ADVANCES IN

Immunology

VOLUME 2

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VOLUME 2

1962

ACADEMIC PRESS New York and London

ACADEMIC PRESS INC.
111 FIFTH AVENUE
NEW YORK 3, N. Y.

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

Library of Congress Catalog Card Number 61-17057

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

W. H. TALIAFERRO
J. H. HUMPHREY

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 commonsense 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

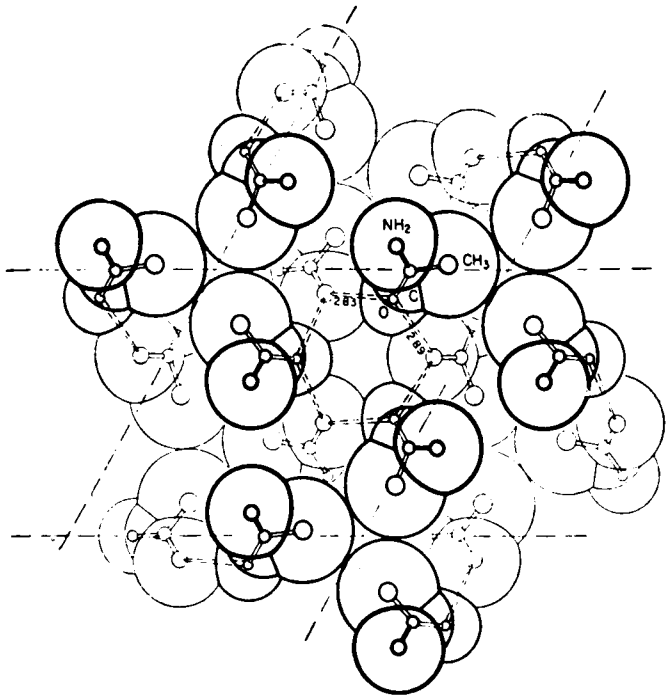

FIG. 1. Acetamide crystal viewed along the threefold axis showing two layers of molecules knit together by hydrogen bonds between oxygen atoms and $-NH_2$ groups. (From Corey, 1948.)

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 napten, 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