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A THEORY OF ANTIBODY-ANTIGEN REACTIONS

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LIGHT SCATTERING ARISING FROM COMPOSITION FLUCTUATIONS IN MULTI-COMPONENT SYSTEMS

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Abstract

Part I describes a theory of antibody-antigen reactions employing bivalent and univalent antibody molecules and multivalent antigen molecules. All species in a system of this kind are defined by a distribution function which has been derived on the basis that the most probable distribution is the appropriate one. Some of the features of antibody-antigen reactions are discussed in the light of this theory.

In Part II experiments are described which measured the increase in turbidity of an antibody-antigen system with increasing time of reaction. It was found that the rate of aggregation of antibody and antigen molecules into large aggregates is dependent on the composition of the system. The existence of soluble aggregates in the antigen excess region is indicated.

A general theory of Rayleigh scattering due to composition fluctuations in multi-component systems has been developed with the aid of the grand canonical ensemble of Gibbs. It is found in Part III. The equation developed contains previously neglected terms arising from thermodynamic interactions between solutes in systems of more than two components.

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Part I

A Theory of Antibody-Antigen Reactions

1. Introduction

For a long time it has been attractive to consider antibody-antigen reactions as involving combination of specific sites by which very large aggregates are attained. To require the existence of aggregates of this kind, one must necessarily assume that the antibody and antigen molecules responsible for the size of the aggregate are multivalent with respect to each other. If one is to require further that the antibody-antigen molecular ratio of these aggregates be variable and no less than unity, then he must consider antibody molecules to be bivalent and antigen molecules to be greater than bivalent. It should be noted that the existence of univalent antibody molecules in the system is still permitted. They cannot, however, be responsible for the specific growth of the aggregate to a size involving more than one antigen molecule, since wherever they occur they end chains which might otherwise have grown longer than they are.

Marrack has suggested that natural protein antigen molecules have several determinant groups per molecule. He has reasoned that if this is true, then antibody sites can be expected to repeat in the antibody protein molecule. In a system of multivalent antibody and antigen molecules he feels that specific combination of the reactive sites must play a part in the formation of aggregates. He pictures these aggregates as coarse lattices(1).

Heidelberger and Kendall have adopted the viewpoint of multivalence on the basis of variable antibody-antigen ratios and a curve fitting equation useful for the precipitin reaction, which they derived on the assumption of multivalent antibody and antigen molecules. They regard the final precipitate as consisting of antibody molecules held together in three dimensions by antigen molecules(2,3,4). Pauling has propounded a detailed theory for the formation of bivalent antibody molecules. In the same paper he has explained several observations of antibody-antigen reactions on the basis of bivalent antibody molecules and multivalent antigen molecules, the maximum valence of the antigen being given by the "ratio of its surface area to the area effectively occupied by one antibody molecule, if all regions of the antigen surface were active"(5). He pictures the aggregate as a three dimensional antibody-antigen network, the ratio of its components depending on the relative amounts of antibody and antigen in the system. From a theoretical treatment of antibody-antigen systems, Hershey prefers the lattice or network hypothesis employing antibody molecules of low valence, probably two(6).

Experimental evidence favoring the formation of large specific aggregates has been discussed so extensively in the past by such capable investigators as Pauling and Heidelberger that it will not be reviewed here. However, a general theory presenting the features of a

system involving reactions between bivalent antibody molecules and multivalent antigen molecules has not yet been achieved. It is the purpose here to present such a theory, which, it is hoped, will be of use not only for predictions based on real systems but also for understanding the characteristics of antibody-antigen reactions.

Although theoretical treatments have been developed in the past, they have not been sufficiently general to predict the common characteristics of the precipitin reaction. Variable antibody-antigen ratios of the precipitate, which depend on the preparation of the system, are of fundamental importance to a good theory. Inhibition to precipitation in regions of antigen excess and also antibody excess should be accounted for without relying on artificial assumptions of solubility. One should attempt to describe the relative amounts of precipitate corresponding to the composition of the system. One should be able to explain the relative differences of the system arising from the manner in which composition is varied. The quantitative function of blocking antibody molecules has yet to be described for the ordinary systems.

Heidelberger and Kendall have developed an equation which relates the total amount of combined antibody to the composition of the system and the equivalence zone antibody-antigen ratio(3). Their equation, however, is only good in regions of antibody excess. It is not applicable for many systems. Furthermore, the assumptions

they used in the derivation are so unrealistic that one would think of their equation as hardly anything more than empirical. Fortunately, Kendall derived the equation on a much more sound basis many years later(7). It is interesting to note that he used bivalent antibody molecules, and he approached the problem with probability considerations. Although he supposed irreversibility of the antibody-antigen reactions was necessary assumption for his derivation, such was not the case.

Hershey has developed a theory for reactions of multivalent antibody and antigen molecules. He assumed, however, that the reactions leading to the formation of aggregates composed of one antigen and several antibody molecules are not influenced by further aggregation. He came finally to the expected conclusion that "no great disturbance of the initial equilibrium occurs during the formation of precipitates"(6). It will be shown presently that this assumption and its conclusion are not justified.

Pauling and his colleagues have applied the principles of chemical equilibrium directly to a theory of the precipitin reaction for relatively simple systems(8,9). They only considered systems composed of bivalent antibody and antigen, univalent hapten, certain soluble complexes, and one insoluble species. It was found that the experimental points showing the dependence of the amount of precipitate on the amount of hapten added to the system

did not fall on a straight line as the theory predicted (8,10,11). Pauling explained this deviation from linearity on the basis of heterogeneity of the antiserum, which he described by an error function of the free energy of interaction of antibody and hapten in competition with the precipitating antigen(12). The theory was found to be in satisfactory agreement with experiment.

Teorell has developed a theory of the precipitin reaction patterned after the treatment of polybasic acid equilibria. He, therefore, required antibody to be univalent and antigen multivalent. He was able to express, in the usual manner, the concentration of each aggregate indirectly in terms of total amount of antibody and antigen and the dissociation constants. In order to obtain agreement with experiment he was forced to make assumptions regarding solubilities of the aggregates formed(13,14). These are highly questionable and rather artificial.

2. A Theory for Reactions of Multivalent Antigen Molecules with Bivalent and Univalent Antibody Molecules

The theory presented here uses as a basis the concept of the most probable distribution. It will be assumed as is generally done, that the most probable distribution is the appropriate one. The number of ways of forming a given distribution of aggregates in the system, corresponding to a given number of antigen sites reacted, is

maximized with respect to the occupation numbers(the number of each kind of aggregate). The occupation numbers, which are then evaluated, define the most probable distribution. Hence, an expression is obtained giving the number of every kind of aggregate in the system corresponding to the extent of reaction in the system, that is, the fraction of antigen sites which have reacted. The remaining problem is that of using this distribution to obtain information about the system for different compositions.

Flory was the first to discuss this kind of a distribution for large aggregates. He studied molecular size distributions of three dimensional polymers(15,16,17). Stockmayer later obtained the most probable distribution of molecular sizes for certain types of branched-chain polymers(18). It is this method of Stockmayer which is used in the following presentation. Consequently, the two assumptions used by Flory and Stockmayer also characterize this work. In this theory it is assumed that intra-aggregate reactions yielding cyclical structures cannot occur. One result of this is that a fixed number of bonds is required for the formation of an aggregate of given composition no matter how the aggregate is put together. This number of bonds is one less than the total number of antibody and antigen molecules of which the aggregate is composed. It is next assumed that any unreacted site is as reactive as any other site regardless

of the size or shape of the aggregate to which it is attached.

An antibody site is the reactive area on an antibody molecule which permits combination of the latter with an antigen molecule at one of its reactive areas. The antigen site is defined in an analogous way. An aggregate is defined as a group of antibody and antigen molecules, any two of which are connected by only one chain consisting of alternating antibody and antigen molecules bound to each other by their respective reaction sites, provided these two molecules are not bound together by their reaction sites(see Appendix). Therefore, if a bond in a single aggregate is broken the antibody and antigen molecules on either side of the bond are in no way then connected to those on the other side. Two aggregates exist. An antibody-antigen reaction involves the combination of one antibody site with one antigen site in the formation of a bond. An aggregate consisting of two molecules must be composed of one antibody molecule and one antigen molecule with one bond between them. Furthermore, there con be only one bond holding any antibody molecule to an antigen molecule.

The following terminology will be used throughout the discussion.

G = number of antigen molecules in the system.

A = number of antibody molecules in the system with two reactive sites(bivalent antibody).

D = number of antibody molecules in the system with one reactive site (univalent or blocking antibody).

M = number of aggregates in the system plus the number of free antibody and antigen molecules.

f = number of effective reaction sites on each antigen molecule (f-valent antigen).

m_{ijk} = number of aggregates each of which is composed of i bivalent antibody molecules, j univalent antibody molecules, and k antigen molecules.

W_{ijk} = number of ways to construct a single i,j,k-aggregate containing no cyclic structures from i given bivalent antibody molecules, j given univalent antibody molecules, and k given antigen molecules.

q = number of free antibody sites on an aggregate.

p = fraction of antigen sites in the system which have reacted; it is also called the extent of reaction.

ρ = fraction of antibody sites in the system which belong to bivalent antibody molecules.

$r = fG/2A$

M_G = molecular weight of the antigen.

M_A = molecular weight of the bivalent antibody.

M_D = molecular weight of the univalent antibody.

The total number of ways to form the number of aggregates m_{ijk}, for all appropriate i,j, and k values out of the A, D, and G molecules is

$$\Omega = G!\,A!\,D! \prod_{i,j,k} \left[\left(\frac{w_{ijk}}{i!j!k!} \right)^{m_{ijk}} \frac{1}{m_{ijk}!} \right] \tag{1}$$

In order to find the most probable distribution, that is, the set of the numbers m_{ijk}, corresponding to the maximum value of Ω, one must set the derivative of Ω with respect to the variables m_{ijk}, equal to zero for constant A, D, G, and M, which are expressed by

$$\sum_{i,j,k} i\, m_{ijk} = A \quad ; \quad \sum_{i,j,k} j\, m_{ijk} = D \tag{2}$$

$$\sum_{i,j,k} k\, m_{ijk} = G$$

and

$$\sum_{i,j,k} m_{ijk} = M \tag{3}$$

The sums and products of Equations 1, 2, and 3 correspond to all values of k in the system. On account of the fact that antibody sites can react only with antigen sites the indices i and j are related to k in the following way, as demonstrated in the Appendix.

$$\begin{aligned} i &= k - 1 + q \\ 0 \leq q &\leq fk - 2k + 2 \\ 0 \leq j &\leq fk - 2k + 2 - q \end{aligned} \qquad (4)$$

In the sums which follow, these relations give the limit unless otherwise specified. fk-2k+2 is the number of free antigen sites on an aggregate consisting of k antigen molecules and k-1 bivalent antibody molecules, the minimum number required to hold the aggregate together.

The condition given by Equation 3 implies a constant reacted fraction of antigen sites p. This can be shown by expressing p in terms of m_{ijk} as

$$p = \frac{1}{fG} \sum_{i,j,k} [2(k-1) + q + j] m_{ijk} = \frac{A + D + G - M}{fG} \qquad (5)$$

since the number of reacted antigen sites in an i,j,k-aggregate is 2(k-1)+q+j.

The differentiation is performed with the help of Stirling's approximation yielding

$$\sum_{i,j,k} \left(\log \frac{W_{ijk}}{i!\,j!\,k!} - \log m_{ijk} \right) \delta m_{ijk} = 0 \qquad (6)$$

On account of the restrictions imposed by Equations 2 and 3, the number of independent variables m_{ijk}, is reduced by four. Hence, four of the increments dm_{ijk}, are functions of the remaining ones and can be eliminated with the use of Equations 2 and 3 in differential form. This can be accomplished by adding the following equations to Equation 6 and choosing the four constants τ, η, ξ, and B, known as Lagrangean undetermined multipliers, so that the coefficients of four of the increments vanish(19).

$$\begin{aligned} \log \tau \sum_{i,j,k} i\, dm_{ijk} &= 0 \\ \log \eta \sum_{i,j,k} j\, dm_{ijk} &= 0 \\ \log \xi \sum_{i,j,k} k\, dm_{ijk} &= 0 \\ \log B \sum_{i,j,k} dm_{ijk} &= 0 \end{aligned} \tag{7}$$

Since all of the remaining increments are independent, their coefficients can be made to vanish separately. Therefore, the most probable distribution becomes

$$m_{ijk} = \frac{W_{ijk}}{i!j!k!} \tau^i \eta^j \xi^k B \tag{8}$$

The constants $\tau, \eta, \xi,$ and B can be evaluated after performing the summations indicated in Equations 2 and 3. To sum these expressions W_{ijk} is needed. In the Appendix it is found to be

$$W_{ijk} = f^k 2^i \frac{(fk-k)!}{(fk-2k+2-g-j)!} \frac{i!}{g!} \tag{9}$$

If the running index i is replaced by q the summing can be accomplished in the following manner, where Equation 3 is used as a typical example.

$$M = \frac{B}{2\tau} \sum_{k=0}^{\infty} \frac{(f\xi 2\tau)^k (fk-k)!}{(fk-2k+2)!\, k!} \tag{10}$$

$$\times \sum_{g=0}^{fk-2k+2} \frac{(2\tau)^g (fk-2k+2)!}{(fk-2k+2-g)!\, g!} \sum_{j=0}^{fk-2k+2-g} \frac{\eta^j (fk-2k+2-g)!}{(fk-2k+2-g-j)!\, j!}$$

Extending the maximum value of k to infinity for the purpose of summation involves negligible error. After evaluating the term for k zero, which yields $\tau + \eta$, the sums over j and q are accomplished with the use of the binomial theorem.

$$\sum_{g=0}^{fk-2k+2} \frac{(2\xi)^g (fk-2k+2)!}{(fk-2k+2-g)!\,g!} \sum_{j=0}^{fk-2k+2-g} \frac{\eta^j (fk-2k+2-g)!}{(fk-2k+2-g-j)!\,j!}$$

$$= (1+\eta)^{fk-2k+2} \sum_{g=0}^{fk-2k+2} \left(\frac{2\xi}{1+\eta}\right)^g \frac{(fk-2k+2)!}{(fk-2k+2-g)!\,g!} \qquad (11)$$

$$= (1+\eta+2\xi)^{fk-2k+2}$$

Equation 10 then becomes

$$M/B = \xi+\eta + \frac{(1+\eta+2\xi)^2}{2\xi} \sum_{k=1}^{\infty} \frac{y^k (fk-k)!}{(fk-2k+2)!\,k!} \qquad (12)$$

$$y = f\xi 2\xi (1+\eta+2\xi)^{f-2}$$

The sum over k in Equation 12 as well as the corresponding ones in Equation 2 can be expressed in the following way.

$$S_i \equiv \sum_{k=1}^{\infty} k^i y^k \frac{(fk-k)!}{(fk-2k+2)!\,k!} \; ; \quad i = 0, 1 \qquad (13)$$

Stockmayer has summed this expression for i zero, one, and two in the paper in which he describes this method for the most probable distribution of branch-chain polymers(18). He obtained the results

$$S_0 \equiv \frac{\alpha(1-\alpha f/2)}{(1-\alpha)^2 f};$$

$$S_1 \equiv \frac{\alpha}{(1-\alpha)^2 f}; \quad y = \alpha(1-\alpha)^{f-2} \tag{14}$$

$$S_2 \equiv \frac{\alpha(1+\alpha)}{f(1-\alpha)^2[1-(f-1)\alpha]};$$

Consequently, Equations 2 and 3 yield

$$M/B = \jmath + \eta + \frac{(1+\eta+2\jmath)^2}{2\jmath} \frac{\alpha(1-\alpha f/2)}{(1-\alpha)^2 f}$$

$$G/B = \frac{(1+\eta+2\jmath)^2}{2\jmath} \frac{\alpha}{(1-\alpha)^2 f} \tag{15}$$

$$D/B = \eta + \eta \frac{(1+\eta+2\jmath)}{2\jmath} \frac{\alpha(f-2)}{(1-\alpha)^2 f} + \eta \frac{(1+\eta+2\jmath)}{\jmath} \frac{\alpha(1-\alpha f/2)}{(1-\alpha)^2 f}$$

$$A/B = \jmath + \frac{(1+\eta+2\jmath)}{2\jmath}[1+\eta+2\jmath(f-1)] \frac{\alpha}{(1-\alpha)^2 f}$$

$$-(1+\eta-2\jmath) \frac{1+\eta+2\jmath}{2\jmath} \frac{\alpha(1-\alpha f/2)}{(1-\alpha)^2 f}$$

The Lagrangean undetermined multipliers are found rather tediously to be

$$\zeta = \frac{\rho p\,(1-\rho p r)}{2\,(1-p)}$$

$$\eta = (1-\rho)\,\frac{p}{1-p}$$

$$\xi = \frac{\alpha(1-p)^{f-1}}{f\,(\rho p-\alpha)} \qquad (16)$$

$$B = \frac{fG(1-p)(1-\rho p r)}{\rho p r}$$

in which

$$\alpha = \rho^2 p^2 r \qquad (17)$$

α is the probability that an antigen site has reacted with a bivalent antibody molecule, the other site of which has also reacted.

With the use of Equations 16 and 17, the distribution given in Equation 8 becomes

$$M_{ijk} = fG\,\frac{(fk-k)!}{(fk-2k+2-g-j)!\,k!\,g!\,j!}\; r^{k-1}\,\rho^{k+i-1}$$

$$\times\, p^{k+i+j-1}\,(1-p)^{fk-k-i-j+1}\,(1-\rho p r)^{i-k+1}\,(1-\rho)^{j} \qquad (18)$$

$$i = k-1+g$$

$$0 \le g \le fk-2k+2$$

$$0 \le j \le fk-2k+2-g$$

Therefore, the number of every kind of aggregate in the system including the free antibody and antigen molecules can be determined if the composition of the system, valence of the antigen, and extent of reaction are known.. The distribution reduces to one for a system consisting of bivalent antibody molecules and f-valent antigen molecules alone, if ρ and j are given the values unity and zero respectively.

It should be noted that the terms in the sum of Equation 13 have important properties. These properties will be explained on the basis of their physical implications. From Equation 14, one finds that y has a maximum value given by

$$\alpha_c = \frac{1}{f-1} \qquad (19)$$

$$y_c = \frac{(f-2)^{f-2}}{(f-1)^{f-1}}$$

The point at which this occurs will hereafter be designated the critical point and indicated with the subscript c. The most probable distribution m_{ijk}, was obtained for a fixed extent of reaction, or a fixed value of y. Once m_{ijk} was obtained, however, y could take on many values each corresponding to the system for a definite value of p. Therefore, as the antibody-antigen reactions proceed, p becomes larger and larger. The value of y

increases in a corresponding fashion up to the critical point where it passes through a maximum. In order to understand the nature of the system at the critical point it is convenient to evaluate the total number of aggregates m_k, containing k antigen molecules. This can be done by summing the distribution m_{ijk}, over all allowed values of i and j.

$$m_k = \sum_{i,j} m_{ijk} = fG \frac{(fk-k)!}{(fk-2k+2)!\,k!} \times \alpha^{k-1}(1-\alpha)^{fk-2k+2} \tag{20}$$

Then the rate of change of m_k with respect to α is found to be

$$\left(\frac{\partial m_k}{\partial \alpha}\right)_k = fG \frac{(fk-k)!}{(fk-2k+2)!\,k!}\, \alpha^{k-2}(1-\alpha)^{fk-2k+1} \times \left\{ k[1-\alpha(f-1)] - (1+\alpha) \right\} \tag{21}$$

Equation 21 shows that the number of k-aggregates for k unity is decreasing from the very start of the reaction. The numbers of a ggregates for all other values of k are increasing for sufficiently small values of α. As the reactions proceed, that is, for a somewhat larger value for α, m_2 begins to decrease, later m_3 begins to

decrease, and so on. In other words, the aggregates continue to build up into larger aggregates as the reactions proceed. Just preceding the critical point all m_k except those for the largest k values are decreasing. Finally, at the critical point and beyond, $(\partial m_k/\partial\alpha)_k$ is negative for all values of k. This means, ofcourse, that all sizes of aggregates are disappearing at the critical point. In a real system this cannot be true, however, since the very largest of the aggregates must be growing in size. The reason for this difficulty is that the sum over all finite values of k was replaced by the sum extending the k values to infinity. This implies that aggregates can be infinite and for these, $(\partial m_k/\partial\alpha)_k$ would not be negative. So, although the physical picture is clear, there is this difficulty with the model. It can be avoided to some extent by discussing the relative magnitudes of the rates of disappearance of the aggregates. With the use of Stirling's approximation Equation 21 becomes

$$\left(\frac{\partial m_k}{\partial\alpha}\right)_k = G\left(\frac{y}{y_c}\right)^k \frac{1}{k^{5/2}} \cdot \frac{(1-\alpha)}{\alpha^2} \qquad (22)$$

$$\times \left\{k\left[1-\alpha(f-1)\right]-(1+\alpha)\right\} \frac{f e^2 (f-1)^{1/2}}{\left[2\pi(f-2)^5\right]^{1/2}};$$

$$k \gg 1$$

It is obvious that at the critical point the rate of disappearance of the very largest aggregates is negligible compared to the rate of disappearance of relatively small aggregates. The difference beyond the critical point is even greater, since y is a maximum at y_c. Therefore, all aggregates are growing into a few exceedingly large ones. The bulk of the system is in these few. Equation 20 yields with the use of Stirling's approximation

$$m_k = G\left(\frac{y}{y_c}\right)^k \frac{1}{k^{5/2}} \cdot \frac{(1-\alpha)^2}{\alpha} \frac{f^2(f-1)^{1/2}}{[2\pi(f-2)^5]^{1/2}}; \qquad (23)$$

$$k \gg 1$$

The changes in the numbers of aggregates which occur in the region of the critical point are relatively little for small aggregates, while they are tremendous for large aggregates. The critical point is, therefore, characterized by the fact that the system at this point is changing from one composed chiefly of small aggregates into one composed of relatively few exceedingly large aggregates.

It should be mentioned that on account of the $k^{5/2}$ in the denominator of Equation 23, S_0 and S_1 of Equation 14 can be used beyond the critical point, but S_2 cannot since it becomes infinite at that point, that is, the corresponding series diverges.

The fact that neither the fraction of reacted antigen

sites nor the fraction of reacted antibody sites can exceed unity is expressed by the following relations

$$p \leq 1$$
$$\rho p n \leq 1 \qquad (24)$$

Furthermore, from Equation 17, p_c is found to be

$$p_c = \left(\frac{\alpha_c}{n \rho^2}\right)^{1/2} = \frac{1}{\rho}\left[\frac{2}{f(f-1)} \cdot \frac{A}{G}\right]^{1/2} \qquad (25)$$

The extent of reaction at which the material passes into the form of very large aggregates is dependent on the valence of the antigen and the composition of the system. Equations 19, 24, and 25 yield the interesting result

$$\frac{f}{2(f-1)} \leq \frac{A}{G} \leq \frac{f(f-1)}{2}\rho^2 \qquad (26)$$

for the attainment of p_c. If the system is prepared in such a manner that the bivalent antibody-antigen ratio lies outside the limits given by Equation 26, then p_c can never be reached. Analogous limits exist for any other fixed value of α. If the attainment of p_c is required for precipitation to occur, then Equation 26

predicts that the regions outside the above limits are the antigen excess and antibody excess inhibition zones, in which precipitation does not occur. It further predicts that the beginning of the inhibition zone of antibody excess but not antigen excess is altered by altering the value of ρ. An increase in the amount of univalent antibody in the system decreases the range of antibody-antigen ratios over which precipitation occurs. The univalent antibody acts as an inhibitor. Equation 26 also predicts that in a system of bivalent antibody and bivalent antigen there is only one antibody-antigen ratio, namely, unity, for which the critical point can be reached. It, therefore, gives theoretical grounds for the interesting experimental fact that it is difficult to obtain precipitation in a system of this kind. Figure 1 illustrates how these limiting antibody-antigen ratios are affected by the valence of the antigen for values of unity and one-half. The differences between the corresponding ordinate values for the upper and lower limits give the range of ratios for which the critical point can be attained. This range increases as the valence of the antigen increases.

Another interesting but not surprising feature of these reactions is given by Equation 20. The number of aggregates m_k, each of which has the same number of antigen molecules, is independent of the amount of antibody in the system at the critical point. This is also true for

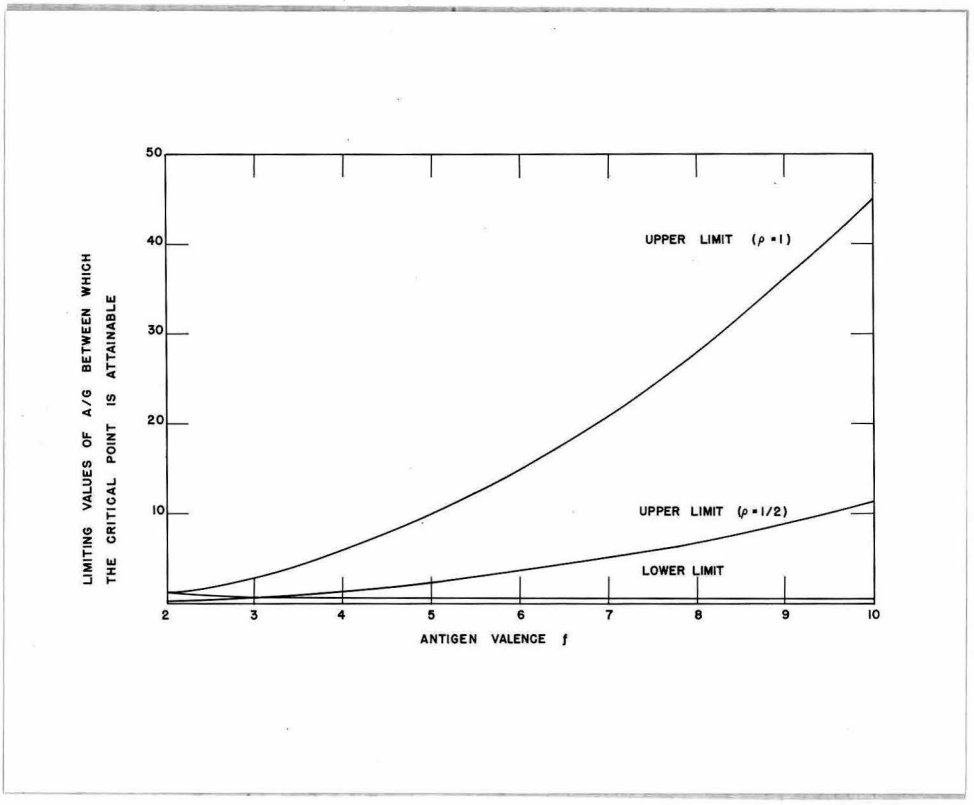

Figure 1

The effect of antigen valence on the critical point limits. These limits are defined by

$$\text{(lower limit)}, \quad \frac{f}{2(f-1)} \leq A/G \leq \frac{f(f-1)}{2}\rho^2, \text{(upper limit)}$$

any other fixed value of α. It can be seen there that each system of a set with the same constituents having a different value of A but the same value of G and α, has the same number of aggregates m_k, for each k. The differences lie only in the numbers of antibody molecules in these aggregates occupying positions other than between two antigen molecules. These differences can be determined from the average numbers of bivalent and univalent antibody molecules, $\bar{i}_k$ and $\bar{j}_k$ respectively, in a k-aggregate. They are

$$\bar{i}_k = \frac{\sum_{i,j} i\, m_{ijk}}{\sum_{i,j} m_{ijk}} = k-1+(fk-2k+2)\frac{\rho\beta-\alpha}{1-\alpha}$$

$$\bar{j}_k = \frac{\sum_{i,j} j\, m_{ijk}}{\sum_{i,j} m_{ijk}} = (fk-2k+2)\beta\frac{1-\rho}{1-\alpha} \qquad (27)$$

As the reactions proceed $\bar{i}_k$ and $\bar{j}_k$ both increase. They depend on the extent of the reaction p, which is different for each of the systems in the set considered above. The average number of antibody molecules of both kinds found in a k-aggregate is

$$\bar{i}_k + \bar{j}_k = k-1+(fk-2k+2)\frac{\beta-\alpha}{1-\alpha}, \qquad (28)$$

the same number as there would be for $\bar{i}_k$ alone if there

were no univalent antibody present. In other words, the univalent antibody acts like bivalent antibody of which only one site is used. This means that one can determine the correct average total antibody in a k-aggregate by using a hypothetical system which contains no univalent antibody. In this hypothetical system, however, the k-aggregate may not be the one of interest since it is formed more easily than that in the real system. That is to say, in order to attain the same value of α in a system with ρ unity as in one with ρ less than unity, p need have a correspondingly smaller value in the former than in the latter, since ρ and p are inversely proportional to one another.

An interesting expression obtainable from Equation 27, which gives the average fraction of the free sites on an aggregate belonging to antibody molecules, is

$$\frac{\bar{g}_k}{fk-2k+2-\bar{j}_k} = \frac{\rho p - \alpha}{1-\alpha-p(1-\rho)} \; ; \; \bar{g}_k = \bar{i}_k - k + 1 \qquad (29)$$

This fraction is independent of k, and it is, therefore, the same for all aggregates no matter how many antigen molecules are in them. It can be used to determine the effect of composition on the probability of combination of two aggregates. When this fraction is unity, or when it vanishes, the probability for combination vanishes

and Equation 26 is deduced.

The average antibody-antigen ratios of all aggregates containing k antigen molecules are also obtainable from Equation 27. They are

$$\bar{i}/k = 1 + (f-2)\frac{\rho p - \alpha}{1-\alpha};$$

$$\bar{j}/k = (f-2)p\frac{1-\rho}{1-\alpha}; \quad k >> 1 \qquad (30)$$

$$(\bar{i}+\bar{j})/k = 1 + (f-2)\frac{p-\alpha}{1-\alpha};$$

It should be noted that these ratios are independent of k. Therefore, in a given system for a particular extent of reaction the average antibody-antigen ratio is the same for all large aggregates. These ratios increase as the extent of the reaction increases and attain their maximum values when p has its maximum value, over most of the range of composition of interest. This value is f-1 for the last ratio and also for the first ratio if ρ is unity.

Equation 4 can also be used to calculate certain ratios.

$$(i/k)_{max} = 1 + \frac{g_{max} - 1}{k} = f - 1 + \frac{1}{k}$$

$$(j/k)_{max} = f - 2 + \frac{2 - g_{min}}{k} = f - 2 + \frac{2}{k} \qquad (31)$$

$$g_{max} = fk - 2k + 2 \; ; \; g_{min} = 0$$

These ratios are exact for all values of k and can be used to determine the valence of the antigen f, from experimental data. An equivalence ratio can be defined from Equation 4 as

$$(i/k)_e = 1 + \frac{g_e - 1}{k} = \frac{f}{2}$$

$$g_e = g_{max}/2 \qquad (32)$$

Therefore, from Equation 31

$$(i/k)_{max} = 2\,(i/k)_e - 1 + \frac{1}{k} \qquad (33)$$

which for large values of k strongly resembles Pauling's expression which relates the antibody-antigen molecular ratio of a precipitate in the antibody excess region to the corresponding ratio in the equivalence zone(5).

At the critical point Equations 30 reduce to

$$(\bar{i}/k)_c = \left[\frac{2(f-1)}{f} \cdot \frac{A}{G}\right]^{1/2};$$

$$(\bar{j}/k)_c = \left(\frac{1}{p}-1\right)\left[\frac{2(f-1)}{f} \cdot \frac{A}{G}\right]^{1/2}; \quad k \gg 1 \qquad (34)$$

$$\left[(\bar{i}+\bar{j})/k\right]_c = \frac{1}{p}\left[\frac{2(f-1)}{f} \cdot \frac{A}{G}\right]^{1/2};$$

These ratios for i,j,k-aggregates are independent of concentration and increase as the bivalent antibody-antigen ratio for the system increases. Variations in the number of univalent antibody molecules present do not affect the top ratio. This appears reasonable since a k-aggregate at the critical point would have required a particular number of bivalent antibody molecules to form it.

If an expression for the theoretical maximum value of p is substituted into Equation 30 the corresponding ratios designated with the subscript max can be obtained. The maximum value of p is, from Equation 5, obviously

$$p_{max} = \frac{A + G + D - M_{min}}{fG} \qquad (35)$$

$$= \frac{1}{n}\left(\frac{1}{p} - \frac{1}{2}\right) + \frac{1}{f}\left(1 - \frac{M_{min}}{G}\right),$$

where M_{min} is the lowest possible value of M which can be calculated for the system. For example, when ρ is unity,

$$M_{min}/G \simeq 0 \; ; \qquad 1 \leq A/G \leq f-1$$

$$M_{min}/G \simeq A/G-(f-1) \; ; \qquad A/G \geq f-1 \qquad (36)$$

$$M_{min}/G \simeq 1 - A/G \; ; \qquad A/G \leq 1$$

When univalent antibody is not present in the system, it is found that

$$(\bar{i}/k)_{max} = \frac{(f-3)(A/G)^2 - 2(f-1)(A/G) - (f-1)}{2(f-1)A/G - (A/G)^2 - 1} \; ; \qquad 1 \leq A/G \leq f-1 \qquad (37)$$

$$(\bar{i}/k)_{max} = f-1 \; ; \qquad A/G \geq f-1$$

The relation between $\bar{i}/k$ and A/G is shown for the critical extent of reaction p_c, and the maximum extent of reaction p_{max}, in Figure 2.

The Heidelberger-Kendall equation, which expresses the total amount of antibody combined in terms of the composition of the system and the valence of the antigen, is in the notation here

$$A_b = fG - \frac{f^2G^2}{4A} \; , \qquad (38)$$

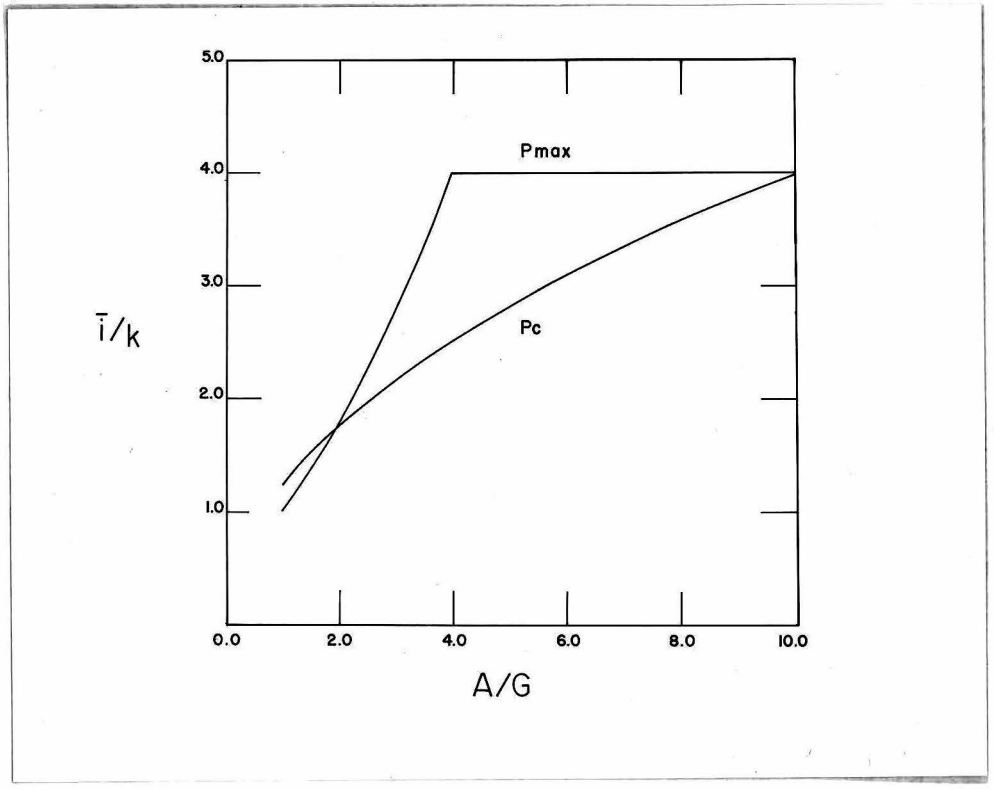

Figure 2

The relation between $\bar{i}/k$ and A/G is shown for the critical extent of reaction p_c, and the maximum extent of reaction p_{max}. $\bar{i}/k$ is the average antibody-antigen ratio of all aggregates containing k antigen molecules. A/G is the antibody-antigen ratio of the entire system. The antibody molecules referred to here are bivalent. A valence of five is assumed for the antigen.

where A_b is the total number of bivalent antibody molecules bound in one form or another(7). It is usually written in terms of grams rather than numbers of molecules and A_b/G is assumed to be the antibody-antigen ratio of the precipitate. The distribution m_{ijk} can be used quite simply to obtain their result

$$A_b = A - m_{100} = fG\rho\beta - \frac{f^2G^2}{4A}\rho^2\beta^2$$

$$D_b = D - m_{010} = \frac{D}{2A+D} fG\beta \qquad (39)$$

If univalent antibody is present, Equation 38 cannot be obtained since p cannot have a value greater than unity. If ρ_p is taken to be unity, however, the top one of the Equations 39 reduces to the Heidelberger-Kendall equation. p can be unity only for the region of extreme antibody excess, namely, where the antibody-antigen ratio of the system is no less than the maximum ratio f-1, of an i,k-aggregate. This is readily shown from Equations 35 and 36. Even in this region of extreme antibody excess the attainment of p_{max} is certainly questionable.

Equations 39 at the critical point reduce to

$$(A_b/G)_c = \left[\frac{2f}{f-1} \cdot \frac{A}{G}\right]^{1/2} - \frac{f}{2(f-1)}$$

$$(D_b/G)_c = \left[\frac{f}{2(f-1)} \cdot \frac{D^2}{AG}\right]^{1/2} \qquad (40)$$

The top equation indicates that the total number of bound bivalent antibody molecules is not dependent on the number of univalent antibody molecules present. This should be expected in the light of Equation 34. In a linear plot of $(A_b/G)_c$ against G, as is generally the case with experimental results in the antibody excess region, Equations 40 predict that the negative slope of the curve should increase as the corresponding values of G decrease. Figure 3 illustrates this point well with the curve labeled G. Therefore, on this basis one can expect increasing deviation from the Heidelberger-Kendall equation with decreasing amounts of G(as antibody excess increases). Since this point has been verified, it places the Heidelberger-Kendall equation on a rather weak foundation. Equations 40 are not required to obtain this result. Any other set of values for p in Equation 36 will give the same effect, provided α is assumed to be independent of composition, an assumption which is not unreasonable in view of the interpretation of α. The appropriate

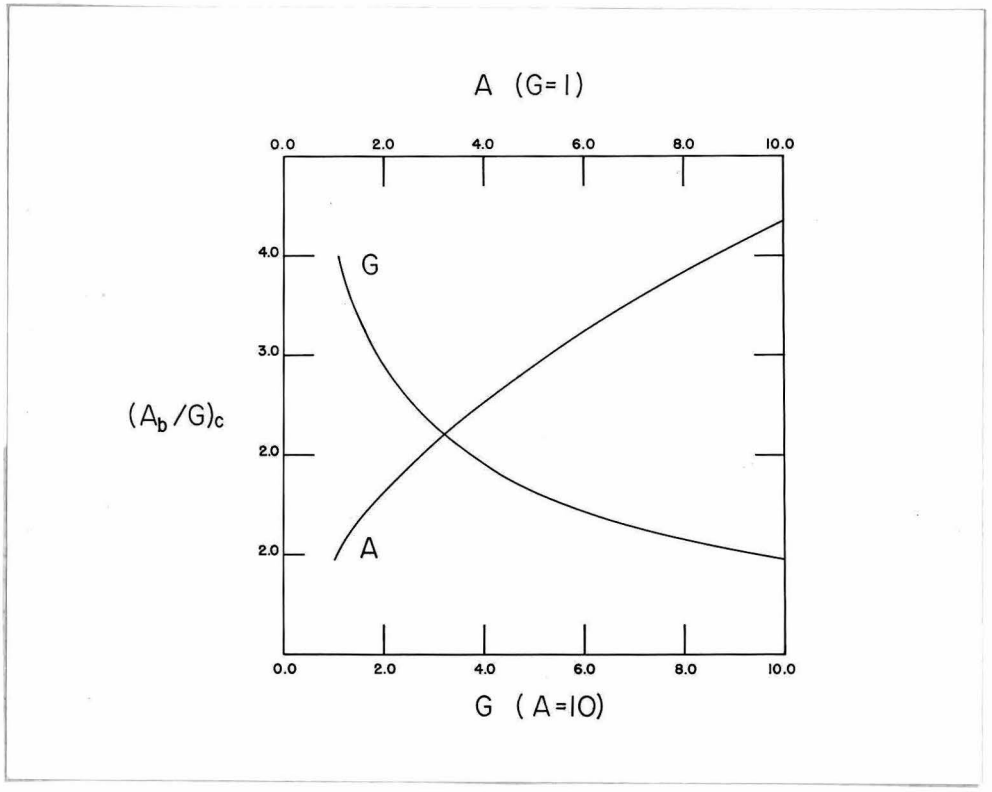

Figure 3

Variations in $(A_b/G)_c$ with increasing amounts of A and G are shown for the critical extent of reaction. $(A_b/G)_c$ is the ratio of bound bivalent antibody to total antigen at the critical point. The curve labeled A refers to the upper abscissa which represents additions of bivalent antibody to the system. The curve labeled G refers to the lower abscissa which represents additions of antigen to the system. A valence of five is assumed for the antigen.

dilutions of antigen in an experiment of this kind should be determined, therefore, from the fact that A_b/G depends on G in an inverse manner.

Although there is a theoretical basis for the use of Equation 40 over the entire range of precipitation in contrast to the Heidelberger-Kendall equation, nevertheless the quantity A_b/G is not the one of interest since, obviously, considerable antigen as well as bound antibody is not precipitated in the region of antigen excess. The expressions which would be more appropriate, however, are those for $\bar{i}/k$ or $(\bar{i}+\bar{j})/k$ given by Equations 30, 34, and 37, since they determine the antibody-antigen ratios for aggregates. They do not require the number of free antigen molecules in the system to be negligible. They have the same dependence on A/G as A_b/G does, so that the criticism mentioned above is still valid.

The weight-average molecular weight of a system composed of bivalent antibody molecules and f-valent antigen molecules is defined by

$$\langle M \rangle_w = \sum_{i,k} M_{ik} f_{ik}^{(w)} \quad ; \tag{41}$$

$$f_{ik}^{(w)} = \frac{M_{ik} M_{ik}}{\sum_{i,k} M_{ik} M_{ik}} \quad ; \quad M_{ik} = i M_A + k M_G$$

The double sum here can be expanded and evaluated from Equation 14. The result, after considerable algebra, is

$$\langle M\rangle_w \sum_{i,k} M_{ik}\, w_{ik} = M_A^2 (A-G) - 2 M_A M_G G + \frac{(M_A+M_G)^2 G}{1-(f-1)\,r\rho^2}$$
$$+ \left[\frac{M_A^2 + M_A M_G}{1-(f-1)\,r\rho^2} - M_A^2\right] 2 f G \rho + M_A^2 f G r \rho^2$$
$$+ \left\{ M_A^2 f (1-r\rho)^2 \left[\frac{1+4r\rho^2-(r\rho^2)^2}{(1-r\rho^2)^2} + f\right]\right.$$
$$\left. + M_G^2 - M_A^2 (2f-1) - 2 M_A M_G (f-1) \right\} \frac{G\rho^2}{1-(f-1)r\rho^2} \qquad (42)$$

The number-average molecular weight of the system is

$$\langle M\rangle_n = \sum_{i,k} M_{ik}\, f_{ik}^{(n)} = \frac{A M_A + G M_G}{A + G - f G \rho}\ ;$$

$$f_{ik}^{(n)} = \frac{w_{ik}}{\sum_{i,k} w_{ik}} \qquad (43)$$

3. A Theory for Reactions of Multivalent Antigen Molecules with Univalent Antibody Molecules

It is of interest to discuss a theory here for reactions of a system containing univalent antibody molecules and f-valent antigen molecules. Although such theories of reaction have been discussed before, they have not been approached from the point of view adopted here(20). The results of this theory, together with those of the previous one, will indicate how valid the basis for Hershey's theory is, in which it is assumed that the reactions leading to the formation of specific aggregates are not influenced by further aggregation.

As before

$$\Omega = D!\,G! \prod_{j,k} \left[\left(\frac{w_{jk}}{j!} \right)^{m_{jk}} \frac{1}{m_{jk}!} \right] \qquad (44)$$

$$k = 0, 1$$

$$j = kg + 1$$

$$-1 \leq g \leq f-1$$

where all symbols except q have the same significance as before. The most probable distribution is determined under the following conditions

$$\sum_{j,k} j m_{jk} = D \; ; \; \sum_{j,k} k m_{jk} = G$$

$$\sum_{j,k} m_{jk} = M \qquad (45)$$

where the values for the running indices are described in Equation 44. Again constant D, G, and M imply constant p.

$$p = \frac{D + G - M}{+G} \qquad (46)$$

With the aid of Lagrangean undetermined multipliers ε, β, and γ, the most probable distribution is found to be

$$m_{jk} = \frac{w_{jk}}{j!} \varepsilon^{j} \beta^{k} \gamma \qquad (47)$$

Since there are f(f-1)...(f-j+1) ways to place j antibody molecules on one antigen molecule,

$$W_{jk} = \left[\frac{f!}{(f-j)!}\right]^{k} \quad ; \quad k = 0, 1 \qquad (48)$$

In straight forward fashion Equations 45 are evaluated using Equations 47 and 48.

$$\begin{aligned} D/\gamma &= \varepsilon + 2\beta f (1+\varepsilon)^{f-1} \\ G/\gamma &= \beta (1+\varepsilon)^{f} \\ M/\gamma &= \varepsilon + \beta (1+\varepsilon)^{f} \end{aligned} \qquad (49)$$

In terms of p and r, where now r = fG/D

$$\begin{aligned} \varepsilon &= \frac{p}{1-p} \\ \beta &= \frac{rp(1-p)^{f-1}}{f(1-rp)} \\ \gamma &= \frac{(1-p)(1-rp)+G}{rp} \end{aligned} \qquad (50)$$

Therefore, the most probable distribution becomes

$$m_{jk} = fG\left[\frac{(f-1)!}{j!\,(f-j)!}\right]^{k} p^{j+k-1}(1-p)^{fk+k-j+1} \times r^{k-1}(1-rp)^{1-k} \qquad (51)$$

$$k = 0, 1$$
$$j = kg + 1$$
$$-1 \leq g \leq f-1$$

If for thermodynamic equilibrium values of m_{jk} and p, K_j is defined by

$$K_j = \frac{m_{j1}}{m_{10}\, m_{j-1,1}}, \qquad (52)$$

then with the use of Equation 51, one obtains the familiar results(20)

$$K_j = \frac{K_1}{f} \cdot \frac{f-j+1}{j}; \qquad (53)$$

$$\frac{K_1}{f} = \frac{p}{m_{10}(1-p)}$$

and

$$\frac{\sum_{j=1}^{f} j m_{j1}}{\sum_{j=1}^{f} m_{j1}} = \frac{K_1 m_{10} (1 + K_1 m_{10}/f)^{f-1}}{(1 + K_1 m_{10}/f)^{f} - 1} \qquad (54)$$

Equation 54 gives the equilibrium ratio of the number of bound antibody molecules to the number of bound antigen molecules. It is clear that this system at equilibrium is a special case of Equation 51.

The weight-average and number-average molecular weights are found, with the aid of Equation 51, to be

$$\langle M \rangle_w \sum_{j,k} M_{jk} m_{jk} = D M_D^2 + G M_G^2 + 2 M_D M_G f G \beta + M_D^2 f(f-1) G \beta^2 \qquad (55)$$

$$\langle M \rangle_n = \frac{D M_D + G M_G}{D + G - f G \beta}$$

Differences between the two theories presented here are manifested in a graph of weight-average molecular weight plotted against the extent of reaction. Figure 4 shows such a graph. It is convenient to subtract the weight-average molecular weight due to the system before

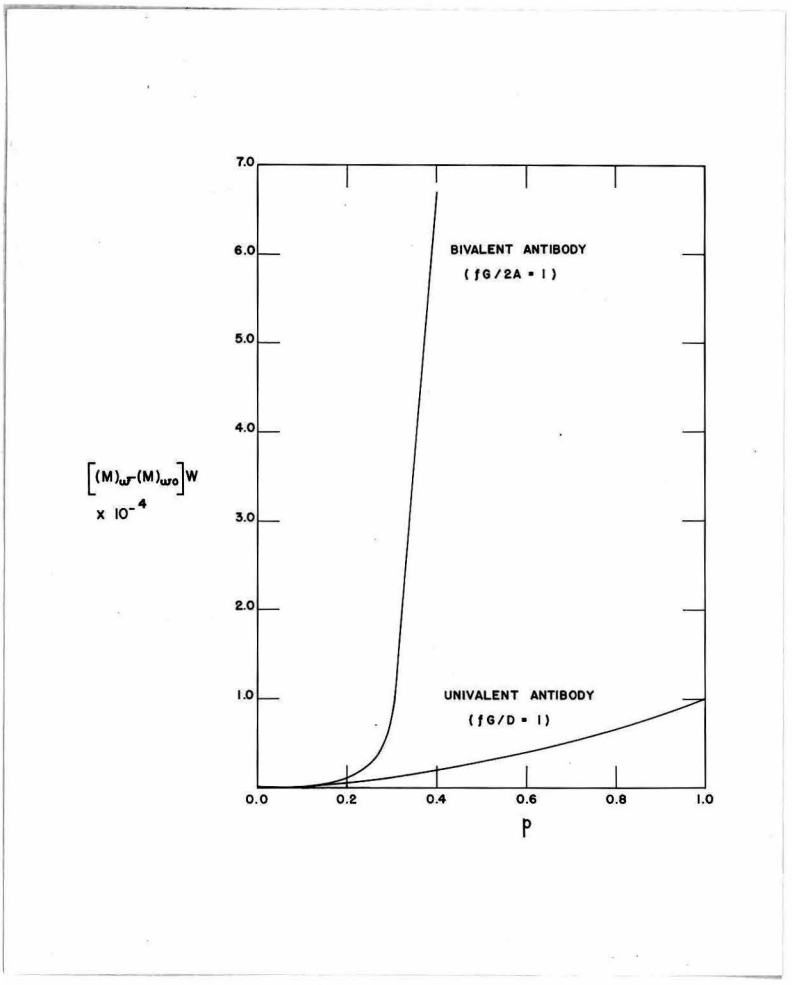

Figure 4

The effect of the extent of the reaction p, on a function of the weight-average molecular weight, $[\langle M\rangle_w - \langle M\rangle_{wo}]W$, for a system containing bivalent antibody and one containing univalent antibody. $\langle M\rangle_{wo}$ is the weight-average molecular weight of the system for p zero. W is the mass of the system.

any reaction has taken place $\langle M \rangle_{wo}$, and multiply the result by the mass of the system W. It is obvious from Figure 4 that Hershey's assumption is not realistic, since the large aggregates have a predominant effect on the system even at small extents of reaction.

4. Experimental Evidence for the Theory for Reactions of Multivalent Antigen Molecules with Bivalent and Univalent Antibody Molecules

Experimental evidence from four antibody-antigen systems has been selected for examination in the light of the bivalent antibody theory presented here. These systems were chosen because they are sufficiently well defined to be treated theoretically. All antibody present in each system is assumed to be bivalent. The antibody-antigen ratio of the precipitate is compared with values for $\bar{i}/k$ for the maximum extent of reaction and the critical extent of reaction. If the critical point is the point at which precipitation is initiated, then the value of the antibody-antigen ratio of the precipitate should lie between the corresponding values of $(\bar{i}/k)_{max}$ and $(\bar{i}/k)_c$. In order to evaluate the last two ratios, the composition of the system and the valence of the antigen must be known. The experimental data used here were obtained from the usual kind of titration experiments (21,22,23). A titration experiment is generally set up in the following manner. A series of test tubes is set

up, each with a known amount of antigen nitrogen. The amount of antigen varies logarithmically with the tube number. The same amount of antiserum is added to each tube. The system in each tube differs from the others, therefore, only in the amount of antigen present, and in a definite way. Systems differing only in this way will be referred to as a set. The number of antigen molecules G, in each system was determined from the amount of antigen nitrogen added and the amount of nitrogen per antigen molecule(the nitrogen factor). The number of antibody molecules in each system was estimated in the following way. The total nitrogen precipitated was plotted against the antigen nitrogen in the system for a particular set of systems. The point on the curve which represented the maximum amount of antibody precipitated in the set was used to calculate a trial value for the number of antibody molecules in the system A', with the aid of the appropriate nitrogen factor. This trial value for A and the corresponding value for G were substituted into the following equation to determine the fraction of antibody molecules in the system which were free.

$$m_{10}/A = (1 - r\rho_{max})^2 = \frac{1}{4}\left(1 - \frac{G}{A'}\right)^2 \qquad (56)$$

Equation 56 follows from Equations 18, 35, and 36 where M_{min} is negligible. As a second approximation it was assumed that the antibody molecules were either free or in the precipitate. Therefore, the fraction of antibody molecules in the precipitate $1-m_{1o}/A$, was calculated from Equation 56 and put equal to A'/A. The value determined for A was then used as a new trial value for A', and the process was repeated until the value for A no longer changed.

The valence of the antigen f, was in all but one case determined from the experimental data, or the extrapolation of them which led to values for antibody-antigen ratios of the precipitates in the greatest possible antibody excess(24). From this information the antigen valence was calculated according to

$$(i/k)_{max} = f-1 \quad ; \quad k \gg 1 \qquad (57)$$

In the case of Diphtheria Toxin-Antitoxin the data on soluble complexes in antibody excess taken by Pappenheimer, Lundgren, and Williams(25) were applied to

$$(i/k)_{max} = f \quad ; \quad k = 1 \qquad (58)$$

Two horse antibody systems and two rabbit antibody

systems have been selected. The experimental values for the antibody-antigen ratios of the precipitates R, are compared to the values for $(\overline{i}/k)_{max}$ and $(\overline{i}/k)_c$, as shown in Tables 1, 2, 3, and 4.

The value for the valence of the antigen was used to calculate the theoretical limits imposed on the antibody-antigen ratio for attaining the critical point from Equation 26, with ρ unity. These limits are compared to those beyond which precipitation did not occur, in Table 5. It should be noted that precipitation can occur in both of these rabbit antibody systems for compositions at which the critical point is not attainable. The horse antibody systems show no such behavior. In fact, for these systems the precipitation limits are more restricting than the critical point limits. The theory is not to be interpreted, however, as requiring the precipitation limits to coincide with the critical point limits. If the critical point is required for precipitation to occur then the theory implies that precipitation cannot occur outside these limits. It does not predict what the limits will be. From the point of view of this theory the limits for the horse antibody systems may be as restricting as they are either on account of equilibrium requirements or the presence of univalent antibody. It is clear, however, that precipitation can occur before the critical point is reached in the rabbit antibody

Table 1

Theoretical and Experimental Values of Precipitate Ratios for Egg Albumin-Horse anti Egg Albumin

A/G	R	$(\bar{i}/k)_{max}$	$(\bar{i}/k)_c$
3.1	2.4*	2.8	2.2
2.6	2.3	2.3	2.0
2.2	2.0	2.0	1.9
2.1	1.9	1.9	1.8
1.8	1.6	1.7	1.7

*Precipitation was probably incomplete. The value of $(\bar{i}/k)_{max}$ corresponds to 88% of the antigen precipitating.

Table 2

Theoretical and Experimental Values of Precipitate Ratios for Diphtheria Toxin-Horse Antitoxin

A/G	R	$(\bar{i}/k)_{max}$	$(\bar{i}/k)_c$
2.9	2.7	2.5	2.2
2.6	2.4	2.2	2.1
2.3	2.1	2.0	2.0
1.7	1.6	1.6	1.7
1.3	1.2	1.2	1.5

Table 3

Theoretical and Experimental Values of Precipitate Ratios for Egg Albumin-Rabbit anti Egg Albumin

A/G	R	$(\bar{i}/k)_{max}$	$(\bar{i}/k)_c$
21	3.9	4.0	-
13	3.5	4.0	-
7.6	3.3	4.0	3.5
4.8	2.9	4.0	2.8
3.8	2.7	3.7	2.5
2.9	2.4	2.6	2.2
2.6	2.3	2.3	2.0
2.3	2.1	2.1	1.9

Table 4

Theoretical and Experimental Values of Precipitate Ratios for Horse Serum Albumin-Rabbit anti Serum Albumin

A/G	R	$(\overline{i}/k)_{max}$	$(\overline{i}/k)_c$
17	6.0	6.0	5.3
11	5.6	6.0	4.3
8.2	5.1	6.0	3.8
5.1	4.0	4.7	3.0
4.4	3.6	3.8	2.8
4.1	3.3	3.5	2.7
3.6	3.1	3.1	2.5
3.3	2.9	2.8	2.4

Table 5

A Comparison of Inhibition Zone Limits

and Critical Point Limits

System	Experimental limits beyond which precipitation does not occur	Theoretical limits beyond which the critical point is not attainable
Egg albumin-horse anti egg albumin	1+ ≤ A/G ≤ 9+	5/8 ≤ A/G ≤ 10
Diphtheria toxin-horse antitoxin	1 ≤ A/G ≤ 5	7/12 ≤ A/G ≤ 21
Egg albumin-rabbit anti egg albumin	1+ ≤ A/G ≤ 21	5/8 ≤ A/G ≤ 10
Horse serum albumin-rabbit anti serum albumin	? ≤ A/G ≤ ~41	7/12 ≤ A/G ≤ 21

systems studied here. One can interpret this to mean that this rabbit antibody is more insoluble than the horse antibody. Boyd has mentioned that horse anti-protein antibody molecules are more soluble than the corresponding rabbit antibody molecules, since a higher concentration of sodium or ammonium sulfate is required to precipitate the former than the latter(26). This feature of the critical point can, therefore, explain the differences in inhibition of these rabbit and horse systems. That is, one would expect to encounter much more difficulty in observing antibody excess inhibition in these rabbit antibody systems than in the horse antibody systems.

Figures 5 and 6 present an interesting experiment which might just briefly be mentioned. They represent somewhat of a three dimensional diagram of an Rh agglutination test(27). The abscissas give the ratio of inhibiting to agglutinating antibody molecules, a variable not ordinarily available to the experimenter. The ordinates give the usual antiserum dilutions. The amount of agglutination is expressed by the different kinds of crosshatching. In these figures the prozone is increased with increasing amounts of inhibiting antibody molecules until, finally, complete inhibition exists. This trend is not apparent when the relative amount of agglutinating serum present is small(the upper values of the ordinates). This is just the effect predicted by Equation 26 and Figure 1. Figures 5 and 6 correspond

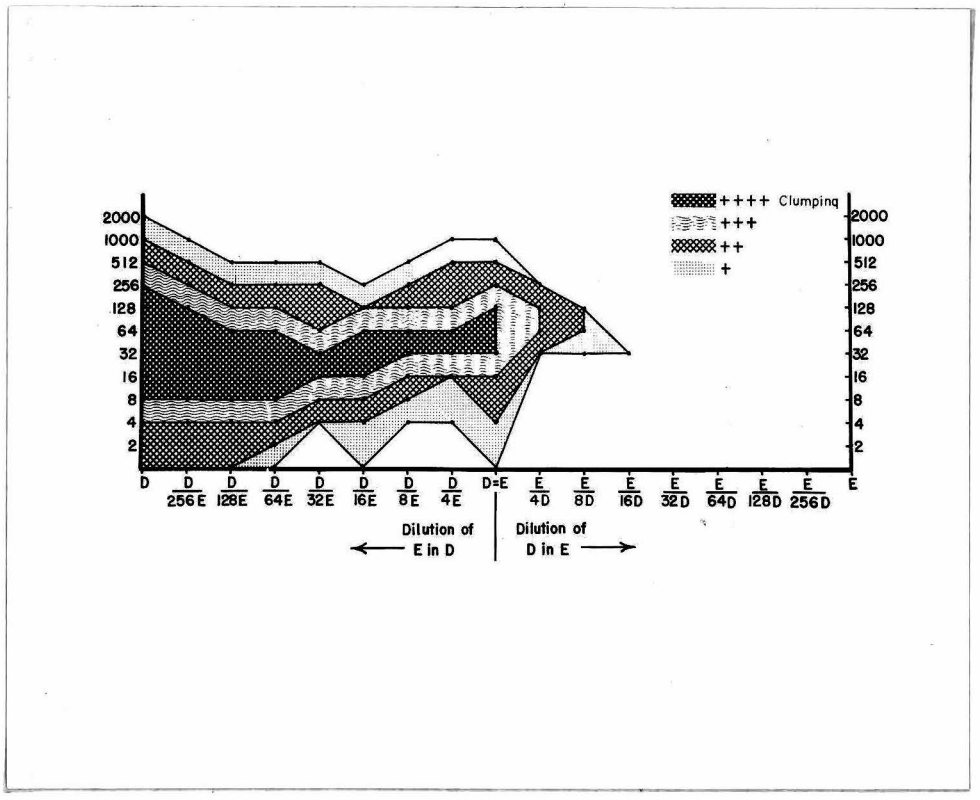

Figure 5

The effect of inhibiting antibody on Rh agglutination. D represents the serum containing agglutinating antibody. E represents the serum containing inhibiting antibody. Antiserum dilutions are indicated on the ordinate. The red blood cells used in this test were considered by Sturgeon to have the usual combining power.

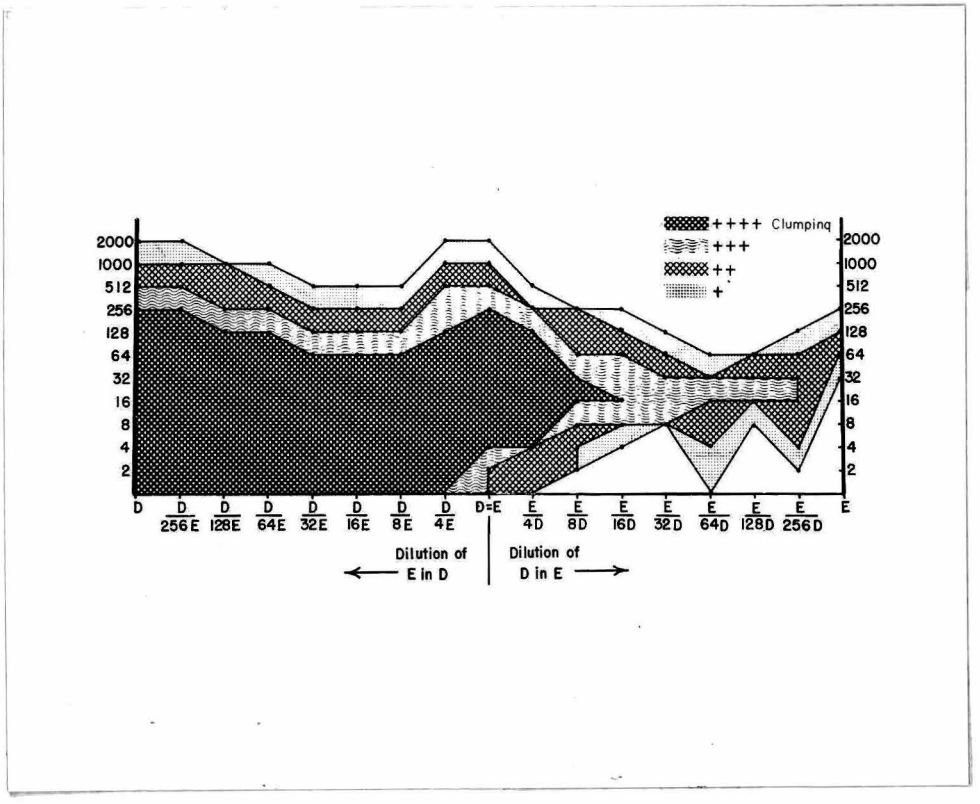

Figure 6

The effect of inhibiting antibody on Rh agglutination. D represents the serum containing agglutinating antibody. E represents the serum containing inhibiting antibody. Antiserum dilutions are indicated on the ordinate. The red blood cells used in this test were considered by Sturgeon to have an abnormal combining power.

to red blood cells of different origins. In performing these experiments, Sturgeon has noted that the red blood cells used in preparing the tests for Figure 6 had considerably more combining power than those corresponding to Figure 5(27). It is obvious that they cause a tremendous decrease in the prozone and, hence, tend to counteract the effect of the inhibiting antibody molecules. The point at which agglutination occurs at the upper end of the ordinate remained the same, however. Equation 26 and Figure 1 are again in full agreement. They demonstrate the insensitivity of the critical point composition to changes in the combining power of the antigen in the antigen excess region(lower limit). They do show a very large effect at the other end. An increase in antigen combining power f, for a fixed composition, reduces the antibody excess inhibition zone or prozone considerably. Therefore, the Sturgeon diagrams appear to be in good qualitative agreement with this theory.

Part II

The Light-Scattering Properties of an Antigen-Antibody Reaction

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THE LIGHT-SCATTERING PROPERTIES OF AN ANTIGEN-ANTIBODY REACTION[1]

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The utility of light scattering measurements on protein solutions has been demonstrated in the past for the determination of molecular weights. The results obtained by this method agree satisfactorily with those obtained by the usual procedures. An important early investigation was that of Putzeys and Brosteaux, who found that the light scattered by ovalbumin, amandin, excelsin and haemocyanin solutions, was proportional to the protein molecular weight. (1) More recently investigations of serum globulins and albumins have been made by Blaker (2). It has been shown that information concerning the size and shape of a protein molecule any dimension of which approaches the wave length of the light used can be obtained by scattering experiments (3). The equation developed by Einstein for a random arrangement of particles, small compared to the wave length of the scattered light (4) has been modified by Debye for the convenient treatment of real two-component systems (5). Debye modified Einstein's equation by introducing the osmotic pressure; his final equation may be written in the form:

$$H\frac{C}{\tau} = \frac{1}{M} + BC \tag{1}$$

where

$$H = \frac{32\pi^3}{3\lambda^4 N_0} n^2 \left(\frac{\partial n}{\partial c}\right)^2_{T,p}$$

C = weight concentration of solute.
n = refractive index of the system.
λ = wave length of light.
N_0 = Avogadro's number.
B = solvent-solute interaction constant (related to that in the osmotic pressure equation).
τ = excess turbidity of the solution over that of the solvent.
M = molecular weight of the solute.

When the particles whose average molecular weight is desired are not small compared to the wave length of the light, a correction factor is needed which may be obtained from light scattering data taken at angles symmetrical about an axis perpendicular to the incident light, if the approximate model for the

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[2] Contribution number 1440.

particle shape is assumed. These models in relation to the data required have been previously described by Zimm, Stein and Doty (6).

The turbidity of a solution can be defined as the fractional decrease in the incident light after transmission through one centimeter of fluid, provided that absorption is negligible. The excess turbidity arises from spontaneous concentration fluctuations; it should be a minimum when measured at 90° to the incident light. There is an increased amount of scattered light at 90°, however, on account of a small depolarization resulting from orientation fluctuations of the solute molecules. In fact, conclusions can be drawn about the anisotropy of polarizability of these molecules from depolarization data. This subject has been discussed by Born (7) and Mark (8). The correction for depolarization has been omitted from the equations presented in this paper.

Since Equation 1 was developed for a two-component system, the only justification for its use for protein solutions is that it appears to give the right answer for molecular weights, which turn out to be weight averaged. Hermans has discussed the reasons why this equation can be used for more than two components when electrostatic effects are considered (9). Usually B is positive, and in cases where B does not vanish data must be taken at different concentrations in order that the extrapolation to infinite dilution can be made in the plot of $H\frac{C}{\tau}$ against C, so that the molecular weight can be calculated. Theoretical treatments have been given for the turbidity of high molecular weight solutes polydispersed in solution (10, 11, 12). In these cases electrostatic effects have been subtracted and the result found by Kirkwood and Goldberg is the following:

$$H\frac{C}{\tau} = \frac{1}{<M>_{AV}} + \frac{C}{<M>^2_{AV}} \sum_{i,k=1}^{p} M_k A_{ik} f_i f_k$$

$$<M>_{AV} = \sum_{k=1}^{p} f_k M_k; \qquad C = \sum_{k=1}^{p} C_k \tag{2}$$

$$H = (32\pi^3/3N_0\rho_0\lambda^4)n^2(\partial n/\partial C)^2_{T,p}$$

$<M>_{AV}$ is the weight average molecular weight, and C is the total concentration of solute given in weight per unit weight of solvent; ρ_0 is the mass of solvent in unit volume, and A_{ik} is the thermodynamic interaction constant originating in the expansion of excess chemical potentials in terms of concentrations. A_{ik} expresses the influence of component k on the activity of component i. It is seen that if the coefficient of C on the right side of the equation remains constant in a given system, then the above expression reduces to Equation 1. In general, however, this is not the case.

It has been found that the pH of a solution affects its turbidity (2). In the case of borate buffer at pH 8.3, B vanishes for rabbit globulin, whereas in acetate buffer at pH 4, B is significantly positive. Accordingly with borate buffer only one light scattering measurement is necessary for the determination of the molecular weight of this protein. These facts might be viewed in the light of Equation 2. If changes in molecular size such as dissociation, occur during the

dilution process, then it is predicted by this equation that B does not remain constant.

The purpose of the present paper is the determination of some properties of an antigen-antibody reaction from turbidity measurements. We shall represent one mole of antigen by Ag and one of antibody by Ab throughout the discussion.

EXPERIMENTAL

The serological system used throughout this set of experiments consists of crystalline bovine albumin as the antigen, and partially purified rabbit antibody against crystalline bovine albumin as the antibody. All solutions were made up in borate buffer at pH 8.3 and ionic strength 0.15. The antibody preparation was made by a triple precipitation of globulin by one-third saturation with ammonium sulfate at pH 7.8 and room temperature. After dissolving the final precipitate in 1% saline solution, the solution was dialyzed against distilled water at 0 C and the water-soluble protein was dissolved in 1% saline and dialyzed against borate buffer. The albumin solution was prepared by dissolving the crystalline protein in borate buffer and dialyzing against the same buffer. Total protein concentrations were determined colorimeterically by Nessler's method according to the procedure adopted by Lanni and Campbell (13), the factor 6.25 being used in converting nitrogen concentration to protein. Antibody concentration was determined in the usual manner by adding serial dilutions of antigen to a constant amount of antibody solution. The resulting water-soluble fractions contained about 17 per cent antibody in one preparation and 33 per cent in another (see Tables I and II). All solutions were centrifuged for twenty minutes at 40,000 G to eliminate dust particles. The cell in which the turbidities were to be measured and the pipettes used for transferring solution from the centrifuge tubes to the cell were flushed with acetone vapors before use. The light scattering apparatus has been described earlier (14). All turbidities were determined at room temperature (approximately 25 C). The turbidity of the antibody solution was determined first in each instance, and then the selected amount of antigen was added to the cell. An attempt was made at uniformity of mixing by keeping the ratio of volumes of albumin and antibody the same in each experiment, and by swirling all mixtures in a like manner.

Experiments were first performed in which the mole ratio of antibody to antigen was varied from 13 to 0.4, the quantity of antigen being held constant. In other experiments the mole ratio of antibody to antigen was kept constant and the total concentration was varied.

RESULTS

Figure 1 shows the increase of light scattered by the mixture of antigen and antibody with time. The mixing was started at zero time. The ordinate indicates relative values of reduced turbidities, which were obtained by subtracting the solvent readings from those of the solution and dividing the result by the weight of antibody protein in the system. The curves represent different mole ratios of antibody to antigen, as given in Table I. The amount of non-specific protein present was twice the amount of precipitable antibody. The amount of antigen used was the same in all cases, and the amount of antibody was varied to give the desired mole ratios. Precipitation was observed in the systems with Ab/Ag = 13, 3.2, 2.5, and 1.3; the last system had formed visible particles before the readings were taken. Visible particulation occurred in the first three at approximately 15, 25, and 50 minutes respectively. Systems with Ab/Ag = 0.8 and 0.4, in which no precipitation occurred during twenty-four hours, showed much more light scattered than those of the corresponding antibody

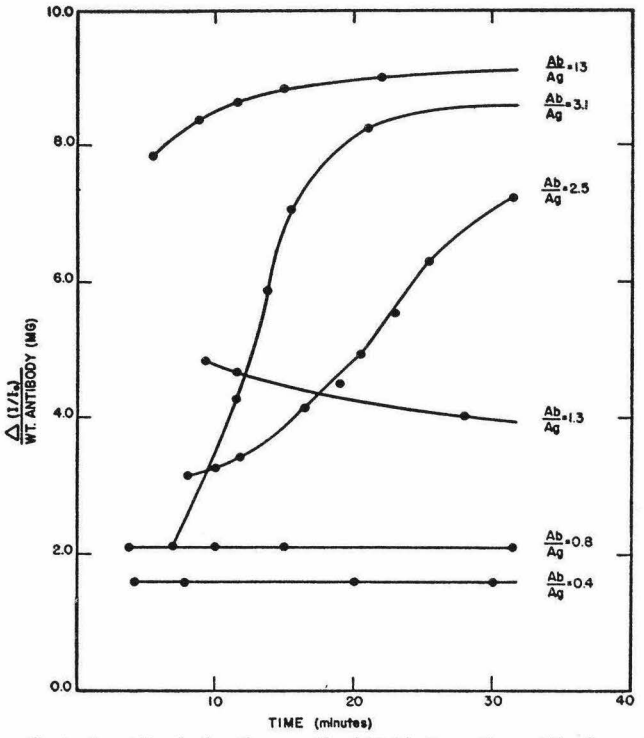

FIG. 1. The effect of antibody/antigen ratio (*Ab/Ag*) on the antibody-antigen reaction for bovine albumin and partially purified rabbit antialbumin antibody.

TABLE I

The effect of Ab/Ag ratio on the antibody-antigen reaction

Ab/Ag (MOLE RATIO)	ALBUMIN	ANTIBODY*	TOTAL VOLUME†	REMARKS
	mg	*mg*		
13.0	0.10	3.0	30.0	Precipitation
3.1	0.10	0.75	30.0	Precipitation
2.5	0.10	0.60	30.0	Precipitation
1.3	0.10	0.30	30.0	Precipitation
0.8	0.10	0.20	30.0	No precipitation, Turbidity increase
0.4	0.10	0.10	30.0	No precipitation, Turbidity increase

* The antibody protein was approximately 33% of the total globulin.

† In every instance 1.0 ml of albumin solution was added to 29.0 ml of the antibody solution.

solutions whose reduced turbidities were about 0.2. Controls made with normal rabbit globulin prepared in the same manner as the purified antiserum showed less than one per cent increase on the addition of antigen.

Another set of measurements was taken on systems with constant antigen-

antibody ratio and total concentrations differing by the factor 2. Figure 2 shows the results for these systems, described in Table II.

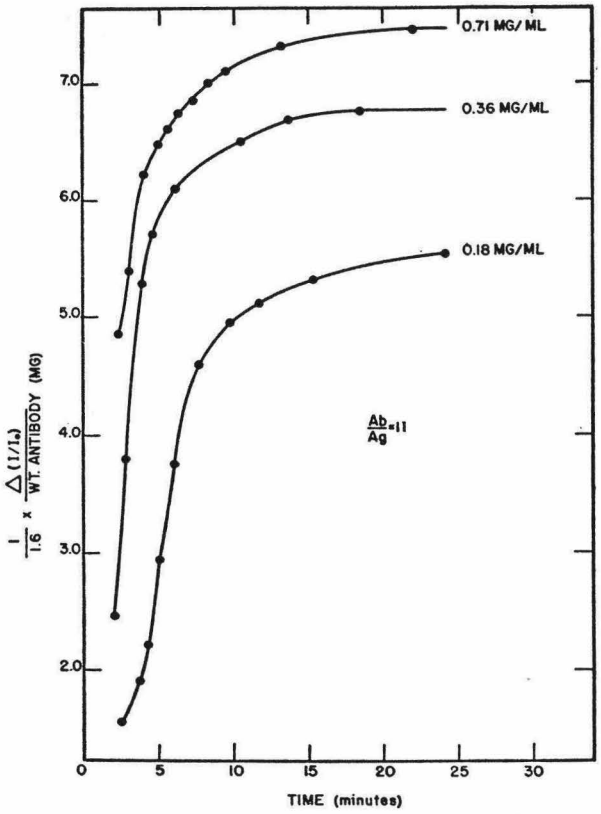

FIG. 2. The effect of total concentration on the antibody-antigen reaction for bovine serum albumin and partially purified antiablumin antibody. The concentrations given are for total protein.

TABLE II

The effect of total concentration of antigen and antibody on the Ab-Ag reaction

Ab/Ag (MOLE RATIO)	ALBUMIN	ANTIBODY*	TOTAL VOLUME†	REMARKS
	mg	*mg*	*ml*	
11	0.10	2.5	21.0	Precipitation
11	0.050	1.25	21.0	Precipitation
11	0.025	0.63	21.0	Precipitation

* The antibody protein was approximately 17% of the total globulin.

† In every instance 1.0 ml of antigen solution was added to 20.0 ml of antibody solution.

DISCUSSION OF RESULTS

The combination of bovine albumin and its homologous rabbit antibody appears to be complete in a few minutes. If we consider the average initial

slopes of the curves to be a measure of the rate of reaction, then we can conclude that the antibody-antigen ratio greatly affects the rate of this combination. Since the data presented are for scattered light 90° to the incident light, they are not sufficient to permit the determination of the molecular weight of interest in the region where the particles are no longer small. As mentioned earlier, light scattering data at other angles would be required for this purpose. If the ordinate values were directly proportional to molecular weight, the slope in the region of large aggregates would probably not decrease so rapidly with increasing time as it does. The negative slope of the curve for the system with Ab/Ag = 1.3 can be interpreted to represent the settling out of precipitate. Since visible particles had formed before measurements could be made, the rate of increase of reduced turbidity in the early part of the reaction, although not evidenced experimentally, undoubtedly would have a larger average value than that of the curve for the system with Ab/Ag = 2.5. Hence the rate of the antibody-antigen reaction discussed here appears to pass through a minimum in the region of the equivalence zone. For other rabbit antibody systems, Boyd, in a study of optimal proportions ratios, has reported times of flocculation which also indicate minima (15).

The curves representing the systems having Ab/Ag ratios less than unity indicate in the antigen excess region the existence of aggregates which we find by observation to be soluble. If we neglect intramolecular interference of scattered light and A_{ik} interaction terms, for the systems with Ab/Ag values of 0.4 and 0.8, we find by Equation 2 molecular weights of 850,000 and 1,300,000 respectively. By calculating a weight-average molecular weight for the former system on the assumption that all antibody is bound in aggregates $AbAg_2$, i.e. a solution containing uncombined antigen, rabbit globulin, and $AbAg_2$, we find 218,000. The neglect of intramolecular interference always leads to a lower value of the weight-average molecular weight than that obtaining in the absence of this interference. Although we have consistently found molecular than they should be whenever we have assumed $A_{ik} = 0$, it is possible that the A_{ik}'s will not always have the same sign. It seems not unlikely that some aggregates such as $Ab_3\ Ag_2$, etc. are present.

The effect of changing the concentration with Ab/Ag kept constant is shown in Figure 2. Let us first discuss the vertical spread of the asymptotes. It is possible that the different values of these asymptotes represent differences in weight – average molecular weight, but it seems not unlikely that they reflect differences in the enormous correction factors which must be applied to Equation 2 in order to include intramolecular interference.

It seems reasonable that little error would be introduced by assuming that the ordinate values, one for each curve, which correspond to the same fraction of the asymptotic values represent the same weight-average molecular weight for the three systems at these points. We can expect that by taking fractions of this kind we eliminate to a large extent the effects of differences in the factors which account for intramolecular interference of the scattered light. The values of the time corresponding to ordinates equal to 50% of the asymptotic values

are approximately inversely proportional to the concentrations. This proportionality is characteristic of any complex of bimolecular reactions. Lanni, using a turbidimeter, has obtained curves very similar to those of Figure 2 in his study of the specificity of serological reactions (16). His curves also roughly show this property of bimolecular reactions. Pauling (17) has pointed out that data in a table of times of flocculation for mixtures of various dilutions of antigen and antiserum presented by Boyd in a study of optimal proportions ratios (15) are suggestive of bimolecular reactions. Boyd's data for fixed Ab/Ag lead to the equation

$$t \propto \frac{1}{C^{1.18}} \tag{3}$$

where C is the concentration of the system and t is the time for flocculation to occur. For a two-fold dilution the time of flocculation increases 2.27 times. The fact that this factor is 14% larger than that required by the bimolecular rate equation can reasonably be explained as a dilution effect; namely, in a more dilute solution the reactions may well have to proceed beyond the apparent flocculation point for a more concentrated system before the observer recognizes that the flocculation stage has been reached. Equation 3 was arrived at by first constructing straight lines 45° to the axes, which give the dilution factors on the same scale. These lines were drawn through the points labeled with the first eight antigen tube numbers; they intersect six contour lines (isochrones) representing the range one minute to 32 minutes for the flocculation time. The distance between the points of intersection of the first and the last contour lines with each one of the 45° lines was measured, and the average distance for the eight lines was calculated. This average distance was then converted to a concentration factor. The exponent, 1.18, in Equation 3 is the power to which the concentration factor, 19, had to be raised to give the time factor, 32. We conclude that as a first approximation antibody-antigen reactions involve collisions of two particles. This conclusion gives justification to the assumption of a series of successive bimolecular reactions upon which Heidelberger and Kendall (18, 19) and Hershey (20, 21) base their quantitative theories.

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SUMMARY

The utility of light scattering measurements on protein systems is discussed. Light scattering data for bovine serum albumin and its homologous rabbit antibody are presented. They describe the turbidity of the system as a function of time. It was found that the rate of aggregation of antibody and antigen molecules into large complexes is dependent on the antibody-antigen ratio. The existence of soluble aggregates in the antigen excess region is verified. Evidence is discussed which indicates that all reactions occurring in an antibody-antigen system are bimolecular.

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Part III

Light Scattering Arising from Composition Fluctuations in Multi-Component Systems

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Light Scattering Arising from Composition Fluctuations in Multi-Component Systems

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A general theory of Rayleigh scattering due to composition fluctuations in multi-component systems is developed with the aid of the grand canonical ensemble of Gibbs. It reduces to the usual expression for systems of two components, but contains previously neglected terms arising from thermodynamic interactions between solutes in systems of more than two components. The theory is used to interpret the turbidity measurements of polystyrene in benzene-methanol mixtures of Ewart, Roe, Debye, and McCartney.

I

THE utility of light scattering measurements in the determination of molecular weights and in the study of thermodynamic interactions in solutions of macromolecules has been clearly demonstrated in recent years. Correct theoretical interpretation of the measurements has been achieved for two-component systems composed of one macromolecular solute in a solvent of low molecular weight. However, attempts to extend the two-component theory to multi-component systems

have led to certain errors and misconceptions, the correction of which is one of the purposes of the present article.

A general theory of composition fluctuations in multicomponent systems will be developed with the use of the grand canonical ensemble of Gibbs. The theory provides a complete thermodynamic description of Rayleigh scattering without the use of supplementary molecular assumptions, although the latter may be of importance in interpreting the thermodynamic information obtained from the light scattering measurements.

It is found that thermodynamic interaction between a macromolecular solute and a solute of low molecular weight may cause the former to induce composition fluctuations with respect to the latter of the same order of magnitude as composition fluctuations with respect to the macromolecular species itself. This effect, which has been neglected in previous theories, is shown to be of importance in interpreting turbidity measurements of solutions of macromolecules in mixed solvents. To illustrate the use of the theory, an analysis is made of the turbidity measurements of solutions of polystyrene in benzene-methanol mixtures obtained by Ewart, Roe, Debye, and McCartney.[1]

II

The turbidity τ_0 of a fluid arising from Rayleigh scattering of light of wave-length λ is determined by the well-known relation,[2] based on the theory of Einstein,

$$\tau_0=(8\pi^3/3\lambda^4)V\langle\Delta\epsilon^2\rangle_{Av}, \tag{1}$$

where $\langle\Delta\epsilon^2\rangle_{Av}$ is the dielectric constant fluctuation in a region of volume V. We shall be concerned here only with those contributions to $\langle\Delta\epsilon^2\rangle_{Av}$ arising from composition and density fluctuations. If we denote by $m_0, \cdots m_\nu$ the average masses and by $N_0, \cdots N_\nu$ the numbers of molecules of the several components in the region V, and define

$$\begin{aligned} c_i&=m_i/m_0; \quad i=1, \cdots \nu,\\ m_i&=M_i\langle N_i\rangle_{Av}/N,\\ \Delta N_i&=N_i-\langle N_i\rangle_{Av},\\ \xi_i&=\Delta N_i/\langle N_i\rangle_{Av}-\Delta N_0/\langle N_0\rangle_{Av},\\ \xi&=\sum_{k=0}^{\nu}\bar{v}_k\Delta N_k/NV; \end{aligned} \tag{2}$$

where N is Avogadro's number and $\bar{v}_k$ the partial molar volume of component k, we may write,

$$\langle\Delta\epsilon^2\rangle_{Av}=\frac{\langle\xi^2\rangle_{Av}}{\kappa^2}\left(\frac{\partial\epsilon}{\partial p}\right)^2_{T,c} +\sum_{\substack{i,k\\=1}}^{\nu} c_ic_k\langle\xi_i\xi_k\rangle_{Av}\left(\frac{\partial\epsilon}{\partial c_i}\right)_{T,p,c_j}\left(\frac{\partial\epsilon}{\partial c_k}\right)_{T,p,c_j}, \tag{3}$$

where κ is the compressibility of the fluid, and the sum extends over all solute species, $k=1, \cdots \nu$, the subscript zero denoting solvent. The first term of Eq. (3) arises from density fluctuations at constant composition, and the second from composition fluctuations. Except for critical phases, Eq. (3) is exact to terms of statistically negligible order of magnitude. We now define in the customary manner the turbidity τ due to composition fluctuations,

$$\begin{aligned} \tau_0&=\tau+\frac{8\pi^3V}{3\lambda^4}\frac{\langle\xi^2\rangle_{Av}}{\kappa^2}\left(\frac{\partial\epsilon}{\partial p}\right)^2_{T,c}\\ \tau&=\frac{8\pi^3V}{3\lambda^4}\sum_{\substack{i,k\\=1}}^{\nu} c_ic_k\langle\xi_i\xi_k\rangle_{Av}\left(\frac{\partial\epsilon}{\partial c_i}\right)_{T,p,c_j}\left(\frac{\partial\epsilon}{\partial c_k}\right)_{T,p,c_j}, \end{aligned} \tag{4}$$

where the second term of the first of Eqs. (4) is the turbidity arising from pure density fluctuations. In Eqs. (3) and (4), we have anticipated the result, $\langle\xi\xi_i\rangle_{Av}=0$, presently to be proved.

In order to determine the composition fluctuations we employ the theory of the grand canonical ensemble in a manner which has been earlier described by one of us.[3] The probability that an open region V in an infinite mass of fluid contain exactly $N_0, N_1, \cdots N_\nu$ molecules of the several components, considered as an example of a grand canonical ensemble, is

$$\begin{aligned} P&=\exp([\Omega+\sum_{i=0}^{\nu}N_i\mu_i'-A(N_0\cdots N_\nu)]/kT),\\ \Omega&=-pV+kT\log\sigma, \end{aligned} \tag{5}$$

where k is Boltzmann's constant, T the temperature, μ_i' the chemical potential of component i, per molecule, and A is the Helmholtz free energy of the region when it contains the specified numbers of molecules. The term $kT\log\sigma$ is of statistically negligible magnitude relative to pV, but is important for normalization in the order required for the calculation of the composition fluctuations. Expansion of the exponent of the right-hand side of Eq. (5) in the variables $N_i-\langle N_i\rangle_{Av}$ yields, with the neglect of terms of higher degree than quadratic, which, except for critical phases make statistically negligible contributions to mean values,

$$\begin{aligned} P&=\sigma\exp(-\tfrac{1}{2}\sum_{\substack{i,k\\=0}}^{\nu}\beta_{ik}{}^0\Delta N_i\Delta N_k),\\ \Delta N_i&=N_i-\langle N_i\rangle_{Av},\\ kT\beta_{ik}{}^0&=\left(\frac{\partial^2A}{\partial N_i\partial N_k}\right)_{T,V,N_j} =\left(\frac{\partial\mu_i'}{\partial N_k}\right)_{T,V,N_j}=\left(\frac{\partial\mu_k'}{\partial N_i}\right)_{T,V,N_j}. \end{aligned} \tag{6}$$

[1] Ewart, Roe, Debye, and McCartney, J. Chem. Phys. **14**, 687 (1946); P. Debye, J. Phys. Coll. Chem. **51**, 18 (1947).

[2] See, for example, Doty, Zimm, and Mark, J. Chem. Phys. **13**, 159 (1945).

[3] J. G. Kirkwood, mimeographed notes, "Lectures on statistical mechanics" delivered at Princeton University (Spring term, 1947).

We now make use of the mathematical relation,

$$\left(\frac{\partial \mu_i'}{\partial N_k}\right)_{T,\,V,\,N_j} = \frac{\bar{v}_i \bar{v}_k}{N^2 \kappa V} + \frac{M_k}{N^2 m_0}\left(\frac{\partial \mu_i}{\partial c_k}\right)_{T,\,p,\,c_j}, \tag{7}$$

where N is Avogadro's number, $\bar{v}_i$ the partial molar volume of component i, M_i the molecular weight, and μ_i is the chemical potential per mole of that component. The coefficients $\beta_{ik}{}^0$ may then be expressed in the form,

$$\begin{gathered}\beta_{ik}{}^0 = \frac{\bar{v}_i \bar{v}_k}{N^2 \kappa V k T} + \frac{N m_0}{2}\frac{\beta_{ik}}{\langle N_i\rangle_{Av}\langle N_k\rangle_{Av}}, \\ \beta_{ik} = \frac{c_i c_k}{M_i R T}\left(\frac{\partial \mu_i}{\partial c_k}\right)_{T,\,p,\,c_j} = \frac{c_i c_k}{M_k R T}\left(\frac{\partial \mu_k}{\partial c_i}\right)_{T,\,p,\,c_j}, \\ \sum_{k=0}^{\nu} \beta_{ik} = 0; \quad \beta_{00} = \sum_{i,\,k=1}^{\nu} \beta_{i0}\beta_{k0},\end{gathered} \tag{8}$$

where the sum rules for the coefficients β_{ik} follow from the Gibbs-Duhem equation. Introducing the composition fluctuation variables ξ_i and the reduced density fluctuation variable ξ, of Eq. (2)

$$\begin{gathered}\xi_i = \Delta N_i/\langle N_i\rangle_{Av} - \Delta N_0/\langle N_0\rangle_{Av}, \\ \xi = \sum_{k=0}^{\nu} \bar{v}_k \Delta N_k / N V;\end{gathered} \tag{9}$$

we obtain from Eqs. (6) and (8) the fluctuation distribution function,

$$\begin{gathered}P(\xi_1 \cdots \xi_\nu, \xi) = \frac{(2\pi)^{-(\nu+1/2)}(\kappa V k T)^{-\frac{1}{2}}}{|\beta|^{\frac{1}{2}}} \\ \times \exp\left(-(N m_0/2)\sum_{i,\,k=1}^{\nu} \beta_{ik}\xi_i\xi_k - V\xi^2/2\kappa k T\right) \\ |\beta| = |\beta_{ik}|,\end{gathered} \tag{10}$$

where $|\beta|$ is the determinant of the thermodynamic coefficients β_{ik}. It will be remarked that the transformation, Eq. (9), has eliminated non-diagonal terms in the Gaussian distribution involving the composition fluctuations ξ_i and the density fluctuation ξ.

The distribution function, Eq. (10), yields with the aid of the theory of quadratic forms the following mean values,

$$\begin{gathered}V\langle \xi_i \xi_k\rangle_{Av} = (V/N m_0)(|\beta|_{ik}/|\beta|); \quad i, k = 1 \cdots \nu, \\ V\langle \xi^2\rangle_{Av} = \kappa k T, \\ \langle \xi \xi_i\rangle_{Av} = 0,\end{gathered} \tag{11}$$

where $|\beta|_{ik}$ is the appropriate co-factor of the determinant $|\beta|$. Substitution of the density and composition fluctuations of Eq. (11) into Eqs. (1) and (3) yields the following expressions for the turbidity,

$$\begin{gathered}\tau_0 = \tau + (8\pi^3/3\lambda^4)(kT/\kappa)(\partial \epsilon/\partial p)^2{}_{T,c}, \\ \tau = \frac{8\pi^3}{3\lambda^4}\frac{1}{N\rho_0}\sum_{i,\,k=1}^{\nu} c_i c_k \frac{|\beta|_{ik}}{|\beta|}\left(\frac{\partial \epsilon}{\partial c_i}\right)_{T,\,p,\,c_j}\left(\frac{\partial \epsilon}{\partial c_k}\right)_{T,\,p,\,c_j},\end{gathered} \tag{12}$$

where ρ_0 is the mass of solvent in unit volume. Equations (12) give a complete description of Rayleigh scattering arising from density and composition fluctuations in terms of thermodynamically defined quantities and the derivatives $(\partial \epsilon/\partial c_i)_{T,\,p,\,c_j}$. The non-diagonal terms in which have previously been neglected in the analysis of Rayleigh scattering in multi-component systems, make it possible for a solute of high molecular weight to induce significant composition fluctuations with respect to a second solute of low molecular weight as the result of strong thermodynamic interaction between the two.

III

We shall now present several applications of Eq. (12), which illustrate the manner in which the turbidity of a multi-component fluid may be used to obtain thermodynamic information relating to the dependence of the chemical potentials of the components on composition. For the case of two components, Eq. (12) of course reduces to the expression,

$$\tau = \frac{8\pi^3 R T}{3\lambda^4 N \rho_0}\left(\frac{\partial \epsilon}{\partial c_1}\right)^2_{T,\,p} M_1 \Big/ \left(\frac{\partial \mu_1}{\partial c_1}\right)_{T,\,p}, \tag{13}$$

given by the elementary theory of composition fluctuations and which has been extensively used in light scattering studies. In order to simplify Eq. (12) in the multi-component case, we suppose all solutes to be non-electrolytes and expand the excess chemical potentials in power series in the concentrations c_i,

$$\begin{gathered}\mu_i = RT \log \gamma_i c_i + \mu_i{}^0(T, p), \\ \mu_i{}^0 = \lim_{c_1 \cdots c_\nu} [\mu_i - RT \log c_i], \\ \log \gamma_i = \sum_{k=1}^{\nu} A_{ik} c_k + 0(c_k c_j),\end{gathered} \tag{14}$$

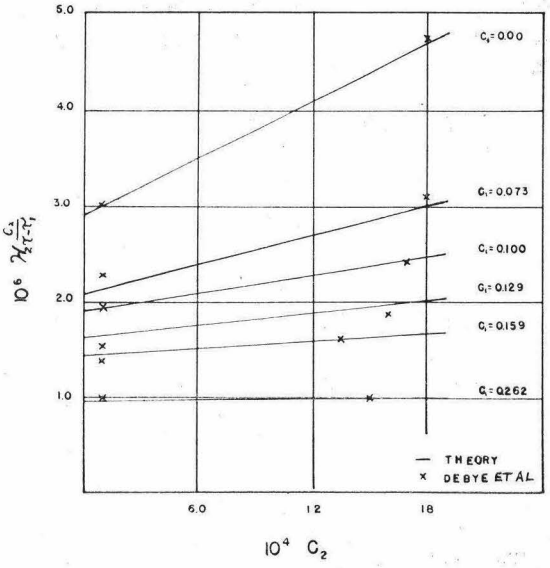

FIG. 1. Turbidity-composition curves of solutions of polystyrene in benzene-methanol mixtures.

retaining only linear terms in the expansions of $\log\gamma_i$. In this approximation, we may write,

$$(\partial\mu_i/\partial c_k)=(RT/c_i)\delta_{ik}+A_{ik},$$
$$M_kA_{ik}=M_iA_{ki}, \tag{15}$$

where, as henceforth, we abbreviate the derivatives $(\partial\mu_i/\partial c_k)_{T,\,p,\,c_j}$ as $\partial\mu_i/\partial c_k$. Using Eq. (15), we find

$$|\beta|_{ik}/|\beta|=M_i\delta_{ik}/c_i-M_iA_{ki},$$
$$\tau=\frac{8\pi^3}{3N\rho_0\lambda^4}\left\{\sum_{k=1}^{\nu}M_kc_k\left(\frac{\partial\epsilon}{\partial c_k}\right)^2-\tfrac{1}{2}\sum_{\substack{i,\,k\\=1}}^{\nu}M_kA_{ik}\frac{\partial\epsilon}{\partial c_i}\frac{\partial\epsilon}{\partial c_k}c_ic_k\right\}, \tag{16}$$

where we abbreviate $(\partial\epsilon/\partial c_i)_{T,p,c_j}$ by $\partial\epsilon/\partial c_i$. In the case of a polymer with a molecular weight distribution, where it is appropriate to treat $\partial\epsilon/\partial c_i$ as independent of i in first approximation, we obtain

$$\frac{Hc}{\tau}=\frac{1}{\langle M\rangle_{Av}}+\frac{c}{\langle M\rangle_{Av}^2}\sum_{\substack{i,\,k\\=1}}^{\nu}M_kA_{ik}f_if_k,$$
$$c=\sum_{k=1}^{\nu}c_k;\quad\langle M\rangle_{Av}=\sum_{k=1}^{\nu}f_kM_k, \tag{17}$$
$$H=(8\pi^3/3N\rho_0\lambda^4)(\partial\epsilon/\partial c)^2,$$

where f_i is the weight fraction of component of molecular weight M_i. In a previous attempt to adapt two-component fluctuation theory to this case,[4] the non-diagonal terms in the sum of Eq. (17) were overlooked.

We now turn our attention to the system of three components. In this case Eq. (12) may be put in the form,

$$\Delta\tau=\frac{H_2M_2RT}{\partial\mu_2/\partial c_2}\frac{1-2\alpha\omega_1+\alpha^2\omega_1^2}{1-(M_2/M_1)\omega_1\omega_2},$$
$$\Delta\tau=\tau-\tau_1;\quad H_2=\frac{32\pi^3n^2}{3N\rho_0\lambda^4}\left(\frac{\partial n}{\partial c_2}\right)^2;\quad\epsilon=n^2, \tag{18}$$
$$\omega_1=(\partial\mu_1/\partial c_2)/(\partial\mu_1/\partial c_1),$$
$$\omega_2=(\partial\mu_1/\partial c_2)/(\partial\mu_2/\partial c_2),$$
$$\alpha=(\partial n/\partial c_1)/(\partial n/\partial c_2),$$

where τ_1 is given by Eq. (13). If all components are non-electrolytes, and we employ the power series,

$$\log\gamma_i=\sum_{k=1}^{2}A_{ik}c_k+\sum_{\substack{j,\,k\\=1}}^{2}B_{ijk}c_jc_k+\cdots,$$
$$\alpha=\alpha_0+\alpha_1c_1+\alpha_2c_2, \tag{19}$$

for the activity coefficients, γ_i, and the refractive index ratio, α, we obtain the following expansion of Eq. (19),

$$H_2c_2/\Delta\tau=1/M_2\{1+G_{10}c_1+G_{01}c_2+G_{20}c_1^2+G_{11}c_1c_2+G_{02}c_2^2\};$$
$$G_{10}=2\alpha_0A_{12};\quad G_{01}=A_{22};\quad G_{02}=2B_{222};$$
$$G_{20}=4\alpha_0B_{112}-2\alpha_0A_{11}A_{12}+2\alpha_1A_{12}+3\alpha_0^2A_{12}^2; \tag{20}$$
$$G_{11}=2(1+2(M_1/M_2)\alpha_0)B_{212}-(M_2/M_1)A_{12}^2+2\alpha_0A_{12}A_{22}+2\alpha_2A_{12}.$$

TABLE I. Thermodynamic interaction coefficients in benzene-methanol-polystyrene solutions.*

A_{12}	A_{22}	B_{212}	B_{222}
1.1	340	5100	0.0

* Subscript 1, methanol; subscript 2, polystyrene; $M_2=3.45\times10^5$.

We have analyzed the light scattering data of Ewart, Roe, Debye, and McCartney[1] on solutions of polystyrene in benzene-methanol mixtures, by means of Eq. (20), supplemented by an additional cubic term of the order $c_1^2c_2$, the coefficient of which we do not interpret theoretically, although this could easily be done. The curves from which the coefficients are determined are compared with experiment in Fig. 1. It will be observed that the measurements are reasonably well reproduced. The coefficients of the refractive index increment ratio α were estimated to be $\alpha_0=-1.9$; $\alpha_1=4.8$; $\alpha_2=0$. The values of the thermodynamic interaction coefficients, A_{ik} and B_{ijk}, of Eq. (20), calculated from the experimentally determined coefficients G_{ik} of Eq. (20) are presented in Table I. The coefficients A_{ik} and B_{ijk} are, of course, dimensionless, but it should be remembered that the numerical values are appropriate to concentrations c_i expressed in grams of solute per gram of benzene.

The calculations which have been presented exhibit the manner in which turbidity measurements may be used in conjunction with the present theory to obtain thermodynamic data in multi-component systems containing at least one macromolecular component. The positive value of the interaction coefficient A_{12} for polystyrene and methanol, when interpreted from the molecular standpoint, means, as Debye surmised, that a polystyrene molecule exhibits a preference for benzene molecules in its statistical environment. Such qualitative considerations should, however, be regarded as supplementing the thermodynamic theory presented here, rather than the basis for an exact analysis of turbidity.

[4] B. Zimm, and P. Doty, J. Chem. Phys. **12**, 203 (1944).

Appendix

The Determination of the Combinatorial Factor W_{ijk}

W_{ijk} is defined as the number of ways in which i bifunctional units(to be called S_i-units), j unifunctional units(called S_j-units), and k f-functional units(called S_k-units) can be formed into a single i,j,k-aggregate containing no cyclic structures. All units and all functional sites thereon are distinguishable. All sites on the S_k-units are equivalent. All sites on the S_i and S_j-units are equivalent. Furthermore, sites on S_i and S_j-units are permitted to react only with sites on S_k-units and vice versa.

This problem can be solved by the device invented by Mayer and Mayer(28) and adopted by Stockmayer in similar problems(18). S_k-units are represented by mechanical frames containing f holes. Indistinguishable bolts are required to hold the frames together, each bolt passing through a pair of holes belonging to different frames. Bolts are also required to fill all other holes. These, however, do not connect different frames with each other. Each of them has one free end.

The number of ways to bolt all the frames together into a so-called k-aggregate, containing no cyclic structures, is W_k. It should be noted that the insertion of i S_i-units and j S_j-units into the k-aggregate does not change the number of ways of forming the latter. k-1 of the S_i-units must take the place of those bolts connecting

two frames together. The rest of the S_i-units and the S_j-units must replace bolts which have one end free. The number of ways of inserting the i S_i-units and j S_j-units into the k-aggregate is defined as R_{ijk}.

Therefore,

$$W_{ijk} = W_k R_{ijk} \qquad (A1)$$

W_k is determined in the following manner. Since a k-aggregate requires k-1 bonds, k-1 bolts are required for this purpose. Since bolts are required to fill all other holes, the total number of bolts used is then

$$fk-(k-1) = fk-k+1$$

Any one of the bolted arrangements can be dissociated into k separate frames, each containing f-1 holes occupied by bolts and one empty hole. There will be one free bolt left over. The bolt chosen as the free bolt uniquely determines the empty hole in each of the k frames. Since there are fk-k+1 bolts altogether, there are likewise fk-k+1 different dissociated arrangements of the required kind which correspond to the same bolted arrangement. Now, if P is the number of possible dissociated arrangements of this kind, and if Q is the number of ways of bolting each dissociated arrangement together, the

number of different bolted arrangements is

$$W_k = \frac{PQ}{fk-k+1} \tag{A2}$$

Since any one of the holes on the frames can be the empty one,

$$P = f^k \tag{A3}$$

To find Q, k-1 indistinguishable washers are introduced, no more than one being placed on any bolt. The number of ways to choose k-1 out of the fk-k+1 bolts, on which to place washers, is

$$\frac{(fk-k+1)!}{(fk-2k+2)!\,(k-1)!}$$

Washered bolts are now inserted into holes in frames with which they are not already connected. The free bolt is kept for last. That is, the first washered bolt can select any one of k-1 empty holes(excluding the one on its own frame). There are then k-2 single frames and one double frame. In a like manner the second washered bolt can select any one of k-2 empty holes. This process continues

until only the one free bolt remains. If the free bolt has a washer there remain two structures, each with a hole, which must be bolted together. If it does not have a washer there remains just one hole on one structure which must be filled. Therefore, washered bolts can be inserted in (k-1)! ways. This number of ways combined with the number of ways of assigning washers is

$$Q = \frac{(fk-k+1)!}{(fk-2k+2)!} \qquad (A4)$$

Therefore, the substitution of Equations A3 and A4 in A2 gives

$$W_k = \frac{f^k (fk-k)!}{(fk-2k+2)!} \qquad (A5)$$

This proof for W_k is the same as that given by Stockmayer(18).

R_{ijk} can be obtained in the following manner. k-1 of the i S_i-units must be selected for the bonding positions now occupied by bolts. These can be selected in

$$\frac{i!}{(i-k+1)!} \text{ ways.}$$

The remainder of the S_i-units, i-k+1, and all the S_j-

units must replace any of the fk-2k+2 bolts each of which has one end free. This selection can be accomplished in

$$\frac{(fk-2k+2)!}{[fk-2k+2-(i-k+1)-j]!} \text{ ways.}$$

Now, since each of the S_i-units has two distinguishable functional sites, R_{ijk} will contain the factor 2^i. Therefore,

$$R_{ijk} = 2^i \frac{(fk-2k+2)!}{(fk-k-i-j+1)!} \cdot \frac{i!}{(i-k+1)!} \qquad (A6)$$

Let the number of S_i-units of which only one functional site is used be defined by

$$g = i - k + 1 \qquad (A7)$$

With the use of Equations A5, A6, and A7, Equation A1 becomes

$$W_{ijk} = f^k 2^i \frac{(fk-k)!}{(fk-2k+2-g-j)!} \cdot \frac{i!}{g!} \qquad (A8)$$

Since the numbers of S_i-units and S_j-units cannot exceed the total number of bolts, it is clear that

$$
\begin{aligned}
i &= k-1+\ell \\
0 \le \ell &\le 4k-2k+2 \qquad (A9) \\
0 \le j &\le 4k-2k+2-\ell
\end{aligned}
$$

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Propositions

1. I propose an interesting relation which occurs between the number of ways of forming a branch-type aggregate (containing no cyclical structures) out of k particles, each with the same number of reaction sites, and the number of ways of forming smaller branch-type aggregates.
2. I propose a method for obtaining the number of ways of forming a branch-type aggregate. This method produces results more rapidly than that of Stockmayer and is useful for aggregates which his method has apparently not been able to handle(1).
3. I suggest that three dimensional polyesterifications like those carried out by Flory(2) should be carried out in a manner prescribed by the standard procedures of the precipitin reaction.
4. The assumptions involved in the use of iodine as a label in antibody-antigen reactions have not been justified. It is not unreasonable to suppose that antibody-antigen ratios determined by means of this kind of labeling device are incorrect. The use of double labels may avoid this difficulty.
5. I believe that the determination of the composition of the precipitate of an antibody-antigen reaction, as a function of the time of the reaction, may give information regarding the valence of the antibody.

6. Calorimetric measurements of an antibody-antigen reaction as a function of the composition of the system may yield information regarding the state of the antibody-antigen aggregates in the system.
7. A new expression relating the antibody-antigen ratio of the precipitate to the composition of the system is the following.

$$R = a(Ab/Ag)^{1/2}$$

R is the antibody-antigen molecular ratio of the precipitate, Ab/Ag is the molecular ratio of antibody to antigen for the system, and a is a constant. This equation can be used for the entire region of precipitation for either the α or β titration procedure.

8. (a) I believe that it is more helpful to one attempting to grasp the meaning of entropy to stress the expression

$$dS \sim \frac{dW}{W}$$

rather than

$$S \sim \log W$$

(b) It is interesting to regard the energy of a particular ideal system as a problem of balls in boxes. Each box represents a degree of freedom and each ball represents

the smallest unit of energy available. When the system is at the absolute zero there are no balls in the boxes. It is easy, therefore, to see how the entropy of the system is increased by raising the temperature, i.e., by adding more balls to the boxes.

9. The time rate of a goldfish opening its mouth closely obeys the Arrhenius equation for a monomolecular reaction with a constant activation energy. Since this energy is the same at both high and low temperatures, there may not be a reversible inactivation of the enzyme responsible for the rate-limiting reaction.

10. It appears that strength-duration diagrams may better be represented by

$$i = \frac{i_r}{1 - e^{-t/c}}$$

than by

$$i = \frac{a}{t} + b ,$$

where i is the threshold current, i_r and b are the rheobase currents, t is the time of the impulse, and c and a are time constants.

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