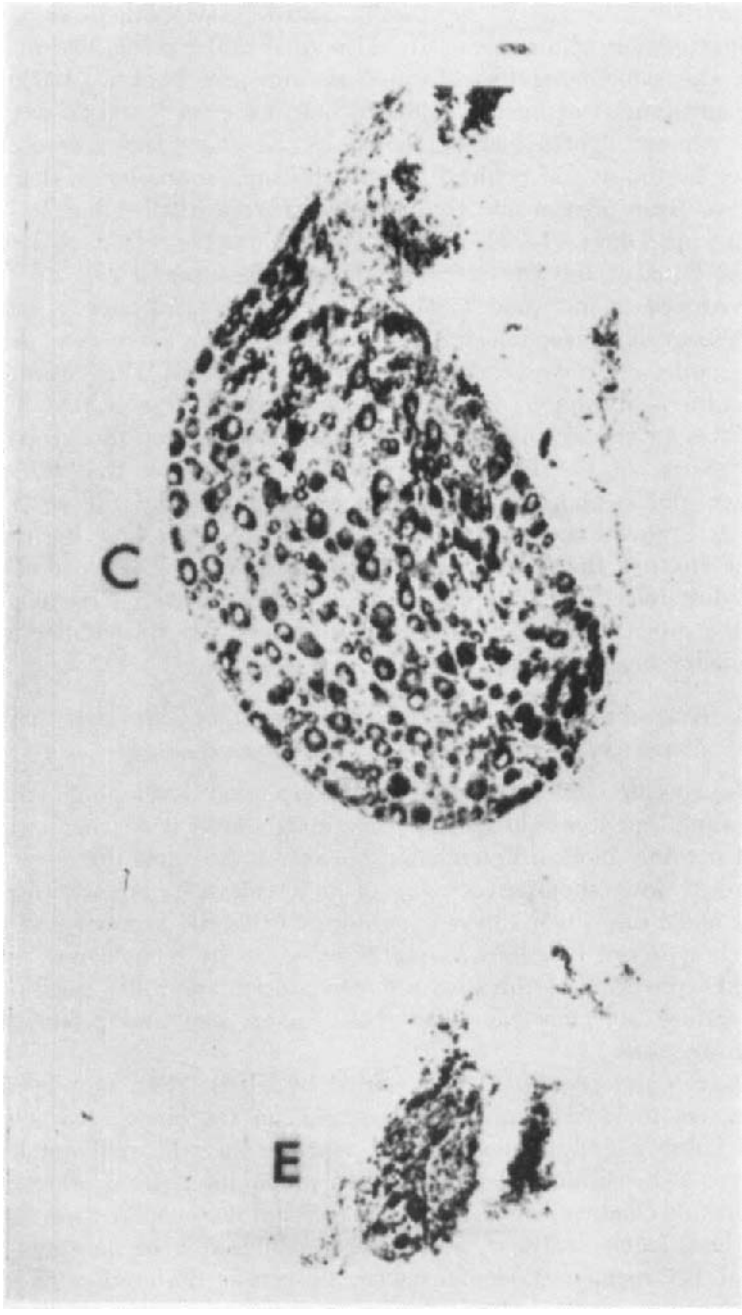
Bishop *et al.* (1960) and Katsch (1960) could not obtain aspermatogenesis in very young guinea pigs, and Katsch (1960) attributed this finding to the absence of certain antigens in the young animals. However, Bishop *et al.* (1960) discovered that sensitization could occur in neonatal guinea pigs. Injection of the testis homogenate and adjuvant between 1 and 10 days after birth caused damage much later when the germ cells matured.

5. *Nerve Growth Factor*

One of the most striking specific actions of an antiserum upon developing embryos comes from recent work dealing with a factor that acts upon nerve growth. Recent papers by Cohen (1958), Levi-Montalcini (1958), Levi-Montalcini and Angeletti (1960, 1961), and Levi-Montalcini and Cohen (1960) have reviewed the work in which a factor isolated from mouse sarcoma and submaxillary glands, as well as from snake venom and young connective tissue, stimulates growth of spinal and sympathetic nerves of chick and mouse embryos, both *in vivo* and *in vitro*.

Cohen (1959, 1960) found that the nerve growth promoting protein from snake venom and the mouse salivary gland, after alcohol precipitation and separation in a cellulose column, yielded a single component in the analytical ultracentrifuge. Antisera were developed against the purified mouse salivary gland protein and were injected subcutaneously for 21 days into newborn mice and rats and into adult mice, cats, and rabbits at a dosage of 0.05 ml./1.5 gm. of body weight (Cohen, 1960).

FIG. 12. (*opposite*) A comparison of a control superior cervical ganglion (C) of a 4-month-old mouse with a ganglion from an experimental mouse (E) which had been injected from birth to the eighth day with an antiserum to the purified nerve growth factor from the mouse salivary gland (Levi-Montalcini and Booker, 1960a).


C
E

Such antiserum treatment specifically destroyed sympathetic nerve cells. No other abnormalities occurred and normal rabbit serum had no effect.

In the same laboratory, Levi-Montalcini and Booker (1960b), the same antiserum was injected into newborn mice for 8 and 20 days after birth. Mitotic figures had decreased by 12 hours and decreased still further by the second or third day. At this time, a number of degenerating cells were present and the neuroblasts were smaller than in control animals. At 8 days, 97–99% of the sympathetic nerve cells were destroyed and, at 20 days, the level of nerve cells in the superior cervical ganglia was reduced to less than 1% of the level in control mice. Apparently the damage is irreversible since the damage to the nerve cells persisted 3–4 months after cessation of antiserum injections. The effect of the antiserum is illustrated in Fig. 12. The authors suggest that a factor circulates in the normal animal that is necessary for the growth and maintenance of the sympathetic nervous system and that the mouse salivary glands and the homologous venom glands of the snake store the nerve growth factor but do not produce it. This view is supported by the fact that the nerve growth factor was found in the blood of young and adult mice (Levi-Montalcini and Booker, 1960a). It is interesting that the growth factor also acted upon sensory and sympathetic ganglia of human fetuses cultured *in vitro*.

F. Attempts to Direct Differentiation *in Vitro* with Specific Antisera

The specific inhibitory action of antisera upon developing embryos is interesting, but it would be much more significant if specific antibodies could not only block differentiation but could also allow these cells to be redirected into another pathway of differentiation. Sonneborn (1948, 1950) and Beale (1952) have demonstrated that the expression of ciliary antigen type can be controlled in *Paramecium* by homologous antisera. The interpretation of this action depends upon controlled conditions of temperature and nutrient since these factors can also produce these transformations.

Clayton and Okada (1959; reported by Ebert, 1959) employed anti-organ sera to block normal differentiation of the homologous organ in chick cultures and then attempted to rechannel the differentiation of these cells by adding ribonucleic acid preparations from other organs. Cultures of chick embryo heart, cartilage, and mesonephros were treated with homologous antisera, and cytolysis, inhibition of outgrowth, and several heteromorphic changes were observed. Restoration of growth of such antisera-inhibited cultures occurred if the homologous ribonucleic

acid (RNA) (prepared by the phenol method) was added to the culture, but heterologous RNA had no effect in restoring growth and, further, RNA did not alter the pathway of differentiation.

Nace and Inoue (1957) cultured explants of *R. pipiens* dorsal, neurula, trunk tissues in the presence of γ-globulin from antifrog neurula serum. Control explants cultured for 7 to 14 days showed differentiation of notochord, neuroblasts, fibroblasts, mesenchyme, epidermis, and pigment cells. The cytotoxic activity of the unabsorbed antisera was removed by absorption with neurula pseudoglobulin (yolk livetin). The dorsal explants cultured in these absorbed antisera only showed differentiation of epidermis and mesenchyme. This result indicates that the differentiation of certain cell types was inhibited by the pseudoglobulin-absorbed antisera without any effect on the development of mesenchyme and epidermis.

G. Developmental Significance of the Response of Embryos to Antisera

It was stated previously that the responses of embryonic cells to antigens and antisera would allow the embryo itself to answer some of the questions asked in this review. The response of the embryo to antigens has already been considered in Section V. Perhaps the most important question of all those asked is the following: Are antigens end products of differentiation or are they part of the causal mechanisms?

Both Schechtman (1955) and Ebert (1959) have emphasized that adult antigens are the end products of differentiation and Schechtman (1955) states that "in no case has it yet been demonstrated that the new antigens or other chemical entities which appear in the course of development are components of the mechanisms of differentiation." It has been possible to show that the exposure of developing embryos to various antisera can selectively block the development of various tissues and organs. Such inhibitions are exemplified by the blocking of heart development in the chick embryo with antiadult heart serum (Ebert, 1950), the inhibition of sea urchin cleavage by the use of antibodies to fertilizin (Tyler and Brookbank, 1956a,b), the prevention of reaggregation of sponge cells and amphibian embryo cells by specific antisera (Spiegel, 1954a,b), the autoimmune reaction to brain (Gluecksohn-Waelsch, 1957) and testis (Freund *et al.*, 1953; Katsch and Bishop, 1958), the destruction of the property of nerve growth stimulation by an antiserum to a purified protein fraction (Cohen, 1960; Levi-Montalcini and Booker, 1960a), the cytolysis of ectoderm and optic cup of chick embryos by antilens sera and anti-α-crystalline (Langman, 1959), the

inhibition of lens induction by antibody treatment of chick embryo optic vesicles (W. M. Clark and Fowler, 1960), and the inhibition of notochord and neuroblast differentiation by an antiserum to a centrifugal supernate of frog neurulae (Nace and Inoue, 1957).

In some of these experiments there is selective destruction of specific tissues after their characteristic antigens have formed. These results would fit the concept that adult tissue antigens are end products of differentiation. In other cases where the differentiation of certain tissue types is inhibited, with no apparent cytotoxic effects, the antisera could inhibit by interfering with end-product antigens or by blocking antigens that play an integral causal role in differentiation.

VII. Future Goals in Immunological Studies of Development

The major aim of biologists working in the developmental field is to control differentiation and growth selectively in a manner which is explicable in a causal sense. One method of approach is that of interfering with tissue-specific proteins in tissues which experimentally are known to have two or more alternate pathways of differentiation.

The most desirable kind of experiment to demonstrate the causal role of antigens in embryonic differentiation and growth would be one in which cells, blocked in one pathway of differentiation by a highly specific antiserum, would not be destroyed but would follow another pathway of differentiation. To date, an experiment of this type has not yet been performed with the developing embryo. One obvious difficulty to these proposed experiments would be that of entry of a sufficient number of antibodies into the cells. This problem might necessitate using cells with the property of pinocytosis.

If the differentiation of a specified group of cells can be directed by using specific antisera, this would imply that tissue-specific proteins are more than mere tracers or products of embryonic development and that their synthesis is directly concerned with the differentiation process. This task remains as a goal for the future.

Acknowledgment

The author wishes to thank Dr. James D. Ebert, Dr. Hans Laufer, Dr. George W. Nace, and Dr. Albert Tyler for reading a preliminary copy of this review and offering numerous valuable suggestions.

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