

## MOLECULAR STRUCTURE AND BIOLOGICAL SPECIFICITY

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By Linus Pauling  
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The most striking and characteristic property of biological substances is the specificity of activity which they show - the power to combine selectively with or to influence the behavior of one substance, rejecting others with a precision and certainty seen in few physical and chemical phenomena. This specificity of activity is shown by genes in their ability to reproduce themselves, usually unchanged, and to produce the enzymes or other products through which they determine character<sup>S</sup>; also by enzymes, which select from a mixture the molecules upon which they exert their catalytic action, by hormones, by therapeutic agents, and, in an especially striking way, by antibodies. A complete and reliable understanding of the physicochemical basis of biological specificity would bring us much nearer to the solution of the great fundamental problem of biology, that of the nature of life.

My own interest in biological problems was formed first by a study of hemoglobin made a dozen years ago, which led to the discovery that hemoglobin itself (ferrohemoglobin) is paramagnetic, whereas oxyhemoglobin and carbonmonoxyhemoglobin are diamagnetic. It was found that the magnetic properties of hemoglobin provide a simple physicochemical method of measuring

equilibria and rates of reaction involving hemoglobin and its derivatives, and the magnetic technique has since been found useful also in the study of cytochrome c and other iron-containing proteins. Dr. Alfred Mirsky and I then developed a theory of the denaturation of proteins,<sup>1</sup> based upon the concepts that in a native protein the polypeptide chains are coiled together into a definite structure, with a configuration which determines the specific properties of the protein, and that heat, alkali, urea, and other denaturing agents and conditions may cause the configuration of the polypeptide chain or chains to be altered, without necessarily breaking any peptide bonds or otherwise changing the covalent-bond structure of the molecules. We pointed out that some protein molecules in the native state might have that configuration of coiled polypeptide chains which is the most stable of the configurations accessible to the chains, and that these proteins might be capable of undergoing reversible denaturation, with the chains coiling back into the stable configuration characteristic of the native protein as the denaturing agent or condition is slowly removed. Other native proteins, however, might be built originally into a configuration which is not the most stable of those accessible to the polypeptide chains; these proteins, when denatured by uncoiling their chains, would not, on removal of the denaturing agent, settle into the original native configuration but instead into the postulated more stable configuration; accordingly a protein of this sort would not undergo reversible denaturation, regenerating the original native protein, although it might be denatured and then renatured to give a well-defined and crystallizable protein, with properties different from those of the native protein.

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<sup>1</sup>A. E. Mirsky and L. Pauling, Proc. Nat. Acad. Sci., 22, 439 (1956).

When, in 1936, I became interested in the problem of the structure of antibodies, as the result of conversations with Dr. Karl Landsteiner, I found that the complex and at first confusing reported phenomena of immunology could be clarified and brought into order by a theory of the structure of antibodies based upon the idea of the folding of polypeptide chains into the most stable of the accessible configurations.<sup>2</sup> The theory of the structure and process of formation of antibodies developed in this way involved the acceptance of the suggestion that antibody and antigen have complementary structures, originally made by Breinl and Haurowitz, J. Alexander, and Stuart Judd.<sup>3</sup> The picture of the serological precipitate as a framework <sup>(lattice)</sup> of multivalent antibody and multivalent antigen, developed by Marrack and by Heidelberger,<sup>4</sup> also seemed to be so reasonable and so in accord with most of the observational data as to require its acceptance.<sup>4</sup>

The theory of the structure of antibodies and the process of antibody formation depends upon the assumption that the antibody precursor is a polypeptide chain of such a nature as to be able to fold into a large number of alternative configurations, which have nearly equal free energy, and hence nearly equal stability. In the absence of an antigen in the region in which the antibody precursor is being formed the polypeptide chain will fold into one of the most stable of the configurations acceptable to it, producing a molecule of normal gamma globulin. However, if an antigen molecule is present, it must be considered as part of the environment acting upon the folding of the polypeptide chain, and the most stable configuration of those accessible to the folding

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<sup>2</sup>L. Pauling, *J. Am. Chem. Soc.*, 62, 2645 (1940).

<sup>3</sup>S. Breinl and F. Haurowitz, *Zeit. physiol. Chem.*, 192, 45 (1930); J. Alexander, *J. Proteoplasma*, 14, 296 (1931); Stuart Judd, *J. Immunol.*, 23, 423 (1932).

<sup>4</sup>J. R. Marrack, "The Chemistry of Antigens and Antibodies," Report 250 of the Medical Research Council, H.M. Stationery Office, London, 1934; 2nd ed., 1938; M. Heidelberger et al., *J. Exp. Med.*, 61, 563 (1935); *Chem. Rev.*, 24, 523 (1935).

chain now become; different from those in the absence of the antigen; they are now configurations which take the greatest advantage of the opportunity of interaction with the antigen, of such a nature as to stabilize the system - that is, of the opportunity of assuming such a structure as to lead to attraction between the forming antibody and the antigen, and hence to the formation of an antigen-antibody bond. A structure of this sort would be one in which the surface atoms of the antibody molecule are able to come into the closest possible proximity to the surface atoms of the antigen molecule. This could be achieved in case that the folding antibody molecule were to mold itself over a portion of the surface of the antigen molecule, reproducing the configuration of the antigen in the same way as a coin does its die. The principal forces of attraction which are operative are the general van der Waals forces (electronic dispersion forces), the forces described as hydrogen-bond forces, and the electrostatic forces between positively charged and negatively charged ionized groups. A very high degree of specificity can be obtained if the surface area over which the complementarity in structure is exercised is great enough to include a good number of interacting structural units.

The assumption that antibodies are bivalent, or have still greater valence - that is, that each antibody molecule has two or more surface regions capable of combining specifically with the homologous antigen - is necessary in <sup>that</sup> case, the framework theory of the serological precipitate is accepted. The general evidence, of varied nature, for the framework theory, as summarized by Marrack and Heidelberger, is strong but not complete. Further evidence was obtained by studies made by my collaborators (Professor Dan H. Campbell, Dr. David Pressman, Dr. Carol Ikeda, Dr. M. Ikawa, Mr. David R. Brown, Mr. A. L. Crossberg, Dr. Stanley M. Swingle, Dr. John T. Maynard) and myself by the study of

the precipitation of antibodies with simple chemical substances of known structure. It was discovered by Landsteiner and van der Scheer<sup>5</sup> that a precipitate is formed when a dye made by coupling two or more haptenic groups with resorcinol or tyrosine is added to an antiserum obtained by injecting an animal with an asoprotein containing the same haptenic group. We investigated the interaction of many substances containing the para-asobenzeneearsonic acid group and anti-para-asobenzeneearsonic acid serum, and found that all dyes containing two or more of these haptenic groups were able to form a precipitate with the serum, whereas those containing only one haptenic group were not. It was also found that under certain conditions the ratio of the number of molecules of dihaptenic precipitating antigen to the number of molecules of antibody in the precipitate was equal to unity, and if the assumption is made that each of the two haptenic groups is operative in bond formation the bivalence of the antibody is proved. However, it was also found that the same molecular ratio of unity held for the precipitate formed by trihaptenic and tetrahaptenic dyes. This confusing result can be explained by the reasonable assumption that the steric interaction of the large antibody molecules about a small dye molecule is so great as to prevent more than two antibodies usually from combining with the haptenic groups of the same dye molecule, the steric repulsion thus effectively limiting the valence of the polyhaptenic substance to two, and the data then indicate bivalence of the antibody. A determinative experiment has also been carried out, involving the simultaneous precipitation of antibodies from two different antisera by a single substance, which is incapable of precipitating either of the antisera alone. The substance is a dye containing one haptenic group of each of two different kinds, and the two

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<sup>5</sup>K. Landsteiner and J. van der Scheer, Proc. Soc. Expt. Biol. Med., 29, 747 (1932); J. Exptl. Med., 56, 399 (1932); 57, 655 (1933); 67, 79 (1933).

specific antisera which when mixed are simultaneously precipitated by the dye are those made by injecting separate rabbits with azoproteins containing, respectively, the two haptenic groups. This experiment provides very strong evidence for the framework theory of serological precipitation.<sup>6</sup>

The precipitation of antibody and antigen is closely similar to that of, say, silver ion by cyanide ion, and the similarity extends also to the re-resolution of the precipitate in an excess of one of the reactants (cyanide ion or antigen). The cyanide precipitate dissolves in an excess of cyanide ion because of the formation of a silver cyanide complex  $\text{Ag}(\text{CN})_2^-$ , and similarly the antibody-antigen precipitate redissolves in an excess of antigen because the antigen molecules combine with both (or all) of the combining groups of the antibody, saturating them and forming a soluble complex. It would be of interest to physical chemists to investigate this reaction quantitatively, and to find whether the same simple laws of chemical equilibrium apply as to the silver cyanide precipitation and re-resolution. It is found that these simple laws do not apply, but that instead the behavior of antisera and antigens is that which would be expected if the antiserum contained antibody molecules of many different kinds, with their combining groups differing by several kilocalories per mole in free energy of combination with haptens, corresponding to a several hundred-fold or thousand-fold range in equilibrium constants for the combination reaction. The data indicate clearly that natural antibodies are very heterogeneous. This is, of course, to be expected from the theory of antibody production described above.

The nature of the specific forces operative between antigen and antibody has been investigated especially by the quantitative study of the phenomenon of hapten inhibition. A monohaptenic substance is able to combine with antibody, but not to form a precipitate. Through combination with the antibody, however, the formation of a precipitate by a polyhaptenic substance can be inhibited. This

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<sup>6</sup>L. Pauling, D. Pressman, and D.H. Campbell, *J. Am. Chem. Soc.*, 66, 330 (1944).

phenomenon of hapten inhibition was discovered by Landsteiner. Quantitative studies of the inhibiting power of different haptens of known structure have been made, and subjected to physicochemical interpretation by the use of a theory of heterogeneous antibody. This theory is based upon the assumption that the distribution function for the heterogeneous antibody is an error function in the free energy of interaction of antibody and haptenic group. The assumption of an error function in the free energy (that is, in the logarithm of the equilibrium constant) is seen to be a reasonable one by the argument that the total free energy of combination of the combining group of an antibody with the hapten may depend upon several structural features, which may be present or absent independently of one another; if the number of structural features were large, there would result an error function distribution in the free energy of interaction with antibody, to which they make their independent additive contributions.<sup>7</sup>

It has been found that the hapten inhibition constants of different haptens depend very strongly upon the degree of conformity in shape of the haptens to the immunizing haptenic group. The requirement for similarity in shape is such that the conclusion can be drawn that the antibody reflects or reproduces, in a negative way, the shape of the haptenic group of the immunizing antigen to within about  $1 \text{ \AA}$ .<sup>8</sup> Moreover, it has been found for a series of related haptens containing substituent groups in the position para to the charged group (the arsonic acid group) that the average equilibrium constants for combination with antibody depend significantly upon the optical polarizability of the para group, in the way indicated by the London theory of electronic dispersion forces, the magnitude of the effect being such as to indicate approximation of the antibody to within  $1 \text{ \AA}$  of the haptenic group. A third test has been made, that of the contribution

<sup>7</sup>L. Pauling, D. Pressman, and A.L. Grossberg, J. Am. Chem. Soc., 66, 784 (1944), and later papers.

<sup>8</sup>L. Pauling and D. Pressman, J. Am. Chem. Soc., 67, 1003 (1945).

of an electrical charge to the antibody-antigen forces.<sup>9</sup> This test involves comparison of a hapten containing the trimethylammonium ion group and one containing the uncharged tertiary butyl group, and the determination of their hapten inhibition constants. The difference in free energy of combination indicated by these hapten constants can be expressed in terms of a distance between the positive charge of the charged haptenic group and a complementary negative charge of the antibody, with the use of effective dielectric constants as indicated by the investigations of Schwarzenbach.<sup>10</sup> The distance so found is 7.0 Å. Since the radius of the phenyltrimethylammonium ion is 5.5 Å, and the minimum distance to which a negative charge could approach the surface of an antibody is 1.4 Å (the radius of an oxygen atom), the value 7.0 Å shows that the complementary negative charge of the antibody is within 2.1 Å of the minimum possible distance from the positive charge of the immunizing haptenic group. This evidence also accordingly supports the thesis that the forces of specific attraction between antibody and antigen depend upon the very close approximation of the antigen and antibody molecules.

For a long time there remained unrecognized a striking analogy between the highly specific phenomenon of serological interaction and another highly specific phenomenon of the chemistry of simpler substances; namely, the phenomenon of crystallization. The process of the crystallization of a substance from a complex solution is in general highly specific - often a very pure substance can be grown as crystals from a complex mixture, as is shown by the example of the formation of pure crystals of cream of tartar from grape jelly. It is clear that the specificity of crystallization is the result of the same interatomic and intermolecular forces and the same striving toward complementarity that are responsible for the specificity of antibodies. A molecular crystal

<sup>9</sup>D. Pressman, A.L. Grossberg, L.H. Pence, and L. Pauling, *J. Am. Chem. Soc.*, 68, 250 (1946).

<sup>10</sup>G. Schwarzenbach, *Z. physik. Chem.*, A176, 133 (1956).

is stable because all of the molecules pile themselves into a configuration such that each molecule is surrounded as closely as possible by other molecules, in such a way as to make the forces of attraction of the molecules within the crystal as great as possible. This result is achieved if the cavity in the crystal into which each molecule fits conforms as closely as possible to the shape of the molecule, and if also there is a complementariness in structure, with respect to hydrogen bond formation and ionic interactions, between the molecule and the surrounding molecules. Other molecules, with different shape and structure, would not fit into this cavity nearly so well, and in consequence other molecules would not in general be incorporated in the growing crystal. Only if the other molecules were very similar to the molecules of the crystal would deviation from specificity occur, leading to the formation of solid solutions. It is well known, for example, that organic compounds containing methyl groups tend to form solid solutions with those containing chlorine atoms substituted in the corresponding positions. The replacement of methyl groups by chlorine atoms similarly leads to biological cross-reactivity - a hapten containing a methyl group interacts nearly as strongly with the serum homologous to a hapten containing a chlorine group in this position as does the chlorine-substituted hapten itself.

The value of a physicochemical attack on the biological problem of the nature of the structure and properties of antibodies has been indicated. The usefulness of this attack is limited by the complication introduced by the heterogeneity of antibody. Other natural proteins with specific properties may be far more homogeneous, and might be still more profitably subjected to physicochemical study. For example, the enzymes, for which the value of the physicochemical attack has been shown by the early work of Michaelis, are in general

homogeneous proteins, and a quantitative study of such phenomena as substrate inhibition and competition might well provide very valuable information about the configuration of the active region of the enzyme molecules and the nature of the phenomenon of enzymatic catalysis.

The theory of biological specificity described above, which is strongly supported by the results of immunochemical experiments, requires that the molecules which attract one another specifically do so only when they are in immediate juxtaposition, that is, when their surfaces are within a few Angstroms of one another. These specific forces, depending on the atomic-scale complementariness of structure of the two interacting molecules, decrease in magnitude very rapidly with increasing intermolecular distance, and are negligible at distances greater than about  $10 \text{ \AA}$ . between the surfaces of the molecules. Long-range non-specific forces between molecules exist, of course, but it is very difficult to develop any theory of long-range specific forces on the basis of our present knowledge of molecular structure.<sup>11</sup> The discovery of long-range intermolecular forces of such great specificity as is shown in biological phenomena would be of the greatest interest.

Inasmuch as the existence of short-range specific forces, dependent upon the contact between the molecules, must be accepted, in view of the strength of the evidence that has been accumulated for it, the discovery that long-range specific forces between antibody molecules and antigen molecules also exist would be very surprising. The experiments reported during the last two years by Rothen and interpreted as indicating the existence of these long-range specific forces are accordingly of great interest.<sup>12</sup> Rothen has shown that a

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<sup>11</sup>L. Pauling and M. Delbrück, Science, 92, 77 (1940).

<sup>12</sup>A. Rothen, J. Biol. Chem., 165, 345 (1946); 167, 299 (1947); 168, 75 (1947).

layer of protein antigen, such as bovine albumin, deposited on a metal plate is able to combine with its homologous antibody, or to be hydrolyzed through the enzymatic action of trypsin, even after some other material, such as Formvar (a polyvinyl-formal resin), has also been deposited on the plate. On the assumption that the Formvar or other screening materials is spread uniformly over the surface of the plate, and thus covers the antigen, these experimental results have been interpreted as showing that the forces of specific attraction between antigen and antibody or the forces of catalytic action of the enzyme on the protein are able to operate over distances as great as 100 or 200 Å., the average thickness of the screening layers.

I think it is highly probable that it will be found that the specific interaction of antigen and antibody and of enzyme and substrate in these experiments requires the atomic contact of the interacting molecules, and that the original deposit of serum albumin is not completely covered by the screening layer, even though it has an average thickness of 100 or 200 Å. It seems to me very likely that a 9-Å. film of serum albumin on the surface of water, in which the protein molecules are spread out into layers one polypeptide chain thick, does not retain the uniform spread-out configuration very long after the film has been transferred to a metal plate, but that instead the protein molecules fold up again into either their original globular native configuration, or into other configurations than the single polypeptide layer. The "film" of serum albumin on the metal plate would then consist of isolated molecules or clumps of molecules, rising perhaps 100 Å. or more above the surface of the plate. A deposit of Formvar or other screening material might well fill in the valleys between these globular molecules, leaving the tops of the molecules available for direct contact with antibody molecules or enzyme molecules. The many interesting experimental

results of Rothen seem all to be compatible with this interpretation, and hence not to require that specific forces between antigen and antibody or between enzyme and substrate exert themselves over distances greater than those involved in direct molecular contact.

The success that has greeted our efforts to understand the properties of serological systems in terms of physical chemistry and structural chemistry may well give us hope that with the passing of time it will be possible to obtain a sound insight into the mechanisms by which a complex organism grows, undergoes morphological differentiation, and carries on all its functions. I believe that chemistry will play a very important part in the golden age of biology that is now beginning.