

The Extrapolation of Light-Scattering Data to Zero Concentration

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INTRODUCTION

The problem of protein interactions has been a focus of research over a long period of time. It is well known that such systems as enzyme-substrate and antigen-antibody interactions involve the association of the active protein with other molecules. Furthermore, the degree of molecular dispersion of a protein may greatly affect its biological properties as shown, for example, by Nord and co-workers (1-5).

Bier and Nord (5, 6) were among the first to show that light scattering could be used for the study of protein interactions. In general, this technique has gained wide acceptance for the study of protein associations and the thermodynamics of macromolecular interacting systems (7-17). The interactions which a protein undergoes in solution, however, lead often to serious complications in the analysis of light-scattering data. These complications usually manifest themselves in rendering the extrapolation to zero protein concentration very difficult or in introducing a certain amount of uncertainty into the meaning of the intercept. As a result, a certain amount of confusion has existed as to whether the correct molecular weight of polyelectrolytes, such as proteins, can be obtained only in the presence of salt or, to the contrary, only in the salt-free case. It is the purpose of this paper to present an analysis of various factors affecting the extrapolation of light-scattering data to zero concentration and to outline by typical examples the proper procedure to be used in various cases.

EXPERIMENTAL

Light-scattering measurements on α -lactalbumin and β -lactoglobulin were carried out on the Brice photometer, using the Dintzis technique of dilution (16). In the case of β -lactoglobulin the temperature of the solutions was adjusted by equilibrat-

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ing them in a cold bath of the given temperature, and measurements were taken rapidly, the instrument being located in a room of the appropriate temperature. The proteins were prepared by the standard techniques and provided by Dr. W. G. Gordon (β -lactoglobulin) and Dr. C. A. Zittle (α -lactalbumin). The refractive index increment of HgCl_2 at 436 $m\mu$ was determined on the Brice photoelectric differential refractometer.²

Two-Component Systems

Using the multicomponent theory of light scattering (18–20), it has been shown that in the case of a two-component system, such as a macromolecule (component 2) in water (component 0), the light-scattering equation is:

$$H \frac{C_2}{\tau} = \frac{1}{M_2} \left[1 + \frac{C_2}{RT} \left(\frac{\partial \mu_2^{(e)}}{\partial C_2} \right) \right]$$

$$\mu_2 = RT \log C_2 + \mu_2^{(e)} + \mu_2^0(T, p) \quad (1)$$

$$H = \frac{32\pi^3 n^2 (\partial n / \partial C_2)^2}{3\lambda^4 N}$$

where C_2 is the concentration of the macromolecule in grams/ml., τ is the excess turbidity of the solution over that of the solvent, M_2 is the weight-average molecular weight of the macromolecule, R is the gas constant, T is the thermodynamic temperature, $\mu_2^{(e)}$ is the excess chemical potential of the macromolecule, n is the refractive index of the solution, λ is the wavelength of the light, and N is Avogadro's number.

Examination of Eq. (1) reveals immediately that, in a two-component system, extrapolation to zero concentration *does* give the true molecular weight of the macromolecule. In such a system, however, extrapolation to zero concentration may be quite difficult and a correct value of $1/M_2$ may be almost impossible to obtain. Thus, great caution has to be exerted that the measurements are extended to a sufficiently low concentration range to permit the correct extrapolation. This complication is due to the fact that the function $\partial \mu_2^{(e)} / \partial C_2$ may often take on a complicated form, with the result that the data follow neither a straight line nor, at times, any simple curve. Three typical cases will be described here: (a) an associating protein; (b) an isoionic protein in ion-free water; and (c) a protein system at high charge in ion-free water.

Associating Protein. If, under the conditions of measurement, the protein undergoes the reaction $nP \xrightleftharpoons{k} P_n$, and if its activity coefficient, γ_2 , is represented by the product of two terms, f_2 , the fraction of protein which

² B. A. Brice, unpublished.

is not associated, and γ_2' , the activity coefficient of the nonaggregated protein, then:

$$\frac{1}{RT} \frac{\partial \mu_2^{(o)}}{\partial C_2} = \frac{\partial \log \gamma_2'}{\partial C_2} + \frac{\partial \log f_2}{\partial C_2} \quad (2a)$$

$$\frac{\partial \log f_2}{\partial C_2} = -\frac{Kn(n-1)f_2^n C_2^{n-2}}{M_2^{n-1} f_2 + Kn^2 f_2^n C_2^{n-1}} = \frac{1}{C_2} \left(\frac{M_2 - \bar{M}_w}{\bar{M}_w} \right) \quad (2b)$$

$$K = \frac{(1-f_2)M_2^{n-1}}{nf_2^n C_2^{n-1}} \quad (2c)$$

where M_2 is the monomer molecular weight and \bar{M}_w is the weight-average molecular weight at concentration C_2 . Combining with Eq. (1), and assuming that $\partial \log \gamma_2' / \partial C_2$ remains constant over the concentration range in question, the light-scattering equation becomes

$$\begin{aligned} H \frac{C_2}{\tau} &= \frac{1}{\bar{M}_w} + \frac{2B_0 C_2}{M_2} \\ 2B_0 &= \frac{\partial \log \gamma_2'}{\partial C_2} \end{aligned} \quad (3)$$

Thus, at any given protein concentration, the scattering value is the sum of the weight-average molecular weight at that concentration, described exactly by the chemical equilibrium, and the linear term in concentration of the nonaggregated species. When $2B_0$ is sufficiently small, the second term may be neglected and the data interpreted directly in terms of the association. In the case of proteins, this has been found to be true in a number of cases at conditions close to the isoionic point.

An example of data treated in this manner is given in Fig. 1, which forms part of a study on the association of β -lactoglobulin as a function of temperature (21-23). It is known that in the pH region between 3.7 and 5.2, 66% of the β -lactoglobulin molecules present in a normal protein preparation can associate to form a tetramer at temperatures close to 0°C. The data presented in Fig. 1 were obtained at pH 4.14 in an acetate buffer of 0.1 ionic strength. It is true that under these conditions the system is more than two component (it is actually a four-component system). If it is assumed, however, that the thermodynamic interactions between protein and buffer are very small, the system may be treated as a two-component system.³ In the concentration range studied, no detectable association occurs

³ As will be shown below, at conditions of low charge on the protein, high ionic strength, and little binding of buffer components to protein, the system behaves as a pseudo two-component one and may be formally treated as such.

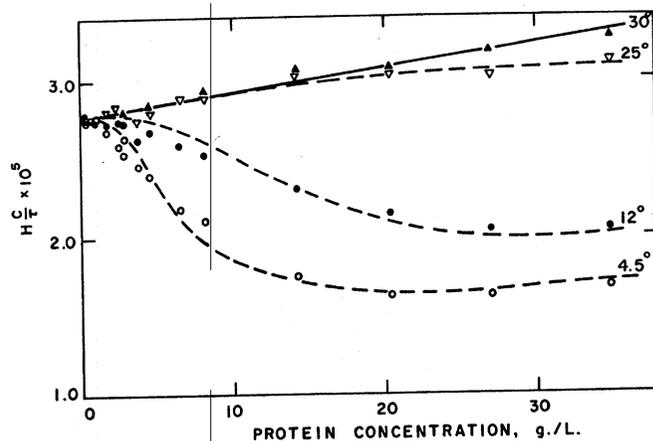


FIG. 1. Light-scattering data of the association of β -lactoglobulin from pooled milk in a pH 4.14 acetate buffer of 0.1 ionic strength.

at 30° so that the slope of the line at that temperature can be used to determine the term $2B_0/M_2$ of Eq. (3). This turns out to be 0.015 l./g. Subtraction of the product of this value with the concentration from the observed $H(C_2/\tau)$ at the given concentration gives $1/M_w$ at that concentration. Then, using Eqs. (2) and properly taking into account the fact that only 66% of the protein can enter into the reaction, $\partial \log f_2/\partial C_2$ was obtained and the equilibrium constants were calculated. It was found that, for the $4P \rightleftharpoons P_4$ reaction, the values of K were 6.0×10^{10} l.³ mole⁻³ at 4.5° , 5.0×10^9 at 12° , and 4.5×10^7 at 25° . Using these values, the light-scattering curves shown by dotted lines on Fig. 1 were calculated. It can be seen that the data are well represented by this analysis. Examination of the curves drawn in dashed lines shows them to have a complicated shape, making their extrapolation to zero concentration almost impossible. Taking the data at 12° , for example, it is seen that as the concentration increases, the curve at first goes up reflecting the presence of nonassociating protein and the second virial coefficient, then, after passing through a maximum, falls, reflecting the association reaction, and finally, after passing through a minimum, starts to rise again, reflecting the combination of a high degree of association with the positive second virial coefficient. The fact that this treatment yields good agreement between experimental data and calculated curves shows that, in this case $\partial \log \gamma_2'/\partial C_2$ indeed, is identical for the monomer and the aggregate. The same has been found for the case of the mercury dimer of serum albumin (24, 25).

Isoionic Protein in Ion-Free Water. In this case it has been shown (16, 26) that Eq. (1) assumes the form

$$H \frac{C_2}{\tau} = \frac{1}{M_2} \left[1 + \frac{C_2}{M_2} \left(-\frac{\pi N e^4 \langle Z_2^2 \rangle_{AV}^2}{(DkT)^2 \kappa (1 + \kappa a)^2} + \frac{7}{6} \pi N a^3 + \frac{\bar{Z}_2^2}{[\text{H}^+]} \frac{1}{[\text{H}^+] + K_w/[\text{H}^+]^2 - \frac{m_2}{2.303 [\text{H}^+]} \frac{d\bar{Z}}{dp\text{H}}} + 2B' \right) \right] \quad (4)$$

$$\kappa^2 = \frac{4\pi N e^2}{DkT} \left(\frac{\langle Z_2^2 \rangle_{AV} C_2}{M_2} \right)$$

where $\langle Z_2^2 \rangle_{AV}$ is the mean square charge of the protein in protonic units e , D is the dielectric constant of the medium, k is Boltzmann's constant and κ and a are the Debye-Hückel parameters. The first term in concentration which represents the contribution of charge fluctuations on the protein molecule, in the limit, is linear in the square root of protein concentration (27); the second term is the excluded volume and is linear in the first power of protein concentration; the third term, which reflects the ionization of protein with dilution, is a function which passes through a maximum at very low values of protein concentration; $2B'$ reflects the combined effect of all other types of intermolecular force.

The resulting curve is very complicated. It assumes a shape such as shown in Fig. 2 in which the light-scattering curve at low concentrations has been calculated for isoionic conalbumin, using published titration data (28) as well as light-scattering measurements obtained at higher concentrations (29). In this case, due to the fact that the isoionic point of conalbumin is very close to pH 7.0, the protein ionization term becomes important in a concentration range which, at present, is one order of magnitude below the practical range of light-scattering measurements. Thus, a simple extrapolation, linear in $C_2^{1/2}$, gives a value of the molecular weight which deviates by less than 1% from the value obtained after proper correction of the experimental data for the progressive ionization. In other cases, however, neglect of correction for the ionization term, or failure to carry out experiments at sufficiently low concentrations to determine the maximum of the light-scattering curve and the slope of the falling portion below the maximum may result in apparent molecular weight values which are wrong by as much as a factor of two. This has been shown to be the case for isoionic serum albumin in ion-free water (16).

Protein System at High Charge, in Ion-Free Water. When protein molecules are present in solution at pH values such that they carry a high charge and the screening is very low, strong repulsive forces are set up between the individual molecules. As has been shown, these lead to an ordering

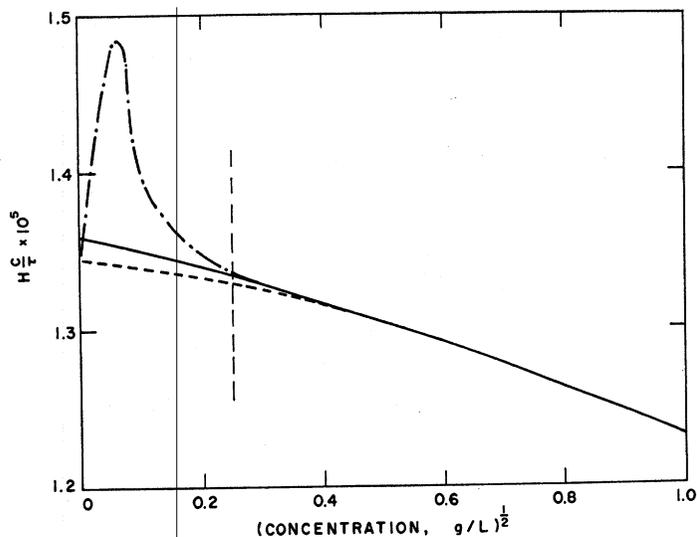


FIG. 2. Light-scattering curve of a protein at its isoionic point in ion-free distilled water. Calculated for conalbumin. —: least-squares curve of experimental data. Vertical dashed line: lowest concentration limit of experimental measurements. - - -: extrapolated experimental data corrected for ionization term. —·—: Course of light-scattering curve in low concentration range calculated from Eq. (4). The difference between the —·— curve and the - - - curve is the contribution of the ionization term.

effect and a nonrandom distribution of the scattering centers in the solution (30, 31). This results in an effectively very large excluded volume. The term $\partial\mu_2^{(e)}/\partial C_2$ of Eq. (1) becomes very large and a function of protein concentration, since with an increase in protein concentration the screening increases the Debye-Hückel parameter increasing directly with the square root of protein concentration. Thus, if the gegenion is hydrogen,

$$\kappa^2 = \frac{4\pi N e^2}{DkT} \left(\frac{\bar{Z}_2^2 C_2}{M_2} + \frac{|\bar{Z}_2| C_2}{M_2} \right) \quad (5)$$

Qualitatively, such a behavior should lead to an initial step increase in $H(C_2/\tau)$ values with protein concentration, followed by a gradual leveling off of the light-scattering curve as the screening becomes sufficiently large. This effect has been observed with several systems, for example, serum albumin (31) and silicotungstic acid (32).

A similar study has been made with α -lactalbumin. The measurements were taken on protein that had been adjusted to pH 6.47 with 0.1 *N* NaOH. The diluting solvent was double-distilled water. Under these conditions, α -lactalbumin has a finite negative charge, and the screening is low. The

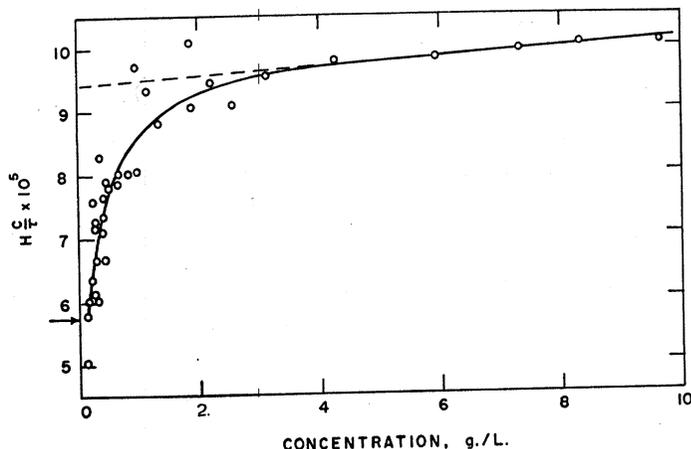


FIG. 3. Light-scattering data of α -lactalbumin in distilled water at pH 6.47.

data are presented in Fig. 3. As can be seen, in the region above a concentration of 1.5 g./l., the points appear to fall on a straight line that would extrapolate to an intercept of 9.4×10^{-5} , corresponding to a molecular weight of 10,600. The molecular weight of this protein, 17,500 (33), corresponds to an $(H(C_2/\tau))_{C_2=0}$ value of 5.75×10^{-5} . At concentrations below 1.5 g./l., however, the scattering assumes a marked concentration dependence, $H(C_2/\tau)$ decreasing almost twofold in the range 0.15–1.5 g./l. At the lower concentrations, the excess turbidity is comparable to that of pure solvent and is in a range inaccessible to normal light-scattering measurements. Thus, had measurements been stopped at the normal lower limit, only the flat region of the curve would have been obtained, leading to the erroneous conclusion that, at pH 6.47 in distilled water, the weight-average molecular weight of α -lactalbumin is 10,600.

A similar error could be made from the literature data on bovine serum albumin (31) and silicotungstic acid (32). Scattering curves for these, normalized to the formula molecular weight, are presented in Fig. 4. These show that extrapolation of the flat portion of the curve to zero protein concentration would lead to intercepts 2.5–3 times the correct value. Indeed, in silicotungstic acid, the downward curvature appears in a region in which the excess turbidity is less than the turbidity of the pure solvent. In the case of the serum albumin at pH 4.1, measurements down to a concentration of 1 g./l. (which is the usual lower limit of measurement) would show the beginning of the curvature.

Matijevic and Kerker (34) have reported light-scattering measurements on 9-phosphotungstic acid in distilled water. Their data, shown in a nor-

LIGHT-SCATTERING DATA

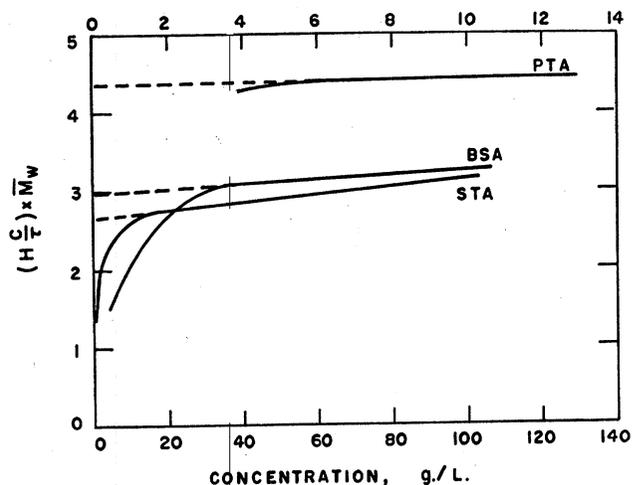


FIG. 4. Normalized light-scattering data of charged macromolecules at low ionic strength. PTA: 9-phosphotungstic acid in distilled water. BSA: bovine serum albumin at pH 4.1 in water of same pH. STA: potassium silicotungstate in distilled water. Upper abscissa refers to concentration of BSA. Solid lines: best curve through experimental points, ending at lowest concentration measured. Dashed lines: extrapolation of linear portion of the solid lines.

malized form in Fig. 4, were not extended to the very low concentration range due to experimental difficulties. As a result, they state that they could not observe the expected sharp drop in $H(C_2/\tau)$ with a decrease in concentration. They found that values on the linear portion of their curve were much higher than the value expected from the formula molecular weight and yielded on extrapolation an "apparent molecular weight" of 1000, instead of 4369, the formula molecular weight.

At the present time, no rigorous quantitative treatment of such behavior in light scattering is available. The *shape* of the curve obtained is that predicted by the approximate treatment of Doty and Steiner (31), but the data cannot be accounted for quantitatively in terms of that treatment. The four cases just described, however, can serve as good examples of the extremely large errors that can be made in the determination by light scattering of the molecular weight of proteins in aqueous solution, under conditions of high charge.

Three-Component Systems

In a three-component system (such as solvent = component 0, dissolved protein = component 2, and other solute = component 1), the light-

scattering equation is:

$$H \frac{C_2}{\tau} = \frac{1}{(1 + D)M_2} \left\{ 1 + \left[\frac{\partial \mu_2^{(e)}}{\partial C_2} - \frac{M_2 (\partial \mu_1 / \partial C_2)^2}{M_1 (\partial \mu_1 / \partial C_1)} \right] \frac{C_2}{RT} \right\} \quad (6a)$$

$$D = -2\alpha \frac{(\partial \mu_1 / \partial C_2)}{(\partial \mu_1 / \partial C_1)} + \left[\alpha \frac{(\partial \mu_1 / \partial C_2)}{(\partial \mu_1 / \partial C_1)} \right]^2 \quad (6b)$$

$$\alpha = \frac{(\partial n / \partial C_1)}{(\partial n / \partial C_2)} \quad (6c)$$

$$\mu_i = RT \log C_i + \mu_i^{(e)} + \mu_i^0(T, p) \quad (6d)$$

where C_i is the concentration of component i in grams/ml., M_i is the molecular weight of component i and $\mu_i^{(e)}$ is the excess chemical potential of that component.

As seen from Eq. (6), in a three-component system the intercept of the usual $H(C_2/\tau)$ vs. concentration plot is *not* the reciprocal of the weight-average molecular weight, but rather it is a function of *both* the molecular weight and the thermodynamic interaction between the macromolecule and component 1.

In the case when there is no interaction between the third component and the protein, i.e., when $(\partial \mu_1 / \partial C_2) = 0$, the term D becomes equal to zero, and the intercept yields the true reciprocal of the weight-average molecular weight. In the case of proteins, at low charge and high ionic strength, if there is no binding of the salt ions to the protein molecules, the term D becomes negligibly small and the system behaves as a pseudo-ideal system (11, 16). The intercept in such a case corresponds to the formula molecular weight within experimental error and the system can be treated as a pseudo two-component system.⁴

When strong protein-component 1 interactions are present, however, the situation becomes quite different. The term D may assume significant values, which are positive in the case of attractive forces and negative in the case of repulsion.⁵ The problem becomes one of the proper evaluation of the term D . Three different approaches have been described. These are:

(1) The thermodynamic treatment, (2) the molecular binding treatment, and (3) the approach where the reciprocal of the intercept is taken to be the sum of the molecular weights of the protein and the component 1 bound per molecule of protein. While it will be shown that methods (1)

⁴ This was the case found for the β -lactoglobulin described above. At 30°, the points extrapolate to an apparent molecular weight of 36,200, which is in good agreement with the accepted value.

⁵ This statement applied to cases where the ratio of refractive index increments (α) is positive [cf. Eq. (6c)]. Should α be negative, the opposite is true. In the remainder of this paper, it will be assumed that α is positive.

and (2) reduce to identical forms in practice, method (3) has no basis in theory and may lead to serious errors.

1. *Thermodynamic Treatment.* In order to calculate the term D , it is necessary to have knowledge of $\partial\mu_1/\partial C_2$. From Eq. (6d), it is evident that when the concentration of component 1 is completely independent of component 2, $\partial\mu_1/\partial C_2$ reduces to the derivative of the excess chemical potential:

$$\frac{\partial\mu_1}{\partial C_2} = \frac{\partial\mu_1^{(e)}}{\partial C_2} = \frac{M_1}{M_2} \frac{\partial\mu_2^{(e)}}{\partial C_1} \equiv RTA_{12} \quad (7)$$

The problem then reduces to the determination of the interaction constant A_{12} .

If it is assumed that the change in the excess chemical potential of the protein with respect to component 1 is due to the binding of substance a ,⁶ which is part of component 1, then A_{12} may be expressed as (35):

$$A_{12} = \frac{M_1}{M_2 RT} \frac{\partial\mu_2^{(e)}}{\partial C_1} = \frac{M_1}{M_2} \left(\frac{\partial \log f_2}{\partial C_1} + \frac{\partial \log \gamma_2'}{\partial C_1} \right) \quad (8)$$

As in the derivation of Eq. (3), the activity coefficient, γ_2 , of the protein is expressed as the product of two terms, i.e., f_2 representing the fraction of protein molecules without bound substance a , and γ_2' representing the activity coefficient of the protein making up that fraction.

In the case of salt binding, by setting $\partial \log \gamma_2'/\partial C_1$ equal to zero (i.e., neglecting long-range electrostatic interaction) and making use of Scatchard's equation of binding (36):

$$\begin{aligned} A_{12} &= \frac{M_1}{M_2} \frac{\partial \log f_2}{\partial C_1} = - \frac{10^3}{M_2} \sum_{i=1}^s \frac{n_i k_i e^{-2w(\bar{z}_2 + \bar{\nu}z_a)z_a}}{1 + m_a' \gamma_1 k_i e^{-2w(\bar{z}_2 + \bar{\nu}z_a)z_a}} \frac{\partial(\gamma_1 m_a)}{\partial m_1} \\ &= - \frac{10^3}{M_2} \sum \nu_i \frac{\bar{\nu}}{m_1} (\sum \nu_i + A_{11} C_1) \end{aligned} \quad (9)$$

$$m_a' = m_a - \bar{\nu} m_2$$

where γ_1 is the activity coefficient of component 1, n_i is the number of binding sites with an intrinsic binding constant k_i , m_j is the molar concentration of component j , $\bar{\nu}$ is the average number of particles of substance a bound per molecule of component 2, and the exponential term describes

⁶ Where component 1 is a neutral molecule (e.g., urea), substance a is component 1 itself. If the binding, however, is that of a portion of component 1, such as that of chloride ion derived from calcium chloride, substance a is chloride ion, while component 1 is calcium chloride.

the electrostatic contribution defined by Scatchard for the case when component 1 is an electrolyte. The term D becomes then:

$$D = \frac{-2\alpha A_{12}}{\sum \frac{\nu_i}{C_1} + A_{11}} + \left(\frac{\alpha A_{12}}{\sum \frac{\nu_i}{C_1} + A_{11}} \right)^2 \quad (10)$$

where $\sum \nu_i$ is the number of particles into which component 1 dissociates, and

$$A_{11} = \frac{1}{RT} (\partial \mu_1^{(e)} / \partial C_1).$$

Making use of Eqs. (9) and (10) and of binding data from the literature, values of D have been calculated for serum albumin in the presence of urea, of NaCl, and of NaSCN. Since the value of A_{12} required is that at infinite dilution of protein, $m_a' = m_a$ [Eq. (9)]. The values of A_{11} used, taken from the literature (38), were found to make a negligible contribution for the cases treated here. Likewise, the square term of Eq. (10) is small enough to be neglected. The numerical values of D calculated in this manner are shown in col. 7 of Table I. From these calculations one can see that in the case of serum albumin in a medium containing Cl^- or SCN^- ions, the contribution of the binding to the apparent molecular weight (col. 8, Table I) is less than 2%, or well within the experimental error of light scattering. Since bovine serum albumin (BSA) is known to be a strong binder of ions, these values of D may be considered as maximal for most proteins in salt solution. Thus, it becomes reasonable to assume that this effect will be small when the light-scattering molecular weight of a protein is to be measured. This does not mean, however, that the thermodynamic interaction can be neglected arbitrarily without knowledge of the degree of protein-component 1 interaction. Certainly, in cases such as BSA-urea (25, 39) or sodium thymonucleate- HgCl_2 (40), this factor assumes large values and results in apparent molecular weights differing by as much as a factor of 3 from the formula molecular weight.

2. *Molecular Binding Treatment.* In this treatment, suggested by Stockmayer (19) from consideration of the multicomponent theory, and applied by Katz (39), by Casassa (41) and by Kay and Edsall (25), the term D is expressed as

$$D = 2\alpha \frac{M_1}{M_2} \left(\frac{\partial m_1}{\partial m_2} \right)_{\mu_1} + \left[\alpha \frac{M_1}{M_2} \left(\frac{\partial m_1}{\partial m_2} \right)_{\mu_1} \right]^2 \quad (11)$$

$$= 2\alpha \frac{M_1}{M_2} \frac{\bar{\nu}}{\sum \nu_i} + \left(\alpha \frac{M_1}{M_2} \frac{\bar{\nu}}{\sum \nu_i} \right)^2$$

where the terms have the same meaning as above. Examination of Eq. (11) shows that the two treatments yield identical results, since Eq. (11) can be obtained directly by combining Eqs. (9) and (10).⁷

3. *Addition of Molecular Weight of Bound Substance a to Molecular Weight of Protein.* In this empirical treatment, which has been used on occasion, D is expressed as

$$D = \frac{\bar{\nu}M_1}{M_2} \quad (12)$$

Comparison of this with Eq. (10) or (11) shows this treatment immediately to be wrong, since it neglects completely the optical properties of the components [α of Eqs. (6), (10), and (11)] as well as the dissociation of component 1, if it is an electrolyte. Sample calculations for the cases of the binding of NaCl, NaSCN, and urea to serum albumin (BSA) and HgCl₂ to sodium thymonucleate have been carried out using Eq. (12), and the results are listed in cols. 9 and 10 of Table I. The apparent agreement between Eq. (12) and Eqs. (10) and (11) in the case of BSA–NaCl and BSA–NaSCN is purely a coincidence. It is simply due to the fact that in those cases $\sum \nu_i = 2$ and cancels with the coefficient of the numerator of Eqs. (10) and (11), while the ratio of the refractive-index increments (α) is close to unity. In the case of binding of mercury to sodium thymonucleate, however, the calculated values of D from Eqs. (10) or (11) (theoretical) and Eq. (12) (empirical) differ by a factor of 2.5. This results in a 25% error in molecular weight if the empirical approach of Eq. (12) is used. In the case of BSA–urea this error is 10% in the opposite direction, as shown in col. 11 of Table I.

It should be noted that attribution to molecular binding of the deviation of the intercept from the reciprocal of the molecular weight is an idealization of the actual picture. Light scattering, being a thermodynamic measurement, gives a measure of changes in the chemical potential of one component with respect to another independently of the mechanism of the interaction or the nature of the forces operative. Thus, attractive forces between components 2 and 1 lead to a decrease in

$$(H(C_2/\tau))_{C_2=0},$$

while repulsive forces raise that value. According to the molecular binding interpretation, when the net force acting between components 1 and 2 is repulsive and D is negative, there is negative binding of component 1 to component 2 ($\bar{\nu}$ is negative).

⁷ By examination of Eqs. (9), (10), and (11), it can be seen that light-scattering data can be used conversely to characterize molecular binding. Thus, from a measurement of the deviation of the intercept from the reciprocal of the weight-average molecular weight at a set of concentrations of component 1, values of $\bar{\nu}$ at each concentration may be obtained. By plotting these in the manner described by Scatchard (36), the values of the n_i 's and k_i 's may be obtained.

TABLE I
Calculation of Intercepts in Three-Component Systems

(1) Macro- molecule ^e	(2) Compo- nent 1	(3) m_1	(4) $\frac{1}{\tau}$	(5) A_{12} <i>ml./g.</i>	(6) α	(7) $D_{\text{theor.}}^b$ $\times 10^8$	(8) $(1 + D)M_s^b$ theor. = Q	(9) $D_{\text{emp.}}^c$ $\times 10^8$	(10) $(1 + D)M_s^c$ emp. = S	(11) S/Q^d
BSA	NaCl	0.200	9.4 ^e	-0.68	0.90	7.17	69,496	7.97	69,550	1.0008
BSA	NaCl	0.020	4.5 ^e	-3.26	0.90	3.43	69,237	3.81	69,263	1.0004
BSA	NaSCN	0.200	19 ^e	-1.38	0.85	18.9	70,304	21.1	70,458	1.0022
BSA	NaSCN	0.020	7 ^e	-5.07	0.85	7.0	69,483	8.2	69,567	1.0012
SDNA	HgCl ₂	0.0503	1.5 $\times 10^4$	-37.2	0.59	196	9.69 $\times 10^8$	501	12.1 $\times 10^8$	1.249
BSA	Urea	8.0	960 ^f	-1.74	0.62 ^f	1040	141,000	835	127,000	0.901

^a Molecular weight of bovine serum albumin (BSA) taken to be 69,000; that of sodium thymonucleate (SDNA) taken to be 8.1 $\times 10^5$ (40).

^b Calculated according to Eq. (10) or (11).

^c Calculated according to Eq. (12).

^d Error in light-scattering intercept resulting from the use of Eq. (12).

^e Binding calculated from constants given in Ref. (37).

^f Values taken from Kay and Edsall (25) at pH 6.

This fact has been explained by the statement that light scattering measures only the preferential binding of component 1 as compared with solvent (39). Thus, negative values of \bar{v} mean that protein binds solvent preferentially over component 1. Adopting for the sake of simplicity the concept that molecular binding describes completely the thermodynamic interactions in the system, let us consider that each binding site can be occupied either by solvent or component 1. Then, the binding of one molecule of component 1 will involve the displacement of one molecule of component 0, and the free energy of binding, ΔF^b , will be the difference between the free energy of interaction of component 2 with 1 (ΔF^{12}) and component 2 with 0 (ΔF^{02}).

$$\Delta F^b = \Delta F^{12} - \Delta F^{02}$$

$$\frac{\partial \mu_1^b}{\partial m_2} = \frac{\partial \mu_2^b}{\partial m_1} = \frac{\partial^2 \Delta F^b}{\partial m_1 \partial m_2} = \frac{\partial^2 \Delta F^{12}}{\partial m_1 \partial m_2} - \frac{\partial^2 \Delta F^{02}}{\partial m_1 \partial m_2} \quad (13)$$

Thus, the sign of ΔF^b and of $\partial \mu_1^b / \partial m_2$ will depend on the relative magnitudes of the two free energies of interaction. If ΔF^{12} is larger than ΔF^{02} , component 1 is bound preferentially to component 2, ΔF^b is negative, A_{12} is negative, and the intercept is lower than $1/M_2$. Should solvent be bound preferentially, the opposite is true, and the intercept is raised above $1/M_2$.

An interesting example of preferential interaction of component 2 with solvent, reported recently by Palmer and co-workers (42) is that of araban fractions in aqueous solution in the presence of small amounts of dimethyl formamide (DMF). In the presence of 1% DMF, the light-scattering intercept for their fraction 9 was $1/20,700$, while in the absence of DMF a value of $1/36,300$ was obtained. Furthermore, the molecular weight, 38,400, as obtained by sedimentation-viscosity, was independent of solvent composition. Thus, the difference in light-scattering intercepts could be attributed only to the existence of repulsive forces between araban and DMF (preferential binding of water). The value of A_{12} obtained was 32 ml./g. ($\alpha = 0.89$; $\sum v_i = 1$), representing a very strong repulsion of DMF by araban. If interpreted in terms of molecular binding, this would correspond to $1/3$ of the water molecules not being available to DMF at a 1% araban concentration. Indeed, it is not surprising then to find that a small increase in the DMF concentration results in aggregation of the araban.

The three-component treatment can be applied also to the interpretation of the interaction between two proteins in aqueous solution under conditions where protein-salt interactions are small. In such cases, however, it is usually found that the concentrations of both proteins decrease to zero, so that the intercept yields the weight-average molecular weight of the two proteins (43).

CONCLUSIONS

The determination of the molecular weight of a protein by the light-scattering method can present great difficulties and, as a consequence,

great caution has to be exercised in selecting proper conditions for carrying out such measurements. Thus, while in a two-component system (protein dissolved in water), the value of $H(C_2/\tau)$ formally *does* extrapolate to the correct value of $1/M_2$, in practice this extrapolation may be extremely difficult or practically impossible, as shown above for three different cases. In order to obtain the molecular weight of a protein in an aqueous salt-free system, it is vital to extend the measurements to very high dilutions and to take into account properly the various effects which lead to the complex concentration dependence of $\partial\mu_2^{(s)}/\partial C_2$. In the case of a three-component system (protein dissolved in water with another solute present, e.g., a neutral electrolyte), the value of $H(C_2/\tau)$ *formally* does *not* extrapolate to the correct value of $1/M_2$. In such cases, in order to obtain the correct molecular weight, it is necessary to have additional information as to the thermodynamic interaction of the protein with the second solute. In the case of proteins in the presence of salts at moderate ionic strengths the deviation of the intercept from the correct formula molecular weight is usually smaller than the experimental error in light scattering and is not observed. Where interactions are very strong, however, this need not be the case and large deviations may be observed. Since the magnitude of the deviation is also a function of the optical properties of the solution, it is possible to have three-component systems in which the term D disappears simply because $\alpha \approx 0$. An example of such a system is one in which water is component 0, and methanol is component 1. Thus, in order to be certain that the interaction term in the evaluation of the molecular weight of a protein may be neglected, it is necessary to have independent information as to the magnitude of A_{12} and the ratio of the refractive-index increments of components 1 and 2.

REFERENCES

1. NORD, F. F., AND RANKE-ABONYI, O. M. V., *Science* **75**, 54 (1932).
2. NORD, F. F., *Ergeb. Enzymforsch.* **2**, 23 (1933).
3. WEISS, G., AND NORD, F. F., *Z. physik. Chem.* **A166**, 1 (1933).
4. RAMPINO, L. D., AND NORD, F. F., *J. Am. Chem. Soc.* **63**, 2745 (1941).
5. BIER, M., AND NORD, F. F., *Proc. Natl. Acad. Sci. U. S.* **35**, 17 (1949).
6. BIER, M., AND NORD, F. F., *Rev. Sci. Instr.* **20**, 752 (1949).
7. TIMASHEFF, S. N., BIER, M., AND NORD, F. F., *Proc. Natl. Acad. Sci. U. S.* **35**, 364 (1949).
8. TIMASHEFF, S. N., BIER, M., AND NORD, F. F., *J. Phys. & Colloid Chem.* **53**, 1134 (1949).
9. NORD, F. F., BIER, M., AND TIMASHEFF, S. N., *J. Am. Chem. Soc.* **73**, 289 (1951).
10. TIMASHEFF, S. N., AND NORD, F. F., *Arch. Biochem. Biophys.* **31**, 309 (1951).
11. EDSALL, J. T., EDELHOCH, H., LONTIE, R., AND MORRISON, P. R., *J. Am. Chem. Soc.* **72**, 4641 (1950).
12. HALWER, M., *J. Am. Chem. Soc.* **76**, 183 (1954).

13. KRONMAN, M. J., STERN, M. D., AND TIMASHEFF, S. M., *J. Phys. Chem.* **60**, 829 (1956).
14. KRONMAN, M. J., Doctoral Dissertation, Temple University, 1955.
15. DOTY, P., GELLERT, M., AND RABINOVITCH, B., *J. Am. Chem. Soc.* **74**, 2065 (1952).
16. TIMASHEFF, S. N., DINTZIS, H. M., KIRKWOOD, J. G., AND COLEMAN, B. D., *J. Am. Chem. Soc.* **79**, 782 (1957).
17. YASNOFF, D. S., AND BULL, H. B., *J. Biol. Chem.* **200**, 619 (1953).
18. KIRKWOOD, J. G., AND GOLDBERG, R. J., *J. Chem. Phys.* **18**, 54 (1950).
19. STOCKMAYER, W. H., *J. Chem. Phys.* **18**, 58 (1950).
20. BRINKMAN, H. C., AND HERMANS, J. J., *J. Chem. Phys.* **17**, 574 (1949).
21. TOWNEND, R., AND TIMASHEFF, S. N., *Arch. Biochem. Biophys.* **63**, 482 (1956).
22. TIMASHEFF, S. N., AND TOWNEND, R., *J. Am. Chem. Soc.* **80**, 4433 (1958).
23. TIMASHEFF, S. N., AND TOWNEND, R. E., *4th Intern. Congr. Biochem., Vienna, 1958, Abstr. Commun.*, p. 25.
24. EDELHOCH, H., KATCHALSKI, E., MAYBURY, R. H., HUGHES, W. L., JR., AND ED-SALL, J. T., *J. Am. Chem. Soc.* **75**, 5058 (1953).
25. KAY, C. M., AND EDSALL, J. T., *Arch. Biochem. Biophys.* **65**, 354 (1956).
26. KIRKWOOD, J. G., AND TIMASHEFF, S. N., *Arch. Biochem. Biophys.* **65**, 50 (1956).
27. KIRKWOOD, J. G., AND SHUMAKER, J. B., *Proc. Natl. Acad. Sci. U. S.* **38**, 863 (1952).
28. LOWEY, S., *Arch. Biochem. Biophys.* **64**, 111 (1956).
29. TIMASHEFF, S. N., AND TINOCO, I., JR., *Arch. Biochem. Biophys.* **66**, 427 (1957).
30. KIRKWOOD, J. G., AND MAZUR, J., *Compt. Rend. 2° Reunion Chim. Phys., Paris, 1952*, p. 143.
31. DOTY, P., AND STEINER, R. F., *J. Chem. Phys.* **20**, 85 (1952).
32. KRONMAN, M. J., AND TIMASHEFF, S. N., *J. Phys. Chem.* **63**, 629 (1959).
33. EDSALL, J. T., in "The Proteins" (NEURATH, H., AND BAILEY, K., eds.), Vol. 1B, p. 634. Academic Press, New York, 1953.
34. MATIJEVIC, E., AND KERKER, M., *J. Am. Chem. Soc.* **81**, 1307 (1959).
35. COLEMAN, B. D., Doctoral Dissertation, Yale University, 1954
36. SCATCHARD, G., *Ann. N. Y. Acad. Sci.* **51**, 660 (1949).
37. SCATCHARD, G., COLEMAN, J. S., AND SHEN, A-L., *J. Am. Chem. Soc.* **79**, 12 (1957).
38. HARNED, H. S., AND OWEN, B. B., "The Physical Chemistry of Electrolytic Solutions," 2nd ed. Reinhold, New York, 1950.
39. KATZ, S., Doctoral Dissertation, Harvard University, 1950.
40. KATZ, S., *J. Am. Chem. Soc.* **74**, 2238 (1952).
41. CASASSA, E. F., *J. Phys. Chem.* **60**, 926 (1956).
42. TOMIMATSU, Y., PALMER, K. J., GOODBAN, A. E., AND WARD, W. H., *J. Polymer Sci.*, in press.
43. PEPE, F. A., AND SINGER, S. J., *J. Am. Chem. Soc.*, in press.