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Physical Chemistry of Protein Solutions. VIII. The Effect of Temperature on the Light Scattering of Serum Albumin Solutions

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The light scattering of bovine serum mercaptalbumin solutions was measured at 7, 25 and 44° at *p*H's from 4 to 7.5 in 0.003 and 0.1 *m* NaCl solutions. The osmotic second virial coefficient and the corresponding enthalpy contributions are determined from the measurements and the Donnan and electrostatic interaction terms are calculated from theory. The non-electrostatic residuals of both enthalpy and entropy may be independent of the *p*H and ionic strength in this range.

The temperature coefficients of the osmotic pressure and the light scattering of protein solutions are of special interest because of the insight they might offer into the deviations from the ideal Donnan term when the ionic charge is large, noted by Scatchard, Batchelder and Brown[2] and confirmed by Edsall, Edelhoch, Lontie and Morrison[3] and by Scatchard, Gee and Weeks.[4] The facts that the deviation is negative and proportional to the Donnan term, and therefore inversely proportional to the salt concentration indicate that the main deviation is electrostatic. Then the enthalpy term should be positive and about minus one-third the free energy term.[5] The remainder of the deviation which causes the minimum second virial coefficient to be different from zero is also of interest. For this reason A. Brown and Jeanette Weeks of this Laboratory spent many months between 1945 and 1951 trying to measure the temperature coefficient of the osmotic pressure both with the thimble osmometer[2] and with the Hepp osmometer.[4] Their results for isoionic albumin have been published briefly,[4] but precise measurements at high charge are so uncertain that their results may be summarized by the statement that the enthalpy contribution is fixed between limits not much more positive than the total virial coefficient and not very negative.

We therefore attempted the study by means of light scattering, which has the advantages that the ratio of the second virial coefficient term to the first is twice as great as for osmotic pressure and that it is experimentally much more convenient to make measurements at different temperatures on identically the same solution. The results are not very conclusive. They do show the difficulties of the problem, and some of our experimental techniques and our reasoning about such measurements should help other workers.

Dilution Series

To determine the molecular weight, the second virial coefficient and, perhaps, higher virial coefficients from osmotic pressure or light scattering measurements, we make a series of measurements at different protein concentrations w_P, plot P/w_P or w_P/τ against w_P and determine the intercept, the slope and, perhaps, higher derivatives at zero concentration. In polycomponent systems there are many paths of dilution and it is important to choose the right one or one very near it. For non-electrolytes the problem is not difficult. We should dilute at constant activity of every component but the one whose concentration is being varied. We can do this in osmotic pressure measurements by keeping constant the temperature, and the composition and pressure of the solution which contains no non-diffusible component, the dialysate.[6] Since

(1) Polytechnic Institute of Brooklyn. The experimental work reported here was completed in 1953.

(2) G. Scatchard, A. C. Batchelder and A. Brown, THIS JOURNAL, **68**, 2320 (1946).

(3) J. T. Edsall, H. Edelhoch, R. Lontie and F. R. Morrison, *ibid.*, **72**, 4641 (1950).

(4) G. Scatchard, A. Gee and J. Weeks, *J. Phys. Chem.*, **58**, 783 (1954).

(5) G. Scatchard and L. F. Epstein, *Chem. Revs.*, **30**, 211 (1942).

(6) This differs from our previous treatment[2,4] in which the pressure on the solution containing the non-diffusible solute is kept constant.

light scattering is measured at constant pressure, it is impossible to keep the activity of each diffusible species constant during dilution. The best possible path is to allow the activity of the solvent to vary the small amount necessary to keep constant the activity of each solute component except that which is being varied largely. In either osmotic pressure or light scattering we may approximate the desired path by diluting with the dialysate.

With electrolytes the problem is very different because the activities of the neutral components, but not those of the ionic species, are the same in the dialysate as in the solution, and any procedure must be a compromise. As an example let us take the solutions of serum albumin in sodium chloride plus hydrochloric acid or sodium hydroxide, in which we wish to keep the composition of the albumin constant during dilution except for dissociation of possible dimers or higher polymers. To maintain constant the bound hydrogen or hydroxyl ion we should dilute with a solution of the same pH, though the concentration of hydrogen or hydroxyl ion is usually so small that dilution with any pH nearer 7 is satisfactory if there is no buffer in the diluent. The albumin also binds chloride ion, and we should dilute with a solution of the same pCl. However, the ratio of the activity coefficients of two protein species with different valences will depend upon the ionic strength. The effective contribution of protein ions to the ionic strength is usually taken by experimentalists as equal to the equivalent concentration. If this is to be kept constant, the diluent should have the same concentration of sodium ion or of unbound chloride ion, whichever is the larger. The best compromise is probably to dilute with the dialysate, but the compromise will be good only when the difference between the sodium ion concentration and the unbound chloride ion concentration is very small relative to their sum. In this range a fair approximation may be obtained by defining the protein component as $Na_{(\nu-h)/2}[H_hPCl_\nu]Cl_{(h-\nu)/2}$ and diluting at constant concentration of sodium chloride as defined by this definition of the protein component. In the formula $[H_hPCl_\nu]$ represents the average composition of the protein species, P is the isoionic protein, ν the number of bound chloride ions, and h the number of bound protons, which may be positive or negative. In general the number of ions of any species outside the square bracket, ν_i', is

$$\nu_i' = -z_2z_im_i/\Sigma_jz_j^2m_j$$

in which z_2 is the valence of the protein species, and the sum is taken over all small ions.[7,8]

The problem is much less serious if the intercept is known so that each measurement gives an average slope. Fortunately this is the case for enthalpy because the intercept is zero unless the molecular weight varies with the temperature.

Light Scattering Equations.—We start with equation 3.3 of Stockmayer[9]

$$\Delta\tau/H'V^0 = \frac{[\psi_2 - (a_{23}/a_{33})\psi_3]^2}{a_{22} - a_{23}^2/a_{33}} + \psi_3^2\left(\frac{1}{a_{33}} - \frac{1}{a_{33}^0}\right) \quad (1)$$

in which $\Delta\tau = (\tau - \tau_0)$ is the turbidity of the solution minus that of the solution with $m_2^0 = 0$ and $m_3^0 = m_3$

$$H' = 32\pi^3n^2kT/3\lambda^4$$

n is the index of refraction of the solution, λ is the wave length of the light scattered, V^0 is the volume of solution containing unit weight of solvent, $\psi_2 = (\partial n/\partial m_2)_{T,P,m}$, $a_{ij} = RT\,(\partial \ln a_i/\mathrm{d}m_j)_{T,P,m} = RT\,(\partial \ln a_j/\partial m_i)_{T,P,m}$. If we follow our usual custom and define component 2 as

$$Na^{+}{}_{-z_2/2}[H_hPX_\nu]\,X_{z_2\text{ to }x/2}$$

and component 3 as Na^+X^-

$$\ln a_2 = \frac{1}{p}\ln m_2 - \frac{z_2}{2}\ln(m_3 - z_2m_2/2) + \frac{z_2}{2}\ln(m_3 + z_2m_2/2) + \beta_2 \quad (2)$$

$$\ln a_3 = \ln(m_3 - z_2m_2/2) + \ln(m_3 + z_2m_2/)2 + \beta_3 \quad (3)$$

in which p is the average degree of polymerization, which we will consider independent of m_2 and m_3, m_3 is the number of moles of 3 and m_2 is the number of units of P per kilogram of solvent, h is the number of protons and ν the number of anions bound to one unit of P, and $z_2 = h - \nu$.

$$a_{22}/RT = \frac{1}{pm_2} + \frac{z_2^2}{2m_3(1-\eta^2)} + \beta_{22} \quad (4)$$

$$a_{23}/RT = -\frac{z_2^2m_2}{2m_3^2(1-\eta^2)} + \beta_{23} \quad (5)$$

$$a_{33}/RT = \frac{2}{m_3(1-\eta^2)} + \beta_{33} \quad (6)$$

if

$$\eta = z_2m_2/2m_3 \quad (7)$$

If we discard terms in m_2^2 or higher powers of m_2, we may invert equation 1 to give

$$\frac{H'V^0}{RT\Delta\tau} = \frac{\frac{1}{p} + \left(\frac{z_2^2}{2m_3} + \beta_{22} - \frac{\beta_{23}^2m_3}{2} + \beta_{33}m_3\right)m_2}{\psi_2^2\left[1 + \frac{(z_2^2m_2 - 2\beta_{23}m_3^2)\psi_3}{2m_3(2+\beta_{33})\psi_2}\right]^2}m_2 \quad (8)$$

$$\psi_2 = \psi_P + h\psi_H + \frac{\nu-h}{2}\psi_{Na} + \frac{\nu+h}{2}\psi_X = \psi_P + h\psi_{HX} + \frac{\nu-h}{2}\psi_3 = 12.9 + 0.0032h + 0.0048\nu \quad (9)$$

Since we cannot distinguish operationally between the effects of ν and β_{23}, we assume that β_{23} is zero and attribute all interaction between protein and salt to ion-binding. Changing to weight concentration gives

$$m_2 = w_P/\bar{V}_P \qquad \psi_P = \bar{V}_P\left(\frac{\partial n}{\partial w_P}\right) = \bar{V}_P\phi_P \quad \text{and}$$

$$\frac{H'V^0\phi_P^2w_P}{RT\Delta\tau} = \frac{\frac{1}{p\bar{V}_P} + \left(\frac{z_2^2}{2m_3} + \beta_{22}\right)w_P/\bar{V}_P}{\left(1 + h\psi_{HX}/\bar{V}_P\phi_P + \frac{\nu-h}{2}\psi_3/\bar{V}_P\phi_P + \frac{z_2^2w_P\psi_3}{2m_3(2+\beta_{33}m_3)\bar{V}_P^2\phi_P}\right)^2} \quad (10)$$

At the limit of zero concentration, however, the only effect is to change the sign of the term proportional to the compressibility, $-(RT/V_m^0)\,\mathrm{d}(\ln V_m^0)/\mathrm{d}P$, which is usually negligible. So our final equations are unchanged for non-electrolytes or for electrolytes.

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

(8) The dilution for light scattering has been discussed by K. J. Mysels, *J. Phys. Chem.*, **58**, 303 (1954). A similar and more serious problem for equilibrium ultracentrifugation has been discussed by J. S. Johnson, K. A. Kraus and G. Scatchard, *ibid.*, **58**, 1034 (1954), by J. S. Johnson, G. Scatchard and K. A. Kraus, *ibid.*, **63**, 787 (1959), and by J. W. Williams, K. E. Van Holde, R. L. Baldwin and H. Fujita, *Chem. Rev.*, **58**, 715 (1958).

(9) W. H. Stockmayer, *J. Chem. Phys.*, **18**, 58 (1950).

In dilute sodium chloride solutions the denominator may be taken as unity. The first term of the numerator is entirely entropy unless the polymerization changes with temperature, the second is entirely entropy unless the binding changes with temperature, but the third term may also contain contributions from the enthalpy.

Experimental

Apparatus.—The light scattering instrument has been described by Oster[10] and was purchased from the American Instrument Company. It differs from other commercial instruments in that the effects of large variations in light intensity are compensated electrically rather than with neutral filters. The output of the photomultiplier tube is coupled to the grid of an electrometer tube. The grid voltage is held in the desired range despite large variations in photomultiplier current by grounding the grid through the appropriate choice of one of a group of resistors. The electrometer tube itself is one arm of a Wheatstone bridge. A microammeter in the cross-arm of the bridge indicates the unbalance of the circuit due to phototube current. Modifications were made in the electrical system to increase precision and in the scattering chamber for temperature control. In the electrical system the grid circuit resistors in decade steps (0.1–1000 Meg.), were replaced by a set of resistors in the sequence 1:2:5:—, 0.5 to 500 Meg., to permit all measurements to be made on the upper portion of the meter without varying a potentiometer which is in parallel with the meter. This avoided discrepancies as high as 3% due to working on different portions of the characteristic curve of the electrometer tube at different potentiometer settings. In addition, the condenser in parallel with the grid circuit resistors was replaced by a switch with several condensers (7.5, 2, 1 mfd.) and a direct connection to ground in order to control the time constant of the unit.

The microammeter and grid circuit resistors were calibrated *in situ*. To compensate for the non-linear response of the electrometer tube, the meter supplied with the instrument had a non-linear scale, an average of the responses of a dozen tubes. The calibration for the particular tube in the circuit was made by substituting a constant voltage for the photomultiplier tube output, adjusting the voltage for full scale meter deflection and then reducing it by known amounts. The grid circuit resistors were calibrated both by comparing meter readings on different resistors at constant light intensity, and by varying the light intensity with neutral filters of known density. Both meter and resistor calibrations were remade with the instrument in the cold room, checked again at ordinary temperatures, and rechecked every few months. The meter behavior was unaffected by time or temperature, the resistors changed slightly with each.

A thermostat was constructed for scattering measurements above room temperature. A glass cylinder ($2^3/_8$'' diameter, $3\ ^5/_8$'' high) filled with water fitted snugly into a shallow brass base mounted on the axis of the scattering chamber. The cylinder was made from precision bore tubing, a base fused on, and the outside surface ground and polished. Two flat strips, 5/16'' wide and running the length of the cylinder, were ground on the outside surface to minimize reflections where the main beam entered and left the cylinder. A brass lid, from which a heat exchanger and stirrer were suspended, rested on the top of the cylinder on a rubber gasket. It was held in place by pressure at three points, from bars mounted on two 1/4'' rods attached to wings on the cylinder base. The two rods were placed behind the cylinder, leaving unobstructed the region from 0 to 145° through which the photomultiplier housing and slit system moved. The base, rods, under side of the lid, and outside of the glass cylinder, except for an inch wide strip, were painted a flat black.

The heat exchanger was a thin monel metal tube, 3/8'' diameter, 3 3/8'' long, around which 5/65'' o.d. copper tubing was non-inductively wound. Warm water from a constant temperature bath could be circulated through the copper coil, the tubing leading from the bath entered the light scattering chamber through the back wall and connected to the heat exchanger through flair fittings in the thermostat lid. The monel tube also housed the stirrer. Above the lid the stirrer shaft ended in the groove member of a tongue and groove clutch which disengaged as the cover of the scattering chamber opened. In the center of the thermostat lid was a rectangular hole, 1 13/16'' × 12/16'' for the cells. A narrow shelf on which the cell holders rested, extended into it on the underside.

The scattering cells were rectangular, 1 × 3 cm. in cross section, 8.5 cm. high, with 2 mm. walls, and held about 17 ml. of the solution.[3] They had Lucite covers that shielded but did not tightly seal the tops. Brass collars, machined to fit the rectangular opening in the thermostat lid with only a few thousandths of an inch clearance were waxed onto the cells. The back wall, base and collar of each cell were blackened.

The beam collimating system provided with the instrument had to be modified to allow space for the thermostat. Two slits, held 1/32'' apart in a blackened cylindrical holder (formerly a holder for photomultiplier slits) defined the beam. The dimensions of the entrance slit were 0.12'' × 0.22'', of the exit slit 0.16'' × 0.31''. The receiving slits, defining the region seen by the photomultiplier tube, were both 0.275'' × 0.062'' and were mounted as far apart as possible (0.57'') in filter holders which fitted the slots in front of the photomultiplier tube. The requirements on the dimensions were that the slit opening should fall within the area illuminated by the main beam and that corners of the cell should not be seen by the photomultiplier tube in the 45 or 135° position. The latter was the more stringent condition.

The thermostat cylinder was positioned so that the main beam, after traversing the cylinder, was centered on the position of the beam in the absence of the cylinder. The lid was positioned so that the long dimension of the rectangular cells was centered on and parallel to the beam. Small deviations in the position of the cells had negligible effect on the transmitted beam but may have appreciably influenced the amount of stray light due to reflections. The angular distribution of the fluorescence from fluorescein was measured to check the geometry. The envelope was not a smooth function of angle until the back walls of the cells near the heat-exchanger were made opaque. The minimum of the envelope was used to locate the 90° position of the photomultiplier tube. Correcting the fluorescent intensity for the volume seen then gave the theoretical straight line between 45 and 135°.

The transmitted light, reduced by an opal glass diffuser and a neutral filter of density 4, was used as a working standard. The two filters automatically moved into the path of the beam when the phototube viewed 0°. They reduced the intensity of the incident light so that the transmitted beam produced about twice the meter deflection of a water solution viewed at 90°. For calibration, to relate the measurements to absolute turbidities, portions of several bovine albumin solutions were measured both in this instrument and in Professor W. Stockmayer's instrument.[11] In the latter instrument, the turbidities were determined by comparison with the scattering from a purified sample of benzene. Carr and Zimm's value[12] for the intensity of scattering of unpolarized light, $I_u^{4358} = 48.4 \times 10^{-6}$ was used. The stray light and over-all refractive index correction were estimated from measurements of solutions of water, toluene and bovine albumin whose turbidities were known, from the relation

$$\frac{[M^{(90)}/M^{(0)}]_{BA} - S}{[M^{(90)}/M^{(0)}]_{T} - S} R_{W/T} = \tau_{BA}/\tau_{T}$$

where the $M(0)$s are the observed meter readings, S is the contribution to the observed reading due to stray light and is assumed independent of the scatter, the τ's are the turbidities of the scatterers, and $R_{W/T}$ the refractive index correction for comparing solutions of refractive indices of water and toluene. The stray light was found to be about twice the scattering from water, the refractive index correction proportional to $(n_w/n_t)^3$ where n's are the refractive indices.

For measurements above room temperature, the thermostat was filled with water a few degrees above the desired temperature immediately before use. Water, also a little above the desired temperature, was circulated through the copper coil (about 80 ml. per minute of 48° water held the thermostat at 44°). The system was allowed to come to constant temperature with the stirrer running about 30 minutes. Thereafter the stirrer was not used, the bath temperature was controlled within small limits by the rate of water cir-

(10) G. Oster, *Anal. Chem.*, **25**, 1165 (1953).

(11) A. R. Schultz, THIS JOURNAL, **76**, 3422 (1954).

(12) C. I. Carr and B. H. Zimm, *J. Chem. Phys.*, **18**, 1616 (1950).

culation. The solutions to be measured were prewarmed to a few degrees above bath temperature and then allowed to equilibrate in the thermostat for ten minutes before readings were made. The temperature of the bath remained constant to ± 0.2° over several hours. Low temperature measurements, 7°, were made by moving the entire instrument to a 5° cold room.

The bovine mercaptalbumin was kindly supplied by H. M. Dintzis. It was Armour Fraction V, further purified by conversion to the mercury dimer, then three recrystallizations and finally passage over an ion-exchange column to remove the mercury, other salts and lipids before use.[13] After the resin treatment it was stored at −30° either as about 8% solution, isoionic and salt-free, or as the dry protein after freeze-drying.

The solutions were prepared at room temperature from reagent grade NaCl, CO_2-free NaOH, constant boiling HCl, and conductivity water. Non-isoionic stock solutions were made either by dissolving the protein in solutions of the desired *p*H and salt concentration or by dissolving it in sodium chloride solutions and adding 0.002 *M* NaOH until the desired *p*H was reached. Dilutions were made with the appropriate salt or mixed salt and acid or alkali solution. All solutions were stored at −1°. Forced filtration through ultra-fine sintered glass filters under N_2 was used to render the solutions dust free. The filters, after long washing, yielded dust-free water and NaCl solutions. The albumin solutions came through clean except for some large particles which settled out on standing overnight.

Protein concentrations were determined on the filtered solutions after the light scattering measurements. For dilute solutions, a Beckman spectrophotometer was used, the extinction coefficient $[E]^{1\%}_{1\,cm}$. 280 mm. was found to be 6.43 by dry weight (100° at atmospheric pressure). For sufficiently concentrated solutions, dry weights were determined.

The light scattering measurements were made at 45 and 135° as well as 90 and 0°. The measurement of the dissymmetry was used to check for the presence of dust and for deterioration of the solutions. Results from solutions with dissymmetries above 1.08 were discarded. Measurements were obtained at 7, 25 and 44°. For the two higher temperatures, each solution was removed from the cold room only 30–40 minutes per run. The measurements were made on three consecutive days when possible. Attempts to make them at 25 and 44° on the same day sometimes resulted in increased dissymmetries. Keeping the solutions more than a few days also resulted in increased scattering and dissymmetries.

H' for albumin solutions at the several temperatures and salt concentrations were calculated from data in the I.C.T. and from Perlman and Longsworth's values for $\partial n/\partial c$[14] (Table I). The 25° values differ slightly from that calculated by Edsall and collaborators[3] who neglected the slight dependence of $\partial n/\partial c$ on salt concentration evident in Perlman and Longsworth's results. The refractive index increment for this particular preparation of albumin was measured in buffer solution by Dr. Robert Maybury. The value obtained is consistent with Longsworth and Perlman's values. The Cabannes factor for depolarization of albumin was used as 1.04_3 from the results of Edsall and collaborators.[3] It was apparent visually that the concentrated albumin solutions were slightly yellow in color, presumably traces of hematin not removed in purification. An absorption coefficient was determined from 0° measurements at several concentrations, assuming Beer's law, and the ratios $M(90)/M(0)$ were corrected for the effect of path-length difference in the rectangular cells.

Results and Discussion

The results which we have accepted are presented in Table I. A measurement was discarded if the dissymmetry was greater than 1.08. Of the seventy-six measurements accepted, the average dissymmetry was less than 1.02. The first column gives the salt concentration, the second gives the approximate *p*H, the third gives the protein concentration in grams per kilogram of water w_P, and the last three give $Hw_P/\Delta\tau$ at 7, 25 and 44°, with $H = H'V^0\phi_P^2/RT$.

TABLE I

LIGHT SCATTERING MEASUREMENTS

			$Hw_P/\Delta\tau \times 10^6$		
m_3	*p*H	w_P	7°	25°	44°
0.15	7.5	1.31	1.451	1.421	1.453
		2.60	1.501	1.502	1.490
		5.19	1.576	1.569	1.570
		7.88	1.744	1.762	1.717
					1.734
		15.73	1.950	1.978	1.952
		31.56	2.440	2.488	2.394
	6.0	5.09	1.515	1.496	1.543
	5.4	1.23	1.462	1.508	1.499
		2.44	1.563	1.561	1.570
				1.576	
		3.75	1.518	1.569	1.566
		4.86	1.551	1.554	1.593
				1.620	
		15.75	1.801	1.808	1.769
			1.817		
		31.65	2.099	2.106	2.053
	4.0	7.89	1.576	1.619	
		15.75	1.644	1.681	
		31.50	1.801	1.889	
0.003	7.0	3.21	2.111	2.069	
		6.36	3.096	2.994	3.022
	6.0	2.93	1.678	1.648	1.645
		5.86	2.043	1.968	1.920
				2.070	1.946
	5.3	1.35	1.436	1.422	
		2.72	1.446	1.425	1.411
		5.38	1.481	1.455	1.405
0.000	5.1	1.79		1.398	
		2.70	1.420	1.432	1.403
		5.58	1.331	1.357	1.318

The calculated slopes are presented in Table II. The first two columns are again the salt concentration and the *p*H. The third column is a weighted average of the enthalpy contribution to the slope, and the fourth and fifth columns are similarly weighted averages of the slope itself. The average enthalpy contribution to the slope is taken as

$$\Sigma w_P[(Hw_P/\Delta\tau)_{7^0} - (Hw_P/\Delta\tau)_{44^0}]\ 280 \times 317/(298 \times 37\ \Sigma w_P)$$

and the average slope as

$$\Sigma w_P[(Hw_P/\Delta\tau - 1/\bar{W}_P]/\Sigma w_P$$

in which the points at the three temperatures are averaged together. In column 4, $1/\bar{W}_P$ is taken as 1.5×10^{-6}, which is the value determined by the measurements at 0.15 *m* NaCl and *p*H 5.4. This

TABLE II

LIGHT SCATTERING SLOPES

m_3	*p*H	$S_{7\text{-}44} \times 10^5$	$S'_{(1.5)} \times 10^5$	$S'_{(1.4)} \times 10^5$	ν_H	ν_{Cl}	S_D
0.15	7.5	0.010	0.026	0.034	−17	8	0.042
.15	6.0	− .023	.004	.023	− 4	9	.023
.15	5.4	− .009	.015	.023	0	10	.013
.15	4.0	− .044	.012	.017	37	23	.026
.003	7.0	.094	.224	.243	− 8	1	.540
.003	6	.084	.081	.101	− 3	2	.167
.003	5.3	.113	− .011	.009	0	2	.027
.000	5.1	.031	− .030	− .008	0	0	..

(13) H. M. Dintzis, Ph.D. Thesis, Harvard University, 1952.

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

TABLE III

ELECTROSTATIC CONTRIBUTIONS TO β_{22}

m_3	pH	$-\bar{z}_2^2$ term	$-\bar{z}_2^{2^2}$ term	$+\bar{z}_2^{2^3}$ term	$-\bar{z}_2^{2^4}$ term	Measured BMA	Measured BSA	Non-electrostatic BMA	Non-electrostatic BSA
Timasheff, Dintzis, Kirkwood and Coleman									
3×10^{-5}	Iso.	893	6562	725	115	....	−1255	..	5590
1×10^{-4}	Iso.	814	3030	395	64	− 795	−1215	2718	2298
3×10^{-4}	Iso.	705	1491	180	26	....	− 990	..	1050
1×10^{-3}	Iso.	541	453	53	7	− 200	− 570	748	378
3×10^{-3}	Iso.	383	141	11	1	....	5	..	519
1×10^{-2}	Iso.	284	49	2	0	430	150	761	481
3×10^{-2}	Iso.	175	13	0	0	....	305	..	492
1×10^{-1}	Iso.	57	1	0	0	600	635	658	693
1.5×10^{-1}	Iso.	30	0	0	0	....	575	..	605
From Table II[a]									
3×10^{-3}	7.0	2676	6867	3628	5663	−7425		4153	
3×10^{-3}	6.0	1064	1087	228	142	−1642		423	
3×10^{-3}	5.3	460	203	18	5	− 445		205	
1.5×10^{-1}	7.5	185	16	0	0	− 192		9	
1.5×10^{-1}	6.0	52	1	0	0	12		65	
1.5×10^{-1}	5.4	32	0	0	0	242		274	
1.5×10^{-1}	4.0	60	1	0	0	− 228		−167	

[a] To obtain the contribution to a slope of Table II, β_{22} is multiplied by 4×10^{-10}. The last place in Table II corresponds to 25 in β_{22}.

corresponds to a molecular weight of 67×10^3, which is very close to the most probable monomer weight of bovine mercaptalbumin. In column 5, $1/\bar{V}_P$ is taken as 1.4×10^{-5}, which corresponds to the number average molecular weight determined for this sample from osmotic pressure measurements.[15] Both figures are given because the weight average molecular weight can be less than the number average only if the filtration for light scattering removed polymerized molecules. The difference between the two slopes is probably a fair measure of the uncertainty in the free energy. The enthalpy contribution in the third column does not depend upon the choice of molecular weight.

The sixth and seventh columns give estimates of the hydrogen ion bound to 69,000 g. of albumin,[16] and the last column gives the ideal Donnan contribution to the slope, calculated from these values for the bound ions. This is an entropy contribution and is independent of the choice of molecular weight since it is determined by the square of the average charge $\bar{z}^2$ divided by the square of the average molecular weight $\bar{V}_P^2$.

The uncertainty given for the enthalpy terms represents only the computational uncertainty arising from the indicated uncertainties in $Hw_P/\Delta\tau$. Examination of columns 4, 5 and 6 of Table I shows, however, that in twenty-two cases of measurements at three temperatures, seven indicate a maximum and four indicate a minimum. The randomness of the distribution of these extremes indicates that they are due to experimental error.

Our measurements on isoionic albumin at 25° agree in slopes with the very thorough measurements of Timasheff, Dintzis, Kirkwood and Coleman.[17,18] These authors find very good agreement between their measurements with salt-free albumin and the proton fluctuation equation of Kirkwood and Shumaker,[19] which is the same as equation 4 of ref. 2 with the mean square charge $\bar{z}^2$ suggested in footnote 14a. They did not publish a comparison for the solutions with salt, for which Kirkwood and Shumaker's equation corresponds very closely to the first term of 13 of ref. 2 but decreases somewhat less rapidly as the salt concentration increases. We have tried this comparison with our equation, plus the equation of Mayer[20] for the higher terms. We have assumed that the charge fluctuation is still equal to the proton fluctuations when chloride ion is also bound. We have assumed that all terms depend upon $\bar{z}^{2\nu}$, which undoubtedly is correct for all but the third which might be proportional to $\bar{z}^{3^2}$ rather than $\bar{z}^{2^3}$. If so it would be somewhat smaller. Table III contains our calculations for $\beta_{22} = \mathrm{d} \ln \gamma_2/\mathrm{d}m_2$. The first column gives the salt concentration, the second the pH, or "iso," for the isoionic measurements from Yale. The third column, $\bar{z}_2^2$ term, comes from the second term of equation 13, ref. 2 and arises from the difference in size of the protein and the small ions, the fourth column, $\bar{z}_2^{2^2}$ term, comes from the first term of the above equation and corresponds to the Debye–Hückel approximation, the fifth column, $\bar{z}_2^{2^3}$ term, gives the Mayer term for $\nu = 3$, and the sixth $\bar{z}_2^{2^4}$ term, for $\nu = 4$. The higher terms are relatively small. The seventh and eighth columns give the measured β's for bovine mercaptalbumin and for bovine serum albumin, and the ninth and tenth columns give the difference between the measured values and

(15) J. Pigliacampi, Ph.D. Thesis, M.I.T., 1957.

(16) G. Scatchard, J. S. Coleman and Amy L. Shen, THIS JOURNAL, **79**, 12 (1957).

(17) S. N. Timasheff, H. M. Dintzis, J. G. Kirkwood and B. D. Coleman, *Proc. Natl. Acad. Sci., U. S.*, **41**, 710 (1955); THIS JOURNAL, **79**, 782 (1957).

(18) Dr. Julian M. Sturtevant found heat absorption of about 600 cal./mole on diluting a 20% solution of Armour bovine serum albumin with an equal volume of water (private communication). This corresponds to a slope of −0.001 where we found +0.031.

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

(20) J. E. Mayer, *J. Chem. Phys.*, **18**, 1426 (1950).

the corrections for the electrostatic terms in the previous columns.

For the two most dilute solutions the non-electrostatic residual is very large. This may be due partly to failure of the experimentalists to obtain the limiting slope. At 1×10^{-3} and above, the residuals for the isoionic solutions are moderately constant with averages of about 700 for the Yale BMA, 500 for their BSA and 250 for our BMA.

Our results with added acid or base are moderately consistent at 0.15 m salt, but the electrostatic correction is much too large at 0.003 m. The discrepancy must be in the theory and it must apply to at least three of the four terms. It may arise from polarization of one protein molecule by the other due to shift of protons, or perhaps of chlorides, as suggested by Kirkwood and Shumaker.[19] Since we are calculating here the interaction of two like charges, polarization will reduce the electrostatic effect. There are no quantitative calculations of such effects. If the ratio $\kappa a/\sqrt{I}$, ν_{Cl} and the charge fluctuations are all independent of the temperature, the term proportional to $\bar{z}^2$ should have no enthalpy contribution, and the others should have an enthalpy term about $-{}^3/_8$ that of the free energy term. In our measurements in 0.15 m NaCl and at pH 5.3 in 0.003 m NaCl the electrostatic enthalpy term should be negligibly small.

It is quite possible that the difference between the enthalpy terms at 0.003 and 0.15 m NaCl are due to the differences in the electrostatic terms and that the variation of the non-electrostatic residuals of both β_{22} and its enthalpy counterpart are independent of pH and salt concentration in the range of our measurements. This would make the enthalpy contribution about -600 and the entropy contribution about $+1100$. The fluctuation theory explains, at least qualitatively, the fact that the slope at the isoelectric point (minimum slope) is less positive for thiocyanate ion than for chloride ion[3] by the fact that the greater binding shifts the isoelectric point nearer the pH where half the carboxyls are un-ionized, where the fluctuation is largest.

The greatest difficulty with the interpretation of these measurements is in the correction for excluded volume, which is entirely an entropy contribution. It was noted in ref. 2 that Huggin's extension of the lattice theory for chain molecules[21] gives 160,000 for the excluded volume contribution to β_{22}. For a sphere of radius 30 Å., the van der Waals theory as used by Zimm[22] and by Mayer[20] gives 204,000. As used by Kirkwood and Shumaker[19] it gives 180,000.

(21) M. L. Huggins, THIS JOURNAL, **64**, 1712 (1942).
(22) B. H. Zimm, *J. Chem. Phys.*, **14**, 164 (1946).

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Physical Chemistry of Protein Solutions. IX. A Light Scattering Study of the Binding of Trichloroacetate Ion to Serum Albumin

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The turbidity of bovine serum mercaptalbumin has been measured at 25° in isoionic solutions with 0.01 to 2.5 m sodium trichloroacetate and with addition of acid or of base from 0.05 to 0.25 m salt and corrected for large-particle scattering. The salt causes aggregation so that the binding cannot be calculated from the apparent molecular weight. The second virial coefficient yields values for the binding which agree reasonably with other methods at low salt concentrations, but which exceed the total number of basic groups at high salt concentrations. Over the range studied, the addition of acid or base had very little effect on the apparent net charge.

This work was initiated in an attempt to apply to the binding of small ions to proteins the method used by Ewart, Roe, Debye and McCartney[2] for non-electrolytes. This method uses the change with addition of solute 3 in the apparent light-scattering molecular weight of solute 2 at the limit of zero concentration of 2. We start with equation 10 of the preceding paper.[3]

$$\frac{H'V^0\phi_P^2 w_P}{RT\Delta\tau} = \frac{\frac{1}{p\bar{V}_P} + \left(\frac{z_2^2}{2m_3} + \beta_{22}\right) w_P/\bar{V}_P}{\left(1 + h\psi_{HX}/\bar{V}_P\phi_P + \frac{\nu - h}{2}\psi_3/\bar{V}_P\phi_P + \frac{z_2^2 w_P\psi_3}{2m_3(2+\beta_{33}m_3)\bar{V}_P^2\phi_P}\right)^2} \quad (1)$$

If the degree of polymerization p and the number of bound protons h are known, ν may be determined from the intercept of $w_P/\Delta\tau$ *vs.* w_P, and β_{22} may be determined from the slope. If p is unknown we may assume β_{22} is known as in the osmotic pressure studies[4] and calculate ν from the slope at the limit of zero concentration, and determine p from the intercept.

We used sodium trichloroacetate with bovine serum mercaptalbumin because ν and ψ_3 are both larger than for any other anion we have studied. We were unable to use the intercept, however, for the salt caused polymerization of the protein which varied with the details of handling. There were difficulties even with the slope, because of large

(1) Research Department, Philco Corporation, Philadelphia, Pennsylvania. The experimental work reported here was completed in 1955.
(2) R. H. Ewart, C. P. Roe, P. Debye and J. R. McCartney, *J. Chem. Phys.*, **14**, 687 (1946). See also W. H. Stockmayer, *ibid.*, **18**, 58 (1950).
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