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Light Scattering Investigation of Charge Fluctuations in Isoionic Serum Albumin Solutions[1]

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Light scattering investigations have been carried out on Armour bovine plasma albumin (BSA), bovine serum mercaptalbumin (BMA) and human serum mercaptalbumin (HMA) in their isoelectric region. All three proteins were deionized by passing over an ion-exchange column. In the case of salt free aqueous solutions it was found that the function $H(C_2/\Delta\tau)$ is close to linear in the square root of protein concentration for all three proteins, as predicted by the fluctuating charge theory of Kirkwood and Shumaker. Values of the fluctuating charge were found to be 3.58, 3.51 and 3.98 protonic units for BSA, BMA and HMA, respectively. These are in good agreement with values calculated from other types of measurements. An analysis is made of the contribution of the progressive ionization of the three proteins in the isoionic state to the derivative of the excess chemical potential of the protein with respect to its concentration, showing this effect to be not large under the experimental conditions used. It may, however, cause the light scattering plot in the absence of salt to pass through a maximum at low concentrations of protein. An analysis in terms of multicomponent light scattering theory of the data obtained in the presence of sodium chloride revealed that the contribution to the intercept of the thermodynamic interaction term between protein and salt is small in the present case.

Introduction

In recent years the technique of light scattering has received wide application in physico-chemical investigations of proteins.[5,6] In addition to yielding information on molecular weights[7,8] and state of molecular aggregation[7,9-15] of a system, it is particularly well adapted to the study of the thermodynamic interactions which occur in solutions of macromolecules.[16] Thus, in a multicomponent system,[16-18] the turbidity is given by an equation of the form[16]

$$\tau = \frac{8\pi^3}{3\lambda^4\rho_0}\sum_{i,k=1}^{\nu} c_i c_k \frac{|\beta|_{ik}}{|\beta|}\left(\frac{\partial\epsilon}{\partial c_i}\right)_{T,p,c_l}\left(\frac{\partial\epsilon}{\partial c_k}\right)_{T,p,c_l}\left(\beta_{ik} = \frac{c_i c_k}{M_i \bar{R}T}\left(\frac{\partial\mu_i}{\partial c_k}\right)\right) \quad (1)$$

where λ is the wave length of the light, N is Avogadro's number, ρ_0 is the density of the solvent, c_i is the concentration of the i-th component in moles per mole of solvent, ϵ is the dielectric constant of the solution, $|\beta|_{ik}$ is the appropriate cofactor of the determinant $|\beta|$, M_i is the molecular weight of species i, μ_i is the chemical potential per mole of that component, and T is the thermodynamic temperature.

In the case of a three-component system (such as water = component 0, neutral salt = component 1, and protein = component 2), equation 1 becomes

$$H\frac{C_2}{\Delta\tau} = \frac{1}{1+D}\left\{\frac{1}{M_2} + \left[\frac{A_{22}}{M_2} - \frac{A_{12}}{M_1}\left(\frac{A_{12}}{\frac{2}{C_1} + A_{11}}\right)\right]C_2\right\} \quad (2)$$

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

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

$$A_{ij} = \frac{1}{RT}\frac{\partial\mu_i^{(e)}}{\partial C_j}$$

$$\mu_i = RT\log C_i + \mu_i^{(e)} + \mu_i^0(T,\rho)$$

$$\alpha = \frac{\partial n}{\partial C_1}\Big/\frac{\partial n}{\partial C_2}$$

where $\Delta\tau$ is the excess turbidity over that of the solvent, C_i is the concentration of component i in grams per ml., n is the refractive index of the solution, M_i is the molecular weight of component i, and $\mu_i^{(e)}$ is the excess chemical potential of that component.

According to this equation, in the case of a three-component system, the intercept of the usual $H(C_2/\Delta\tau)$ *vs.* concentration plot is not the reciprocal of the molecular weight, but rather the reciprocal of the sum of the molecular weight and a term representing the thermodynamic interaction between the macromolecule and the third component. This has been demonstrated previously for the case of the system polystyrene-benzene–methanol.[16,19] In order to determine the value of the molecular weight in the presence of a third component, it is necessary to evaluate the term D from independent thermodynamic measurements. This term is found to be negligible for many protein systems.

In a solution of two components, such as a salt-

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(3) Lilly Post-Doctoral Fellow, 1953–1954.

(4) This work is drawn in part from the dissertation of Bernard D. Coleman submitted in partial fulfillment of the requirements for the Ph.D. degree, Yale University, 1954.

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(7) M. Halwer, G. C. Nutting and B. A. Brice, THIS JOURNAL, **73**, 2786 (1951).

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(12) H. Edelhoch, E. Katchalski, R. H. Maybury, W. L. Hughes and J. T. Edsall, THIS JOURNAL, **75**, 5058 (1953).

(13) P. Doty, M. Gellert and B. Rabinovitch, *ibid.*, **74**, 2065 (1952).

(14) R. F. Steiner, *Arch. Biochem. Biophys.*, **44**, 120 (1953).

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(16) J. G. Kirkwood and R. J. Goldberg, *J. Chem. Phys.*, **18**, 54 (1950).

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

(18) H. C. Brinkman and J. J. Hermans, *ibid.*, **17**, 574 (1949).

(19) R. H. Ewart, C. P. Roe, P. Debye and J. R. McCartney, *ibid.*, **14**, 687 (1946).

free aqueous solution of an isoionic protein, equation 1 yields

$$H\frac{C_2}{\Delta\tau} = \frac{1}{M_2}\left[1 + \frac{C_2}{RT}\left(\frac{\partial\mu_2^{(e)}}{\partial C_2}\right)_{T,p}\right] \qquad (3)$$

In this case the solute molecular weight, M_2, may be determined directly from the intercept of the $H(C_2/\Delta\tau)$ *vs.* C_2 plot, while from the slope of the curve, the derivative of the excess chemical potential of the protein with respect to concentration may be calculated. This is a measure of the departure of the solution from ideal behavior. Attractive forces between the solute molecules lead to negative values of this derivative, whereas repulsive forces give rise to positive values.

Kirkwood and Shumaker[20] have shown that in protein solutions an attractive force should arise between protein molecules from fluctuations in charge and charge distribution associated with fluctuations in the number and configuration of the protons bound to the protein molecules. According to the fluctuating charge theory of Kirkwood and Shumaker, for an isoionic protein with a mean net charge of zero, but a non-zero mean-square charge, the derivative of the excess chemical potential with protein concentration is given by the expression

$$\frac{1}{RT}\frac{\partial\mu_2^{(e)}}{\partial C_2} = \frac{-\pi Ne^4\langle Z^2\rangle_{av}^2}{M_2(DkT)^2\kappa(1+\kappa a)^2} + \frac{7\pi Na^3}{6M_2} + 2B'$$

$$\kappa^2 = \kappa_0^2 + \kappa_2^2$$

$$\kappa_0^2 = \frac{4\pi Ne^2}{1000DkT}\sum_j C_jZ_j^2 \qquad (4)$$

$$\kappa_2^2 = \frac{4\pi Ne^2}{DkT}\left[\frac{\langle Z^2\rangle_{av}}{M_2}C_2\right]$$

where $\langle Z^2\rangle_{av}$ is the mean square charge of a protein molecule in protonic units e, D is the dielectric constant of the medium, k is Boltzmann's constant, and κ and a are the Debye–Hückel parameters. The second term of the first of equation 4 is the excluded volume, while $2B'$ is the contribution of the non-electrostatic and dipole and higher multipole interactions.

Binomial expansion of $(1 + \kappa a)^{-2}$ in equation 4 and combination with equation 3 results in a power series in $C_2^{1/2}$ for the function $H(C_2/\Delta\tau)$. For the case of a salt-free isoionic protein solution with an average charge of zero, this is

$$H\frac{C_2}{\Delta\tau} = \frac{1}{M_2}\left\{1 - \frac{\pi^{1/2}N^{1/2}e^3\langle Z^2\rangle_{av}^{1/2}}{2(DkT)^{3/2}M_2^{1/2}}C_2^{1/2} + \left[\frac{2\pi Ne^4\langle Z^2\rangle_{av}^2 a}{M_2(DkT)^2} + \frac{7\pi Na^3}{6M_2} + 2B'\right]C_2 + 0(C_2^{3/2})\ldots\right\} \qquad (5)$$

According to equation 5, $H(C_2/\Delta\tau)$ should be linear in $C_2^{1/2}$ at high protein dilutions. Furthermore, since the only unknown parameter in the coefficient of $C_2^{1/2}$ is the value of the fluctuating charge, $\langle Z^2\rangle_{av}$, the latter may be calculated from a direct measurement of the limiting slope of $H(C_2/\Delta\tau)$ plotted as a function of $C_2^{1/2}$, if the average charge of the protein is zero.

It is the purpose of the present investigation to test further[21] the theory of charge fluctuation, to compare values of the fluctuating charges of some proteins as measured by light scattering with values calculated from other types of measurements, and to examine other factors contributing to the light scattering of isoionic proteins. The proteins chosen for this investigation were Armour crystalline bovine plasma albumin (BSA), bovine plasma mercaptalbumin (BMA) and human plasma mercaptalbumin (HMA).

(20) J. G. Kirkwood and J. B. Shumaker, *Proc. Natl. Acad. Sci., U. S.*, **38**, 863 (1952).

(21) S. N. Timasheff, H. M. Dintzis, J. G. Kirkwood and B. D. Coleman, *ibid.*, **41**, 710 (1955).

Experimental

Light Scattering.—The light scattering measurements were carried out in a Brice-Phoenix photometer,[22] using an opal glass primary standard for the determination of absolute turbidities. The measurements were carried out in 3-ml. square cells with the introduction of 2.5 mm. wide slits into the optical system and the use of a cell holder specially designed to eliminate stray light from the view of the photomultiplier.[23] (In the case of some preliminary measurements carried out in 30-ml. cells, the normal optics of the instrument were used.) When used with 3-ml. cells, the photomultiplier was protected from illumination at all times, except during actual measurements, by the introduction of a camera shutter into its nose piece. This shutter was operated from outside the cell housing by means of a cable plunger. An instrumental constant relating the two types of cells and optics was determined by direct comparison of identical protein and fluorescin solutions in the two systems.

The light scattering instrument was calibrated with the opal glass standard of known opacity furnished by the instrument manufacturer and was found to give a Rayleigh's ratio of 49.1×10^{-6} with three times redistilled C.P. benzene. The linearity of the instrument was checked over the range used in the protein light scattering experiments with a solution of fluorescin. As shown in Fig. 1, both the 3-ml. cell with slit optics and the 30-ml. cell with wide optics yielded horizontal lines when the ratio of the light intensity at 90° to fluorescin concentration was plotted against the concentration of fluorescin.

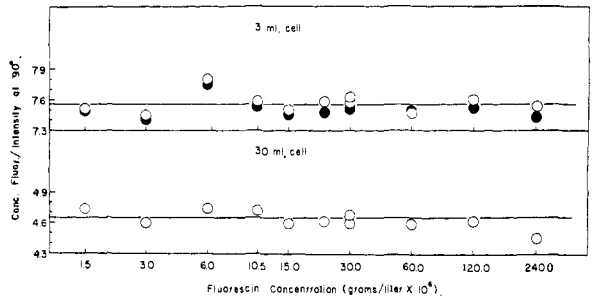

Fig. 1.—Calibration of the linearity of the light scattering instrument with fluorescin solutions. The light intensities observed at 90° cover the range measured with the protein solutions.

Protein Solutions.—An 8–10% solution of Armour BSA was prepared by dissolving it in twice distilled water. The resulting solution was dialyzed against several changes of distilled water for four days at 5°. At the end of this period the *p*H of the solution was found to be 4.9 and its specific conductance at 0° was 1.66×10^{-5} ohm^{-1} cm.$^{-1}$, indicating that mobile ions other than hydrogen were still present. The remaining ionic impurities were removed by passing the protein solution through an ion-exchange column at 4° as described by Dintzis.[24] This column contained 21 ml. of a 2:1 mixture of anionic and cationic resins above 7 ml. of cationic resin. The anionic resin was Amberlite I.R. 120 which had been converted to the hydrogen form by extraction with 6 *N* HCl in a Soxhlet extractor. The cationic resin was Amberlite I.R. 400 which had been con-

(22) B. A. Brice, M. Halwer and R. Speiser, *J. Optical Soc. Am.*, **40**, 768 (1950).

(23) H. M. Dintzis, in preparation.

(24) H. M. Dintzis, Doctoral Dissertation, Harvard University, 1952.

verted to the hydroxyl form by extraction with a 100-fold excess of 3 *N* carbonate-free sodium hydroxide. After passing through the column, the specific conductance of the protein solution at 0° was found to be 0.30×10^{-5} ohm^{-1} cm.$^{-1}$. The *p*H was 4.9. This *p*H corresponds to an hydrogen ion concentration of 1.26×10^{-5} *M* and yields a value of 0.30×10^{-5} ohm^{-1} cm.$^{-1}$ for the contribution of the hydrogen ions to the specific conductance at 0°. Therefore, the conductance of the column treated solution could be accounted for quantitatively in terms of hydrogen ions. This solution was then lyophilized and the protein was stored at 5° for use in the light scattering experiments.

Measurements.—For light scattering measurements, the lyophilized protein was dissolved in twice distilled water to make an 8–10% solution and again passed over the ion-exchange column. This solution was then clarified by centrifuging it for three hours in a Spinco Model L centrifuge at 40,000 r.p.m., removing the middle portion of the liquid out of the centrifuge tube, and filtering it through a sintered glass filter similar to that designed for light scattering by Bier.[25,26] This filter consists of two glass reservoirs joined by a glass connection at the bottom. A sintered glass disk of ultrafine porosity is sealed into the lower portion of one of the reservoirs. The solution is introduced into the filter so that one reservoir and the portion of the other one below the filter disk are filled. Nitrogen pressure of one to three pounds is applied, causing the solution to pass through the filter upward. In this manner, a minimum surface is presented to the atmosphere, minimizing the possibility of surface denaturation and of catching stray dust from the air. The filtered solution was then transferred to a glass vial and stored in the refrigerator.

The actual measurements were carried out in 3 ml., 1 cm.-square Pyrex-glass cells with closely fitting Teflon cover.[23] In each set of measurements, the turbidity of the solvent was first determined and then a series of small increments of concentrated (5–8%) stock solution was added from a Gilmont ultramicroburet. After each addition the solution was mixed by inverting the cell several times. Each series of measurements usually consisted of six to ten concentrations. In every case, each concentration range was covered several times and points obtained in overlapping dilution series were found to agree to better than 2%, indicating that this technique was free of errors due to protein denaturation or the introduction of small amounts of dust during protein addition or mixing. In the case of runs in the presence of salt, after each addition of salt-free stock protein, the necessary amount of aqueous salt solution, one hundred times more concentrated than the conditions of the run, was added to keep the electrolyte concentration constant throughout a concentration series. (In the experiments carried out in 30-ml. cells, each concentration point was measured in an individual cell, all additions being made from volumetric pipets.)

All the glassware used in the light scattering measurements was washed with a mixture of hot nitric and sulfuric acids, with the exception of the cells which were washed with detergent, and then rinsed many times with distilled water and finally doubly distilled water.

BMA and HMA.—The BMA and HMA were prepared according to the methods developed by Hughes and Dintzis.[27,28] Stock 8–10% protein solutions were prepared again, deionized and cleared for light scattering in the same manner as the BSA.

Auxiliary Measurements.—All salt solutions were prepared by making a 1.0 *M* salt solution from a reagent grade chemical. This was filtered through the sintered glass filter described above and diluted to the working concentrations with doubly distilled water. The water used as diluting solvent in all experiments was doubly distilled water prepared in an all-Pyrex glass still.

The value for the refractive increment of all three proteins was calculated for the wave length of 436 μ and 25° from values reported by Perlman and Longsworth for Armour BSA[29] dissolved in a salt-free aqueous solution, by using their dispersion equation. This resulted in a value of 0.1953 for $\partial n/\partial c_2$, which was used in all calculations both in the presence and absence of salt.

The change in *p*H of BSA was followed with dilution in the salt-free system and also with addition of NaCl to a 1.5% solution of the column deionized protein. In the salt-free preparations, the *p*H was calculated from conductivity measurements with the assumption that the measured conductivity was due solely to hydrogen ions. It was found that in the salt-free solutions, the *p*H changed from 4.6 at 3.8% protein to 5.2 at 0.06% protein. Addition of salt caused the *p*H to shift from 4.9 in the deionized solution to 5.5 in a solution in 0.15 *M* NaCl.

All concentrations were measured by dry weight determinations. An aliquot of the stock salt-free solution was weighed out in each case into a weighing bottle. The solution was evaporated in an oven at 105° and atmospheric pressure until it had attained constant weight. In all the cases checked, good agreement was found with concentrations measured by means of ultraviolet absorption at 279 mμ in a Beckman Model DU spectrophotometer. In some preliminary experiments, the concentration was measured by the micro-Kjeldahl technique and also by the method of Koch and McMeekin[30] using a factor of 6.31 for conversion to protein concentration.

The ultracentrifugal analyses were carried out at room temperature in a Spinco Model E analytical ultracentrifuge at a speed of 59,780 r.p.m.

All the light scattering data were calculated according to the method of least squares, and errors of estimate were calculated.

Results

Salt-free Measurements.—The results of light scattering measurements with one preparation of salt-free isoionic BSA have been reported previously.[21] In Fig. 2 are presented the light scattering data for two preparations of BSA, plotted as a function of the square root of protein concentration. Since the intercepts of these two sets of measurements differed by 2.5%, the data have been normalized with respect to the intercepts.[31] The results from the two sets of measurements fall on the same plot, which is very close to linear in $C_2^{1/2}$ over the 600-fold concentration range of 0.005 to 3% protein. (The set of points represented by filled circles is the same as the one previously reported.[21]) Least squaring of the data yields the following equation

$$H\frac{C_2}{\Delta\tau} = \left(H\frac{C_2}{\Delta\tau}\right)_{C_2=0}(1 - 2.08C_2^{1/2} - 1.94C_2) \quad (6)$$

The standard error of estimate in $H(C_2/\Delta\tau)$ was found to be 0.024×10^{-5}.

The results obtained with a fresh preparation of isoionic salt-free BMA are presented in the usual light scattering plot in Fig. 3. The data, which cover a concentration range of 0.005 to 3.5% protein, reveal a marked upward curvature in the dilute region. Just as with BSA, this curvature is most pronounced in the region below a concentration of 0.3%, which in this case is determined by a set of 36 points. When plotted against the square root of the concentration, as is shown in Fig. 4, the points fall very closely to a straight line over the 700-fold range of concentration covered by these measurements. The curve is well repre-

(25) M. Bier, Doctoral Dissertation, Fordham University, 1950.

(26) F. F. Nord, M. Bier and S. N. Timasheff, THIS JOURNAL, **73**, 289 (1951).

(27) W. L. Hughes, Jr., *ibid.*, **69**, 1836 (1947).

(28) W. L. Hughes, Jr., and H. M. Dintzis, unpublished experiments.

(29) G. E. Perlman and L. G. Longsworth, THIS JOURNAL, **70**, 2719 1948).

(30) F. C. Koch and T. L. McMeekin, *ibid.*, **46**, 2066 (1924).

(31) It should be pointed out that the second preparation was permitted to remain in solution in the deionized form for one week at refrigerator temperature prior to the measurements. Therefore, the slightly higher value of the apparent molecular weight is not unexpected.[7,8,10,11,32]

(32) P. Bro, S. J. Singer and J. M. Sturtevant, THIS JOURNAL, **77**, 4924 (1955).

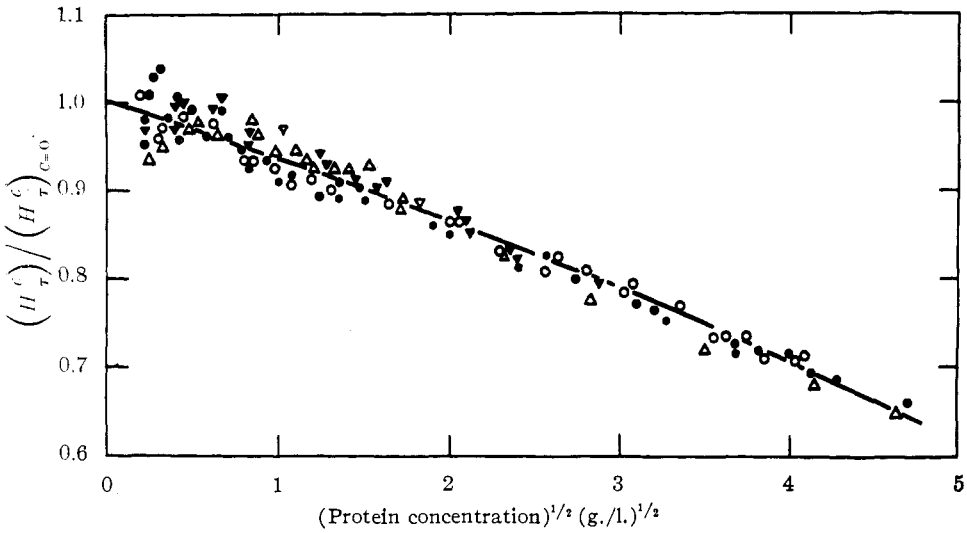

Fig. 2.—Normalized light scattering data for isoionic Armour bovine serum albumin plotted as function of the square root of protein concentration: ●, sample I in dist. H_2O (reported in ref. 17); ○, sample II in dist. H_2O; △, BSA in 1×10^{-5} *M* HCl; ▼, data of Dandliker.[45] Due to too great overlapping, *ca.* 20% of the points have been omitted.

sented by a quadratic in $C_2^{1/2}$ with the following coefficients determined by the method of least squares

$$H\frac{C_2}{\Delta\tau} = 1.51 \times 10^{-5}(1 - 2.70C_2^{1/2} + 5.52C_2) \quad (7)$$

The standard error of estimate in $H(C_2/\Delta\tau)$ was found to be 0.023×10^{-5}.

In the case of isoionic salt-free human serum mercaptalbumin (HMA), the light scattering data when plotted in the usual manner also displayed a marked upward curvature in the low concentration range, defined by 40 points as shown on Fig. 5.

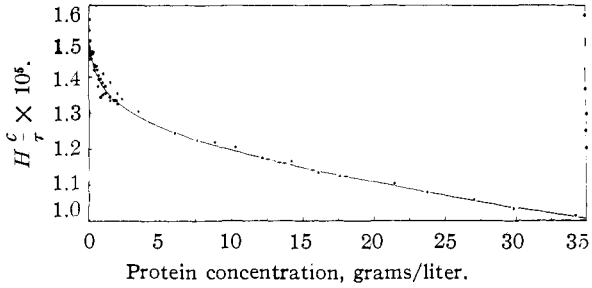

Fig. 3.—Light scattering data for isoionic bovine serum mercaptalbumin in dist. H_2O plotted in the usual manner.

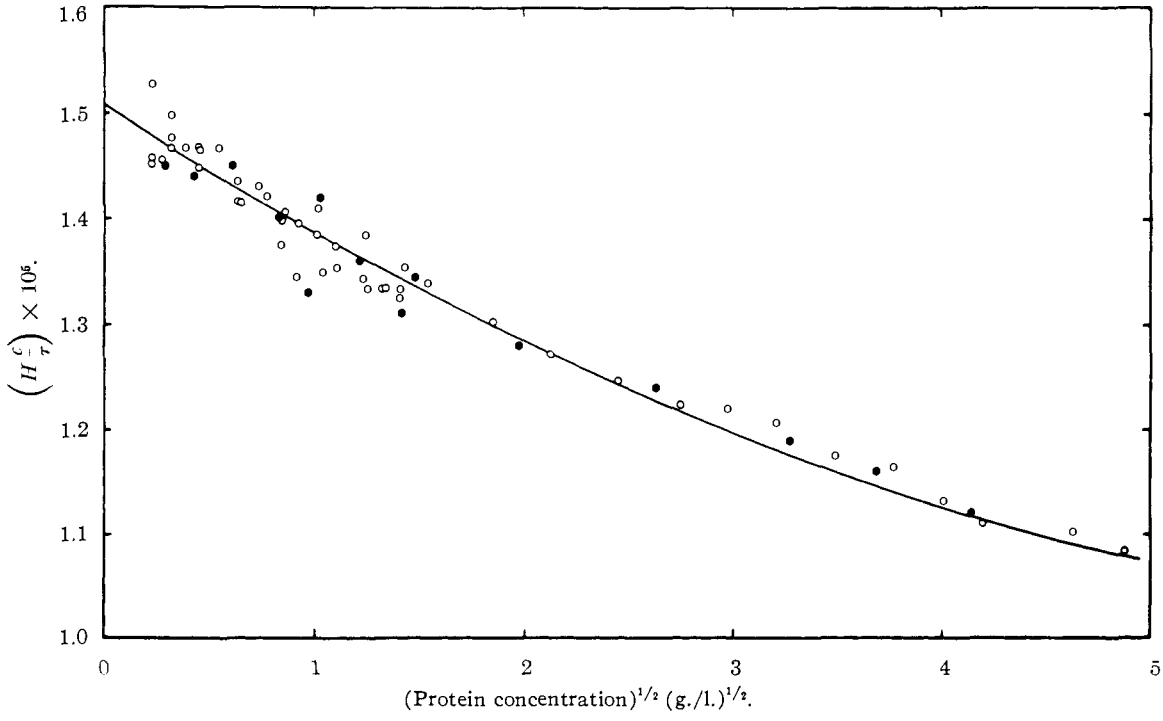

Fig. 4.—Light scattering data for isoionic bovine serum mercaptalbumin plotted as a function of the square root of protein concentration: ○, BMA in dist. H_2O; ●, BMA in 1×10^{-5} *M* HCl.

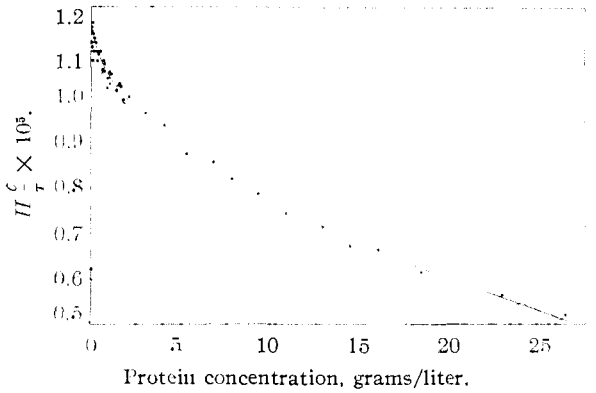

Fig. 5.—Light scattering data for isoionic human serum mercaptalbumin in dist. H_2O plotted in the usual manner.

A plot of the same data as a function of the square root of protein concentration again resulted in very nearly a straight line (Fig. 6). This is represented

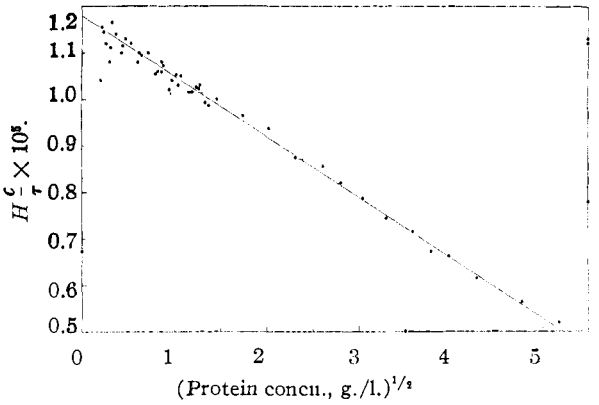

Fig. 6.—Data of Fig. 5 plotted as a function of the square root of protein concentration.

by the following equation obtained by least squaring of the data

$$H\frac{C_2}{\Delta\tau} = 1.17 \times 10^{-5}(1 - 3.00C_2^{1/2} - 2.97C_2) \quad (8)$$

The standard error of estimate in $H(C_2/\Delta\tau)$ was found to be 0.014×10^{-5}.

In all three proteins, the term in the first power of the concentration makes only a small contribution to $H(C_2/\Delta\tau)$.

Measurements in the Presence of Salt.—A series of measurements was carried out on each preparation of BSA and BMA in solutions of various concentrations of NaCl, ranging from 1×10^{-5} M to 1.5×10^{-1} M salt. The results obtained with one preparation of each protein are presented in Fig. 7 and 8.

As can be seen, a series of straight lines is obtained at higher salt concentrations when the light scattering data are plotted as a function of the first power of protein concentration. In both cases, the curve obtained in the salt-free solutions is shown by the dotted line. The slopes of these plots are negative at low salt concentrations, becoming increasingly positive with an increase in the concentration of electrolyte. This is in agreement with previous observations[8] and with light scattering theory,[16] since the progressive binding of chloride

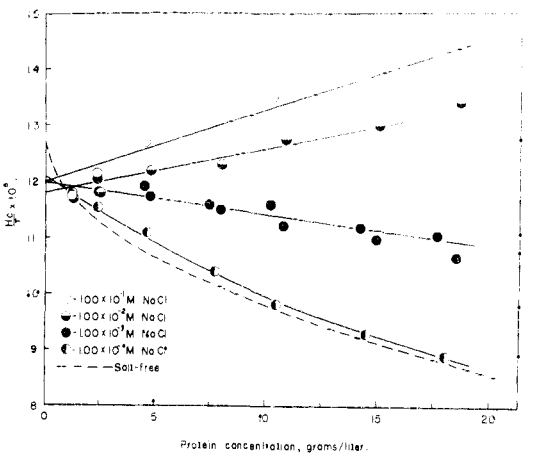

Fig. 7.—Light scattering data of isoionic Armour bovine serum albumin in various concentrations of NaCl.

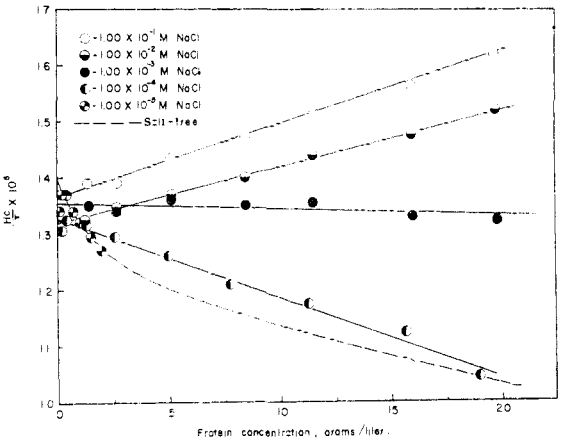

Fig. 8.—Light scattering data of isoionic bovine serum mercaptalbumin in various concentrations of NaCl.

ions, as the salt concentration rises, results in an increasing negative charge on the protein molecules. It is the swamping out of the attractive force due to fluctuations in charge and the electrostatic repulsion resulting from chloride binding which leads to the positive value of the second virial co-

TABLE I

LIGHT SCATTERING DATA FOR ISOIONIC SERUM ALBUMIN SOLUTIONS

NaCl concn. (M)	Armour BSA Mol. wt.	Armour BSA $\frac{2BM_2^2}{1000}$	Armour BSA β_{22}*	Bovine mercaptalbumin Mol. wt.	Bovine mercaptalbumin $\frac{2BM_2^2}{1000}$	Bovine mercaptalbumin β_{22}*
Salt-free	80,900	....	....	69,500[b]	...	...
Salt-free	82,500[a]	....	....	76,000	...	...
1×10^{-5}				72,500	...	...
3×10^{-5}	85,200[a]	−1240	−1255	....	...	...
1×10^{-4}	84,000[a]	−1200	−1215	75,500	−780	−795
3×10^{-4}	82,200	− 880	− 990	....	...	...
1×10^{-3}	83,300[a]	− 445	− 570	73,700	− 75	−200
3×10^{-3}	80,200	175	5	....	...	...
1×10^{-2}	86,600[a]	570	150	76,600	850	430
3×10^{-2}	80,100	725	305	....	...	...
1×10^{-1}	84,200[a]	975	635	75,800	940	600
1.5×10^{-1}	78,400	875	575	....	...	...
Acetate, pH 4.8, $\Gamma/2$ = 0.01			....	73,500		
Human mercaptalbumin						
Salt-free;				90,700		
Acetate, pH 5.3, $\Gamma/2$ = 0.01				89,900		

[a] Solution remained one week in deionized form at 4°.
[b] Different preparation of protein.

efficient of equation 2. It is interesting to note that in the case of BSA there is an incipient curvature in the data obtained in 1×10^{-4} M NaCl, while in the BMA the points obtained in 1×10^{-5} M NaCl fall exactly on the salt-free curve.

From the least squaring of the data in the presence of salt, values have been obtained for the apparent molecular weights and for the slopes of these solutions. From the slopes, the values of the functions $2BM_2^2/1000$ and β_{22}*, defined by Edsall, *et al.*,[8] have been calculated. These data are summarized in Table I, and are in good agreement with previously reported values.[8]

As a further check on the linearity of the instrument and the absence of experimental artifacts, a set of measurements in the presence of salt over the same protein concentration range and turbidity range as that in the salt-free case was carried out with each of the three proteins. The results obtained with BSA in 1×10^{-3} M NaCl over a concentration range of 0.005 to 1.8% protein have been reported previously[21] while data obtained over a similar concentration range with BMA in 1×10^{-3} M NaCl and in pH 4.82 acetate buffer of 0.01 ionic strength are presented in Fig. 9. In all three cases the plot of $H(C_2/\Delta\tau)$ *vs.* concentration is linear as expected from equation 2, indicating that the curvature obtained in the salt-free case is a real property of the protein solution.

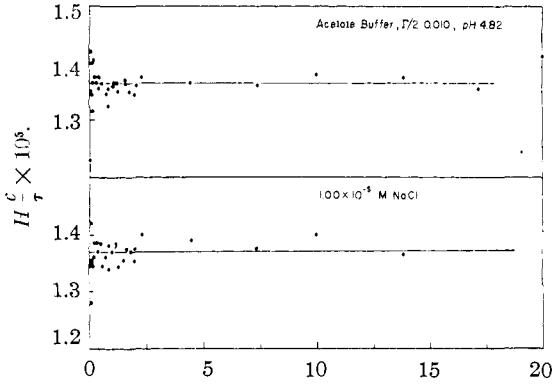

Fig. 9.—Light scattering data of isoionic bovine serum mercaptalbumin in pH 4.82, $\Gamma/2$ = 0.010 acetate buffer and in 1.0×10^{-3} M NaCl measured over the same concentration and turbidity range as the salt-free solutions.

From Table I it can be seen that the values of the molecular weights are higher than those normally assumed for the monomers of BSA and HMA (although in good agreement with other light scattering values[7,8]). This difference can be accounted for largely in terms of the presence of "dimer" in these preparations as shown by ultracentrifugal analysis (Fig. 10). Thus, while the BMA has a pattern typical for a monodisperse protein, the BSA contains *ca.* 5% of "dimer" and the HMA *ca.* 15%. The light scattering molecular weight being weight average, it is quite reasonable to conclude that this content of heavy material is sufficient to account for the high values of the molecular weights. The customary correction for depolarization has not been applied since it has been shown by Geiduschek[33] to be negligibly small for serum albumin.

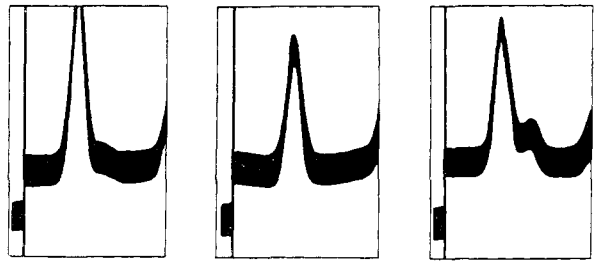

Fig. 10.—Ultracentrifugal patterns of the proteins used in this study: (sedimentation proceeds from left to right); time, 4380 sec.

Discussion

Charge Fluctuations.—As has been shown above, light scattering measurements on isoionic BSA, BMA and HMA in salt-free aqueous solutions resulted in plots which were nearly linear in the square root of protein concentration. Using equation 5, which applies to isoionic proteins with a mean charge of zero, it is possible to obtain apparent values of 3.56 ± 0.05, 3.75 ± 0.05 and 4.08 ± 0.04 protonic units for the fluctuating charges on BSA, BMA and HMA, respectively. The corresponding values for the apparent molecular weights are 77,000 and 78,900 for the two preparations of BSA, 66,300 for BMA and 85,800 for HMA.

The three serum albumins described in this investigation, however, are not neutral at their isoionic points, but carry a net negative charge the value of which increases with dilution of the protein solutions. This is due to the fact that these proteins have isoionic points close to pH 5.0 and must act as gegenions to the hydrogen ions present in solution.

It has been shown recently[34] that such a progressive ionization can make a substantial contribution to the excess chemical potential of the protein. This results in an extra positive term in C_2 in the expression for the derivative of the excess chemical potential of the protein with respect to concentration. The gradual increase of this term with dilution may give rise to a pseudo square-root dependence of $H(C_2/\Delta\tau)$ on concentration. The magnitude of this effect can be calculated[34] using expressions developed by Kirkwood.[35] Thus, if the activity coefficient of the protein is expressed as the product of its activity coefficient in the neutral state and the fraction of the protein in the neutral state at any given concentration, the derivative of the excess chemical potential with respect to protein concentration becomes[34]

$$\frac{1}{RT}\frac{\partial\mu_2^{(0)}}{\partial C_2} = -\frac{\pi N e^4 \langle Z^2\rangle_{av}^2}{M_2(DkT)^2\kappa(1+\kappa a)^2} + \frac{\bar{Z}^2}{M_2[H^+]}\frac{1}{1+\frac{K_w}{[H^+]^2}+m_2\frac{d\bar{Z}}{d[H^+]}} + \frac{7\pi N a^3}{6M_2} + 2B' \quad (9)$$

(33) E. P. Geiduschek, *J. Polymer Sci.*, **13**, 408 (1954).

(34) J. G. Kirkwood and S. N. Timasheff, *Arch. Biochem. Biophys.*, **65**, 50 (1956).

(35) J. G. Kirkwood in E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publ. Corp., New York, N. Y., 1943, p. 290-294.

where $\bar{Z}$ is the mean charge of the protein, and m_2 is its concentration in moles per liter.

When the second term on the right of equation 9 was evaluated[34] for BSA in distilled water it was found to assume a large value even at a protein concentration of 0.5 g./l., and it becomes larger than the intercept at 0.05 g./l.

In the data presented in Figs. 2, 4 and 6, no positive term of such magnitude is observed. This is not surprising in view of the fact that the above calculation was based on the assumption that no ions other than protein, hydrogen and hydroxyl are present in the system at any dilution and that extrapolation to zero concentration constitutes a true extrapolation to *p*H 7.0, a set of conditions not attainable in the experimental environment used. An examination of our conductivity data on the stock protein solution and the distilled water along with the data obtained in 1×10^{-5} *M* NaCl and the *p*H measurements on some of our dilute solutions suggest that our "salt-free" solutions are more correctly described as having an ionic strength of no more than 1×10^{-5} and a *p*H in the vicinity of 5.0. Indeed, at no dilution at which the *p*H was measured, was it found to depart significantly from 5. Measurements, carried out in 1×10^{-5} *M* HCl on BSA and BMA, shown in Fig. 2 and 4, confirm this conclusion since good agreement is obtained between the points obtained in 1×10^{-5} *M* HCl and in "salt-free" solution.

A calculation of the effect of progressive ionization in 1×10^{-5} *M* HCl showed that under that set of conditions this effect is greatly suppressed, never amounting to more than 3.5% of the total scattering. Furthermore, as shown in ref. 34 and in Fig. 11, this effect has the property of passing

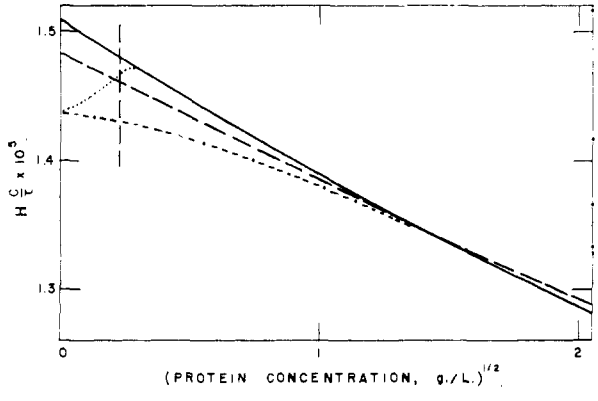

Fig. 11.—Corrections of light scattering data of bovine serum mercaptalbumin for the progressive ionization and added electrolyte effects. Solid line (—), least-square curve of the experimental points; dashed line (---), same data after correction for progressive ionization; long-dash line (– –), same data after correction of points both for progressive ionization and added electrolyte, according to equation 10; dotted line (. . .), curve obtained by adding calculated values of ionization term to the dashed line. (The vertical dashed line represents the lower limit of experimental data, 0.005% protein.)

through a maximum at a concentration of 0.1 g./l. for BSA, resulting in an extrapolation to a higher value for the molecular weight than would be obtained from extrapolation according to equation 5.

In the calculation of the fluctuating charge of the protein from light scattering data corrected for progressive ionization, the introduction of the 1×10^{-5} *M* HCl into the system must also be taken into consideration, since at low protein concentrations the contribution of the protein and of the added acid to ionic strength, and therefore to the Debye-Hückel κ, are of similar magnitude. Therefore, the simple square root relation (eq. 5) cannot be used, setting κ_0 equal to zero. Under such conditions, after binomial expansion of $(1 + \kappa a)^{-2}$ in equation 4, the light scattering equation becomes[36]

$$H\frac{C_2}{\Delta\tau} = \frac{1}{M_2}\left[1 - \frac{\pi^{1/2}N^{1/2}e^3}{2(DkT)^{3/2}M_2^{1/2}} \frac{\langle Z^2\rangle_{av}^{3/2}\, C_2^{1/2}}{\left(1 + \frac{2Mm_{HCl}}{\langle Z^2\rangle_{av}\, C_2}\right)^{1/2}} + \frac{C_2}{M_2}\left(\frac{\bar{Z}^2}{[H^+]} \frac{1}{1 + \frac{K_w}{[H^+]^2} + m_2\frac{d\bar{Z}}{d[H^+]}} + 2B\right) + 0\,(C_2^{3/2})\ldots\right] \quad (10)$$

where $2B$ represents the contribution of all other terms to the slope.

An examination of this equation reveals that the two effects act in opposite directions, correction for progressive ionization lowering the light scattering curve, while accounting for the contribution of the added electrolyte raises the points by a comparable amount.

In order to calculate the value of the fluctuating charge of a protein such as those described in this paper, it is necessary to reduce the experimental data to a form which may be treated according to equation 5. Correction of the data for this purpose involves first subtraction from the experimental points of the values of the progressive ionization term, then reduction of the resulting curve to a form independent of the added electrolyte. The second step is carried out by addition to the curve, corrected for ionization, of the difference between the $C_2^{1/2}$ terms of equations 5 and 10.

The magnitude of the additional terms in equation 10 has been calculated point by point for the light scattering data for BSA, BMA and HMA, shown in Fig. 2, 4 and 6. The calculation of the first term was carried out by the method of successive approximations varying the value of $\langle Z^2\rangle_{av}^{1/2}$ while the second term was evaluated from Tanford's titration data.[37] In these calculations it was assumed that the effect of progressive ionization is identical for these three closely related proteins. Reduction of the data in this manner, shown in Fig. 11 for BMA, has resulted in the following least-square expressions for the three proteins, which may be treated according to equation 5

$$\text{BSA: } H\frac{C_2}{\Delta\tau} = \text{Intercept}\,(1 - 2.11\,C_2^{1/2} - 1.41C_2)$$

$$\text{BMA: } H\frac{C_2}{\Delta\tau} = \text{Intercept}\,(1 - 2.22C_2^{1/2} + 2.99C_2) \quad (11)$$

$$\text{HMA: } H\frac{C_2}{\Delta\tau} = \text{Intercept}\,(1 - 2.79C_2^{1/2} - 4.47C_2)$$

(36) Since at the concentration of HCl used, the values of A_{12} and D in equation 2 are very small, this system is being treated as a two-component rather than a three-component one.

(37) C. Tanford, S. A. Swanson and W. S. Shore, THIS JOURNAL, 77, 6414 (1955).

The values of $\langle Z^2\rangle_{av}^{1/2}$ corresponding to these coefficients of $C_2^{1/2}$ are 3.58, 3.51 and 3.98 protonic units for BSA, BMA and HMA, respectively. That these values are not seriously different from those calculated with the use of equation 5 is demonstrated in Table II by direct comparison.

TABLE II

FLUCTUATING CHARGES AND DIPOLE MOMENTS OF SERUM ALBUMINS

Protein	$\langle Z^2\rangle_{av}^{1/2}$, protonic units (Light scattering) Uncor.[b]	Cor.[c]	(Titration)	$\langle \Delta\mu^2\rangle_{av}^{1/2}$, Debye units (Light scattering)[a]	(Dielectric increments) (ref. 40)
BSA	3.56	3 58	3.46	515	350
BMA	3.75	3.51	..	510	350
HMA	4.08	3.98	..	575	700
HMA/BMA	..	..	..	1.1	2.0

[a] Assuming a spherical molecule, with a radius of 30 Å. [b] From slopes of equations 6, 7 and 8. [c] From slopes of equations 11.

Values for the mean square fluctuating charges can also be calculated from the titration curves[38] and amino acid composition[20] of isoionic proteins. Thus, from the titration curve[37] of Armour BSA in salt-free solution, it is possible to calculate a value of 3.46 protonic units for $\langle Z^2\rangle_{av}^{1/2}$. This is obviously in very good agreement with the value of 3.58 obtained from light scattering.

Kirkwood and Shumaker[39] also have shown that fluctuations in charge and configuration of protons on a protein molecule will give rise to a fluctuating dipole moment which they have shown from amino acid composition to be of similar magnitude to that calculated from dielectric increment measurements. Therefore, it becomes of great interest to calculate the values of fluctuating dipole moments from light scattering data and to compare them with the values obtained from dielectric increment measurements. Neglecting electrostatic interaction and assuming the protein molecule to be a sphere with a uniform distribution of ionizable sites on its surface, the two quantities are related according to equation 12

$$\Delta\mu^2 = e^2 \langle Z^2\rangle_{av} b^2 \qquad (12)$$

where b is the radius of the spherical protein molecule.

A comparison of the dipole moments calculated from fluctuating charge values, assuming the three albumins to be spheres 30 Å. in radius, and from dielectric increment measurements is presented in Table II. It can be considered that the values of 510 and 575 Debye units calculated according to equation 12 for BMA and HMA, respectively, are in fair agreement with the experimental values of 350 and 700 Debye units.[40] Such agreement is satisfactory in view of the assumptions made in these calculations. It can be concluded, therefore, that the fluctuations are of sufficient magnitude to account for the dielectric increment measured for BMA. A comparison of the dipole moment values for HMA and BMA reveals that the ratio of these values for the two proteins (HMA/BMA) is equal to 2.0 when measured directly. The same ratio, when calculated from light scattering data, is equal to only 1.1. This suggests that the value of the fluctuating dipole may not be sufficiently large to account for the entire measured dipole moment of human serum mercaptalbumin but that for HMA the measured dipole moment represents the sum of a fluctuating dipole and a permanent dipole.

Alternate Interpretations.—The observed curvature in the light scattering measurements has been attributed to the contribution of the fluctuating charge terms in equations 5 and 10. Possibilities of other interpretations also have to be considered. Three alternatives to charge fluctuations were considered. These were: (1) formation of a surface layer of protein which reduced significantly the bulk concentration at high dilutions; (2) progressive ionization of the protein as its self-buffering power diminished with decreasing concentration; (3) a rapidly reversible concentration-dependent association of the protein. These will be discussed in order.

In order to determine whether there was a significant loss in bulk protein concentration at high dilutions due to the formation of a surface layer, the following experiment was designed. Solutions of deionized BMA were made up in distilled water and in 1×10^{-3} M NaCl. The concentrations of these solutions corresponded to the lowest concentration used in the light scattering measurements, namely, *ca.* 0.005% protein. Each solution was introduced into a quartz Beckman ultraviolet absorption cell (1 cm.) of identical size and shape as the light scattering cells. The ultraviolet absorption at 279 mμ was measured. The cell was inverted five times as is done in the mixing procedure in the light scattering experiments and the absorption was measured again. The solution was then transferred to another cell and the same operations were repeated. Altogether each solution was transferred in this manner four times, the absorption measurements being repeated each time. The results, shown in Table III, demonstrate that in the course of these transfers there was no decrease in the ultraviolet absorption of the protein solutions either in distilled water or in 1×10^{-3} M NaCl. Had any loss in protein out of solution occurred due to the formation of a monolayer on the glass or Teflon surfaces, a decrease in the ultraviolet absorption would have been observed after each transfer. Therefore, the observed curvature is not due to the

TABLE III

SURFACE LAYER DATA (BMA)

Operation	Optical density at 279 mμ Salt free 1	Salt free 2[a]	Salt free 3[a]	1×10^{-3} M NaCl[a]
Original	0.122	0.126	0.123	0.123
Mixing	.122	.126	.123	.123
Transfer	.123	...	...	...
Mixing	.123	.125	.123	.123
Transfer	.125	...	...	...
Mixing	.126	.124	.124	.124
Transfer	.124	...	...	...
Mixing	.123	.125	.124	.124

[a] Each transfer was followed immediately by mixing. No readings were taken before inversion of the cell.

(38) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publ. Corp., New York, N. Y., 1943, p. 402.

(39) J. G. Kirkwood and J. B. Shumaker, *Proc. Natl. Acad. Sci., U. S.*, **38**, 855 (1953).

(40) J. L. Oncley and H. M. Dintzis, unpublished results.

loss of protein *via* the formation of a monolayer on the glass or Teflon surfaces of the cell and the cell cover. Furthermore, recent data of Bull[41] indicate that the loss of protein out of solution due to adsorption on the glass surface could be no greater than 1% of the total protein at any of the concentrations used in this study.

The possibility that the upward curvature in the salt-free solution was due entirely to the progressive ionization of the protein as its self-buffering power diminished with decreasing concentration has been discussed above and has been properly accounted for in the analysis of the data. Furthermore, a similar study carried out with conalbumin,[42] which has an isoionic point very close to *p*H 7.0, and therefore a negligibly small contribution from progressive ionization,[34] has also revealed the presence of a marked curvature in the salt-free measurements, which can be interpreted in terms of a reasonable value of the fluctuating charge.

The theoretical possibility that these results should be attributed to a concentration dependent association arising from specific short range forces may be rejected, since such an effect should always result in light scattering measurements which are linear in the protein concentration at high dilutions. This is not the case in the present investigation. Furthermore, the small value of the first power of C_2 term in equations 5, 6, 7, 8 and 11 points to the existence of a close balance between the short-range attractive and repulsive forces, the latter making a positive co-volume contribution to B which is almost equal to the negative contribution of the former. In order to explain these results on the basis of a concentration dependent aggregation one would have to fit the data with a series of appropriately fitting equilibrium constants and invoke a mechanism for a rapidly adjusted equilibrium which is very strongly dependent on ionic strength. In the case of charge fluctuations, it is possible to explain the light scattering results using a single parameter which is quantitatively in agreement with other types of measurements.

From these considerations it can be concluded that of the possible explanations which have been offered for the observed curvature, all can be rejected with the exception of the contribution of charge fluctuations on the protein molecule as proposed by Kirkwood and Shumaker. This yields a reasonable interpretation of the light scattering measurements and, conversely, these experiments demonstrate the validity of the method of light scattering for the determination of the magnitude of $<Z^2>_{av}^{1/2}$ on an isoionic protein molecule.

Intercepts.—An examination of the data for all three proteins reveals that the intercept of the deionized protein, when the data are extrapolated according to equation 5, is always higher (by 3–7%) than that in the presence of salt, except for the run on BMA in 1×10^{-5} M NaCl.

Since the correct molecular weight in the "salt-free" case is given by the intercept of the curve corrected for ionization, subtraction of that term and subsequent extrapolation to zero concentration gives the following values for the molecular weights of the three albumins: 82,500 and 80,900 for the two preparations of BSA, 69,600 for BMA and 90,700 for HMA. These are in good agreement with the intercepts obtained in the presence of salt summarized in Table I. The molecular weight of BMA, furthermore, is in good agreement with the value obtained by Dintzis from mercury titration.[43]

As pointed out above, in the case of light scattering measurements in the presence of a third component, theory[16] predicts that the intercept will not be equal to the reciprocal of the molecular weight, but rather to the reciprocal of the sum of the molecular weight and a term dependent on the thermodynamic interaction constant of the third component with the protein and the refractive increment of the two solutes, as shown in equation 2. The term D of equation 2 will have a positive value in the case of salting in and a negative value for salting out. For BSA, using data of Scatchard, *et al.*,[44] on the binding of chloride ions to albumin, it is possible to calculate for the case of 0.15 M NaCl (the highest salt concentration used in these studies) a value of 7×10^{-3} for the term D. This means that the reciprocal of the intercept of the $H(C_2/\Delta\tau)$ *vs.* C_2 plot will be 0.7% greater than the true molecular weight of the protein. From this calculation, it can be concluded that for serum albumin the contribution of the interaction term is no greater than the experimental error in the determination of $1/M_2$ and, therefore, can be neglected.

Dandliker,[45] who has also reported on the difference in intercepts between measurements in salt-free BSA and those in the presence of salt, interpreted this difference in terms of the presence in his sample of BSA of a contaminant which aggregates in water and is removed from salt-free solutions during the clearing of the solution for light scattering. In the present study all solutions were filtered in the salt-free state, the salt being added only in the light scattering cell, eliminating this explanation for our solutions. Furthermore, in a specially designed experiment, stock solutions of deionized BSA were made up in distilled water and in 0.15 M NaCl prior to clarification. Light scattering measurements were carried out on a series of protein concentrations both in distilled water and in 0.15 M NaCl.[46] The usual curved plot was obtained in the salt-free case while the run in the presence of salt gave a straight line plot which extrapolated to a lower value of $H(C_2/\Delta\tau)$ than the deionized protein. When sufficient concentrated NaCl was added to each of the cells containing salt-free protein to bring the NaCl concentration up to 0.15 M in each cell, the value of the turbidity shifted immediately in every cell to such a value that the points fell on the straight line obtained in the 0.15 M NaCl run, as shown in Fig. 12. This is further evidence that, in the present case, the apparent difference in intercepts is due not to

(41) H. B. Bull, *Biochim. Biophys. Acta*, **19**, 464 (1956).

(42) S. N. Timasheff and I. Tinoco, *Arch. Biochem. Biophys.*, in press.

(43) H. M. Dintzis, unpublished results.

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

(45) W. B. Dandliker, *ibid.*, **76**, 6036 (1954).

(46) These experiments were carried out in a series of 30-ml. cells using volumetric pipets for dilution, one dilution of protein being studied in each cell.

an impurity in the protein, but to a rapidly reversible phenomenon, namely, progressive ionization.

Furthermore, in his publication, Dandliker has been unable to conclude whether his data approached proportionality to the square root or the first power of protein concentration at infinite dilution. When his data were normalized to the same intercept as our measurements on BSA, however, it was found that his points agreed well with the results obtained in the present studies, as shown in Fig. 2. His data, however, cover too small a concentration range and his number of points is not sufficient to determine the shape of the curve.

Conclusions

The results presented above may be considered as a reasonable experimental confirmation of the fluctuating charge theory of Kirkwood and Shumaker.[20,39] The values of the fluctuating charges measured by light scattering are in good agreement with titration data on BSA and can account to a large extent for the dielectric increments measured on BMA and HMA. This experimental verification of the theory of charge fluctuations further leads to the consequence that in studies on protein interactions the attractive force arising from fluctuations in charge and proton configuration on protein molecules has to be considered and evaluated on an equal basis with other types of force. Thus, Kirkwood[47] recently has shown that this type of mechanism can account for the bell-shaped *p*H *vs.* activity curves obtained with many hydrolytic enzymes, and it has been shown that a good fit for experimental data may be obtained on the basis of this theory.[48] This type of attractive force should also be kept in mind when considering other types of both specific and non-specific protein interactions and in interpreting mechanisms of biological reactions.[49]

It should be pointed out further that, in light scattering measurements on protein systems, it is necessary to exert great care in the selection of the conditions for the measurement and in the interpretation of the results. In salt-free isoionic solutions, it becomes necessary to extend the measurements to the region of high dilution and correct properly for the ionization term, since otherwise the curvature to the contribution of the $C_2^{1/2}$ term may be missed with the result that the intercept will be drawn at a wrong value of the molecular weight.[50] In the presence of salt or buffer,[51] however, the intercept cannot be interpreted unequivocally as being the reciprocal of the molecular weight. It is true that in many instances the contribution of the thermodynamic interaction term will be very small and can be essentially neglected as is the case with isoionic BSA and NaCl. In order to be certain that the omission of this term is valid, however, it is necessary to have information on the magnitude of A_{12} of equation 2. This can be obtained from other types of thermodynamic measurements.

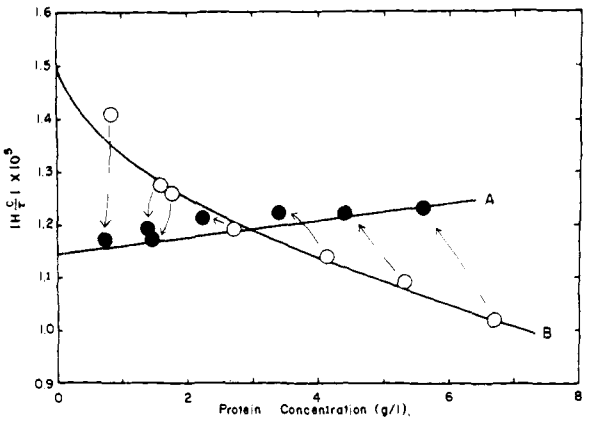

Fig. 12.—Light scattering data of Armour bovine serum albumin: A, least-squared curve of data obtained in independent run in 0.15 *M* NaCl; B, least-squared curve of data obtained in dist. H_2O; O, points measured in dist. H_2O; ●, values assumed by open circles immediately after addition of NaCl to make salt concentration 0.15 *M*.

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(47) J. G. Kirkwood, *Disc. Faraday Soc.*, **20**, 78 (1955).

(48) J. G. Kirkwood and I. Tinoco, unpublished data.

(49) J. G. Kirkwood, in W. D. McElroy and B. Glass, "The Mechanism of Enzyme Action," Johns Hopkins, Baltimore, Md., 1954, p. 4.

(50) Dandliker[45] found in the salt-free case a molecular weight which was 3% higher if obtained from a straight line plot in C_2 than if the same quantity had been determined from a plot in $C_2^{1/2}$.

(51) In the case of a buffer, the solution becomes a more than three component system. In such a case, the light scattering equation assumes a much more complicated form than the three-component system (equation 2).