

ANTIBODY-ANTIGEN REACTIONS : MODEL SYSTEMS FOR THE SPECIFIC INTERACTIONS OF BIOLOGICAL MACROMOLECULES

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GENERAL ASPECTS

There is a close similarity between antibody-antigen interactions and enzyme-substrate associations. Each of these systems involves at least one macromolecule, the antibody or the enzyme molecule respectively, and, as in most biological systems, the most important physico-chemical feature underlying these reactions is their specificity¹⁻⁴.

With reference to enzymes, the specificity of their catalytic action on a distinct substrate has been interpreted as being due to a unique interaction between the substrate and an "active site" on the enzyme molecule, resulting in the formation of an intermediate enzyme-substrate complex and in an increased reactivity of the appropriate bonds in the substrate. This concept of steric complementariness between the substrate molecule and the active site on the surface of the enzyme molecules, as represented by the classical "lock and key" hypothesis of Emil Fischer, is obviously an oversimplification. However, this idea has proved a useful operational concept, particularly in providing an explanation for the high degree of stereoselectivity of enzymes in their reactions with substrates capable of existing as distinct stereoisomers, and for the competitive inhibition by structurally closely related substrate materials⁵. Nevertheless, in spite of the great advances made over the last decade in developing methods for the determination of the amino-acid sequence and structure of polypeptides and proteins^{6, 7}, a detailed description of the conformational geometry of the "active sites" of most enzymes is still a matter of conjecture⁸.

All these concepts are well known and require no further elaboration. Let us now turn to antibody-antigen systems, which represent the simplest examples of specific interactions of biological molecules and which can, with the aid of well defined and tailor-made compounds, serve as experimental models for the elucidation of the factors involved in these interactions. The great advantage of antibody-antigen reactions is that they are not complicated and obscured by secondary chemical processes, as is the case of enzymatic or hormonal reactions.

The introduction of a foreign substance, usually a natural macromolecule such as a protein, a polysaccharide or a conjugated lipid, into an animal induces the production of globular serum proteins which possess the unique property of combining specifically with the foreign substance⁹. These

serum proteins are known as antibodies and the foreign substances responsible for their production are known as antigens. Most antibodies have been shown to have the electrophoretic mobility of serum γ -globulins and a sedimentation coefficient of 6.5 Svedberg units corresponding to a molecular weight of about 160,000^{10, 11}. Instances of antibodies with molecular weights of the order of about 1,000,000 have been also reported¹².

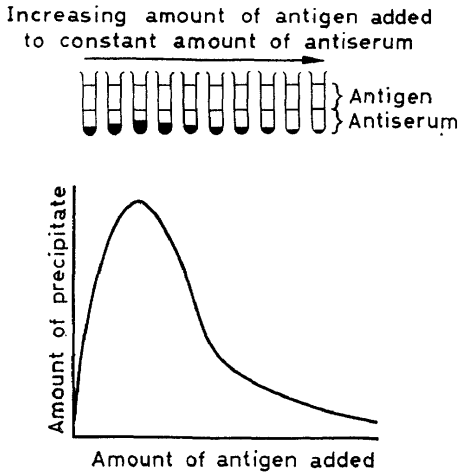


Figure 1. Precipitin curve

The interaction between antibodies and their appropriate antigens may result in different *in vivo* and *in vitro* manifestations^{9, 11}, the most common detectable *in vitro* reaction being the formation of a flocculent precipitate. In general, the amount of precipitate formed on addition of increasing amounts of antigen to a constant amount of antiserum is represented quantitatively by a typical curve, known as the precipitin curve (*Figure 1*). It is evident from this curve that the amount of precipitate consisting of both antigen and antibody increases at first in the antibody excess zone, reaches a maximum in the equivalence zone where both reactants are precipitated quantitatively, and then decreases in the antigen excess zone where precipitation is progressively inhibited. On the supposition that both antibody and antigen molecules were polyvalent*, Pauling was able to explain the general features of this curve in terms of his framework theory¹⁵. Accordingly, in the region of antibody excess the precipitate would consist of small aggregates composed primarily of antibody molecules crosslinked by the small number of antigen molecules. In the region of maximum precipitation the antibody-antigen complexes would be crosslinked into larger and more compact aggregates, consisting of an alternating and recurring antibody-antigen pattern. Addition of more antigen than that required to combine with all antibody sites would result in the disruption and loosening of this compact, regular framework, and in the formation of smaller aggregates. In the limit, in excess antigen only small complexes would be

* The concept of valency refers here to the number of combining sites on either the antibody or antigen molecule^{13, 14}.

formed consisting of one antibody molecule combined with the number of antigen molecules equivalent to the valency of the former; no crosslinking of these complexes could occur and they would remain in solution. As a corollary, if the antigen, or the antibody molecule were univalent, polymeric aggregates could not be formed and the corresponding antibody-antigen complexes would be soluble. All these relationships are illustrated with appropriate diagrams in Pauling's original paper¹⁵.

This theory has been fully confirmed by more recent experimental data; most antigens were shown to be polyvalent (*e.g.* the valencies of ovalbumin, thyroglobulin and viviparus haemocyanin were calculated¹⁶ as 5, 40 and 231 respectively) and precipitating antibodies were shown to possess two combining sites¹⁷⁻²⁰.

Antibody-antigen reactions are highly specific inasmuch as antibodies will react only with the antigen used for immunization or with molecules possessing groups which are sterically closely related to the determinant groups of the original antigen used. Obviously, a large protein molecule, such as serum albumin or γ -globulin, will have a complex configuration and may possess various antigenically determinant groups²¹⁻²³. However, because of the structural complexity of natural antigens, it is rather difficult, if not impossible, to delineate precisely the determinant group(s) of a natural macromolecule. Therefore, in an attempt to elucidate the characteristic features of antibody-antigen reactions, notably Landsteiner³, Haurowitz²⁴, Pressman²⁵, Campbell²⁶, Karush²⁷ and their associates used chemically well-defined antigens consisting of a small group, such as nitrobenzene, or the benzoate, sulphanilate, arsanilate, or trialkyl anilinium ions, coupled to a protein by covalent bonds. Landsteiner coined the term "hapten" to describe the small molecule which is antigenic only when coupled to a large carrier molecule, usually a protein. The injection of the hapten by itself does not lead to the production of antibodies, unless the hapten becomes coupled to the host's own proteins. Furthermore, if free hapten is added to its homologous antiserum, no precipitation results. On the other hand, if a hapten-protein conjugate possessing more than one hapten per molecule of conjugate is added to the homologous antiserum, precipitation occurs and follows the typical precipitin curve. Moreover, this precipitation can be inhibited or the precipitate formed can be redissolved if the free hapten is added prior to, or after, the addition of the polymeric hapten-conjugate, respectively. In conformity with the framework theory, precipitation will also result with a relatively small trihaptenic molecule provided a three-dimensional polymeric network can be established²⁸. Obviously, the addition of a dimeric haptenic molecule would lead to the formation of long chain polymers, as demonstrated recently by light scattering measurements²⁹.

THERMODYNAMICS AND KINETICS

From this brief description of hapten-antibody reactions it is evident that the study of such systems provides a means of defining complementarity at molecular level and of determining precisely the thermodynamics and the kinetics of these association reactions involving biological macromolecules. The extent of combination of haptens with their homologous

antibodies was determined by many workers using methods such as equilibrium dialysis^{25, 27, 30, 31}, light scattering²⁹, electrophoresis and ultracentrifugation³², polarography³³, and quenching of fluorescence³⁴. Several intrinsic equilibrium constants and the relevant thermodynamic constants, given in *Table 1*, were calculated by the application of the Law of Mass Action.

Table 1. Equilibrium and thermodynamic constants for antibody-hapten associations

| <i>Hapten-antibody system</i> | <i>K</i> (l. mole ⁻¹) | $-\Delta F^\circ$ (kcal mole ⁻¹) | ΔS° (c.u.) | <i>References</i> |
|---|--------------------------------------|---|----------------------------|-------------------|
| ϵ - <i>N</i> -dinitrophenyl-lysine: anti-DNP antibody | 4.2×10^8 | 11.3 | 9 | 34 |
| Benzoic acid: anti- <i>p</i> -azobenzoate antibody | 4.1×10^4 | 6.1 | 7.4* | 92 |
| Terephthalanilide-4,4'-diarsonic acid : anti- <i>p</i> -azo-benzene arsonic acid antibody | 2.0×10^5 | 7.4 ± 2 | 22 ± 9 | 93 |
| Bovine serum albumin-azobenzene arsonic acid: anti- <i>p</i> -azobenzene arsonic acid antibody | $7 \pm 3 \times 10^5$ | 4.8 ± 0.2 | 18 ± 4 | 94 |

* Calculated from data in reference 92.

It should be pointed out that an antibody preparation would be expected to be heterogeneous with respect to the conformational details of the antibody combining sites, different antibody molecules being complementary to different portions of the hapten^{35, 37}. Supporting evidence for this expectation has been obtained experimentally^{35, 38} and, consequently, the equilibrium constants determined for antibody-hapten combinations represent only average values based on the assumption of statistical distribution of combining energies. In some instances, heterogeneity indices for different antihapten antibodies were actually calculated^{36, 37, 39}. For the calculations of the relevant parameters the following equations may be used:

$$\frac{1}{H_b} = \frac{1}{(Ab_t)} + \frac{1}{K(Ab_t)H_c}$$

and

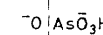
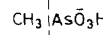
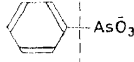
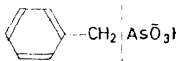
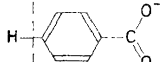
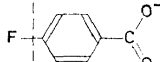
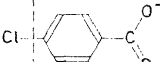
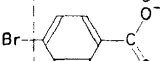
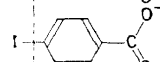
$$\frac{(Ab_t)}{(H_b)} = 1 + \frac{1}{(K_0 H_c)^\alpha}$$

where H_b and H_c represent the concentrations of the bound and free hapten respectively, and Ab_t is equal to the total concentration of antibody combining sites. The constant K corresponds then to an equilibrium constant for the combination of the hapten with the antibody on the assumption that all combining sites have identical binding capacity, and K_0 is an average equilibrium constant corresponding to the apex of the distribution curve, from which the index of heterogeneity, α , can be calculated^{36, 37}. For an antibody system with homogeneous combining sites the index of heterogeneity is obviously unity and it becomes smaller as the distribution of K values broadens. Using the above relations, the values for K_0 and α can be obtained by graphical methods³⁷.

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From a comparison of the relative combining constants calculated for the interaction of one antibody preparation with closely related haptens, it was possible to deduce the dominant structural features responsible for complementarity. For example, as shown in *Table 2*, if the relative association constant of antibodies directed against a small hapten is K'_0 , it is seen that small modifications in the structure of the hapten results in complete loss of binding⁴⁰. Similarly, from the data listed in *Table 3* it is seen

Table 2. Effect of hapten structure on equilibrium constant. (D. Pressman. *Molecular Structure and Biological Specificity*¹, by courtesy Director of Publications, American Institute of Biological Sciences)

| Antibody system | Hapten | K'_0 (l.mole ⁻¹) |
|--|---|-----------------------------------|
| Rabbit antibodies to <i>p</i> -azobenzene arsonic acid |  | 0 |
| |  | 0 |
| |  | 1.0 |
| |  | 0 |
| Rabbit antibodies to <i>o</i> -azobenzoic acid |  | 1.00 |
| |  | 0.6 |
| |  | 0.5 |
| |  | 0.4 |
| |  | 0.5 |

that the only hapten which can fit the antibody site produced against the *p*-azobenzene arsonate ion is the corresponding azobenzene phosphonate derivative which according to the "template theory" of antibody formation^{15, 41} could be fitted into the depression left on the antibody molecule by the larger, but otherwise similar, *p*-azobenzene arsonate ion⁴⁰.

Pictorially, the idea of steric complementarity in antibody-antigen interactions may be illustrated by the diagrams in *Figure 2*. This type of diagrams representing Van der Waals' outlines of the haptens helps us to understand some of the structural characteristics of the combining site. However, it is obvious that configurational factors by themselves would not lead to the formation of stable complexes unless this process were associated with a favourable interaction of opposite electrical charges, hydrogen bonds, Van der Waals' forces⁴⁰, and hydrophobic or apolar bonds^{42, 43} operating between the envelopes of the interacting molecules and leading to a lowering

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Table 3. Effect of the structure of the charged group of the hapten on the equilibrium constant (D. Pressman. *Molecular Structure and Biological Specificity*, by courtesy Director of Publications, American Institute of Biological Sciences)

Specificity of charged groups in the $\text{N}=\text{N}-\text{C}_6\text{H}_4-\text{AsO}_3\text{H}$ system

| Hapten | K_0 |
|--------|-------|
| | 1.0 |
| | 0 |
| | 0 |
| | 0 |
| | 1.0 |
| | 0 |

in free energy of the system. Moreover, one must use such illustrations with caution since they cover up a great deal of our ignorance, in particular in relation to the fate of the water of hydration associated with the combining sites of the antibody and antigen molecules during their interactions⁴⁰. In effect, the thermodynamic studies of antigen-antibody and hapten-antibody systems suggest that the decrease in free energy for these association reactions is governed to a large extent, if not almost entirely, in many though not all instances, by an increase in entropy. This has been interpreted as being due to the release of the water molecules, originally "frozen" on the surface of the combining sites into the bulk of the solution*.

To illustrate this point let us consider the results given in *Table 1*. Most of the binding constants for these reactions are of the order of $10^4-10^5 \text{ l. mole}^{-1}$ and the corresponding values for the free energies (at about 25°) are in the range of -5 to $-9 \text{ kcal mole}^{-1}$. The change in enthalpy has been found to be almost zero and, therefore, the free energy term, ΔF , is almost equal

* Additional evidence for this explanation was provided by experiments in which increased pressure reversed the antigen-antibody reaction, these results suggesting that antigen and antibody molecules separately occupy a smaller volume than the complex plus the water of hydration located in the combining sites¹⁸. However, as implied above, for a number of hapten-antibody systems negative values were recorded for the change in entropy²⁷.

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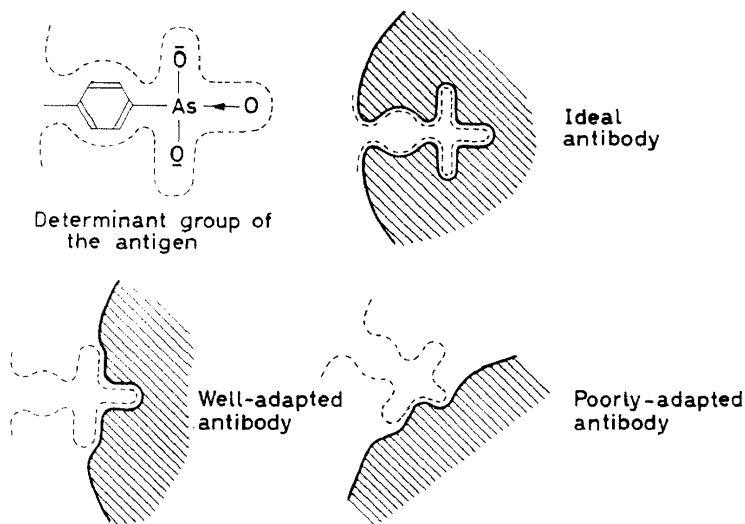


Figure 2. Determinant phenylarsonic acid groups of an antigen and different types of antibodies. (From *Chemistry and Biology of Proteins*, by courtesy Academic Press Inc., New York)

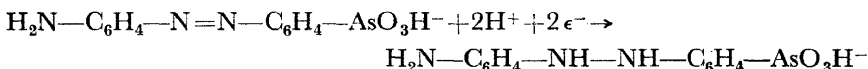
to $-T\Delta S$. This corresponds to a positive value (of about 20 e.u.) for the change in the entropy for these association reactions.

It is usually assumed that such associations are primarily directed by the interaction of opposite electrical charges on the combining sites^{40, 42}. This may be indeed the case with haptens or substrates possessing readily ionizable groups. However, for some non-ionizable haptens, such the dinitrophenyl group³⁴, and the corresponding antibodies, ΔF is also of the same order of magnitude, *i.e.* $-11 \text{ kcal mole}^{-1}$, and it is likely that in these associations the participation of hydrophobic bonds⁴² is the predominant factor driving these reactions. These bonds would result from the tendency of the hydrophobic portions of the combining molecules to aggregate with one another in water and, thus, reduce the surface area exposed to the polar environment.

Although it has become a general practice to utilize the methods of chemical kinetics for the elucidation of the mechanisms of most chemical reactions, only very few studies of the speed of antibody-antigen associations have been reported^{44, 45}. In all these cases, the kinetic data were obtained from measurements of the amount of light scattered as a function of time for systems containing polyvalent antigens and divalent antibodies. It is probable, therefore, that the results obtained with such systems represent complex, over-all reactions leading to the formation of aggregates of different size and that, in particular, the initial phases of these reactions were obscured by fast re-equilibration steps resulting in some statistical distribution of these aggregates with respect to their size.

Recently, in an attempt to establish the mechanism and the factors determining the primary step in the reaction between antibodies and their homologous antigens, a study of the kinetics of the combination of univalent

haptens with their corresponding rabbit antibodies was initiated in the author's laboratory³³. With such systems, the only obtainable complexes would be AbH and AbH₂. Since these reactions were anticipated to be very fast, it was decided to follow their rates with the aid of a cathode-ray polarograph⁴⁶, capable of registering concentration changes in the micromolar range within successive intervals of seconds. The hapten chosen for this study was 4-(4'-aminophenylazo)-phenylarsonate ion (R⁰-hapten), the azo group of which is readily reducible at a dropping mercury cathode according to the equation



This system was chosen on the assumption that the hapten, after combination with the antibody molecule, which has a molecular weight of 160,000, would no longer be available for reduction at the cathode.

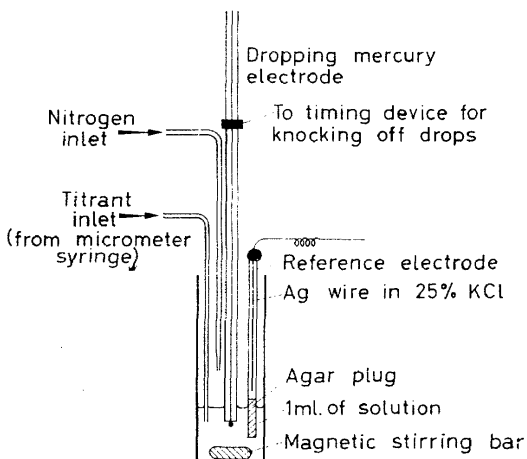


Figure 3. Polarographic cell assembly

A schematic representation of the cell assembly used is given in *Figure 3*. The polarographic waves* were clearly visible on the fluorescent 6 in. screen of the cathode-ray tube and were recorded photographically. Typical polarographic curves are shown in *Figure 4*. The potential at which the peak occurs is characteristic of the material being reduced, and the height of the peak is proportional to its concentration.

Theoretical curves representing the binding between the hapten and antibody were constructed on the assumption of association constants ranging from 10^5 to 10^6 (*Figure 5*). The experimental data obtained for the system, 4-(4'-aminophenylazo)-phenylarsonate ion and its homologous antibody, were represented by similar binding curves and an association constant of $7 \times 10^5 \text{ l. mole}^{-1}$ was calculated. This value is in good agreement

* The antibody and hapten solutions were de-oxygenated prior to each experiment and were kept in a nitrogen atmosphere during the experiment.

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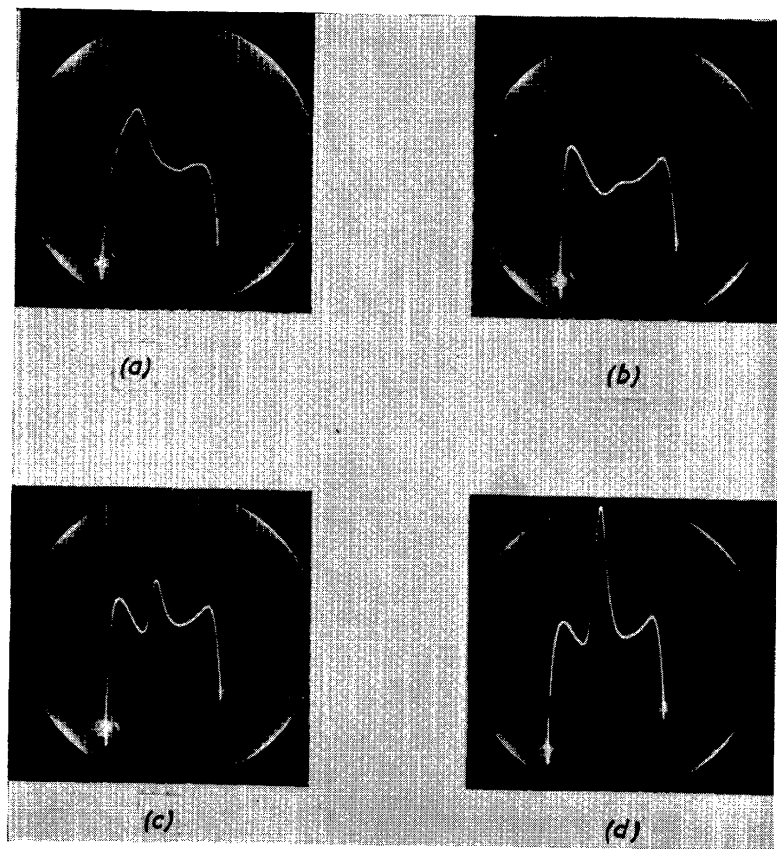


Figure 4. Polarographic curves obtained on the oscillograph: curves (a) and (b) represent oscillographic traces for buffer and antibody solutions (in absence of hapten); curves (c) and (d) were obtained on addition of increasing amounts of the reducible hapten

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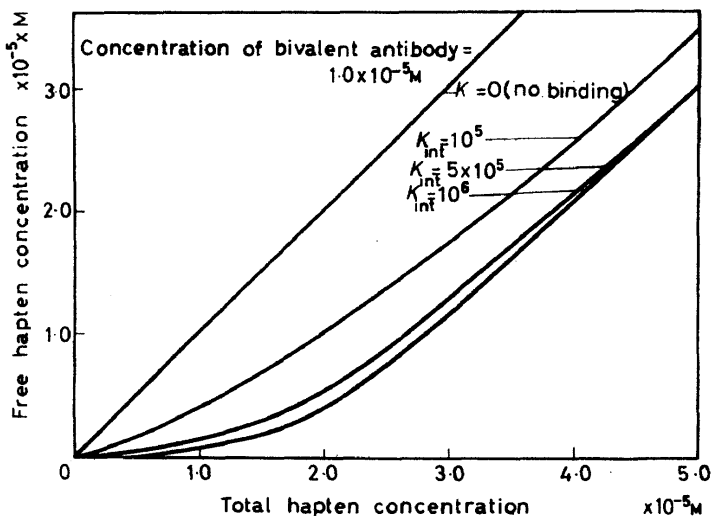


Figure 5. Theoretical titration curves

with equilibrium constants obtained by the equilibrium dialysis method for similar hapten-antibody associations⁹⁵. However, no kinetic data could be deduced directly from this study since the reaction was found to have reached equilibrium within the time of mixing, which was estimated to be less than two seconds.

The benzene arsonate group was considered to represent the major portion of the immunizing hapten, 4-(4'-aminophenylazo)-phenylarsonate ion,

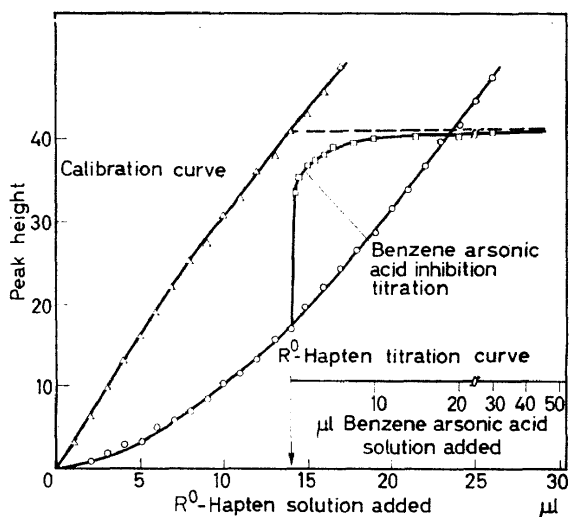
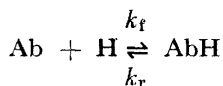


Figure 6. Replacement of R^0 -hapten by inhibitor. The calibration curve with the reducible hapten R^0 was obtained with the antibody solution in the presence of an excess of the non-reducible inhibitor, benzene arsonic acid. The lower continuous line represents the binding curve for R^0 obtained in the absence of the inhibitor

which determined the structure of the complementary site on the antibody. In consequence, it was expected that the smaller group, *i.e.*, the benzene arsonate ion, could be used as an inhibitor. Indeed, it was thus shown that the addition of this smaller ion to a solution containing complexes of antibody with the larger reducible hapten resulted in the release of the latter (*Figure 6*). Furthermore, the replacement of the reducible hapten from the combining site of the antibody by the addition of appropriately large amounts of inhibiting hapten was found to be practically "instantaneous". From these "negative" kinetic data one can deduce that the rate constants for the forward and reverse steps in the reaction



are larger than $10^5 \text{ l.mole}^{-1} \text{ sec}^{-1}$ and 1 sec^{-1} , respectively. For comparison, some rate constants estimated for a few enzyme-substrate reactions⁴⁷ are listed in *Table 4*. As can be seen the rate constants for the formation of these complexes are one or two orders of magnitude larger than the minimum values estimated roughly for antibody-hapten associations. On the basis of the simple theory of bimolecular collisions in solutions, the maximum value for the rate constant of the forward step, if it were only diffusion-controlled, can be estimated as about $10^{10} \text{ l.mole}^{-1} \text{ sec}^{-1}$.

Table 4. Kinetic constants for enzyme-substrate associations

| <i>Enzyme</i> | <i>Substrate</i> | k_f ($\text{mole}^{-1} \text{ sec}^{-1}$) | k_r (sec^{-1}) |
|---------------|------------------------------------|--|--------------------------------|
| Peroxidase | H_2O_2 | 1×10^7 | |
| Catalase | H_2O_2 | 6.6×10^6 | 2×10^{-2} |
| Catalase | CH_3OOH | 8.5×10^5 | 1.6×10^{-2} |
| Catalase | $\text{CH}_3\text{CH}_2\text{OOH}$ | 2×10^4 | 4×10^{-2} |

Obviously due to steric restrictions and since the antibody combining site is probably not more than 1 per cent of the total area of the antibody, the maximum rate constant for the association step might be reasonably expected to be smaller by a factor of at least 10^2 – 10^3 than the maximum value derived from the simple collision theory. It is hoped that the precise definition of the kinetic factors responsible for the speed of antibody-antigen interactions will be possible with the use of one of the special "relaxation" methods developed recently by Eigen in Göttingen^{48, 49} for following rapid reactions in solutions (see Note 1 p. 502).

ISOLATION OF ANTIBODIES WITH ANTIGENICALLY SPECIFIC ADSORBENTS

As mentioned previously, most precipitating antibodies produced in the rabbit, which has been the preferred species for experimental purposes, are associated with normal serum γ -globulins and are indistinguishable from the latter proteins by physical or chemical criteria. The only distinguishing feature of antibody molecules is their ability to combine specifically with the

homologous antigen molecules. Chemical analysis of normal γ -globulins and of various antibody preparations has revealed no significant difference in the type and number of amino-acids⁵⁰, and an identical single *N*-terminal pentapeptide sequence (Ala-Leu-Val-Asp-Glu-) has been demonstrated both for normal rabbit γ -globulins and for different rabbit antibodies^{51, 52}. On the basis of earlier and even more fragmentary evidence, Pauling¹⁵ suggested that an identical amino-acid sequence persists in a considerable portion of the peptide chain of all rabbit γ -globulins, whether antibody or not, and that the configurationally complementary structure of antibodies, as well as their heterogeneity, is the result of a unique folding of the peptide chain (*i.e.* of the secondary and tertiary structure of antibody molecules) which is imprinted or guided by the antigen.

From these remarks it is clear that only by using pure antibody preparations, devoid of normal globulins and other serum contaminants, can one hope to obtain the definitive information necessary for establishing the unique features of the structure of antibody molecules, and for calculating the precise thermodynamic and kinetic constants for antibody-antigen interactions. In principle, antibodies could be isolated in pure form by exploiting their property of combining specifically with their antigens. Such a procedure would involve the following steps: (i) formation and isolation of insoluble antigen-antibody complexes, (ii) dissociation of these complexes, and (iii) separation of the purified antibodies from the antigens. In general, the combination of antibodies with antigens to form insoluble complexes can be effected with ease, and since the combination of most antigens with their antibodies is predominantly due to electrostatic forces, it is not surprising that dissociation of antibody-antigen complexes can be brought about simply by lowering or raising the pH^{53, 54}. However, the isolation of the constituents of antigen-antibody complexes is associated with some difficulty, primarily because of the similarity of their physical properties.

Obviously, specific and general procedures could be applied for the isolation of antibodies in a pure form provided the antigens could be rendered insoluble by attachment through stable, covalent bonds to some solid and inert supporting medium. This principle has been employed by several investigators using different natural and synthetic materials, such as cellulose⁵⁵⁻⁵⁷, red cell stroma^{58, 59}, keratin⁶⁰, fibrinogen⁶¹, ion-exchange resins⁶² and polystyrene⁶³⁻⁶⁶, for the preparation of insoluble antigenically specific adsorbents. The synthesis and the properties of such antigen-resins will be discussed in detail by Professor Manecke⁶⁷ and, therefore, only a short description of the experience gained with some immunosorbents in the author's laboratory will be presented here.

In the belief that supporting media with hydrophilic groups would possess properties resembling those of ion-exchange resins and would, thus, conceivably bind proteins also non-specifically, protein antigens were coupled through azo bonds to a non-polar polystyrene framework by the sequence of reactions⁶⁴ shown in *Figure 7*. In the later phases of this work, a polyaminostyrene preparation supplied by Norsk Hydroelektrisk Co., Oslo, Norway, was used.

For the isolation of antibodies, the polystyrene-antigen conjugates were suspended in the appropriate antisera for 1 to 3 hours with occasional stirring.

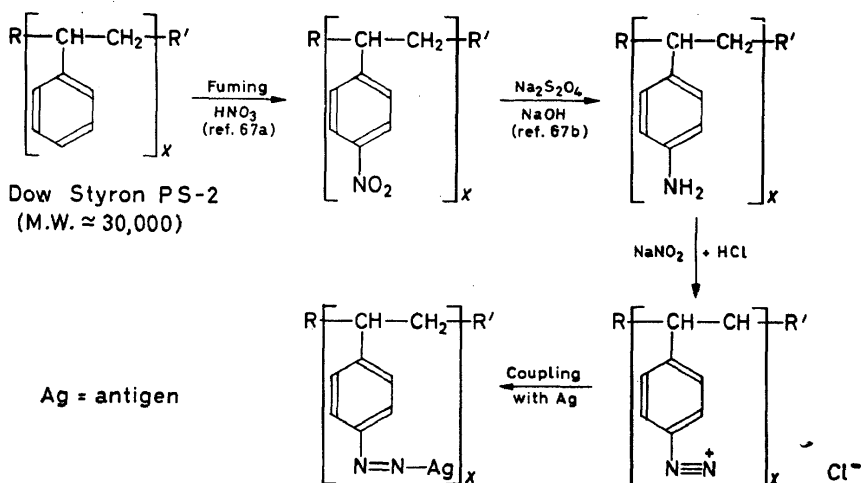


Figure 7. Flowsheet of the preparation of polystyrene-antigen conjugates

The polystyrene-antigen-antibody complex was then filtered off and washed with saline until the filtrate was found to be free of protein. For the dissociation and recovery of the antibody, the polystyrene-antigen-antibody complexes were suspended in saline and the pH was adjusted to 3 with dilute hydrochloric acid. After 90 to 120 minutes the dissociated antibodies were filtered off and the pH of the filtrate was adjusted to 7. After an experiment, the polystyrene-antigen conjugate was resuspended in saline and kept at 4° for subsequent re-use.

The original serum and the eluate were analyzed by Tiselius electrophoresis for their protein distribution and by the quantitative precipitin test for their antibody content. Using several polystyrene conjugates prepared with different antigens, it was shown that these immunosorbents completely removed precipitating antibodies from the homologous rabbit antisera. The specificity of this reaction was further demonstrated by the fact that these antisera were not depleted of their antibodies by conjugates prepared with unrelated antigens and that only negligible amounts of protein, of the order of 0.01 g per cent, were removed from these sera. The results of the electrophoretic analysis of antisera before and after exposure to the related polystyrene-antigen conjugates are shown diagrammatically in Figure 8. It is evident that the antibodies removed by the immunosorbent were localized primarily in the region of γ -globulins and extended also into the region of the faster migrating serum globulins. Whilst this method was found to yield antibody preparations, which were 80 per cent pure, as measured by their ability to form precipitates with the appropriate antigens, the recoveries of antibodies were seldom higher than 35 per cent.

The effect of the extent of the amination of polystyrene on the yield and purity of antibodies was not thoroughly investigated in this study. However, it would seem that the degree of amination would determine the steric configuration of the three-dimensional polystyrene-antigen network. It is

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conceivable that, in an aqueous medium, the polystyrene-antigen conjugates prepared with batches of highly aminated polystyrene retain an unfolded structure, and that antibodies are combined only onto their surfaces. This type of conjugates should not retain proteins non-specifically. On the other hand, polystyrene molecules to which only a small number of antigen molecules are attached may be coiled into "spongy" structures, which are stabilized by the interaction of the hydrophobic benzene residues. These would not allow for the free draining of protein solutions through their matrices and, thus, would lead to a non-specific, irreversible occlusion of proteins.

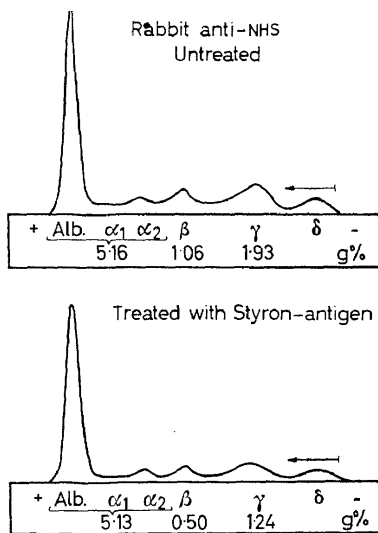


Figure 8. Electrophoretic analysis of a rabbit antiserum before and after exposure to the homologous polystyrene-antigen conjugate (NHS refers to normal human serum)

Findings similar to those obtained in this laboratory were reported by other workers⁶⁵ who used polystyrene-antigen conjugates. More recently^{63, 68, 69} it was also demonstrated that polystyrene-antibody conjugates could be synthesized and that the resulting conjugates had the ability of combining specifically with the corresponding antigens, and could thus be used for the purification of the latter. Grubhofer and Schleith⁷⁰ also showed that enzymes could be coupled to diazotized polystyrene without impairing their enzymatic activity. It would, therefore, seem that poly-amino polystyrene has a wide applicability in studies necessitating the fixation of a biologically active material to an insoluble polymeric framework.

Cellulose-antigen complexes were prepared in the author's laboratory⁵⁶ according to both methods described by Campbell *et al*⁵⁵ and by Gurvich⁵⁷. However, these immunosorbents were found to yield low recoveries (about 35 to 50 per cent) of antibodies with a low degree of purity (about 35 to 50 per cent) contrary to the claim of the former workers that antibody preparations with purities of the order of 90 per cent could be obtained. In a more recent study in this laboratory, red cell stroma-hapten conjugates were used as immunosorbents for the isolation of anti-arsanilic rabbit

antibodies⁵⁹. The antibodies were displaced from the immunosorbents by dissociation with a 5-10 per cent solution of a sodium salt of *p*-arsanilic acid or the sodium salt of *p*-iodobenzene arsonic acid. The dissociating hapten was then removed by extensive dialysis. These antibodies were shown to be also dissociated off the immunosorbents by hydrochloric acid at pH 3.2. The purity of antibody preparations obtained by displacement with hapten was of the order of 60 per cent and their recovery was also about 60 per cent. The apparent lack of purity of these antibody preparations, as demonstrated by the decrease in their precipitability, was attributed to some of the antibody sites having the ability of binding haptens more tenaciously or irreversibly; an effect which would result in some inhibition of precipitation. These conclusions are in agreement with the concept of heterogeneity of antibodies^{36, 37, 71}, suggesting that the hapten could be removed by dialysis only from antibody sites with low combining capacities.*

FRAGMENTATION AND RESYNTHESIS OF ANTIBODY MOLECULES

The detailed mechanism of antibody formation at cellular level, as well as that of normal globulins, is still a matter for speculation⁷². Although Pauling's template theory of antibody formation^{15, 73} has not been found acceptable by most biologists^{74, 75}, his original predictions regarding the structural features of antibodies, have recently received additional experimental support from studies of the degradation of antibody molecules. As referred to earlier in this paper, Pauling¹⁵ assumed that all antibody molecules possessed the same polypeptide chains as normal globulins and differed only in the configuration of the chains. He also assumed that an antibody molecule had, at most, two active sites and that the observed versatility of complementarity to different antigens (and, incidentally, the observed heterogeneity of antibodies) could be explained by postulating the existence of an extremely large number of accessible configurations with nearly the same energy for the end parts of the globulin polypeptide chain. Furthermore, he postulated that the central, backbone portion of the antibody molecule could assume only one possible configuration characteristic of the normal globulin, which would account for the antigenic similarity of antibodies and normal globulins.

Recently, γ -globulins from immune rabbit sera were digested with crystalline papain^{50, 76, 77} and for each antibody preparation three fragments were separated by chromatography on carboxymethyl cellulose. These fragments represented over 90 per cent of the original protein and were non-dialyzable through Visking tubing. Two of these fragments (fractions I and II) appeared to be almost identical in chemical, physical and immunochemical properties and had a molecular weight of about 50,000 (see Note 2 on p. 502). Fraction III had a molecular weight of about 80,000 and crystallized easily in the form of thin, perfectly diamond-shaped plates. The relative yields of the three fractions were found to be almost identical and, therefore, it seems likely that rabbit γ -globulin consists of two nearly identical portions (giving rise to fractions I and II) joined to a third portion of quite different structure.

* These conclusions are also supported by the recent data of Velick *et al.*³⁴.

None of the fragments precipitated with the homologous antigens, but both fragments I and II were shown to inhibit specifically the precipitation of the whole antibody with the homologous antigen. These results suggested that each of these fragments possessed an antibody combining site, which had not been altered by digestion with papain. The correctness of this interpretation was demonstrated by Nisonoff *et al.*^{78,79}, who showed clearly that each of the fractions I and II of a rabbit antihapten antibody was univalent, whilst the intact antibody was divalent. Fraction III, on the other hand, had no inhibitory power and, therefore, was considered to have no antibody site.

When intact rabbit γ -globulin was used as antigen in other species (goat and rat), it was shown that fraction III precipitated most of the antibody formed, this observation demonstrating that most of the antigenic sites of γ -globulin were in fraction III. All three fractions proved to be good antigens (in rat, horse and sheep): fractions I and II were antigenically indistinguishable, but there was little if any cross-reaction with fraction III. More recently Nisonoff *et al.*^{79, 80} have shown that γ -globulin can be degraded by the combined or successive actions of pepsin and reducing agents (such as 2-mercaptoethylamine, cysteine, thioglycolate) into fragments essentially similar to those produced by papain digestion. Treatment with pepsin alone caused the reduction in molecular weight for the bulk of the antibody protein from 160,000 to 106,000⁸¹. The residual molecule was, however, still bivalent, and, on subsequent treatment with one of the reducing agents capable of splitting disulphide bonds, it was split into univalent fragments with a molecular weight of about 56,000. Considerable evidence has been presented by these workers suggesting that the mechanisms of action of papain and pepsin on the antibody are similar and that the fragment having a molecular weight of 106,000 consists primarily of Porter's fragments I and II united through a disulphide bond. This additional data further strengthens the belief that the antibody molecule consists of two nearly identical subunits joined through an S—S bond, and that the third fragment is linked to one or both of them through covalent bonds, which are split by either papain or pepsin. Fragments I and II are thus responsible for the antibody activity and for the individual allotypic variation^{82, 83} of γ -globulins, whereas fragment III appears to be the stable portion of the molecule common to all rabbit γ -globulins.

In further experiments, Nisonoff^{81, 84} showed that dimerization of the univalent fragments occurred readily by the reformation of the disulphide bond when these fragments were subjected to mild oxidation. The resynthesized material had a molecular weight of about 106,000 and possessed the characteristics of a divalent antibody. Moreover, when a mixture of approximately equal amounts of univalent fragments prepared from antibodies with different specificities, *i.e.*, prepared from specifically purified antibodies to ovalbumin and bovine γ -globulin, was subjected to oxidation, hybrid or bispecific molecules were formed⁸⁵. The latter failed to form precipitates with either ovalbumin or bovine γ -globulin alone, but more than half precipitated with an appropriate mixture of the two antigens. The lack of precipitation with one antigen does not indicate that no bivalent molecules of single specificity were present. For random combination of

equal amounts of two univalent fragments, A and B, the ratios of divalent molecules A—A, B—B, and A—B would be expected to be 1 : 1 : 2. Since A—B is univalent with respect to either system, it could block the precipitation of either A—A or B—B with the appropriate antigen. A mixture of antigens, however, could form a three-dimensional continuous framework with hybrid molecules of the type A—B.

When all this evidence is pieced together the picture which emerges is very similar to Pauling's visionary concept for the structure of the antibody molecule, predicted over twenty years earlier¹⁵.

Finally, a few remarks concerning the nature of antibodies in allergic sera might be in order. It has been shown repeatedly that antibodies in sera of individuals allergic to different external agents, such as foods, drugs, inhalents, *etc.*, do not precipitate on incubation with the offending antigen. This failure to precipitate was attributed to different reasons, such as the univalency of these antibodies, their presence in concentrations below the threshold of detectability, or their inability to combine *in vitro* with the appropriate antigens. By the use of immunosorbents, prepared from polystyrene conjugated with the constituents of the pollens of ragweed and different grasses, it was shown⁸⁶ that antibodies in the sera of allergic individuals had the ability of combining firmly and specifically with the appropriate antigens and, furthermore, that the former were present in exceedingly low concentrations, probably 100 to 1000 times lower than that found in experimental sera. On the supposition that these antibodies were also divalent, it was expected that their presence could be demonstrated by an *in vitro* technique provided the combining "mass" could be artificially increased. For this purpose ragweed and grass antigens were linked by covalent azo bonds to rabbit red cells, using *bis*-diazotized benzidine⁸⁷ and more recently, tolylene-2,4-di-isocyanate⁸⁸ as the coupling agents. Suspension of these antigen-coated red cells into the sera of individuals allergic to these pollens, or of animals immunized with the constituents of these pollens, led to the formation of a specific agglutination pattern due to crosslinking of the red cells into a gelatinous, three-dimensional network*. This technique was shown to be at least 100 times as sensitive as the precipitin test for the detection of antibodies⁸⁹. The specificity of this test was demonstrated by an inhibition method: the addition of free soluble antigen inhibited the formation of these networks and the cells sedimented simply to the bottom of the test tube giving a compact "button". The similarity between the mechanisms responsible for the haemagglutination and the precipitin reactions has been further demonstrated⁹¹ using univalent antibody fragments as prepared by Porter⁷⁶ or Nisonoff *et al*⁷⁹. These fragments inhibited also the haemagglutination reaction given by sera obtained from both allergic individuals and from experimentally immunized animals. These results would support, therefore, the view that the antibodies

* For the sake of simplicity no distinction will be made here between the different types of antibodies present in sera of allergic individuals⁹⁰. Suffice it to say, that some of the antibodies in these sera have the ability of attaching themselves to the skin, conjunctiva and mucosae of the allergic individuals, or on passive transfer to the same tissues of normal individuals. Combination of these "skin-sensitizing" antibodies with the appropriate antigen(s) on the "sensitized" tissues results in an inflammatory reaction due to the release of histamine and other pharmacologically active substances⁹⁸.

involved in the haemagglutination reaction obtained with allergic sera are also divalent.*

CONCLUDING REMARKS

This cursory survey of the nature of antibodies and their reactions with the appropriate antigens or haptens is obviously incomplete. The most intriguing question as to how the stereospecificity, exhibited by an antibody molecule for its antigen, is imprinted on the antibody molecule was left out from this discussion primarily because of the complexity of the problem and because of the unavailability of definitive experimental data necessary for establishing the mechanism of antibody formation^{72, 73}.

Antibody production represents an example of the synthesis of a protein molecule, the structure of which is not completely determined by the genetic machinery of the organism producing it. In some way or other, the presence of the foreign, antigenic substance in the organism of the host leads to the production of antibody molecules having physico-chemical properties similar to those of normal γ -globulin molecules. According to the modern views of protein biosynthesis⁹⁹, the specificity of the structure of proteins appears to be determined by the cytoplasmic RNA (ribonucleic acid), which in turn is synthesized under the control of nuclear DNA (deoxyribonucleic acid) possessing the necessary coded information for genetic differentiation. On the basis of this theory, the RNA is assumed to act as a template for the assembly and linkage of the appropriate amino-acids into a specified structure.

Unfortunately, up to the present, the complete sequence of amino-acids in antibody molecules and in normal γ -globulin molecules has not been established, and, therefore, it is not known whether the primary structure of these different molecules is identical. As mentioned previously, it has been shown so far^{51, 52} that these molecules (in rabbit serum) have an identical *N*-terminal pentapeptide. In the absence of more complete information and on the basis of the data presented in the last section of this paper, it is very tempting to assume that the rest of the primary structure of these molecules is also identical. If this were indeed the case, a single RNA template would be implicated in the synthesis of these molecules and the observed wide spectrum of antibodies (and of their physico-chemical properties) might be ascribed to the configurational lability and versatility of these molecules to fold into different shapes dictated by the antigen. This view is in essence similar to that expressed recently by Karush⁹⁶, who assumed that the structure of a newly-formed globulin molecule (on release from its template) was molded by contact with the antigenic group and that it was subsequently stabilized by the formation of intramolecular disulphide bonds determining the secondary and tertiary structures of the antibody molecule. According to Karush⁹⁶ the final step "in the emergence of the antibody is its dissociation from the complex in which it was born". As a result of this dissociation[†] the antigenic determinant is regenerated and can

* In fact, recently, after separation of these haemagglutinating antibodies by chromatography on DEAE-cellulose (100) and after their concentration, it was demonstrated that these antibodies had the properties of normal precipitins¹⁰¹.

† According to this hypothesis, the heterogeneity of antibodies is interpreted as being due to the dissociation occurring before the complete number of disulphide bonds necessary to provide maximum complementarity had been formed.

stimulate the formation of more antibody molecules. Although this mechanism for antibody synthesis seems plausible (it is seldom possible to prove a mechanism as absolutely correct!), it does not provide an answer to the problem as to how the organism recognizes the "non-self" character of the antigen⁹⁷, and this aspect of the problem has been included in Burnet's *clonal selection theory*^{72, 74}.

It would thus appear that antibody-antigen reactions represent model systems not only for *in vitro* studies designed to elucidate the mechanism of the interactions between biological macromolecules at molecular level, but also for the elucidation of some of the genetic factors underlying the biosynthesis of proteins with induced specificities.

Note 1 added in proof

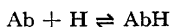
A more detailed account of the results obtained by cathode-ray polarography has been recently published¹⁰². Furthermore, from the results of temperature-jump experiments, values of the order of 10^8 and 10^7 l.mole⁻¹ sec⁻¹ were derived for the interaction of haptens of the R⁰ type with bovine serum albumin¹⁰³ and with the homologous antibodies¹⁰⁴, respectively.

Note 2 added in proof

Nisonoff reported at the 1st Western Conference of Immunology (La Jolla, Calif., Jan. 1962) that both fractions I and II represent mixtures of the two univalent antibody fragments.

Summary

The similarity between antibody-antigen interactions and enzyme-substrate associations was pointed out, and the physico-chemical factors underlying the specificity of these reactions were discussed. Some of the methods available for the calculation of the thermodynamic data for antibody-hapten reactions were reviewed and the polarographic technique used in the author's laboratory was described. It was shown that the minimum rate constants for the forward and reverse reactions in the system



(where H and Ab represent 4-(4'-aminophenylazo)-phenylarsonate ion and the homologous rabbit antibody, respectively) were 10^6 l.mole⁻¹ sec⁻¹ and 1 sec⁻¹.

The problems associated with the isolation of "pure" antibodies were discussed and the results obtained with some antigenically specific adsorbents, synthesized for the recovery of both precipitating (rabbit) and "non-precipitating" (allergic) antibodies, were given.

Using univalent antibody fragments, prepared by the method of Porter, it was shown that haemagglutinating, "non-precipitating" antibodies were also divalent.

The recent literature relating to the structure of antibodies and to the mechanism of antibody formation was briefly surveyed.

This research was supported by grants from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md., and the National Research Council of Canada, Ottawa, Ontario.

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