(expts. 4, 5 and 6) and the rate decreased as the concentration of caproic acid increased (expts. 1, 2 and 3).[23,24] A comparison of the results of expts. 4 and 9 shows the effect of carrying out the reaction in refluxing toluene instead of benzene. When excess malonic ester was used, the rate constants decreased appreciably (*cf.* expts. 2, 7 and 8); again this may be a medium effect. Attempts to determine the effect on the kinetics of a large excess of benzaldehyde were unsuccessful because under these conditions a normally slow side reaction seriously interfered (see Experimental).

Because of this side reaction and the somewhat anomalous effects encountered upon using excess malonic ester and upon varying the para substituent in the benzaldehydes a detailed interpretation of the results in terms of the mechanism is not given here. It should be noted, however, that the weight of the evidence is consistent with the favored mechanism[25] if the over-all rate is chiefly controlled by the rate at which the piperidine removes a proton from the active methylene group. The question of mechanism is, of course, not necessarily involved in using the results for predicting the optimum conditions for condensations of this type.

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Summary

It has been found that the rates of condensation of certain active methylene compounds with benzaldehyde decreased in the order: $CNCH_2COOC_2H_5$ (66), $CH_3COCH_2COOC_2H_5$ (22), $C_6H_5CH_2NO_2$ (1.2), $H_2C(COOC_2H_5)_2$ (1), $C_6H_5CH_2COCH_3$ (0.58) and $C_6H_5COCH_2C_6H_5$ (0.56). The numbers in parentheses are a quantitative measure of the relative reactivities. The rates of condensation of malonic ester with benzaldehydes decreased only gradually in the order: *p*-$CH_3OC_6H_4CHO$, C_6H_5CHO, *p*-ClC_6H_4CHO and *p*-$O_2NC_6H_4CHO$. The over-all decrease was about 40%.

(23) This negative effect of increasing acid concentration and the relatively small effect of varying the para substituent in the benzaldehyde indicate that the dehydration step of the process is not the rate controlling one.

(24) The decrease in catalytic activity of piperidine with increasing acidity has been previously noted for the reaction of formaldehyde with cyanacetamide. See Enkvist, *J. prakt. Chem.*, **149**, 65 (1937) and Enkvist and Andersson, *ibid.*, **153**, 116 (1939).

(25) See Hammett, ref. 19a, p. 343, for a general discussion of the mechanism of these reactions. Also see Coombs and Evans, *J. Chem. Soc.*, 1295 (1940).

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[CONTRIBUTION FROM THE DEPARTMENT OF PHYSICAL CHEMISTRY, HARVARD MEDICAL SCHOOL]

Light Scattering in Solutions of Serum Albumin: Effects of Charge and Ionic Strength[1,2]

BY JOHN T. EDSALL, HAROLD EDELHOCH,[3] RENÉ LONTIE AND PETER R. MORRISON

The determination of molecular weights of proteins from measurement of light scattered by their solutions, was apparently first carried out by Putzeys and Brosteaux[4] who studied egg albumin, serum albumin and certain hemocyanins. They employed amandin as a standard protein, assuming its molecular weight to be 330,000; the relative molecular weights of the other proteins they studied agreed well with those obtained by other methods. More recently, Bücher[5] has used light scattering to measure the molecular weight of enolase, employing edestin as a standard of reference. Heller[6a] has reported measurements on egg albumin; and Halwer, Nutting and Brice[6b] have determined on an absolute scale the molecular weights of β-lactoglobulin, ovalbumin and bovine serum albumin with results in good agreement with the values obtained by other methods. Campbell, Blaker and Pardee[7] have determined the molecular weight of a rabbit antibody. Oster, Doty and Zimm[8] studied tobacco mosaic virus, which, unlike the other molecules previously mentioned, is in its longest dimension comparable with the wave length of visible light; thus they were able to determine not only the molecular

(1) This paper is Number 88 in the series "Studies on the Plasma Proteins" from the Harvard Medical School, Boston, Massachusetts, on products developed by the Department of Physical Chemistry from blood collected by the American Red Cross, and Number XXVII in the series "Preparation and Properties of Serum and Plasma Proteins" from the same Department. A preliminary account of some of this work was presented at the 114th Meeting of the American Chemical Society in Washington, D. C., August 30 to September 3, 1948; see the abstracts of this meeting, page 25C.

(2) This work was originally supported by grants from the Rockefeller Foundation and from funds of Harvard University. It was aided early in 1941 by grants from the Committee on Medicine of the National Research Council, which included a grant from the American College of Physicians. From August, 1941, to July, 1946, it was carried out under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University. Since then it has been aided by a grant recommended by the Panel on Hematology of the National Institutes of Health.

(3) National Cancer Institute Postdoctorate Research Fellow, 1947–1949.

(4) P. Putzeys and J. Brosteaux, *Trans. Faraday Soc.*, **31**, 1314 (1935); *Med. Kon. Vl. Acad. Wetensch.*, **III**, No. 1 (1941).

(5) T. Bücher, *Biochim. et Biophys. Acta*, **1**, 467 (1947).

(6) (a) W. Heller and H. B. Klevens, *Phys. Rev.*, **67**, 61 (1945). (b) M. Halwer, G. C. Nutting and B. A. Brice, Abstracts of Papers, 116th Meeting, American Chemical Society, page 3G, 1949.

(7) D. H. Campbell, R. H. Blaker and A. B. Pardee, THIS JOURNAL, **70**, 2496 (1948).

(8) G. Oster, P. Doty and B. H. Zimm, *ibid.*, **69**, 1193 (1947)

weight, but also—from measurements of the angular dissymmetry of the scattering intensity—the length of the major axis of the molecule.

Thus the determination of the molecular weights of proteins by light scattering is a well-established procedure. However, the intensity of scattering from a protein solution, when studied as a function of protein concentration, often shows very great deviations from the behavior of an ideal solution. A series of experiments by two of us (R. L. and P. R. M.) in the summer of 1947, revealed pronounced effects of variation of *p*H and ionic strength, even at low protein concentrations; and these have been systematically explored, using more precise and sensitive techniques, during the past two years. Analogous effects are well-known in osmotic pressure measurements on protein systems; hence, the results obtained in the light scattering measurements were not unexpected, in view of the intimate relation between the theory of light scattering and that of osmotic pressure (see for instance[9a,b,c,d]). Here we shall present the theoretical background for the interpretation of the experiments, which will then be presented in the later portion of this and in subsequent papers.[10]

Definition of Components

Protein solutions generally contain at least three components and often many more. An isoionic solution of a pure protein in pure water is indeed a two component system; but in general we deal with protein ions carrying a net charge, in the presence of one or more salts, and often also of one or more non-ionic diffusible components. Frequently also it is important to consider systems containing two or more proteins, which may interact strongly with one another.

We shall follow Scatchard[12] in the definition of components, denoting the solvent as component 1, the protein—if only one is present—as component 2, the salt with diffusible ions—if only one is present—as component 3. Other protein components, if present, may be denoted by higher even numbers, and other diffusible components by higher odd numbers.[13] All components are taken as electrically neutral; hence we distinguish between the protein *ion* and the protein *component*. The valence (Z_2) of the protein ion is defined as the mean net proton charge per protein ion, Z_2 being zero for the isoionic protein. It is thus equal to the mols of "bound acid" (Z_2 positive) or "bound base" (Z_2 negative) per mol of protein, determined by a titration with the hydrogen or glass electrode, with suitable corrections.[14] An albumin, soluble in water in the absence of salt, may be taken as approximately isoionic ($Z_2 = 0$) after thorough electrodialysis has removed all diffusible ions except H^+ and OH^- from the solution.[15] If a neutral salt is added to such a solution, it remains isoionic by definition, although the salt addition may cause the electrophoretic mobility to become positive or negative, due to selective binding of cations or anions by the protein. The value of Z_2 is adjusted to positive or negative values by addition of strong acids or bases.[17] We shall consider later the effects of binding of other ions by the protein, which may make the effective net charge of the protein ion considerably different from Z_2.

We shall take the molecular weight of isoionic serum albumin as 69,000, the figure given by the osmotic pressure studies of Scatchard, Batchelder and Brown (see reference 32 below), although the light scattering measurements reported here indicate a small amount of material of higher molecular weight in the preparation employed by us. The specification of molecular weight of the protein, in the definition of the protein component, is largely a matter of convenience. What is essential is that the mass and chemical nature of the protein component (or components) added to the system should be definitely specified. The value of Z_2 must, of course, always be expressed so that it is stoichiometrically correct; the mean number of protons bound to, or removed from, the isoionic protein, *per gram protein*, must obviously depend only on the stoichiometric composition of the system, not on the assumed molecular weight. The values of Z_2 reported here give moles of H^+ bound or removed, per gram protein, multiplied by the factor 69,000.

The protein in solution may undergo association or dissociation reactions; so the actual weight average molecular weight, as revealed by the light scattering measurements,

(9) (a) P. Debye, *J. Phys. and Colloid Chem.*, **51**, 18 (1947); (b) B. H. Zimm, R. S. Stein and P. M. Doty, *Polymer Bulletin*, **1**, 90 (1945); (c) H. Mark in "Molecular Architecture" (Frontiers in Chemistry, Vol. 5) p. 121 (Interscience Publishers, New York, N. Y., 1948); (d) G. Oster, *Chem. Rev.*, **43**, 319 (1948).

(10) Experiments closely related to ours have been recently carried out by Doty and Steiner,[11] who have been concerned particularly with phenomena occurring when the protein carries a large net charge, and the added salt is zero or nearly zero. In the experiments reported here, the amount of added salt was appreciable, and the special effects studied by Doty and Steiner did not appear.

(11) P. Doty and R. F. Steiner, *J. Chem. Phys.*, **17**, 743 (1949).

(12) G. Scatchard, THIS JOURNAL, **68**, 2315 (1946).

(13) The term "diffusible" in this connection, denotes ability to pass through a membrane impermeable to molecules as large as typical proteins.

(14) See for instance E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," New York, N. Y., 1943, Chapter 20; C. Tanford, THIS JOURNAL, **72**, 441 (1950).

(15) If the only ions present in this solution are H^+, OH^- and protein ions, it is clear that the mean net charge on the protein cannot be *exactly* zero unless the isoionic point happens to coincide with the *p*H of neutrality. Thus, if the *p*H of the electrodialyzed solution is 5, we have $[H^+] = 10^{-5}$ *M*, $[OH^-]$ negligible, and the protein must carry a small negative charge to balance the excess of H^+ over OH^- ions. For a serum albumin solution, concentration 7 g./l. (10^{-4} *M*) this requires that Z_2, for the electrodialyzed solution, be -0.1 ($= -10^{-5}/10^{-4}$), instead of zero. The difference is well within the usual experimental error, but it can be corrected for if necessary. The correction obviously becomes more important, the more acid the isoionic point of the protein and the more dilute the protein solution. Scatchard and Black[16] define an isoionic material as one which gives no non-colloidal ions other than hydrogen and hydroxyl. Thus, by their definition, electrodialyzed albumin is isoionic when $Z_2m_2 = (OH^-) - (H^+)$, m_2 being the molar concentration of protein ion; while we have defined it as isoionic when $Z_2 = 0$. In practice, the difference between the two definitions is generally negligible.

(16) G. Scatchard and E. S. Black, *J. Phys. Colloid Chem.*, **53**, 88 (1949).

(17) The use of strong alkali may be avoided by addition of sodium bicarbonate to the protein solution, which is then frozen and dried from the frozen state *in vacuo*. Under these circumstances, CO_2 is evolved according to the reaction: $HCO_3^- \rightarrow CO_2(g) + OH^-$; and on redissolving the dried protein the *p*H of the solution, and the value of Z_2, should be the same as if an equivalent amount of Na^+OH^-, instead of $Na^+HCO_3^-$, had been added in the first place.

may be quite different from the value assumed in defining the protein component. Under the conditions of the experiments reported in this paper, the evidence seems to be quite against the occurrence of such reactions, although they do occur in the presence of certain other substances,[1] as will be reported in detail later. The occurrence of such reactions, if they are reversible, need not affect our definition of the protein component. (If irreversible reactions are occurring, a thermodynamic treatment is obviously inapplicable; such cases will not be considered here.)

The protein component, like all the other components, is so defined as to be electrically neutral. If the protein is being added to the system as an ion of valence Z_2, we must add at the same time some diffusible ions of charge opposite in sign to Z_2, or remove some ions of the same sign of charge as Z_2, or do both of these things, in such a way that the total increment of net charge is zero. Certain amounts of the diffusible ions in the system are thus assigned to the protein component, the amounts being positive if the ion in question is added, negative if it is removed, when the protein ion is added to the system.

The protein component might be defined so as to contain one mole of protein ion and Z_2/Z_1 moles of the diffusible ion of opposite charge to Z_2, where Z_1 is the valence of this diffusible ion. This is perhaps the most obvious definition, but it has the serious disadvantage that the addition of one mole of protein component involves adding $1 + (Z_2/Z_1)$ moles of ions to the system; the resulting effect on the chemical potential of the solvent would be chiefly due to the diffusible ions added, not to the protein ion which primarily concerns us. Therefore, following Scatchard,[12] we choose another definition, which involves the net addition of only one mole of ions to the system per mole of protein component. If only two diffusible ions are present, and both are univalent, this requires adding $Z_2/2$ moles of the diffusible ion with sign of charge opposite to that of the protein, and *removing* $Z_2/2$ moles of the diffusible ion with the same sign of charge as Z_2, when we add one mole of protein ion to the system. Thus the net addition of moles of diffusible ions is zero, and the solution remains electrically neutral after the protein component is added.

Consider the specific case of serum albumin at a concentration of 10^{-4} molar (approximately 7 g./l.), in a solution to which 20×10^{-4} moles/l. of hydrochloric acid has been added, so that $Z_2 = +20$; 0.010 mole/l. of sodium chloride has also been added to the solution. Thus the total concentration of diffusible ions in mole/l. is $(Na^+) = 0.010$; $(Cl^-) = 0.012$. (The concentration of free hydrogen and hydroxyl ions is assumed to be negligible in comparison.) The first definition discussed in the preceding paragraph would include in component 2, per liter solution, 0.0001 mole of protein ion (valence +20) and 0.0020 mole of chloride ion. Component 3 (sodium chloride) would then contain 0.010 mole of both Na^+ and Cl^- ion. By the second definition, which we shall employ in the following discussion, component 2 includes 0.0001 mole of protein ion, 0.001 mole of Cl^- ion, and *minus* 0.001 mole of Na^+ ion. Hence, by this definition, component 3 consists of 0.011 mole of both Na^+ and Cl^-. Obviously, when we add all the constituents of all the components together, the resulting sum must give correctly the actual composition of the system. It is clearly meaningless to ask whether (for example) any individual chloride ion, chosen at random, belongs to component 2 or component 3; but it is essential that the sum of the number of chloride ions assigned to these components, by any definition, should equal the number of chloride ions actually present.

A somewhat more general discussion may be given for the case in which there is only one protein component, and the mean valence of the protein ion is Z_2. Assume the solution to contain in addition to H^+ and OH^- ions (at negligible concentrations), only one other salt, containing only one kind of cation and one kind of anion (although one mole of salt may contain two or more moles of either cation or anion or both). Let m_2 be the molar concentration of protein; then Z_2m_2 is its equivalent concentration as cation or anion, with the appropriate sign. Then the algebraic sum of the total molar concentrations in the solution of other cations and anions, multiplied by their valences, must be equal numerically, and opposite in sign, to Z_2m_2. Some of these ions must be assigned to the protein component in order to satisfy the conditions for this component stated above. Let Z_c be the valence of the cations of the added salt, Z_a that of the anions. Let ν_{2c} be the number of diffusible cations per mole of protein component, and ν_{2a} the number of diffusible anions. These numbers may be calculated as follows. To fulfil the requirement that only one mole of ions be added to the system per mole of protein component, we must have

$$\nu_{2c} = -\nu_{2a} \tag{1}$$

and to fulfil the requirement that the protein component is electrically neutral, the relation must hold that

$$Z_c\nu_{2c} + Z_a\nu_{2a} = -Z_2 \tag{2}$$

combining with (1) above, we have

$$\nu_{2c}(Z_c - Z_a) = -Z_2 \tag{3a}$$

$$\nu_{2c} = \frac{-Z_2}{Z_c - Z_a} = \frac{-Z_2}{Z_c + |Z_a|} = -\nu_{2a} \tag{3b}$$

Thus, if the total molar concentration of diffusible cation in the solution is m_c, and the total molar concentration of anion is m_a, and if one mole of the salt (component 3) contains ν_{3c} moles of cation and ν_{3a} moles of anion, then the molar concentration of component 3 is

$$m_3 = \frac{1}{\nu_{3c}}(m_c - \nu_{2c}m_2) = \frac{1}{\nu_{3a}}(m_a - \nu_{2a}m_2) = \frac{1}{\nu_{3a}}(m_a + \nu_{2c}m_2) \tag{4}$$

These equations imply nothing concerning the nature of the forces between the protein ion and the other ions in solution; they serve simply to define the components stoichiometrically. Other definitions of the components could of course be employed. From the definition given here, it is apparent (equation 3a) that either ν_{2c} or ν_{2a} must be negative, except when both are zero. This system of definition is useful only when m_3 is fairly large compared to $\nu_{2c}m_2$ (or $\nu_{2a}m_2$); some of the possible alternative definitions are discussed by Scatchard.[12]

If another salt, containing a cation X^+ and an anion Y^-, is added to the system, then the ions X^+ and Y^- are taken as forming a new component (component 5 according to our conventions). If they are present in equivalent concentrations in the solution, these ions are not considered as forming part of the protein component.[18]

Light Scattering in Multicomponent Systems of Molecules Small Compared to the Wave Length of the Light Employed.—In dilute solutions the osmotic pressure, P, of a two-component system composed of a solvent (component 1) of low molecular weight, and a solute (component 2) of high molecular weight, can generally be described by the equation[9a,9c]

$$\frac{P}{RTc_2} = \frac{1}{M_2} + Bc_2 \tag{5}$$

Here M_2 is the molecular weight of the solute, c_2 its concentration in g./cc., and B is a coefficient determined by the thermodynamic properties of the system. B may be called the interaction constant.

(18) In this last respect, our definition of the components differs from that of Scatchard. As long as only one diffusible salt (component 3) is present, our definitions of ν_{2c} and ν_{2a} coincide with his (see ref. 12, p. 2317, equation 23). Our definition, in the case when more than one diffusible salt is present, is chosen as convenient for the conditions of our experiments. Some special systems are not covered by our definitions, but the latter may be given natural extensions when necessary (along the lines indicated in Scatchard's treatment) to cover such special cases when they arise.

Similarly, the turbidity, τ, of such a solution is given by the equation

$$\frac{Hc_2}{\tau} = \frac{\partial}{\partial c_2}\left(\frac{P}{RT}\right) = \frac{1}{M_2} + 2B_{22} \qquad (6)$$

H in equation (6) is defined by the equation

$$H = \frac{32\pi^3}{3N\lambda^4} n^2 \left(\frac{n - n_0}{c_2}\right)^2 \qquad (7)$$

N is Avogadro's number, λ is the wave length *in vacuo* of the light employed, n_0 the refractive index of the solvent and n that of the solution. Generally $n - n_0$ is proportional to c_2, so that the ratio in parentheses in equation (7)—that is, the refractive index increment of the solute—is a constant for the particular system and the particular wave length employed.

Therefore, a plot against c_2 for a given two component system of either the function Hc_2/τ or the function P/RTc_2 yields an intercept equal to $1/M_2$; but the slope of the former curve is twice that of the latter.[19]

For systems of more than two components, it is in general no longer permissible to regard the slope terms B in equations (5) and (6) as identical; and a more precise formulation is required. The basis of such a formulation was apparently first given by Zernike[21]; it has recently been further developed by Brinkman and Hermans,[22] by Kirkwood and Goldberg,[23] and by Stockmayer.[24]

The mean square value of the fluctuation in refractive index $\overline{(\Delta n)^2}$, which determines the total turbidity, is a function of the concentrations, and refractive index increments, of each of the components of the system. It also involves cross terms, involving the correlation between the fluctuations of the concentrations of different components. For a given pair of components, i and j, the cross term is zero if the chemical potential of i is unaffected by a variation of the mass of j in the system; but if the chemical potentials are not independent in this manner the cross terms do not vanish.

The general equation, for the turbidity, τ, of systems at constant pressure and temperature, may be written[22,23,24]

$$\tau = H'' \frac{\Sigma_i\Sigma_j \Psi_i\Psi_j A_{ij}}{|a_{ij}|} \qquad (8)$$

where

$$H'' = 32{,}000\pi^3 n^2/3N\lambda^4 \qquad (9)$$

The summation is taken over *all but one* of the components. Generally it is most convenient to omit the solvent from the summation; to compensate for this omission the complete equation should include a term for the turbidity of the pure solvent, arising from density fluctuations in it. This term is generally small, in systems containing large molecules, and for brevity it is omitted from (8). However, in practice, we have generally determined the turbidity of the pure solvent and subtracted it from that of the solution. Ψ_i denotes the *molar* refractive increment of component i; that is, Δn per mole of solute per liter of solution (or per kg. solvent). The terms a_{ij}, in the determinant $|a_{ij}|$ denote the coefficients $a_{ij} = \partial \ln a_i/\partial m_j = \partial \ln a_j/\partial m_i = a_{ji}$; here the a's denote activities, and the m's denote concentrations in mole/l. The term A_{ij}, in the summation in the numerator, denotes the cofactor of the term a_{ij} in the determinant $|a_{ij}|$; that is the determinant derived from $|a_{ij}|$ by striking out the row and column in which the term a_{ij} occurs, and multiplying the resulting determinant of lower order by $+1$ if $i + j$ is even, and by -1 if $i + j$ is odd.

Our equation (8) differs from Stockmayer's[24] in that he defined the terms a_{ij} as $\partial\mu_i/\partial m_j$, where μ denotes chemical potential; whereas we have employed activities. Thus, his expression differs from ours by a factor RT in the denominator. This difference has been taken care of in the formulation given here, so that our working equations are essentially identical with those of Stockmayer. Also the volume factor V, appearing in Stockmayer's equations, does not appear explicitly in equation 8, since the concentrations are here expressed in volume units.

Application to a Two-Component System.—For a two-component system, the summation in (8) involves only component 2. Hence $|a_{ij}| = a_{22} = \partial \ln a_2/\partial m_2$, and $A_{ij} = 1$. By definition of the activity (a_2) and the activity coefficient (γ_2)

$$\ln a_2 = \ln m_2 + \ln \gamma_2 = \ln m_2 + \beta_{22} \qquad (10a)$$

$$a_{22} = \frac{\partial \ln a_2}{\partial m_2} = \frac{1}{m_2} + \frac{\partial \ln \gamma_2}{\partial m_2} = \frac{1}{m_2} + \beta_{22} \qquad (10b)$$

Hence

$$\tau = \frac{H''\Psi_2}{a_{22}} = \frac{H''\Psi_2^2}{\frac{\partial \ln a_2}{\partial m_2}} = \frac{H''\Psi_2^2 m_2}{1 + \beta_{22}m_2} = \frac{H''\phi_2^2 M_2 c_2}{1000(1 + \beta_{22}m_2)} \qquad (11)$$

Here $\Psi_2 = \partial n/\partial m_2$; ϕ_2, the refractive increment per g. of component 2 per cc. of solution, is $1000\ \Psi_2/M_2$.

Rearranging (11) we may then write

$$\frac{H''\phi_2^2 c_2}{1000\tau} = \frac{Hc_2}{\tau} = \frac{1}{M_2}(1 + \beta_{22}m_2) \qquad (12)$$

Since $c_2 = m_2M_2/1000$, this gives the slope factor B in equation (6) as

$$B = 1000\beta_{22}^0/2M_2^2 \qquad (13)$$

where β_{22}^0 is the limiting value of β_{22} at low values of m_2.

(19) This statement assumes that the solute is really a single component; if, like most polymers, it is made up of a number of different individual molecules, the intercept, of the Hc_2/τ curve gives the reciprocal weight average molecular weight, and that of the P/RTc_2 curve yields the reciprocal number average.

Strictly speaking, the weight average to be used in a calculation from light scattering involves not merely the molecular weight of each component, but its molecular weight multiplied by the square of its refractive index increment. This distinction is not important in most polymers, for which the refractive increment per gram is essentially independent of molecular weight in any one preparation. However, it may be of real importance for a mixture of proteins, since the refractive increment per gram may differ significantly from one protein to another.[20]

(20) S. H. Armstrong, Jr., M. J. E. Budka, K. C. Morrison and M. M. Hasson, THIS JOURNAL, **69**, 1747 (1947).

(21) F. Zernike, "L'Opalescence Critique," Dissertation, Amsterdam, 1915; *Arch. Néerland. Sci.*, (3A) **4**, 74 (1918); cited in ref. 22.

(22) H. C. Brinkman and J. J. Hermans, *J. Chem. Phys.*, **17**, 574 (1949).

(23) J. G. Kirkwood and R. J. Goldberg, *ibid.*, **18**, 54 (1950).

(24) W. H. Stockmayer, *ibid.*, **18**, 58 (1950).

Systems of Three and More Components.—For multicomponent systems in general, we shall follow Scatchard's[12] formulation of the expressions for the activities of the components and their derivatives with respect to the masses of the components—that is, the coefficients which enter into equation (8). We shall use the subscript J or K to denote any component made up of small molecules or ions,[25] and i to denote a small ion which is a constituent of one or more components, but is not itself a component. Again denoting the protein as component 2, we have, following Scatchard

$$\ln a_2 = \ln m_2 + \Sigma_i \nu_{2i} \ln m_i + \beta_2 \quad (14)$$
$$= \ln m_2 + \Sigma_i \nu_{2i} \ln (\Sigma_J \nu_{Ji} m_J + \nu_{2i} m_2) + \beta_2$$
$$\ln a_K = \Sigma_i \nu_{Ki} \ln m_i + \beta_K \quad (15)$$
$$= \Sigma_i \nu_{Ki} \ln (\Sigma_J \nu_{Ji} m_J + \nu_{2i} m_2) + \beta_K$$

Here, by definition

$$\beta_2 = \ln \gamma_2 \quad (16)$$
$$\beta_K = \Sigma_i \nu_{Ki} \ln \gamma_K \quad (17)$$

In these equations ν_{2i} denotes the number of moles of ions of species i (for instance sodium or chloride) contained in one mole of protein component; and ν_{Ki} denotes the number of moles of ions of species i contained in one mole of the K'th component (for instance $\nu_{3i} = 1$ if K denotes sodium chloride (component 3), the ion i then being either sodium or chloride). In the special case of a three-component system, in which component 3 is a salt composed of one anion and one cation, we have $\beta_3 = 2 \ln \gamma_3$.

From these relations, we derive the coefficients employed in equation (8), denoting $\partial\beta_2/\partial m_2$ as β_{22}, $\partial\beta_K/\partial m_2 = \partial\beta_2/\partial m_K$ as β_{2K}, and so forth.

$$a_{22} \equiv \frac{\partial \ln a_2}{\partial m_2} = \frac{1}{m_2} + \Sigma_i \frac{\nu_{2i}^2}{m_i} + \beta_{22} \quad (18)$$

$$a_{2K} \equiv \frac{\partial \ln a_2}{\partial m_K} = \frac{\partial \ln a_K}{\partial m_2} = \Sigma_i \frac{\nu_{2i}\nu_{Ki}}{m_i} + \beta_{2K} \quad (19)$$

$$a_{KJ} \equiv \frac{\partial \ln a_K}{\partial m_J} = \frac{\partial \ln a_J}{\partial m_K} = \Sigma_i \frac{\nu_{Ji}\nu_{Ki}}{m_i} + \beta_{JK} \quad (20)$$

Here J denotes any diffusible component other than K.[26] For the three-component system containing one salt (component 3) with two ions, the summation denoted by Σ_i includes only one cation and one anion, and $\nu_{3c} = \nu_{3a} = 1$. From (3a) and (3b) we have $\nu_{2c} = -\nu_{2a} = -Z_2/(Z_c - Z_a)$, which becomes $-Z_2/2$ if the ions denoted by c and a are both univalent. Then, using equation (4), we have

$$m_c = m_3 - \frac{Z_2}{2} m_2 \quad (21)$$

$$m_a = m_3 + \frac{Z_2}{2} m_2 \quad (22)$$

From (18) we obtain

$$a_{22} = \frac{1}{m_2} + \frac{Z_2^2}{4}\left(\frac{1}{m_3 - (Z_2 m_2/2)} + \frac{1}{m_3 + (Z_2 m_2/2)}\right) + \beta_{22} = \frac{1}{m_2} + \frac{Z_2^2}{2m_3\epsilon} + \beta_{22} \quad (23)$$

Here the factor ϵ is defined as

$$\epsilon = 1 - (Z_2 m_2/2m_3)^2 \quad (24)$$

The value of ϵ lies always between zero and unity, and it may be taken as unity when $Z_2 m_2 << 2\ m_3$.[27]

From (19), (21) and (22) we obtain

$$a_{23} = \frac{\nu_{2c}}{m_c} + \frac{\nu_{2a}}{m_a} + \beta_{23} = -\frac{Z_2^2 m_2}{2m_3^2\epsilon} + \beta_{23} \quad (25)$$

Finally for the effect of variation in the logarithm of the activity of component 3 with variations in its own molarity we have, from (15), (21) and (22)

$$a_{33} = (2/m_3\epsilon) + \beta_{33} \quad (26)$$

The value of β_{33} is determined independently, from measurements of electromotive force, freezing point, or vapor pressure, in solutions of the pure salt.

Applied to the three component system just discussed, equation (8) becomes

$$\tau = \frac{H''(\Psi_2^2 a_{33} - 2\Psi_2\Psi_3 a_{23} + \Psi_3^2 a_{22})}{a_{22}a_{33} - a_{23}^2} \quad (27)$$

For protein systems, the first term in the numerator of (27) is generally much the largest, and the second and third may often be neglected.

This point will be considered in terms of the system water–serum albumin–sodium chloride. The molar refractive increment, Ψ_2, of serum albumin, per liter solution, is, from the data of Perlmann and Longsworth[28] and of Armstrong, Budka, Morrison and Hasson,[29] equal to 12.9 for the sodium D line, assuming a molecular weight of 69,000.[29] The data for sodium chloride solutions, at the same wave length (see for instance, Geffcken[30]) when extrapolated to infinite dilution, give $\Psi_3 = 9.5 \times 10^{-3}$. Thus Ψ_2^2 is greater than $2\Psi_2\Psi_3$ by a factor of 680, and exceeds Ψ_3^2 by a factor of nearly 2×10^6. On the other hand, we must consider the relative magnitude of the coefficients a_{22}, a_{23} and a_{33}.

From equations (23) and (25) we obtain

$$\frac{a_{22}}{a_{33}} = \frac{\frac{m_3\epsilon}{m_2} + \frac{Z_2^2}{2} + \beta_{22} m_3\epsilon}{2 + \beta_{33} m_3\epsilon} \quad (28)$$

The denominator is not far from 2 for any of the conditions of our experiments, since m_3 is always less than 0.2, and $\beta_{33}m_3$ is from the Debye–Hückel theory, in aqueous solutions, of the order of $-0.6m_3^{1/2}$ or less. The first term in the numerator is the only one that ever becomes very large. In the extreme case where, for example, $m_3 = 0.2$ and $m_2 = 10^{-6}$, a_{22}/a_{33} is of the order of 10^5; hence, $\Psi_3^2 a_{22}$ is less than 10% of $\Psi_2^2 a_{33}$. This case corresponds to an albumin solution containing 0.07 g./l., whereas the most dilute solutions we have studied are more than twice as concentrated as this. We, therefore, conclude that the third term in the numerator of (27) may safely be omitted.

From equations (25) and (26) we obtain

$$\frac{a_{23}}{a_{33}} = \frac{(-Z_2^2 m_2/2m_3) + \beta_{23} m_3\epsilon}{2 + \beta_{33} m_3\epsilon} \quad (29)$$

Here again the denominator is not far from 2, and the first term in the numerator is the only one that ever becomes important. Hence approximately

$$a_{23}/a_{33} \cong -Z_2^2 m_2/4m_3 \quad (29a)$$

Since, by the definition of components, $|Z_2 m_2| \leqq 2m_3$, it follows that a_{23}/a_{33} cannot be greater than $-Z_2/2$; or -10 as an upper limit for our experimental conditions. Thus $-2\Psi_2\Psi_3 a_{23}$ is always less than 2% of $\Psi_2^2 a_{33}$, and the

(25) A "small" component may be defined here as one which passes readily through membranes impermeable to proteins of molecular weight of the order of 30,000 or above.

(26) For example, J may be sodium chloride and K magnesium sulfate.

(27) In the extreme case when the only diffusible ions present are those required to balance the net charge on the protein, ϵ becomes equal to zero. Under these circumstances, the application of our equations would become meaningless and a different definition of components should be adopted. The general treatment adopted in this paper implicitly assumes that ϵ is not very far from unity.

(28) G. E. Perlmann and L. G. Longsworth, THIS JOURNAL, **70**, 2719 (1948).

(29) From the equation given by Perlmann and Longsworth, the corresponding value for light of wave length 4358 Å., at which most of our measurements were made, is 13.5. However, the calculations given here in the text are for the D line, since data for sodium chloride at $\lambda = 4358$ Å. are not available.

(30) W. Geffcken, *Z. physik. Chem.*, **B5**, 81 (1929).

second term in the numerator of (27) may also safely be neglected.[31]

Omitting these terms, and rearranging the others, we obtain from (27)

$$\frac{\Psi_2^2 H''}{\tau} = a_{22} - (a_{23}^2/a_{33}) \qquad (30)$$

We shall consider at first only the limiting conditions in which $|Z_2 m_2| << 2m_3$; hence $\epsilon = 1$ to a close approximation, and $a_{23} \cong \beta_{23}$. Then (for notation compare equation (11))

$$\frac{\Psi_2^2 H''}{\tau} = \frac{\phi_2^2 M_2^2 H''}{10^6\,\tau} = \frac{1}{m} + \frac{Z_2^2}{2m_3} + \beta_{22} - \frac{(\beta_{23})^2 m_3}{2 + \beta_{33} m_3} \qquad (31)$$

$$\frac{Hc_2}{\tau} = \frac{\phi_2^2 H'' c_2}{1000\,\tau} = \frac{1}{M_n} + \frac{1000}{M_2^2}\left(\frac{Z_2^2}{2m_3} + \beta_{22} - \frac{\beta^2{}_3 m_3}{2 + \beta_{33} m_3}\right) c_2 \qquad (32)$$

Thus, *under these limiting conditions*, the slope $2B$ of the curve for Hc_2/τ as a function of c_2 becomes identical with the slope of the curve for osmotic pressure divided by concentration of protein as a function of c_2, given by Scatchard, Batchelder and Brown.[32]

In the more general case when ϵ is significantly less than unity, and when $Z_2 m_2$ is not negligible compared to $2m_3$, we have

$$\frac{Hc_2}{\tau} = \frac{1}{M_n} + \frac{1000 c_2}{M_2^2}\left[\frac{Z_2^2}{2m_3\epsilon} + \beta_{22} - \frac{\left[\beta_{23} - \dfrac{1000 Z_2^2 c_2}{2M_2 m_3^2 \epsilon}\right]^2}{\dfrac{2}{m_3\epsilon} + \beta_{33}}\right] \qquad (33)$$

In the experimental section, we shall consider the evaluation of the intercept in equation (32), for the determination of molecular weight, and of the slope as a function of Z_2 and m_3. From this, and other available information the activity coefficient derivative, β_{22}, is evaluated as a function of the same variables. The form of the curves obtained also reveals clearly the effects of selective binding of diffusible ions by the protein.

Apparatus and Methods of Measurements

Two different forms of light scattering apparatus have been used. The early one was designed by Hans Mueller and George Rado of the Massachusetts Institute of Technology and was used during the war as a control instrument to determine the stability of human serum albumin solutions which were prepared by various commercial laboratories.[33]

A second instrument incorporated various recent developments. It required only 3 ml. of liquid for a measurement, compared to 20 ml. for the Mueller instrument. This second apparatus will be referred to as the Mueller-Edelhoch-Edsall-Zimm (MEEZ) apparatus.

In the Mueller apparatus, a convergent lens focused the light from a General Electric Type AH-4 mercury arc lamp in the center of a 40-cc. Pyrex centrifuge tube, 2 cm. in diameter. The centrifuge tube was set in the center of a larger vessel which had three plane windows at 0, 45 and 90° and which also could be filled with water to serve as a bath. Several masks, placed so as to minimize the intensity of any stray light, were introduced by covering the vessel with a machined cover. The transmitted light was read at 0°, and the scattered light at 90°, by moving the phototube (RCA 929) along a runner. A darkened film was fixed in front of the window which received the transmitted beam, to reduce the intensity of the incident beam to the same order of magnitude as that of the scattered beam. Monochromatic radiation was obtained by using a Corning blue filter (Corning 3389 and 5113) which transmits 14% of 436 mμ, 1% of 405 mμ, and no mercury lines of longer wave length. The current from the phototube was amplified by a d. c. amplifier tube and its intensity was read from a galvanometer scale. The tubes and galvanometer were a commercial unit obtained from the Photovolt Corporation in New York.

The second (MEEZ) apparatus was modelled after that of Zimm.[34] An additional amplifier stage, fed to the vertical plates of a two-inch oscilloscope, was used to detect the balance point of the potentiometer circuit. The line voltage, stabilized by a Raytheon 500 Watt Voltage Regulator, furnishes the voltage for the second set of plates, producing a symmetrical figure eight at balance, since the mercury arc is modulated at 120 c. p. s. and the line voltage at 60 c. p. s. The electronic part of this apparatus, with minor modification by Dr. Norman Hollies and the authors, was designed and built by H. Mueller and G. Rado as part of the circuit for an earlier model of their Photoelectric Tyndall Compensator.

In the arrangement for observing the transmitted and scattered light, the solution was contained in a 1-cm. Beckman absorption cell, which had all four of its sides carefully finished and polished. The cell could be filled to the proper level with 3 ml. of solution. The cell was held in position by two uprights, one of which had a spring attachment which firmly fixed the position of the cell. Three pairs of masks, suitably placed, prevented any stray light from entering the photocells. A General Electric Type AH-3 mercury arc lamp was employed as a light source and its image was focused in the solution cell by the convergent lens. The width of the beam at the center of the cell was 1.5 mm. A Wratten C5 filter transmitted 42% of the mercury 436 mμ line, 12% of 405 mμ, and none of the other lines. A liquid filter one cm. in depth, of 6% *p*-nitrotoluene in ethanol almost completely removed the 405 mμ line with very little absorption of 436 mμ.[35] A diaphragm was employed to regulate the height of the light beam.

The filtered incident beam produced a d. c. photocurrent from the phototube (RCA 927) of about 2.5 μamp. (This current remains constant, provided that the incident light intensity remains constant.) The voltage on the photomultiplier tube (RCA 1P21) was adjusted for a given set of measurements so that the ratio of the two photocurrents was in a convenient range for measurements on the bridge. A bulb action Packard shutter (obtained from Eastman Kodak Company) was placed in the opening of the phototube housing, while a camera shutter was used in the photomultiplier housing, both of which were operated by long extension coils. In operation, the apparatus was covered with a black cloth and measurements were performed in the dark, slight illumination for readings being obtained by a photographic red lamp.

To test linearity of response of the photomultiplier tube

(31) The molar refractive increment of sodium thiocyanate per liter is 0.0133 for the sodium D line ("International Critical Tables," Vol. VII, p. 75). Thus, for albumin in thiocyanate solutions, the error in neglecting the second and third terms in the numerator of (27) is somewhat greater than in sodium chloride solutions, but is still below 2% for the conditions of the great majority of our measurements.

(32) G. Scatchard, A. C. Batchelder and A. Brown, THIS JOURNAL, **68**, 2320 (1946); see their equation 9.

(33) The apparatus is described in a memorandum on the Photoelectric Tyndall Compensator by H. Mueller and G. Rado dated July 19, 1943, prepared for a conference on the uses of normal human serum albumin at that time (under O. S. R. D. contract O. E. M.-cmr 139). See also G. Scatchard, S. T. Gibson, L. M. Woodruff, A. C. Batchelder and A. Brown, *J. Clin. Invest.*, **23**, 445 (1944).

(34) B. H. Zimm, *J. Chem. Phys.*, **16**, 1099 (1948).

(35) The *p*-nitrotoluene solution slowly decomposes and turns yellow on exposure to light. It is best to prepare it freshly at frequent intervals. See J. T. Edsall and E. B. Wilson, Jr., *J. Chem. Phys.*, **6**, 124 (1938).

and to estimate the maximum sensitivity of the apparatus, a series of runs at voltages from 770–950 volts, produced for this experiment by a series of B batteries, were performed on carefully distilled samples of benzene and *p*-xylene. Below 950 volts, the patterns on the oscilloscope were sufficiently stable to detect a change of 0.001 unit in the experimental reading, R. From 770–950 volts the readings at balance ranged from 0.090–0.400. Over this 4.5 fold range of photomultiplier response, the ratio of the readings of benzene and *p*-xylene remained constant, with an average deviation of 1%. However, under ordinary working conditions, readings tend to be unstable at voltages above 800. The readings on the benzene or *p*-xylene standards, after correction was made for background intensity, were considered reliable within ±2 to 3%.

p-Xylene was generally used as the working standard. The measured value of its reduced intensity appeared to remain approximately constant from day to day, but was found to change somewhat over a period of weeks. There is, apparently, a small amount of drift in the instrument constants with time, which produces this change in the reading of *p*-xylene.

Depolarization measurements were made by inserting polaroids before the solution cell and in front of the phototube housings. Polaroids cast in square form were obtained from the Polaroid Company, Cambridge. Their principal axes, with respect to the direction of the electric vector in the transmitted light, were parallel to the sides, thus facilitating the settings for the polarization measurements. Since the polarizers considerably reduced the incident and scattered light intensities, the experimental error in these measurements was somewhat increased.

The response of the photomultiplier was found to vary with the state of polarization of the light incident on it. This variation was determined by measuring the response of the photomultiplier tube to the fluorescent spectrum of fluorescein, by polarizing the fluorescent light with a polaroid sheet. A yellow filter was used to intercept any scattered radiation due to impurities in the dye solution not removed by centrifugation at 16,000 g. The response to horizontally polarized incident light was found to be approximately 16% greater than that to vertically polarized light of the same intensity.

Corex Beckman cells, obtained from Pyrocell Company in New York and polished by A. D. Jones Optical Company in Cambridge, Massachusetts, gave turbidities for different aliquots of a single protein preparation within 3% of each other (for ten different cells). Assuming the average value to be most accurate, all the cells were calibrated to give this value. The walls of the cells were then marked to permit the incident and scattered beams to pass through the same sides from measurement to measurement, since it was found that the readings varied as the cells were rotated through 90°.

Materials

Bovine serum albumin, obtained from Armour and Company (preparation 37–215) and recrystallized four times by Dr. W. L. Hughes, Jr.,[36] of the Harvard Medical School, by the decanol procedure, was used in all these studies. A 1% solution of this preparation in water contained less than 10^{-4} molar NaCl and had a *p*H of 4.90. Albumin concentrations were determined with a Beckman spectrophotometer. The value of $[E]_{1\text{ cm.}}^{1\%}$ at 280 mμ was taken as 6.6.[36]

Except for a few early experiments, the water used in making up the solutions had been freshly distilled in an all glass apparatus.

The solutions employed for measurements in the Mueller apparatus were freed of particulate impurities by filtering a 10% solution through a Hormann D-10 pad. The Hormann pads were rendered free of cationic impurities by successively washing them with solutions of 0.1 N acetic acid, 0.1 N NaCl and water. Appropriate dilutions were made in a 40-cc. centrifuge tube which was spun at 2000 g immediately prior to measuring its scattering.

The solutions used in the MEEZ apparatus were prepared in a slightly different manner. A solution of 7% albumin was filtered through a Corning "fine" sintered glass funnel and then centrifuged at 16,000 g. A series of solutions of the required protein concentration was prepared from the centrifuged stock solution by dilution with water. A 3-ml. sample was then removed with a pipet and transferred to the square solution cell. A clean, dry pipet was used for each 3-ml. sample. In this way, any trace of albumin that may have been surface denatured remains on the wall of the pipet. Solutions of 0.40 ml. of 0.0255, 0.285 and 1.570 ionic strength were then introduced, with adequate and careful mixing, into the protein solution in the cell to give final ionic strengths of 0.003, 0.033 and 0.183. The turbidity of the solution was measured after each addition of salt solution.

*p*H values acid to the isoionic point of albumin were obtained by the careful addition of 0.01 M HCl to a 7% water solution of albumin. *p*H values alkaline to the isoionic point were obtained by adding 0.01 M $NaHCO_3$, and then lyophilizing the solution to remove carbon dioxide (see footnote 17).

The salts employed were J. T. Baker C. P. materials and were used without recrystallization.

Calibration of Apparatus

Both types of apparatus used by us give readings proportional to the intensity of scattering, at 90° to the incident beam (I_{90}), compared with the intensity of the transmitted light (I_T) emerging from the cell containing the liquid. For a liquid containing only molecules small compared with the wave length of the incident light, the ratio of I_{90} to the intensity of the *incident* beam (I_0) is proportional to the turbidity, τ. For liquids of the turbidities studied by us, I_0 and I_T always differ by much less than 1%, and generally by a factor of 0.1% or less. Hence, we may take the ratio I_{90}/I_T as proportional to the turbidity. To place the values on an absolute basis, it is necessary to determine the factor of proportionality.

If r is the (constant) distance from the point at which the light is being scattered to the observer, Rayleigh's equations give the relation between I_{90} and the turbidity τ, for unpolarized incident light and for isotropic particles small compared to the wave length of the light.

$$\tau = (16\pi/3)\,(I_{90}r^2/I_T) \qquad (34)$$

Both types of apparatus used in our experiments are designed to give a quantity called R, which is proportional to the reduced intensity $I_{90}r^2/I_T$.

In the subsequent discussion we shall use the symbol R to denote the measured values for the relative readings of the scattered light at 90° compared to the intensity of the transmitted beam at 0°. The calibration of R in absolute terms requires precise specification of the volume of illuminated liquid observed.[37] Here we are not concerned with the absolute measurements, but have calibrated the apparatus using carefully purified standard liquids and absolute turbidity values for these liquids determined by other authors.

If the solution employed as a standard does not have the same refractive index as the solutions whose turbidities are being determined, the experimental readings, R, must be multiplied by two correction factors. These originate from the refraction of the scattered light at the boundary of the solution cell. The solid angle of scattered light that is "seen" by the photomultiplier tube decreases as the refractive index of the scattering solution increases. The

(36) E. J. Cohn, W. L. Hughes, Jr., and J. H. Weare, THIS JOURNAL, **69**, 1753 (1947).

(37) For precise definition, see the book by J. Cabannes, "La Diffusion Moléculaire de la Lumière," Les Presses Universitaires de France, Paris, 1929. In two forthcoming reviews (J. T. Edsall and W. B. Dandliker, *Fortschritte der Chemischen Forschung*, in press; P. Doty and J. T. Edsall, *Advances in Protein Chemistry*, **6**, in press) the symbol R is used, following Cabannes, to denote the absolute value of the reduced intensity. In the present paper, however, it denotes simply an experimental reading, corrected by equations 35 and 36, which is proportional to the reduced intensity.

resulting correction factors have recently been discussed in detail by Carr,[38] working in Zimm's laboratory. The first, known as the refractive index correction factor, is given by the equation

$$C_n = \frac{n_1\left[1 - \frac{r_1}{\mathbf{R}}\frac{(n_1 - 1)}{n_1}\right]}{n_2\left[1 - \frac{r_1}{\mathbf{R}}\frac{(n_2 - 1)}{n_2}\right]} \qquad (35)$$

In equation (35), n_1 and n_2 are the refractive indices of water and organic solvent respectively, r_1 is half the cell width measured normal to the incident beam, and **R** is the distance between center of cell and photomultiplier tube.

For a cylindrical cell such as that used in the Mueller apparatus, the correction factor is equal to C_n in equation (35). For a square cell with flat windows, such as that used in the MEEZ apparatus, it is equal to C_n^2. Numerical values of this factor are listed in Table I.

TABLE I

APPARATUS CONSTANTS AND CALCULATED CORRECTION FACTORS

Apparatus	Apparatus constants, cm.			
	r_1	R	l	a
Mueller	1.25	10	0.4	1.5
MEEZ	0.50	10	.4	1.0

	Calculated constants		
	C_n	C_n^2	C_v
Mueller	1.12		0.985
MEEZ		1.27	.99

The second factor is known as the volume correction factor, and is given by Zimm and Carr as

$$C_v = \frac{n_1 l + \frac{r_1}{2}\left(\frac{a+l}{\mathbf{R} - r_1}\right)}{n_1 l + r\left(\frac{a+l}{\mathbf{R} - r_1}\right)} \Bigg/ \frac{n_2 l + \frac{r_1}{2}\left(\frac{a+l}{\mathbf{R} - r_1}\right)}{n_2 l + r\left(\frac{a+l}{\mathbf{R} - r_1}\right)} \qquad (36)$$

where n_1, n_2, r_1, **R** have the same meanings as listed for C_n, a is the aperture of the photomultiplier tube and l is the width of the cell "seen" by the photomultiplier tube at the boundary of the cell.

This takes into account the variation in the total volume of liquid that is "seen" by the photomultiplier tube as the refractive index of the liquid changes. This volume increases as the refractive index decreases. The numerical values for this correction factor are also given in Table I. It is obvious that the volume correction factor is relatively insignificant for either type of apparatus used by us, but we have inserted this correction in calibrating the ratio of reduced intensities for the standard liquids and the albumin solutions.

Calibration with Standard Liquids

Benzene, *p*-xylene and a solution of polystyrene in toluene were used as primary standards of known turbidities.

C. P. benzene and *p*-xylene were distilled five times and about 5 cc. collected in an apparatus made entirely of glass. On one side this consisted of a rectangular observation cell of square tubing, very similar in form to the cells used for the observation of the albumin solutions. This was connected, by sealed glass tubing, with a reservoir containing the standard liquid. A side arm led off from the tubing, midway between the observation cell and the reservoir. This was connected to a vacuum line; after the vacuum pump had been in operation for twenty minutes, and while pumping was still proceeding, the liquid was frozen with Dry Ice and the neck of the side arm sealed off. The solid was then melted and the reservoir gently warmed while the observation cell was cooled, so that the liquid distilled over without boiling. The cell was then tilted so that the liquid flowed back into the reservoir, and the process was repeated four times, so that the liquid in the observation cell was dust free at the end of the process, when the scattered light was observed at angles close to the incident beam.

The standard polystyrene preparation was a portion of a lot originally prepared by the Dow Chemical Company, which has been used as a working standard by a number of laboratories engaged in studies of light scattering. It was supplied to us through the kindness of Prof. P. Debye and Dr. A. M. Bueche of Cornell University. A solution in toluene, containing 0.5 g. of polystyrene per 100 ml., has been assumed in our calculations to have an absolute turbidity of 0.0035 for light of wave length 4358 Å. This figure represents the excess turbidity of the solution over that of the pure solvent.[39]

The polystyrene solutions, after being made up with toluene which had been several times distilled, were centrifuged at 2000 *g* or at 16000 *g* to remove any suspended particles. Centrifuging at 2000 *g* was found quite as effective as at 16000 *g*; and the different solutions made up from the same standard lot at various times gave readings in good agreement.

Albumin Solutions.—The Debye factor H (equation 7) has been calculated for serum albumin,[40] taking dn/dc_2 as 0.195 at 4358 Å. and 25°, and the refractive index of pure water as 1.3403 under the same conditions. This gives $H = 1.04_0 \times 10^{-5}$, and we have employed this value throughout.[41]

Angular distribution measurements of scattered intensity were carried out by Dr. W. B. Dandliker of this department on the same albumin sample used by us, using an apparatus which will be described elsewhere. The plane containing the incident and the observed scattered light beams was horizontal. With unpolarized incident light, the intensity of the vertical component (electric vector vertical) in the scattered light was found to be constant within ±1% as the angle of observation, θ, was varied from 25 to 143°.

The calculated values for the molecular weight of serum albumin, obtained from several runs with the MEEZ apparatus, using several standard liquids, are given in Table II. The values so obtained are somewhat above the value of 69,000 obtained from osmotic pressure,[42] which is probably the most reliable yet obtained. Ultracentrifuge measurements on the albumin preparation employed showed a few per cent. of a fast component which appeared as a shoulder on the main peak of sedimentation constant $s_{20,w} = 4.6S$. Hence, it might be expected that the weight average molecular weight should be somewhat higher than the number average given by osmotic pressure

(38) C. I. Carr, Ph.D. Thesis, University of California, 1949.

(39) This estimate is based on reports we have received from Paul M. Doty, Harvard University; F. W. Billmeyer, du Pont Laboratories, Arlington, New Jersey; G. C. Nutting, Eastern Regional Research Laboratories, Philadelphia; B. H. Zimm, University of California; and Walter H. Stockmayer, Massachusetts Institute of Technology. All their values for the excess turbidity lie in the range 0.0035 ± 0.0002, a value concordant with that obtained in this laboratory recently by the authors in conjunction with Dr. W. B. Dandliker.

(40) G. E. Perlmann and L. G. Longsworth, THIS JOURNAL, **70**, 2719 (1948).

(41) The binding of anions by albumin, which is discussed in more detail below, may well affect the value of H. To take a case actually realized in some of our experiments, the binding of 20 thiocyanate ions to one molecule of albumin increases the molecular weight by 1160, or 1.7%. The increase in the refractive increment should be relatively still greater, on account of the high polarizability of the thiocyanate ion. Perlmann and Longsworth have noted small variations in the refractive increment of albumin in the presence of different salts and buffers. We shall not, however, attempt to introduce such refinements in the treatment given in this paper.

TABLE II

MOLECULAR WEIGHT OF SERUM ALBUMIN, DETERMINED WITH REFERENCE TO SEVERAL STANDARD LIQUIDS

Apparatus	Standard liquid	Albumin $(c/R)c = 0$ c = concd. (g./ml.)	Standard $\frac{R_{std.}}{\tau_{std.}}$	Standard $\frac{R_{std.}}{\left(\frac{I_{90}r^2}{I_0}\right)_{std.}}$	M
Mueller	Polystyrene	9.7×10^{-6}	359/0.0035		84,000
MEEZ	Polystyrene	9.6×10^{-6}	356/0.0035		76,000
	Benzene	9.6×10^{-6}		$76.5/49.5 \times 10^{-6}$	82,500
	Toluene	9.6×10^{-6}		$92.3/55.5 \times 10^{-6}$	77,000
MEEZ	Polystyrene	11.4×10^{-6}	298/0.0035		78,500
	Toluene	11.4×10^{-6}		$74.5/55.5 \times 10^{-6}$	80,000
MEEZ	Polystyrene	12.1×10^{-6}	288/0.0035		74,000
	Benzene	12.1×10^{-6}		$67/49.5 \times 10^{-6}$	75,000
	p-Xylene	12.1×10^{-6}		$94/72.5 \times 10^{-6}$	78,500

The values of reduced intensity for benzene at $\lambda = 4358$ Å. are taken from Carr[38]; the values for toluene and p-xylene, relative to benzene, from Cabannes, p. 191.[37] The excess turbidity of 0.5% polystyrene in toluene was taken as 0.0035 (see footnote 39). The molecular weight of bovine serum albumin was calculated by the equation

$$H\left(\frac{c}{R}\right)_{c=0}\left(\frac{R_{std.}}{\frac{I_{90}r^2}{I_0}}\right)_{std.}\left(\frac{6+6\rho}{6-7\rho}\right)C_n^2C_v\frac{3}{16\pi}=\frac{1}{M}$$

when organic liquids were employed as standards; and by the equation

$$H\left(\frac{c}{R}\right)_{c=0}\frac{R_{std.}}{\tau_{std.}}\frac{6+6\rho}{6-7\rho}C_n^2C_v=\frac{1}{M}$$

when polystyrene was used. H is the Debye factor and for serum albumin in water at 4358 Å. is equal to 1.04×10^{-5}; R denotes the scattered intensity reading (see equation 34 and the accompanying text). The Cabannes factor for serum albumin, $(6 + 6\rho)/(6 - 7\rho)$, is taken as 1.043. The correction factors C_n and C_v are defined in equations 35 and 36, respectively (see Table I). The equations as given apply to the MEEZ apparatus (square cell). The same equations apply to the Mueller apparatus (cylindrical cell) except that the factor C_n is used instead of C_n^2 (compare Table I).

For the definition of the depolarization factor ρ see Table III (footnote) and also the text.

The scattered intensity of benzene at 90° represents the total intensity of both polarized components in the scattered light at 90°. In evaluating the intensity from the observed readings, it is necessary to take into account the different sensitivity of the photocell to the two components (see text). The readings reported here for benzene, toluene, and xylene have been corrected by the appropriate factor to take account of this difference.

measurements. The molecular weight values near 77,000, reported in Table II, agree well with this expectation.

In any case, the values reported in Table II are subject to later revision when more reliable data for the standard liquids used in calibrating the apparatus are obtained. The absolute value for the excess turbidity of the standard polystyrene solution may be uncertain by a few per cent. The discrepancies between the findings of different authors for the pure organic liquids are rather serious,[42] and are not yet fully explained. We have taken the value for benzene from Carr, and have calculated values for toluene and p-xylene assuming the ratio of reduced intensities for toluene and p-xylene relative to benzene to be 1.12 and 1.46, respectively, as given by Cabannes.[37] As working standards, we have found solutions of carefully purified serum albumin to be as satisfactory as anything we have used; they can, in general, be relied on to be reproducible under given conditions to two or three per cent. or better.

Depolarization measurements were made on the standard liquids and are recorded in Table III. With unpolarized incident light, the depolarization factor, $\rho = I_h/I_v$ for 1% albumin was 0.02; it increases slightly at lower concentrations. Here I_h and I_v are the intensities of the horizontal and vertical components, respectively, in the scattered light, when unpolarized incident light is used. The Cabannes factor for albumin, *i. e.*, $(6 + 6\rho)/(6 - 7\rho)$ is 1.04.

Determination of Interaction Constants (B Values) as a Function of Net Charge and Ionic

(42) See for instance the tabulated values in the books of Cabannes[37] and Bhagavantan,[43] and the critical discussion by Carr.[38]

(43) S. Bhagavantan, "Scattering of Light and the Raman Effect," Chemical Publishing Company, New York, N. Y., 1942.

TABLE III

DEPOLARIZATION FACTORS OF STANDARD LIQUIDS

Substance	ρ_u [c]	ρ_v	ρ_H
Benzene	0.39	0.285	0.95
Doty and Kaufman[a]	.41	.25	.97 calcd.
p-Xylene	.58	.445	.92
Gans[b]	.58		
Toluene	.51		
Doty and Kaufman[a]	.49		
Gans[b]	.51		

[a] P. M. Doty and H. S. Kaufman, *J. Phys. Chem.*, 49, 583 (1945). [b] Bhagavantan, ref. 43. [c] ρ_u denotes the ratio H_u/V_u, the intensity ratio of the horizontal to the vertical component in the scattered light, using unpolarized incident light; ρ_v is the corresponding ratio (H_v/V_v), using vertically polarized incident light; ρ_h is the ratio V_h/H_h, using horizontally polarized incident light. For further details see Doty and Kaufman (ref. *a*).

Strength.—If Hc_2/τ is expressed as a power series in (protein) concentration

$$H\frac{c_2}{\tau}=\frac{1}{M}+2Bc_2+3Cc_2^2+\cdots \qquad (37)$$

The magnitude of C, the third virial coefficient, becomes significant as the net charge on the protein increases, as will be reported elsewhere. In solutions of low ionic strength, at larger values of Z_2, the C values are negative and moderately

large, while in solutions of high ionic strengths, over the entire range of Z_2 covered in this paper (+25 to −20), the C values are positive and small. It is thus important, especially in solutions for which Z_2 is large and ionic strength low, to extend measurements to very low concentrations. At times this has required the study of such dilute solutions that the scattering due to the protein is of the same order of magnitude as that of the solvent. The errors which might arise from failure to work at very low concentrations are illustrated in the upper curve of Fig. 1, for which $Z_2 = +25$ and the ionic strength ($\Gamma/2$) is less than 0.0001. If only the three highest points on this curve had been measured, it would not be difficult to draw a reasonably straight line through them, which could then be extrapolated to a value of Hc_2/τ near 3.5×10^{-5}, corresponding to a molecular weight near 37,000, about half of the true value for serum albumin. The points at lower concentrations, however, can reasonably be extrapolated to give a molecular weight near 75,000, the same value obtained from the other curves in Fig. 1 which represent solutions of progressively increasing ionic strength. It should be noted that *all* the data in Fig. 1 are for solutions containing less than 0.4 g. of albumin per 100 cc. A far more detailed study of light scattering in serum albumin at high net charge and extremely low ionic strength has been given by Doty and Steiner,[43] and unpublished

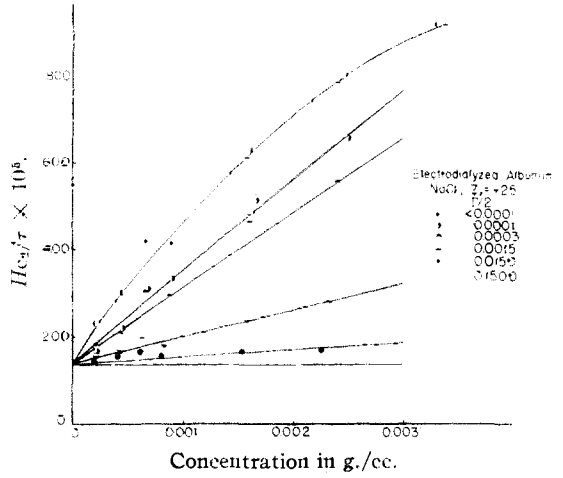

Fig. 1.—Hc_2/τ in bovine serum albumin, as a function of c_2, at several different ionic strengths, in acid solution ($Z_2 = +25$).

Other relations are illustrated in Fig. 1. When the valence of the protein (Z_2) as determined from the titration curve, is +25 and ionic strength is extremely low (10^{-4} or less), the steepest curve in Fig. 1 is obtained, for which a slope factor $2BM^2/1000$ (see equation 39 below) of approximately 10^5 is calculated. When the ionic strength is increased to 0.15, even at the same high value of Z_2, the slope falls almost to zero.[44] For the isoionic protein also, even in the absence of salt, the slope is almost exactly zero.[45]

It is apparent from Fig. 1 that at albumin concentration 0.0025 g./cc., and at $Z_2 = +25$, the turbidity increases more than threefold when $\Gamma/2$ increases from 0.0001 to 0.150. It was considered important to establish the speed with which such changes in turbidity take place when the composition of the medium is changed. Starting with a solution of high Z_2 and very low ionic strength, corresponding to one of the points on one of the upper curves of Fig. 1, salt or buffer was added so as to adjust the composition of the solution to one of the points on the lowest curve, for which the slope B is zero or nearly so. A series of such solutions, corresponding to the different points on the two

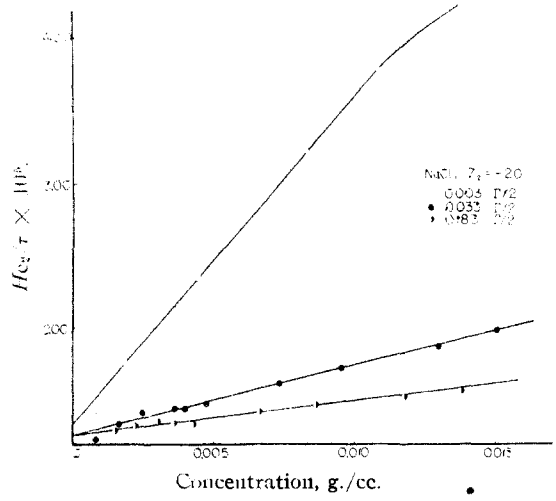

Fig. 2.—Measurements at three different ionic strengths in sodium chloride solutions; $Z_2 = -20$.

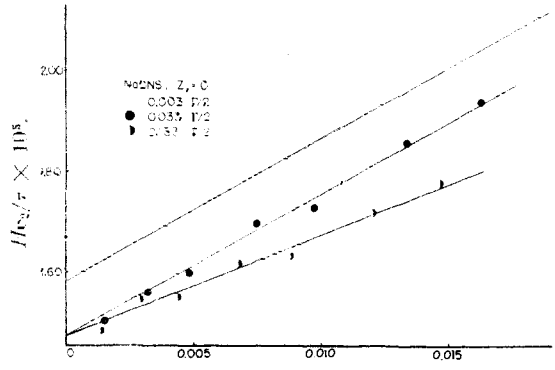

Fig. 3.—Measurements at three different ionic strengths in thiocyanate at $Z_2 = 0$.

(44) The increase of ionic strength is here brought about by the addition of chloride. As shown below, this involves a decrease of the effective net charge of the protein through binding of chloride ion. However, this binding is not enough to neutralize completely the charge, Z_2, due to proton binding.

(45) In the solutions for which the ionic strength of the added salt is 10^{-4} or less, it is clear that the total ionic strength, when $Z_2 = +25$, is very largely determined by the highly charged protein ion and the chloride ions which are present to balance its charge. Consequently, as c_2 increases in such solutions, the ionic strength also increases and the effect of this is to diminish the slope.

curves, were studied. In all cases, the first readings taken, within a minute after adding the salt or buffer, were identical with those for the final equilibrium state of the solution. This would appear to eliminate the possibility that the marked increases in turbidity occurring on addition of salt could be due, for instance, to association of protein molecules. Such association reactions, in protein solutions as dilute as these, would certainly require more than a minute for completion.

Some typical curves for Hc_2/τ as a function of c_2 are shown in Figs. 2 and 3. The choice of the best lines to draw through the experimental points is sometimes a rather difficult matter of judgment. It will be noted that not all the lines are drawn so as to extrapolate to the same limiting intercept, but the deviations are only of the order of magnitude of a few per cent. and probably within the experimental error. It is not certain that the same intercept should be attained in all cases, for the reasons discussed in footnote 41. In general we have given less weight to the experimental points at the very lowest protein concentrations, for which the turbidity is not much greater than that of the pure solvent. In one instance the choice of the slope through a set of eight points at low protein concentrations was determined by the method of least squares and the results agreed within one per cent. with the value for the slope already drawn through the points on a more empirical basis. The slopes of the lines obtained from such data as those of Figs. 2 and 3 form the basis for subsequent figures and the accompanying discussion.

Comparison with Slopes Derived from Osmotic Pressure Data.—According to equations 5, 6 and 32, the slopes, $2B$, of the curves for Hc_2/τ as a function of c_2 should correspond to the slopes of the osmotic pressure curves for P/RTc_2 as a function of c_2. Scatchard, Batchelder and Brown,[32] and Scatchard, *et al.*,[47] have obtained such curves for two different preparations of bovine serum albumin at values of Z_2 from +20 to −20, in sodium chloride at ionic strengths near 0.15. To compare their data with ours, the slopes must be expressed in terms of the same units.

The slope coefficient, B_2, as defined by equation 5 of Scatchard, Batchelder and Brown,[32,46] is related to our interaction constant B by the equation

$$B_2 = BM_2/1000 \qquad (38)$$

Hence, using our equation (32), for dilute solutions

$$2B_2M_2 = \frac{2BM_2^2}{1000} = \frac{M_2^2}{1000}\frac{\partial}{\partial c_2}\left(\frac{Hc_2}{\tau}\right)_{c_2=0} = \frac{Z_2^2}{2m_3} + \beta_{22} - \frac{\beta_{23}^2 m_3}{2 + \beta_{33}m_3} \qquad (39)$$

(46) Elsewhere, as in their equation 9, they write this term as B without a subscript. We denote it here as B_2, to distinguish it from our coefficient B. Their weight concentrations, w_2, are given as g. of albumin/kg. of H_2O. For the dilute solutions considered here, we may write $w_2 = 1000c_2$ to a very close approximation. The error involved in equating these two quantities is small compared with the experimental errors of either type of measurement, for the dilute solutions considered here.

The term $Z_2^2/2m_3$—the first of the three terms contributing to the observed slope—is the exact analog of the Donnan term in the equations for osmotic pressure. The second, β_{22}, involves the effect of the protein on its own activity coefficient; the third involves the protein–salt interaction. In tabulating the data, we have expressed limiting slopes as $2BM_2^2/1000$, so as to give directly the sum of the three terms on the right. The experimental values, and some of the data derived from them, are listed in Tables IV, V and VI.

TABLE IV

DETERMINATIONS OF LIMITING SLOPE IN SODIUM CHLORIDE SOLUTIONS, AS FUNCTION OF Z_2

The results of five different sets of experiments are reported here. The results of the first three, carried out with the Mueller apparatus, are given in the first three columns of the table. The results of the last two sets of experiments, carried out with the MEEZ apparatus, are given in the three right-hand columns.

Z_2	$\frac{BM_2^2}{1000}$	$\Gamma/2$	Z_2	$\frac{BM_2^2}{1000}$	$\Gamma/2$
+27	85	0.15	+20	170	0.183
+20	51		+10	110	
+16	51		+ 5	150	
+13	103		0	310	
+ 9	120		−10	450	
+ 4	170		−20	580	
− 4	290				
−12	410		+20	150	.150
			+ 8.5	65	
0	300	.15	+ 2.0	190	
−12.5	485		+ 1.0	245	
−15	520		− 9	415	
			−20	550	
+16	37	.15			
0	285		+20	1500	.010
−16	420		+ 8.5	220	
			+ 2.0	52	
			+ 1.0	0	
			− 9	1350	
			−20	2620	

Scatchard and his collaborators studied two different preparations of bovine serum albumin, and the values of B_2 which they obtained are somewhat lower in the second preparation.[47] However, this preparation was studied only at Z_2 values between 0 and −13. In plotting Fig. 4 we have extended the curve given by this equation, making use of the data given in their study on the other albumin preparation.[32] On the basis of a discussion with Dr. Scatchard, we have corrected this in two respects: First, for the change in slope with protein concentration, calculated[47] to be 1 + $0.002w_2$, where w_2 denotes g. of albumin per kg. of H_2O (see footnote 46). This requires a division of their numerical values by 1.12, since most of their measurements were made at a protein concentration near 60 g./kg. H_2O, whereas we are

(47) G. Scatchard, A. C. Batchelder, A. Brown and M. Zosa, THIS JOURNAL, **68**, 2610 (1946).

TABLE V

INTERACTION CONSTANTS (B VALUES) AND OTHER RELATED QUANTITIES FOR CHLORIDE AND THIOCYANATE SOLUTIONS

Z_2 is the valence of the protein, calculated from proton binding only; ν is the number of anions bound per mole of albumin, calculated from equation 40; $Z_2^* = Z_2 - \bar{\nu}$, the net charge on the protein; $Z_2^{*2}/2m_3^*$ is the "Donnan term"; $2BM_2^2/1000$ is the slope of the Hc_2/τ curve (see equation 39); and β_{22}^* is obtained from equation 49. The values given in parentheses for sodium chloride at $\Gamma/2$ 0.183 and $Z_2 = +20$ are much higher than other values for solutions under essentially the same conditions (see Table IV). The values in Table IV have been used in plotting Fig. 7. Note that the slope factor, $2BM_2^2/1000$, given here is twice as great as the factor given in Tables IV and VI, and in the figures. The values given here are expressed so that the values of β_{22}^* in the next column may be computed directly, using equation 49.

NaCl							NaCNS						
$\Gamma/2$	Z_2	$\bar{\nu}$	Z_2^*	$Z_2^{*2}/2m_3^*$	$2\frac{BM_2^2}{1000}$	β_{22}^*	$\Gamma/2$	Z_2	$\bar{\nu}$	Z_2^*	$Z_2^{*2}/2m_3^*$	$2\frac{BM_2^2}{1000}$	β_{22}^*
0.003	+20	6.15	13.85	32,000	9,600	−22,400	0.003	+20	13.7	6.3	6,600	6,700	100
	+10	3.00	7.00	8,150	3,000	− 5,150		+10	9.6	0.4	27	830	803
	+ 5	2.00	3.00	1,670	100	− 1,570		+ 5	7.7	− 2.7	1,230	−400	− 1,630
	0	1.04	− 1.04	165	200	35		0	6.0	− 6.0	6,000	1,220	− 4,780
	−10	0.26	−10.26	17,500	3,800	−13,700		−10	3.0	−13.0	28,200	6,600	−21,600
	−20	0	−20	66,500	11,000	−55,500		−20	0.97	−21	73,000	12,000	−61,000
.033	+20	9.85	10.15	1,500	740	−760	.033	+20	21.3	− 1.3	26	−310	−336
	+10	7.4	2.6	100	0	−100		+10	17.3	− 7.3	805	410	−395
	+ 5	6.2	− 1.2	22	200	−178		+ 5	15.6	−10.6	1,710	650	−1,060
	0	5.0	− 5.0	380	550	170		0	14.0	−14.0	2,980	1,220	−1,760
	−10	3.0	−13.0	2,560	1,540	−1,020		−10	10.7	−20.7	6,500	2,200	−4,300
	−20	1.57	−21.57	7,040	2,280	−4,760		−20	8.2	−28.2	12,000	2,800	−9,200
.183	+20	14.3	5.7	90	(340)	(250)	.183	+20	29.9	− 9.9	260	0	−260
	+10	12.1	− 2.1	12	220	208		+10	26.9	−16.9	790	580	−210
	+ 5	11.0	− 6.0	100	300	200		+ 5	25.5	−20.5	1,150	450	−700
	0	10.1	−10.1	275	620	345		0	24.0	−24.0	1,570	1,020	−550
	−10	8.4	−18.4	930	900	− 30		−10	20.9	−30.9	2,600	1,160	−1,440
	−20	6.7	−26.7	1,960	1,160	−800		−20	18.6	−38.6	4,070	1,480	−2,590

TABLE VI

LIMITING SLOPES IN CALCIUM CHLORIDE SOLUTIONS, AT THREE IONIC STRENGTHS

Z_2	$BM_2^2/1000$ $\Gamma/2$		
	0.003	0.033	0.183
+20	5700	410	270
+10	2300	190	
+5	225	0	50
0	−100	50	125
−10	2800	430	300
−20	5500	540	220

concerned with the limiting values at zero protein concentration. Second, we must correct for the absolute differences in the values of B_2 for the two albumin preparations. This has been done by assuming that the B_2 value at any given Z_2 differs by the same absolute amount for the two preparations, at all Z_2 values. Thus values of B_2 were calculated from the osmotic pressure data over the range of Z_2 from +9 to −17. Finally, by using the solid curve shown in Fig. 9 of reference 32 for values of Z_2 greater than +9, and applying the above corrections, a curve has been constructed covering the whole range of Z_2 studied by us. This is shown as the solid curve in Fig. 4. The points, from our own studies in five different sets of experiments (Table IV) over a period of two years, on the same albumin preparation, may be compared directly with the curve from the osmotic pressure data. Most of the light scattering values lie below the osmotic pressure curve, but the differences between the two sets of data are almost certainly within the experimental error of either set of experiments, and certainly within the limits of variation found between different preparations of serum albumin. It should be noted that the agreement would be less good if the data from Scatchard's earlier preparation of albumin had been used in plotting the curve for Fig. 4. This would have resulted in displacing the curve upward by approximately 60 units in the value of $B_2M_2 = BM_2^2/1000$, which is the ordinate of Fig. 4.

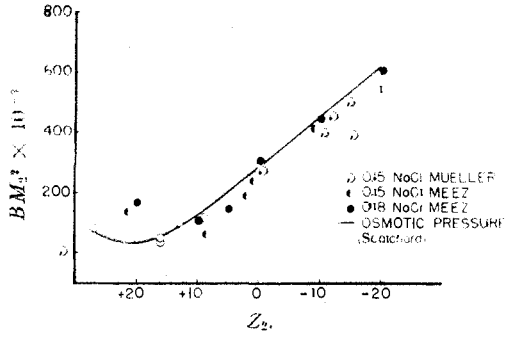

Fig. 4.—Comparison of slopes determined from osmotic pressure (Scatchard, Batchelder and Brown) and from light scattering measurements.

Interaction Constants and the Binding of Small Ions.—The light scattering data have been extended to considerably lower ionic strengths than those reported for the osmotic pressure studies. A systematic comparison of the slopes in solutions of sodium chloride, sodium thio-

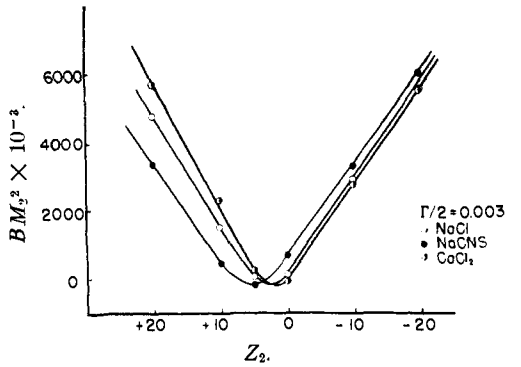

Fig. 5.—Interaction constants at ionic strength 0.003, for solutions of serum albumin in three different salts. Note the difference in scale of ordinates between this and the following figure.

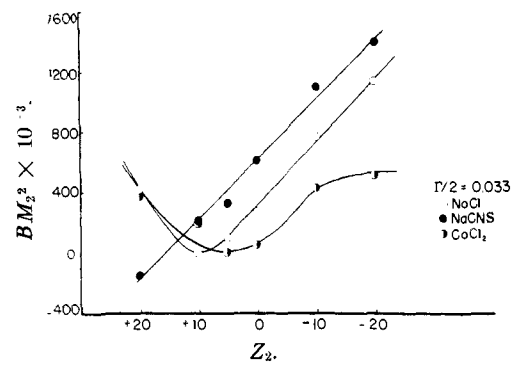

Fig. 6.—Curves similar to those of Fig. 5, at ionic strength 0.033.

cyanate, and calcium chloride, at two ionic strengths, is given in Figs. 5 and 6. In the lower half of Fig. 7 values are given for sodium chloride solutions at four different ionic strengths. Certain features of all the curves are immediately apparent. In general form they are not far from parabolic. The value of B at the lowest point on each curve is generally near zero, and at low ionic strengths this minimum occurs in the neighborhood of $Z_2 = 0$. Qualitatively this is what would be expected from the contribution of the Donnan term $(Z_2^2/2m_3)$ to the slope. Quantitatively, however, the total slope is always much less than would be calculated from the Donnan term alone. It is also obvious that the minimum never lies at $Z_2 = 0$, but is always displaced somewhat to the left. This displacement increases with increasing ionic strength, for the chloride and thiocyanate solutions, and the displacement is much greater for thiocyanate than for chloride. This effect is very pronounced at ionic strength 0.183 (see data in Table V). In such solutions the slope values for thiocyanate have still not reached their minimum, even at $Z_2 = +20$. Qualitatively, this behavior is exactly what might be expected from selective binding of these anions, if it is assumed that the minimum value of B should correspond to the

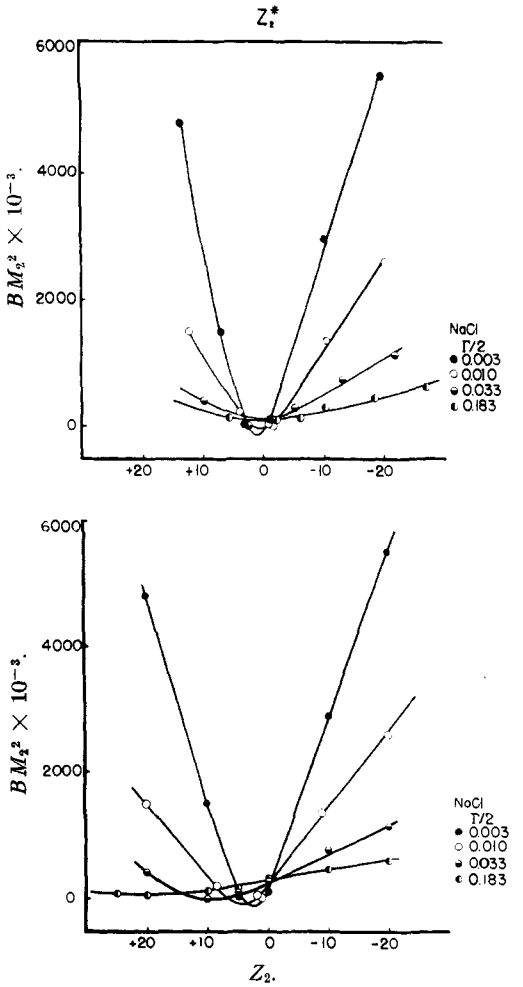

Fig. 7.—Interaction constants for serum albumin in sodium chloride solutions at four different ionic strengths, as a function of the valence Z_2 (lower diagram) and of the corrected net charge Z_2^* (upper diagram).

point at which the actual net charge on the protein is zero, and that the curves should rise symmetrically on either side of this point. The curves for the calcium chloride solutions are definitely displaced with respect to those for sodium chloride. The displacement is in the direction that would be predicted if calcium ions were bound by the protein (see below).

Clear proof of the binding of chloride and thiocyanate ions by albumin, and quantitative values for the amount bound, have been obtained by Scatchard, Scheinberg and Armstrong.[48] Their data are consistent with the assumption that each albumin molecule contains two classes of groups which bind both chloride and thiocyanate: n_1 groups with strong binding, and n_2 groups with a lower affinity for these anions. The amount of bound anion $(\bar{\nu})$ per mole of albumin is given by equation (40).

(48) G. Scatchard, I. H. Scheinberg and S. H. Armstrong, Jr. THIS JOURNAL, **72**, 535, 540 (1950).

$$\bar{\nu} = \frac{n_1 k_1 A\gamma e^{2w(Z_2 - \bar{\nu})}}{1 + k_1 A\gamma e^{2w(Z_2 - \bar{\nu})}} + \frac{n_2 k_2 A\gamma e^{2w(Z_2 - \bar{\nu})}}{1 + k_2 A\gamma e^{2w(Z_2 - \bar{\nu})}} \quad (40)$$

where k_1 is the intrinsic affinity constant for the first set of n_1 groups and k_2 is the constant for the second set of n_2 groups. The values of n_1, n_2, k_1 and k_2 are

	k_1	n_1	k_2	n_2
Cl^-	44	10	1.1	30
CNS^-	1000	10	25	30

γ is the assumed activity coefficient of the anion (Cl^- or CNS^-) in the solution and A is the molar concentration of free anion. w is defined by the equation

$$w = \frac{\mathbf{E}^2}{2DkT}\left(\frac{1}{b} - \frac{\kappa}{1 + \kappa a}\right) = 2.303\left[0.0517 - \frac{0.5085\left(\frac{\Gamma}{2}\right)^{1/2}}{1 + 10.663\left(\frac{\Gamma}{2}\right)^{1/2}}\right] \quad (41)$$

where $\mathbf{E}$ is the proton charge, D the dielectric constant of the medium, b the radius of the protein ion considered as a sphere, a the "collision diameter" for the protein ion and the diffusible ions in the system, and κ has its usual significance in the Debye–Hückel theory. Using these equations and the values of w and γ listed below, we have calculated the values of $\bar{\nu}$ for the various solutions studied by us. We may now express the observed slopes as functions not of the valence, Z_2, but of

$\Gamma/2$	w	γ	$A\gamma$
0.183	0.0286	0.769	0.141
.033	.0465	.860	.0284
.003	.0781	.945	.00284

the total net charge Z_2^*, ($Z_2^* = Z_2 - \bar{\nu}$). When this is done, the curves for chloride and thiocyanate superpose more closely than before, and the minimum value of B_2 is found to coincide rather closely with the value zero for Z_2^*. The increased symmetry of the curves about $Z_2^* = 0$, for sodium chloride solutions, is apparent on comparison of the curves in the upper half of Fig. 7, plotted against Z_2^*, with those in the lower half, representing the same data plotted against Z_2.

The redefinition of the protein ion to include $\bar{\nu}$ ions of Cl^- or CNS^-, making its net charge Z_2^*, involves also redefining the salt concentration, m_3. In the previous set of definitions (see especially equations 21 and 22), $m_3 = {}^1/_2(m_c + m_a)$, where m_c and m_a are the *total* concentrations of univalent diffusible cation and anion, respectively. In the new definition $m_3^* = {}^1/_2(m_c + m_a)_{free}$, the ions bound to the protein being no longer counted in the concentration of component 3. We assume (see ref. 48) that no cation is bound, and that the concentration of bound anion is $\bar{\nu}m_2$. Hence

$$m_3^* = m_3 - (\bar{\nu}m_2/2) \quad (42)$$

In general m_3^* and m_3 are nearly identical in most of the systems we have studied, and they always approach identity as m_2 approaches zero. In practice, therefore, for the calculation of limiting slopes at zero protein concentration, we have taken both m_3 and m_3^* as equal to the molar concentration of added salt, disregarding the excess of anions or cations required to balance the net charge on the protein ion. It should be noted that the chemical potential of component 3 is the same in either system of definitions, and m_2 also is the same in both systems.

Hence we may write, following equation 15

$$\ln a_3^* = \ln\left(m_3^* + \frac{Z_2^* m_2}{2}\right) + \ln\left(m_3 - \frac{Z_2^* m_2}{2}\right) + \beta_3^* \quad (43)$$

From (42) this may also be written

$$\ln a_3^* = \ln\left(m_3 + \frac{Z_2 m_2}{2} - m_2\bar{\nu}\right) + \ln\left(m_3 - \frac{Z_2 m_2}{2}\right) + \beta_3^* \quad (43a)$$

Hence, since $\ln a_3 = \ln a_3^*$, β_3 and β_3^* are related by the equation

$$\beta_3^* - \beta_3 = \ln \frac{\left(m_3 + \frac{Z_2 m_2}{2}\right)}{\left(m_3 + \frac{Z_2 m_2}{2} - \bar{\nu}m_2\right)} \quad (44)$$

The equations for $\ln a_2^*$, a_{22}^* and a_{23}^*, are identical in form with equations 14, 18, 19, 23 and 25, if Z_2 is replaced by Z_2^*, m_3 by m_3^*, and the factor ϵ is redefined as ϵ^* by an equation identical in form with 24. Likewise β_2, β_{22} and β_{23} are replaced by the corresponding starred quantities. However, β_{23} and β_{23}^* require more explicit discussion. Scatchard (12, equation 27) gives, as a limiting value for m_2 approaching zero

$$\beta_{23} = b_{23}M_2(2 + \beta_{33}m_3) \quad (45)$$

$$b_{23}M_2 = -\frac{d}{dm_2}\ln\frac{m_3}{m_3'} \quad (46)$$

where m_3 and m_3' are the values for component 3 at equilibrium inside and outside a membrane containing component 2 on the inside. If it is assumed that m_3 and m_3' differ only by the binding of one or both ions of component 3 by component 2, then $m_3 = m_3' + (\bar{\nu}m_2/2)$ in the limit as m_2 approaches zero, and

$$\lim_{m_2 \to 0} \frac{d}{dm_2}\ln\frac{m_3}{m_3'} = \frac{\bar{\nu}}{2m_3} \quad (47)$$

to a close approximation. Substituting in (45) and (46)

$$\beta_{23} = (-\bar{\nu}/2m_3)(2 + \beta_{33}m_3) \quad (48)$$

This is the value of β_{23} which should appear in equation 25. On the other hand, if we transform to the starred system of definition of components, $\bar{\nu}$ is included in Z_2^*, and the "residual value" of $\bar{\nu}$ to be included in the analog of equation 48 is therefore zero. Hence β_{23}^* is zero, and drops out in the analog of equation 32, which thus becomes

$$\frac{Hc_2}{\tau} = \frac{1}{M_2} + \frac{1000}{M_2^2}\left(\frac{Z_2^{*2}}{2m_3^*} + \beta_{22}^*\right)c_2 \quad (49)$$

In Fig. 8 β_{22}^* is plotted as a function of Z_2^* for chloride and thiocyanate at three different ionic strengths, the ordinate scale being greater by a factor of 10 for the solution at ionic strength 0.003 than for the other two curves. The curves are in general symmetrical about $Z_2^* = 0$—the corresponding curves for β_{22} as a function of Z_2 are of course distinctly asymmetrical—and become increasingly negative as Z_2^* increases in either direction. It appears also from these figures that β_{22}^* is approximately the same function of Z_2^*, at any given total ionic strength, for both chloride and thiocyanate. The form of the curves, at ionic strength 0.003 and 0.033, is very similar, in spite of the tenfold difference in the numerical values of β_{22}^*.

Binding of Calcium Ions by Albumin.—Especially at the two higher ionic strengths studied (see Fig. 6 and the data of Table V), the curves for B as a function of Z_2, in calcium chloride solutions are displaced with respect to those for sodium chloride. The curves are not far apart in acid solutions, but diverge markedly as Z_2 becomes more negative, the calcium curve lying to the right. From the Donnan effect alone, such a displacement would be expected if calcium ions are bound by albumin, the binding increasing as Z_2 decreases. Binding of calcium by serum albumin and other proteins has been clearly established by several methods.[49,50,51] Making certain simplifying assumptions, we may calculate roughly the amount of bound calcium, in the solutions studied, from our data.

The Donnan term in a_{22}, for serum albumin, is very nearly $Z_2^2/6m_3$ in calcium chloride solutions. This may also be written Z_2^2/Γ, where Γ is double the ionic strength due to component 3 ($CaCl_2$); it thus assumes the same form as for a uni-univalent salt for which the term is $Z_2^2/2m_3 = Z_2^2/\Gamma$ (equation 39). The best data relative to calcium binding are probably those at $\Gamma/2 = 0.033$.

The net charge Z_2^*, in calcium chloride solutions, is equal to $Z_2 - \bar{\nu}(Cl^-) + 2\bar{\nu}(Ca^{++})$, where $\bar{\nu}(Ca^{++})$ denotes moles of bound Ca^{++} ion per mole of albumin. We shall tentatively assume that β_{22}^* (equation 49) is the same function of Z_2^* and of ionic strength in sodium chloride and in calcium chloride solutions (compare Fig. 8, for chloride and thiocyanate). Then B is the same function of Z_2^*, at the same ionic strength, for both salts. If we assume that $\bar{\nu}(Cl^-)$ is the same, at the same Z_2 value, for both salts, then the calcium bound is given by displacing any point on the calcium curve horizontally to the left, until it coincides with the point on the sodium chloride curve which has the same B value. The displacement ΔZ_2, divided by two, should then give $\bar{\nu}(Ca^{++})$. The assumptions made in this argument are seriously oversimplified,[52] so that the calculated values are only first approximations.

From the data in Fig. 6, this procedure gives $\bar{\nu}(Ca^{++}) = 0$ at $Z_2 = +20$, and 8 at $Z_2 = -20$, with an approximately linear relation at intermediate Z_2 values. From the experiments and calculations of Weir and Hastings,[50] who deduced the existence of about 10 binding sites for Ca^{++} per mole albumin, with an association constant of about 200 for each site at $Z_2 = -20$, we should calculate $\bar{\nu}(Ca^{++}) = 7$ under the conditions of Fig. 6, at $Z_2 = -20$. The agreement is better than we should have any right to expect, especially as Weir and Hastings worked at very low Ca^{++} concentrations, in the presence of 0.16 M sodium chloride, and our use of their equations involves a large extrapolation from the actual conditions of their experiments. It is probable that the total number of groups in albumin which can bind calcium is actually considerably greater than 10; only the groups which bind Ca^{++} most strongly would have been detected under the conditions of Weir and Hastings' experiments.

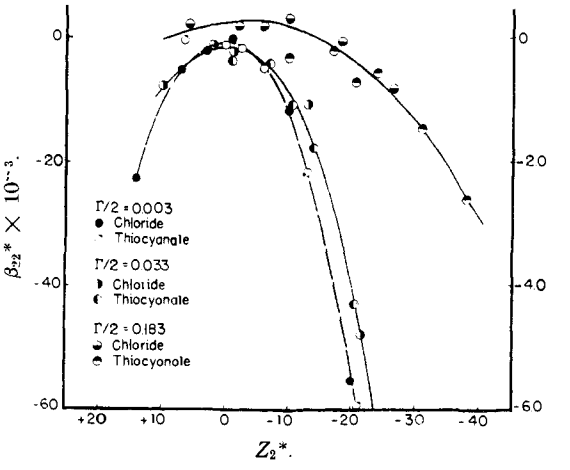

Fig. 8.—Activity coefficient factors for serum albumin, β_{22}^* (equation 49) as a function of the corrected net charge Z_2^*, at three different ionic strengths. The left-hand ordinate scale applies to the dashed curve: the right-hand scale, to the two solid curves.

The decrease in bound calcium, as Z_2 becomes more positive, is determined by at least two effects: (1) the increase of net positive charge on the protein contributes a repulsive electrostatic effect on the calcium ion, (2) the calcium binding groups almost certainly are ionized $^-COO^-$ groups.[53] As the acidity increases, H^+ ions compete with Ca^{++} for these groups, and Ca^{++} binding is thus still further repressed. However, at $\Gamma/2 = 0.183((Ca^{++}) = 0.061m)$ our data indicate that several moles of Ca^{++} may be bound by albumin, even at $Z_2 = +20$, in spite of the unfavorable factors listed above.

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Summary

1. Two different forms of apparatus, for the study of light scattering at 90°, are described.

(49) F. C. McLean and A. B. Hastiugs, *J. Biol. Chem.*, **108**, 285 (1935).

(50) E. G. Weir and A. B. Hastings, *ibid.*, **114**, 397 (1936).

(51) D. M. Greenberg, *Advances in Protein Chemistry*, **1**, 121 (1944).

(52) In a calcium chloride solution of given Γ, the concentration of Cl^- ions is only 2/3 as great as in a sodium chloride solution of the same Γ. On the other hand, the binding of Ca^{++}, by making the net charge more positive, favors the binding of Cl^- for electrostatic reasons. The two effects tend to counterbalance one another, but we shall not attempt a precise calculation of $\bar{\nu}(Cl^-)$ in calcium chloride solutions.

(53) Very probably neighboring hydroxyl groups are also involved. See for instance Greenberg.[51]

One of these permits measurement of the scattering in liquids such as pure benzene, within 2–3%, using 2–4 cc. of liquid.

2. The calibration of the apparatus in terms of several standard liquids is described. The molecular weight of bovine serum albumin, based on these standards, is in good agreement with values derived from osmotic pressure and from sedimentation and diffusion. Serum albumin may serve as a convenient working standard in the determination of molecular weights by turbidity.

3. Relations are stated for turbidity in multicomponent systems containing small ions or molecules and one or more macromolecular ions. The equations predict, among other terms, an effect corresponding to the Donnan factor for osmotic pressure, which becomes large for high net charge on the protein and at low ionic strength. This does not affect the extrapolated molecular weight values, but greatly affects the slope of the curves for c/τ as a function of c.

4. In dilute solutions, the turbidity, τ, of serum albumin obeys Debye's equation $Hc/\tau = (1/M_2) + 2Bc$. The interaction constant B agrees within the limits of error with the values independently obtained from osmotic pressures by Scatchard and his associates. However, the theoretical equations show that such agreement is not in general to be expected in multicomponent systems, and is found here only because some of the terms in the theoretical equations are numerically small under our conditions.

5. The interaction constant B is generally near zero for the isoelectric protein. When the valence (Z_2) of the protein, as determined from the acid–base titration curve, is large, B is large and positive at low ionic strength, but decreases as ionic strength increases.

6. The curves for B as a function of Z_2, at several different ionic strengths, have been studied in solutions of sodium chloride, sodium thiocyanate, and calcium chloride. The form of the curves clearly reveals effects of the specific binding of chloride and thiocyanate ions; the latter being bound much more tightly than the former, in agreement with Scatchard's findings. The curves become far more symmetrical when B is plotted as a function of Z_2^*, which is the net charge per albumin molecule, taking account of *all* ions bound by the protein.

7. Comparison of the curves for calcium and sodium chloride indicates binding of calcium by albumin, the binding increasing with increasing pH. Rough estimates of the amount of calcium bound agree reasonably with those of Hastings.

8. The curves for B, as a function of ionic strength and net charge (Z_2^*) on the protein, agree qualitatively with the predictions from the Donnan effect, but are numerically smaller. This indicates that the factor β_{22}^*—rate of change of activity coefficient of the protein component with its own concentration—is large and negative at low ionic strength, when the net charge on the protein is large. For the isoelectric protein, β_{22}^* is small and sometimes positive.

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A Study of Heterogeneous Equilibria in Aqueous Solutions of the Nickel Selenates at 30°[1]

BY C. S. ROHRER AND H. ROBERT FRONING

Few equilibrium studies have been made involving the hydrates of nickel selenate. The hexahydrate of nickel selenate was first prepared by E. Mitscherlich[2] and later by von Hauer.[3] Andre Klein[4] published an investigation of the two-component system nickel selenate and water, and reported solutions of the hexahydrate stable from the eutectic at −3 to 82.2°, and the tetrahydrate stable to the boiling point of its saturated solution. Dehydration curves gave breaks for the dihydrate at 105° and for the monohydrate at 170°. Mme. Demassieux[5] studied the dehydration of several nickel selenates and found no breaks which would correspond to the tetra-, di- or monohydrates. However, she stated that above 400° the anhydrous salt appeared.

The study on the three component system $NiSeO_4$–H_2SeO_4–H_2O was made at equilibrium to provide data on solubility of the hydrates in varying concentrations of selenic acid and to verify the existence of certain hydrates.

Experimental

Preparation of Selenic Acid.—The selenic acid used in this investigation was prepared by the method of Gilbertson and King.[6]

Preparation of Nickel Selenate.—A nickel carbonate was prepared in accordance with the procedure of Gagnon, Cloutier and Martineau[7] by adding solid C. P. potassium hydrogen carbonate in small quantities to a saturated solution of nickel nitrate, with stirring, until the filtered

(1) Taken in part from a thesis submitted by H. Robert Froning in partial fulfillment of the requirements for the Doctor of Philosophy Degree at Indiana University.

(2) Mitscherlich, *Ann. Physik*, **11**, 326 (1827).

(3) von Hauer, *J. prakt. Chem.*, **80**, 217 (1860).

(4) Klein, *Ann. chim.*, **14**, 263 (1940).

(5) Demassieux, *Compt. rend.*, **221**, 557 (1945).

(6) Gilbertson and King, THIS JOURNAL, **58**, 180 (1936).

(7) Gagnon, Cloutier and Martineau, *Can. J. Research*, **19**, B, 179 (1941).