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Physical Chemistry of Protein Solutions. XI.[1] The Osmotic Pressures of Serum Albumin, Carbonylhemoglobin and their Mixtures in Aqueous Sodium Chloride at 25°

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The osmotic pressures of aqueous solutions of bovine serum mercaptalbumin, carbonylhemoglobin and their mixtures are measured in a modified Hepp osmometer with varying amounts of sodium chloride and of added hydrochloric acid or sodium hydroxide. The results are correlated by simple analytical expressions and are explained approximately by electrostatic theory.

Carbonylhemoglobin has several advantages over the much studied serum albumin for the study of osmotic pressures. It may be prepared reproducibly with the uniform molecular weight of 6.60×10^4 with no apparent dissociation or aggregation; it apparently does not bind either sodium or chloride ions appreciably; its isoionic point is near neutrality; and its molecules are nearly spherical. It has the disadvantage that it is more sensitive to environment. Its color interferes with its study by light scattering. Mixtures of albumin and carbonylhemoglobin have the advantage that the two proteins have almost the same molecular weight. In the isoionic equimolal mixture each albumin molecule loses six protons and each hemoglobin molecule gains six.

The interaction of these proteins is largely due to their charges and is swamped out by moderate salt concentrations. We have therefore devoted particular attention to solutions in 0.003 *m* sodium chloride, which is the most dilute solution for which we had confidence in our measurements.

Materials.—The bovine serum mercaptalbumin was prepared from several times recrystallized mercury dimer, obtained from the Department of Biological Chemistry of the Harvard Medical School through the courtesy of Professor J. L. Oncley, by passing through the Dintzis–Oncley ion exchange column of ammonium, thioglycolate, acetate, mixed hydrogen and hydroxyl, and hydrogen exchangers.[2] The cation exchangers were prepared from Rohm and Haas 1R-120 resin and the anion exchangers from their 1R-400 resin. The albumin was then freeze-dried and stored at about −30°.

The carbonylhemoglobin was prepared from fresh defibrinated horse blood, kindly furnished by the Commonwealth of Massachusetts Department of Public Health Biologic Laboratories, by the method of Steinhardt and Zaiser,[3] which is essentially that of Ferry and Green.[4] The crystals were redissolved by the slow addition of 1 *M* NaOH until the *p*H rose to 7.3. The solution was centrifuged, and the clear supernatant was diluted to about 6%, the *p*H was adjusted to 6.6 by the slow addition of 0.1 *M* HCl and the solution resaturated with CO. The solution was deionized by passing through a column containing the ammonium, acetate, mixed hydrogen and hydroxyl, and hydrogen ion exchangers described above. The solution, approximately 5%, was resaturated with CO and stored at 2° for not more than a month.

The ferrihemoglobin used in the early experiments was prepared from carbonylhemoglobin by air oxidation of a 3% solution in 0.2 *M* NaCl at *p*H 5 and room temperature.[3] The use of ferrihemoglobin was soon abandoned, however, because the molecular weight was 4.5 to 15% lower than the 66,000 of the carbonylhemoglobin from which it was prepared. Apparently there was an irreversible dissociation of a portion of the material to half molecules at the low *p*H. The iron content of this low molecular weight hemoglobin was determined by the method of Klumpp[5] as 0.336%, which indicates that no iron had been lost in the dissociation.

The concentrations of carbonylhemoglobin and ferrihemoglobin were determined spectroscopically with the use of the equations of Zaiser and Steinhardt.[6]

Apparatus and Procedure.—The osmotic pressures were measured in the modified Hepp Osmometer used in this

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

(3) J. Steinhardt and E. M. Zaiser, *J. Biol. Chem.*, **181**, 161 (1951).

(4) R. M. Ferry and A. A. Green, *ibid.*, **81**, 175 (1929).

(5) T. G. Klumpp, *ibid.*, **107**, 213 (1934).

(6) E. M. Zaiser and J. Steinhardt, *J. Am. Chem. Soc.*, **73**, 5568 (1951).

Laboratory,[7] thermostated at 25 ± 0.02°. The membranes were prepared from fresh nitrocellulose (Howe and French, Inc., R.S. 30/40, serial H8318) by the method previously used in this Laboratory.[7] The membranes were washed thoroughly with water and soaked in the appropriate salt solution before use. For measurements with protein solutions of *p*H 6 or greater, the membrane was conditioned before assembling in the osmometer by leaving it in contact with a solution of one or two drops of 0.05 *M* NaOH in the 3 or 4 ml. of salt solution for about 5 minutes, then rinsing with the neutral salt solution. Without thorough washing and this conditioning there was irreversible precipitation on the membrane from solutions of hemoglobin.

With solutions containing carbonylhemoglobin, the space between the protein solution and the lucite cover was filled with CO during the measurement. This retarded the oxidation of the hemoglobin.

A concentration series from about ten to about fifty grams protein per kilogram water in a given salt solution, perhaps with added HCl or NaOH, was prepared by diluting the concentrated solution with a solution of the same weight molality of salt. Acid or base was added slowly as 0.05 *M* HCl or NaOH from a Gilmont micropipet at 2° with stirring.

After each osmotic pressure determination the protein solution was removed from the osmometer, its *p*H was measured and the protein concentration was determined by drying the weighed solution at 105° for at least 24 hr.

Results

Measurement of the osmotic pressures at 25° were made for bovine serum mercaptalbumin (BMA) for equine carbonylhemoglobin (COHb) and for approximately equimolal mixtures of the two proteins, for the isoionic proteins in 0.15, 0.01 and 0.003 *m* NaCl and for the isoionic proteins plus 3 moles HCl, 6 moles HCl, 3 moles NaOH and 6 moles NaOH in 0.003 *m* NaCl. Measurements were also made for isoionic BMA and isoionic COHb in salt-free solution, isoionic BMA in 0.001 *m* NaCl, for the equimolal mixture with 1.5 moles HCl and 1.5 moles NaOH in 0.003 *m* NaCl and for isoionic mixtures of approximately 3:1 and 1:3 BMA to COHb in 0.003 *m* NaCl.

Our measurements with a single protein may be represented by the equation

$$P = Am_p(1 + Bm_p) = aw_p(1 + bw_p) \quad (1)$$

$$A = RT/V^0 \quad (2)$$

$$2B = \bar{z}^2/2m_3 + \beta_{pp}^0 - \frac{\beta_{p3}^0 m_3}{2 + \beta_{33}^0 m_3} \quad (3)[8]$$

V^0 is the volume of the salt solution containing one kilogram of water, w_p the number of grams and m_p the number of moles of protein per kilogram of water and B is an empirical parameter. Obviously $a/A = b/B = 1/\bar{W}_p$, in which $\bar{W}_p$ is the number average molecular weight of the protein, $\bar{z}_p$ is the average charge on the protein, $\beta_{ij} = \partial \ln \gamma_i/\partial m_j = \partial \ln \gamma_j/\partial m_i$, β_{ij}^0 is the limit of β_{ij} as m_p approaches zero, and m_3 is the weight molal concentration of sodium chloride. Since V^0 varies slightly with salt concentration, A and a do also. The density of decane at 25° is 0.7271 and $A \times 10^{-4}$ at 25° is 3.466 for water and 0.003 *m* NaCl, 3.465 for 0.01 *m* NaCl and 3.458 for 0.15 *m* NaCl if the pressure is measured in centimeters of decane.

Most of the measurements with mixtures require a second parameter, which might be introduced in several ways. We have chosen the form

$$P = Am_p[1 + Bm_p - B'm_p/(1 + 2B'm_p)] = aw_p[1 + bw_p - b'w_p/(1 + 2b'w)_p] \quad (4)$$

This leaves the form of equation 3 unchanged if $2B$ is replaced by $2(B - B')$. For very low concentrations B' is equal to K for the reaction $2A = A_2$ for a solution of a single protein and to $K/4$ for the reaction $A + B = AB$ in an equimolal solution of two proteins. For high concentrations $B'm_p/(1 + 2B'm_p)$ approaches 0.5, which is the limit for the formation of double molecules in these two cases. In the intermediate range of our measurements, relations are more complicated. For those solutions which require B', we know the value of $\bar{W}_p$ from other measurements with considerable assurance.

The results are shown in Figs. 1–3, and 5–7 as $\phi = P/Am_p$. The circles are our experimental results and the curves are calculated from equation 4 with the parameters given in Tables I–V. These tables also show the *p*H, the molecular weight, the number of bound protons and chloride ions and, sometimes, other quantities which will be discussed later.

The difference between the measured and calculated pressures is usually less than 0.1 cm. decane or 0.05 mm. mercury, though there are occasional differences which are much larger. The *p*H meter behaved erratically during part of this study and the difficulty was not sufficiently realized at the time. Although many of the measurements are confirmed by measurements with different samples, the only criterion we have for others is their agreement with expectation. Some of the measurements are very erroneous, but we believe that most of them are moderately precise. The average deviation from the averages listed in these tables is 0.03–0.04. Only those with salt-free albumin show a trend.

Discussion

In accordance with the custom of this Laboratory, we will attribute the subscripts 1, 3, 2 and 4 to water, NaCl, BMA and COHb, respectively, and we will define a protein component as $Na_{\bar{z}_i/2}^{+} \cdot [H_{h_i}{}^{+}P_iCl_{\nu_i}{}^{-}]Cl_{\bar{z}_i/2}^{-}$, in which h_i and ν_i are the number of moles of H^+ and of Cl^- bound to one mole of P_i(i is 2 or 4), and $\bar{z}_i = h_i - \nu_i$. Since we cannot distinguish between β_{i3} and the effect of bound Cl^-, we assume that $\beta_{i3} = 0$, so equation 3 becomes

$$2B = \bar{z}_p^2/2m_3 + \beta_{pp}^0 \quad (5)$$

Isoionic Bovine Mercaptalbumin Solutions.—Our results for isoionic BMA are shown in Figs. 1 and 2 and in Tables I and II. The molecular weight usually obtained for BMA is 6.94×10^4, which agrees with the values previously obtained for serum albumin. The measurements in 0.01 *m* NaCl indicate 6.83×10^4, and those in 0.001 *m* NaCl, one series in 0.003 *m* NaCl and perhaps those in salt-free solution give 6.47×10^4, which is approximately equal to the most probable value of the monomer molecular weight,[9] $6.50 \pm 0.07 \times 10^4$. Our values for $2B$ agree well with those from earlier measurements of osmotic pressures of BMA or of BSA (bovine serum albumin) in the

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

(8) G. Scatchard, *J. Am. Chem. Soc.*, **68**, 2315 (1946).

(9) Barbara W. Low, *ibid.*, **74**, 4830 (1952).

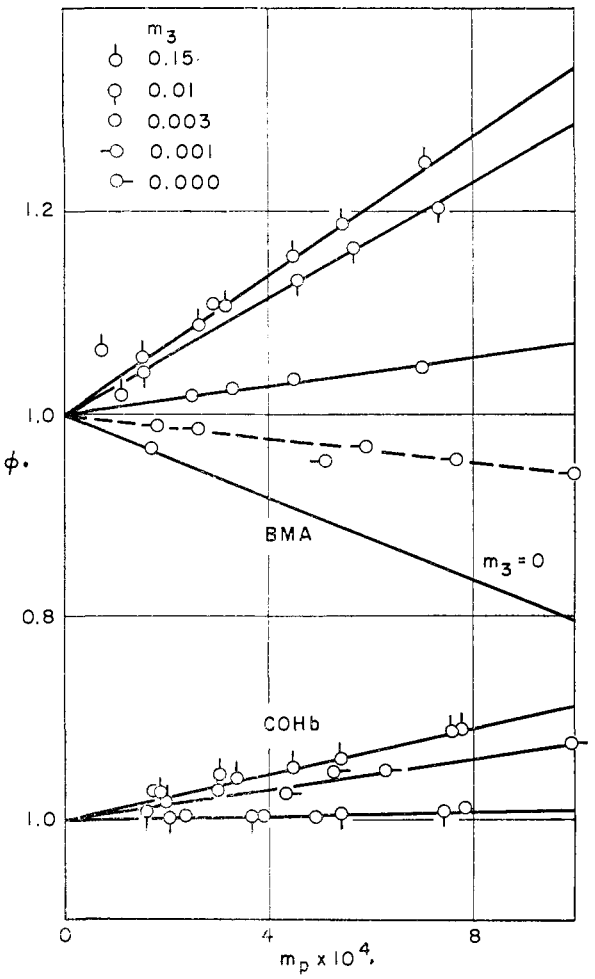

Fig. 1.—Osmotic coefficients.

same range of protein concentration, and of light scattering in much more dilute solutions. The comparison is shown in Table II. The first column is m_3, the concentration of NaCl; the second gives the number of moles of chloride ion bound to one mole of albumin, calculated from the equations of Scatchard, Coleman and Shen,[10] and the other columns show $2B$; the third and fourth are from the light scattering results for BSA, and for BMA of Timasheff, Dintzis, Kirkwood and Coleman[11]; the fifth the results of this paper from the osmotic pressures of BMA, the sixth is the Donnan contribution, calculated with $\bar{\nu}$ of the second column as $-\bar{z}_2$ and the seventh column is this Donnan term plus the electrostatic term calculated from the equations used by Scatchard and Bregman[12] with this $\bar{z}_2$ and $(\bar{z}_2^2 - \bar{z}_2^2)$ as 5.73 taken from our titration curve.[13] The results of other observers are given at the foot of the table. Those of Scatchard, Batchelder and Brown[14] and of Scatchard, Batchelder, Brown and Zoza[15] are from osmotic pressure of BSA, those of Edsall, Edelhoch, Lontie and Morrison[16] are from light scattering of BSA, those of Scatchard and Bregman[12] are from light scattering of BMA, and those of Scatchard, Wu and Shen[17] are from osmotic pressures of BMA. Their results at larger salt concentrations are not included. All B values for albumin are normalized

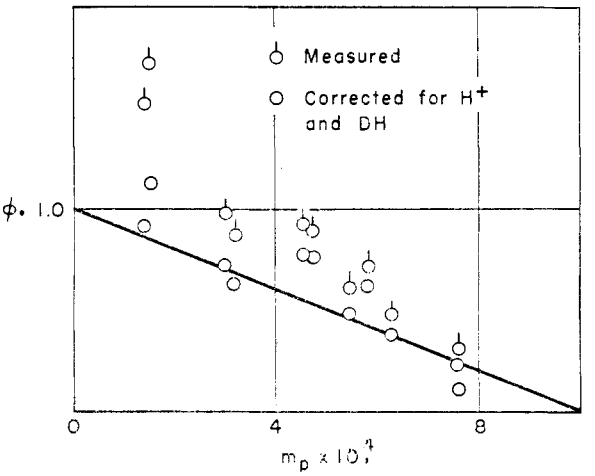

Fig. 2.—Osmotic coefficients, BMA in salt free solution.

TABLE I

PARAMETERS FOR ISOIONIC BOVINE SERUM MERCAPTALBUMIN

$B' = 0$. See Table II for $\bar{\nu}_{Cl}$, B, B_D and B_E

m_3	pH	$\bar{W}_2 \times 10^{-4}$	β_{22}[b]	β_{22}^n[b]
0	4.95[a]	6.94	−400	...
0.001	5.15	6.47	−973	−500
.003	5.32	6.94	−665	−277
.003	5.28	6.47	−661	−273
.01	5.28	6.83	−240	+117
.15	5.36	6.94	+302	+337

[a] $pH = 5.20 - 0.008\ w_p$. [b] Normalized to $\bar{W}_2 = 6.9 \times 10^4$.

TABLE II

PARAMETERS FOR ISOIONIC ALBUMIN[a]

m_3	$\bar{\nu}_{Cl}$	$2B$ BSA[11]	$2B$ BMA[11]	$2B$ BMA-(S + P)	$2B_D$	$2(B_D + B_E)$
0	0			−400	0	
3×10^{-5}	0.1	−814			167	−1605
1×10^{-4}	.3	−810	−651		450	− 572
3×10^{-4}	.6	− 62			600	− 92
1×10^{-3}	1.3	−305	− 66	−128	845	+ 372
3×10^{-3}	2.2	+130		+142	807	419
1×10^{-2}	4.05	362	+690	580	820	463
3×10^{-2}	6.9	538			794	519
1×10^{-1}	9.20	655	779		493	356
1.5×10^{-1}	10.77	678		685	383	348

Other values of $2B$

200,[15] 225[12] at $m_3 = 3 \times 10^{-3}$

1220[16] at $m_3 = 3.3 \times 10^2$

780,[14,15] 590,[15] 600,[16] 620,[12] 700[17] at $m_3 = 1.5 \times 10^{-1}$

[a] All parameters are normalized to $\bar{W}_2 = 6.9 \times 10^4$.

(10) G. Scatchard, J. S. Coleman and Amy L. Shen, *J. Am. Chem. Soc.*, **79**, 12 (1957).

(11) S. N. Timasheff, H. M. Dintzis, J. G. Kirkwood and D. D. Coleman, *ibid.*, **79**, 782 (1957).

(12) G. Scatchard and Judith Bregman, *ibid.*, **81**, 6095 (1959).

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

(14) G. Scatchard, A. C. Batchelder and A. Brown, *J. Am. Chem. Soc.*, **68**, 2320 (1946).

(15) G. Scatchard, A. C. Batchelder, A. Brown and Mary Zoza, *ibid.*, **68**, 2610 (1946).

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

(17) G. Scatchard, Y. V. Wu and Amy L. Shen, *ibid.*, **81**, 6104 (1959).

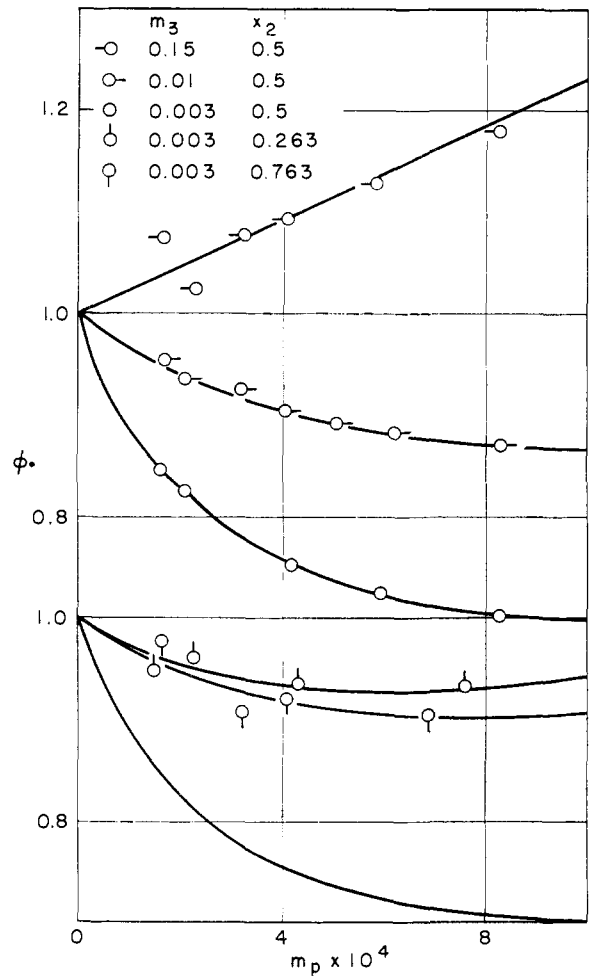

Fig. 3.—Osmotic coefficients, mixtures.

to a molecular weight of 6.9×10^4, that is they are $(6.9 \times 10^4)^2 ab/A$ rather than $(\bar{W}_2)^2 ab/A$.

The numbers in the last two columns are very different from those of Scatchard and Bregman. They used the values of $\tilde{z}_2$ and $\overline{z_2^2}$ given by Timasheff, Dintzis, Kirkwood and Coleman, to whom the measurements of $\tilde{z}_2$ by Scatchard, Coleman and Shen were not available and whose value for $(\overline{z_2^2} - \tilde{z}_2^2)$ was obtained from the measurements with salt-free protein and from dielectric constants. The confirming value obtained from the titration curves of Tanford, Swanson and Shore[18] is in error The revised valences give slightly more positive values for the last column in the more concentrated solutions where the Donnan effect predominates and very much more positive values in the dilute solutions where the negative interaction term predominates.

The difference between an experimental value from the value in the sixth column gives β_{22} and the difference from the last column gives the non-electrostatic contribution to β_{22}, β_{22}^n. Except for the one most dilute solution, β_{22}^n becomes more negative as the concentration decreases. The values in the more concentrated solutions agree moderately well with those calculated from the van der Waals excluded volume,[19,20] 408 or 340 for spherical molecules and somewhat larger for other shapes. Scatchard and Bregman made an error of five hundred fold in calculating this excluded volume. However, their huge value of 160,000 for the Huggins theory is correct.

The measurements in salt-free solution require special treatment in two respects: the hydrogen ions contribute appreciably to the osmotic pressure, and the small electrostatic contribution is more nearly proportional to the concentration than to its square. Our measurements of pH indicate hydrogen concentrations varying from 1 to 2×10^{-5} and the conductance measurements of Timasheff, Dintzis, Kirkwood and Coleman[11] yield the same results. The hydrogen ion concentration should not vary with the protein concentration in the range of our measurements because an average of only 0.07 or 0.15 proton is dissociated from one protein molecule even in our most dilute solution. Our measurements fit the equation $p\mathrm{H} = 5.20 - 0.008\ w_p$ ($\pm$ 0.04). This gives a pH of 4.80 in our most concentrated solutions ($w_p = 50$), where any effect of impurities should be smallest. In Fig. 2 are shown the values of ϕ determined directly and those corrected for 2×10^{-5} m H^+ and for the Debye–Hückel effect. The deviations are somewhat larger than with added sodium chloride. If we follow Timasheff, Dintzis, Kirkwood and Coleman in assuming that the concentration is 1×10^{-5}, we obtain a molecular weight of 6.5×10^{-4} instead of 6.9×10^{-4} and a value of B of -530 instead of -400, with about the same average deviation as for the other assumption. The latter value is quoted in Table II. It is worth noting that the assumption of a larger hydrogen ion concentration would reduce the fluctuation term of these authors and bring their value of the molecular weight closer to that in the solutions containing salt. (See a later discussion, however.)

The electrostatic term is calculated by the Debye theory for two ions with the square of the charge 5.73 and distance of closest approach 60 Å. The contribution to Bm varies from -0.011 in the most dilute solution to -0.013 in the most concentrated.

There is no basis for comparison of these measurements with those in salt-free solution of Timasheff, Dintzis, Kirkwood and Coleman at much smaller concentrations. Their deviations arise almost entirely from the Debye–Hückel electrostatic term which is almost negligible in ours. At their concentrations the negative B term only compensates the effect of finite size in the Debye–Hückel equation and keeps their deviation more nearly proportional to the square root of the concentration.

Isoionic Carbonylhemoglobin Solutions.—Our results for isoionic COHb are shown in Fig. 1 and in Table III. The molecular weight always appears to be 6.60×10^4, which agrees well with the generally accepted 6.68×10^4. The third column is $2B = \beta_{44}$, and the 4th is the non-electrostatic contribution to β, $\beta_{44}^n = 2(B - B_E)$ in which $2B_E$

(18) C. Tanford, S. A. Swanson and W. S. Shore, *J. Am. Chem. Soc.*, **77**, 6414 (1955).

(19) B. H. Zimm, *J. Chem. Phys.*, **14**, 164 (1946).

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

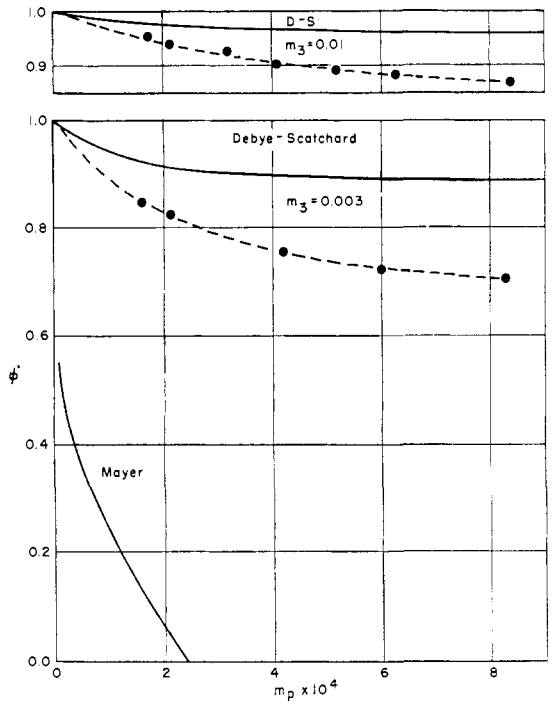

Fig. 4.—Calculated osmotic coefficients of mixtures.

is calculated with a $\bar{z}_4^2$ of 3.5 from the titration curve and a radius of 30 Å. Since there is no small ion binding there is no Donnan term. There is no systematic trend in either β_{44} or β_{44}^n, and the latter is not much less positive than the van der Waals volume exclusion term for this nearly spherical molecule. The corresponding β_{22}^n for albumin becomes negative for the 0.003 m and more dilute salt solutions. The measurement in salt free solution was made with month old carbonylhemoglobin and the pH was high.

TABLE III

PARAMETERS FOR ISOIONIC CARBONYLHEMOGLOBIN

$\bar{W}_4 = 6.60 \times 10^4$, $B' = 0$, $B_D = 0$

m_3	pH	$2B = \beta_{44}$	β_{44}^n
0	6.98	156	156
0.003[a]	6.68	20	136
(.003)[b]	(6.58)	(114)	(230)
.01	6.69	20	69
.15	6.71	230	231

[a] Deionized by ion-exchange. [b] Deionized by electrodialysis.

TABLE IV

PARAMETERS FOR ISOIONIC MIXTURES

m_3	x_2	pH	$\bar{\nu}_{Cl}$	$\bar{W}_p \times 10^4$	$2B$	$2B'$	$2B_D$
0.003	0.763	5.74	1.35	6.83	316	1000	304
.003	.502	5.99	0.7	6.76	142	3000	82
.003	.263	6.61	0.25	6.68	386	1000	10
.01	.504	5.95	1.3	6.76	234	1000	84
.15	.504	6.06	3.5	6.76	434	0	40

Isoionic Mixtures.—The results for the isoionic mixtures are shown in Fig. 3 and in Table IV, in which the first column is the sodium chloride concentration, the second is the mole fraction of albumin in the solute, the third is the pH, the fourth is the average chloride binding per mole of protein (the binding per mole of albumin times the mole fraction of albumin), the fifth is the average molecular weight, the sixth column is $2B$, the seventh is $2B'$ and the eighth is the Donnan contribution to $2B$, $2B_D$.

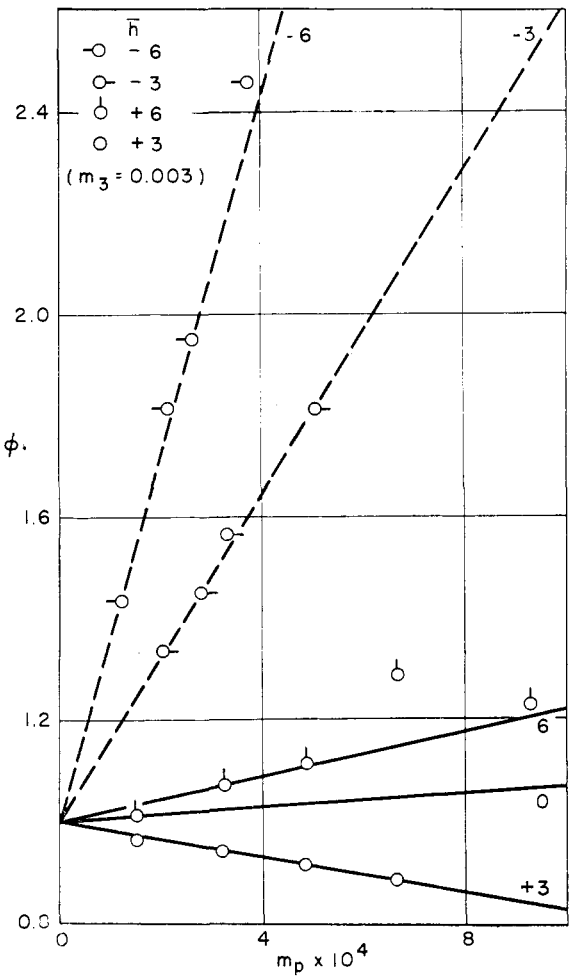

Fig. 5.—Osmotic coefficients, BMA with added acid or base.

Examining first the results for $x = 0.5$, we note that B' is zero in 0.15 m NaCl, and $\beta = 2(B - B_D)$ is more positive than $(\beta_{22} + \beta_{44})/2$. Our results lead to $\beta_{22} = 302$, $\beta_{44} = 230$, $\beta_{24} = 2\beta - (\beta_{22} + \beta_{44})/2 = 522$. $2(B - B_D)$ is also more positive than the average for the pure components in the more dilute salt solutions, but the significance of this relation is dimmed because B' is much greater than B in these cases. The fact that B' is so dependent on the salt concentration shows that it must be largely electrostatic.

We have calculated the electrostatic interaction for a somewhat idealized model which ignores the chloride ion bound to the albumin. At this pH, $\bar{z}_2^2 = \bar{z}_4^2 = 36$, and $(\overline{z_2^2} - \bar{z}_2^2) = (\overline{z_4^2} - \bar{z}_4^2) = 4$. Then $\bar{z}_p^2 = 40$. We assume that the protein ions are rigid, non-polarizable spheres with radius 30 Å. and that Na^+ and Cl^- are rigid, non-polarizable spheres with radii 2.5 Å. and $z^2 = 1$. For this case of two symmetrical salts, the Debye–Scatchard

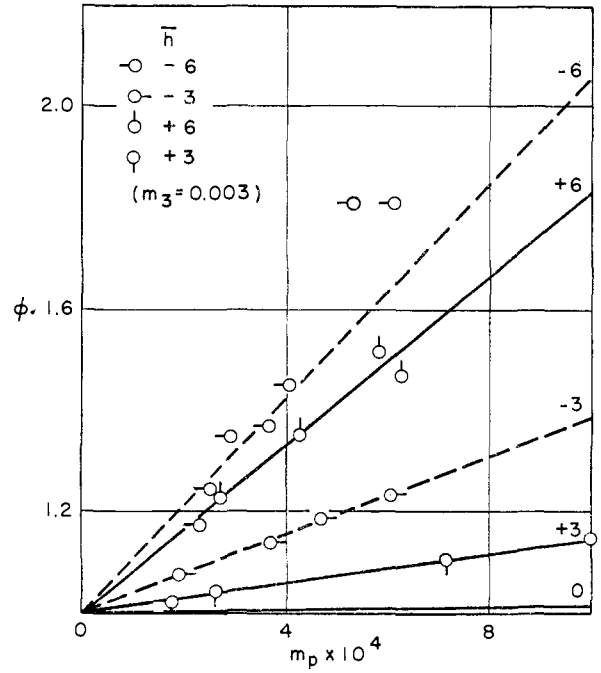

Fig. 6.—Osmotic coefficients, COHb with added acid or base.

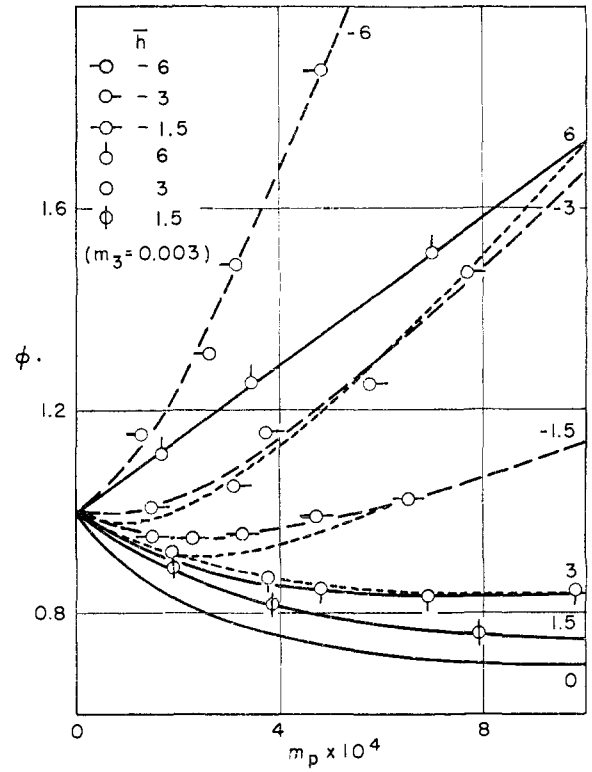

Fig. 7.—Osmotic coefficients, mixtures with added acid or base.

equation[14,21] reduces to

$$\ln \gamma_p = \frac{\frac{-\epsilon^2 \overline{z_p^2} \kappa'}{2DkT}(\kappa' + \kappa' \tan h\, \kappa'\Delta)}{\kappa(\kappa'\tau + \tan h\, \kappa'\Delta) + \kappa'(1 + \kappa'\tau \tan h\, \kappa'\Delta)} \quad (6)$$

in which γ_p is the mean activity of protein ions, κ has its usual significance, $0.3287\sqrt{I_3 + I_p}$ and $\kappa' = 0.3287\sqrt{I_3}$ in water at 25° if I is the ionic strength, $\tau = r_p + r_3$ and $\Delta = r_p - r_3$. It follows that

$$\frac{PV^0}{RTm} - 1 = \phi - 1 = \frac{1}{m^2}\int_0^{m_2} m_2 \, d \ln \gamma_2 \quad (7)$$

$$= \frac{-\epsilon^2 \overline{z_p^2}}{2DkT}\left(\frac{\kappa'^2}{2 \cos h^2 \kappa'\Delta}\right)\left\{\frac{2}{c(\kappa + \kappa')}\left[1 - \frac{d}{c(\kappa - \kappa')}\ln\frac{d + c\kappa}{d + c\kappa'} - \frac{1}{d + c\kappa}\right]\right\} \quad (8)$$

$$c = \kappa'\tau + \tan h\, \kappa'\Delta \quad (9)$$

$$d = \kappa'(1 + \kappa'\tau \tan h\, \kappa'\Delta) \quad (10)$$

We also have calculated $\phi - 1$ from the Mayer equations[22] for the same model in $0.003m$ NaCl. The results of these calculations are shown in Fig. 4. The broken lines represent equation 4. The Mayer equation leads to negative values of ϕ for m_p greater than 2.5×10^{-4}. The cause of this absurd result is that the Mayer equation neglects the difference between κ, the ionic strength of the bulk of the solution, and κ', the ionic strength in the shell from τ to $(\tau + \Delta)$, into which only small ions can penetrate.

The Debye–Scatchard equation on the other hand gives only about a third of the experimental values of $(\phi - 1)$, which correspond to $r_p = 17.5$ instead of 30 Å. So small a size is physically impossible for these proteins, but the result does give an indication of the fault in our model. The proteins are not non-polarizable but have a large polarizability due to the possibility of the protons shifting from one site to another. If two spherical univalent ions with radii 30, the same dielectric constant as water and charges imbedded one Ångstrom from the surface, positive in one ion and negative in the other, are in contact, the probability that these charges will be as close together as possible will be about thirty times that of their being as far apart as possible. The adjustment is made more easily by ionization at sites close to the other ion and deionization at distant sites than by rotation of the large ions. This phenomenon is quite distinct from the charge fluctuation. It should occur between molecules of the same kind with fluctuating charges, however, and would increase the interaction between these molecules. This effect, rather than an underestimate of the hydrogen-ion concentration, may explain the large value of charge fluctuation calculated by Timasheff, Dintzis, Kirkwood and Coleman. Kirkwood and Shumaker[23] recognized that this effect occurs in protein–protein interactions and also that it occurs in an external field and contributes to the dielectric constant. They present a schematic calculation of both effects. The quantitative calculation of either effect would require a detailed knowledge of the distribution of the reactive sites. We must be content with the qualitative understanding that proton polarization contributes largely to the interaction of two proteins with different isoionic

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points and may also contribute to the interaction of molecules of the same protein in isoionic salt free solutions or at very low salt concentrations. We shall see later that it also gives a reasonable qualitative picture of the effects of acid or base added to the isoionic proteins.

The simplest picture of the 1:3 and 3:1 mixtures is to give an average valence of nine to one protein and three to the other and to consider the fluctuation the same as for the equimolal mixtures. Then the dimerization constant should be 0.7 of that for equimolal mixtures, there should also be an *a priori* factor of 0.75 in B'. The asymptotic limit should be 0.75 instead of 0.5. The AB should react further to give A_2B with a constant about half as great as the first, an *a priori* factor of 0.81 and an asymptote of 0.5. The A_2B might react further to give A_3B, with a constant about one fifth of the first, an *a priori* factor of one and an asymptote of 0.25. It is not surprising that the limiting slope $B - B'$ is only -0.2 to -0.25, instead of -0.5, that for the equimolal mixture.

Non-isoionic Solutions.—The results for solutions in 0.003 *m* NaCl with added acid or base are shown in Fig. 5 for BSA, 6 for COHb, 7 for the equimolal mixture and in Table V, which shows $\bar{\nu}_H$ and $\bar{\nu}_{Cl}$, the number of moles of H^+ and of Cl^- bound per mole of protein, the *p*H, $2B$, $2B'$, $2B_D$ and $\beta_{pp}^n = \beta_{pp} - 2\beta_E = 2(B - B_D - B_E)$, with B_E calculated as in Tables II and III, for the single proteins. Figure 8 shows B and B' as functions of $\bar{\nu}_H - \bar{\nu}_{Cl}$.

TABLE V

PARAMETERS FOR NON-ISOIONIC MIXTURES IN 0.003 *m* NaCl NORMALIZED TO $\bar{W}_0 = 6.9 \times 10^4$

$\bar{\nu}_H$	$\bar{\nu}_{Cl}$	*p*H	$2B$	$2B'$	$2B_D$	β_{pp}^n
			Mercaptalbumin			
6	3.5	4.89	448	0	1042	− 142
3	2.87	5.17	349	0	3	− 162
0	2.2	5.32	142	0	807	− 276
−3	1.8	5.89	3211	0	3840	+ 856
−6	1.36	6.71	7233	0	9028	+3365
			Carbonylhemoglobin			
6	0	6.21	1678	0	6000	−1951
3	0	6.49	290	0	1500	− 734
0	0	6.68	20	0	0	+ 131
−3	0	7.02	774	0	1500	− 150
−6	0	7.48	2110	0	6000	−1519
			Mixture $x = 0.5$			
6	1.1	5.45	1470	0	4002	
3	0.9	5.80	330	1800	735	
1.5	.75	5.80	150	1900	93	
0	.7	5.99	142	3000	82	
−1.5	.6	6.34	940	2000	735	
−3	.45	6.68	2080	3000	1950	
−6	.4	7.03	5260	500	6827	

The results with BMA resemble those obtained earlier from osmotic pressure[14] and from light scattering.[12,16] When plotted against $\bar{\nu}_H$, $2B$ gives an unsymmetrical curve, but when plotted against $\tilde{z}_2 = \bar{\nu}_H - \bar{\nu}_{Cl}$ as in Fig. 8, the curve is nearly symmetrical, and somewhat more hyperbolic than a parabola. β_{22} is negative and again approximately parabolic, β_{22}^n is negative and fairly

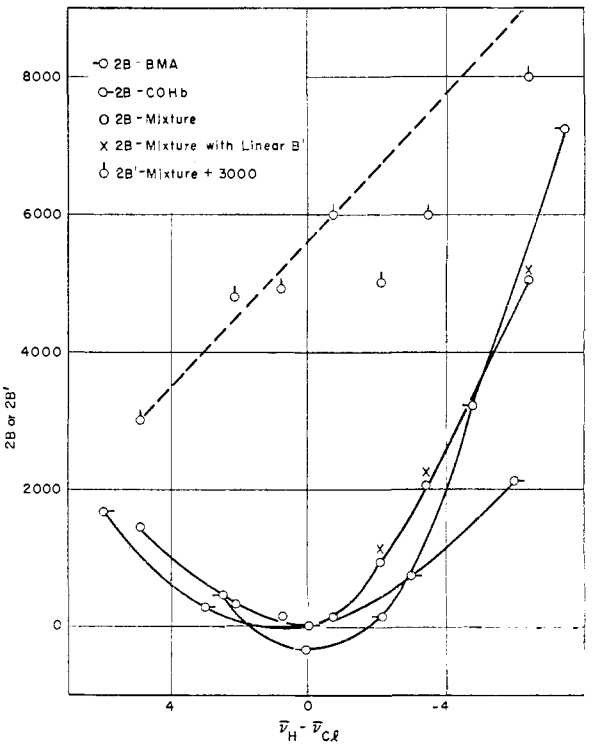

Fig. 8.—Osmotic coefficient slopes.

constant in neutral and acid solutions but rises quadratically on the basic side. The negative values on the acid side may be attributed to proton polarization, but the results in basic solutions must be attributed to error in the theoretical equations.[12]

With COHb, $2B$ is nearly parabolic, but much smaller than for BMA, β_{44} and β_{44}^n are both negative, except in the isoionic solution, and nearly parabolic. The values of β_{44}^n are of the right sign to be explained by proton polarization, but they seem rather large for this effect. However, the *p*H is near the *pK* for imidazoles, of which hemoglobin has 32-36 per molecule. The small and nearly constant (3.5–4) value of $(\overline{z_2^2} - \bar{z}^2)$ indicates a distribution of *pK*'s, which may still give large values of proton polarization. The only other explanation is error in the theoretical equations. If error is the explanation, the sign of the error is opposite to that in alkaline solutions of BMA.

The variations of $2B$ and of $2(B - B_D)$ with average charge for the mixtures resemble those for the single proteins. Both are roughly quadratic, $2B$ is positive and $2(B - B_D)$ is negative. $2B'$, on the other hand, varies approximately linearly with $(\bar{\nu}_H - \bar{\nu}_{Cl})$ from zero in 6 molal acid to 3000 with no added acid or base, but the deviations from this line in alkaline solutions average − 1400. $2B' + 3000$ is plotted in Fig. 8. Changing $2B'$ to the value on the dotted line makes very little difference in the value of $2B$, as shown by the crosses in Fig. 8. Therefore, the uncertainty in $2B'$ leads to almost as great an uncertainty in β_{pp}, which is equal to $2(B - B_D - B')$. Equation 4, although adequate as an interpolation equation, is not good enough to obtain the limiting slope.

This increase in $2B'$ with increasing negative charge would also result from a very large proton polarization in COHb. We have not attempted to calculate any electrostatic effect since the calculations are much more complicated than in the cases considered above and particularly since they depend greatly upon the assumption as to the distribution of protons between the two proteins.

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The Thermally-induced Transition in Fibrin[1,2]

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The thermal transition in fibrin films and fibers was observed by following the changes in three properties as the temperature was changed: the length of the sample at zero force, the retractive force at constant length, and the optical birefringence. These properties were observed to undergo sharp changes at a well-defined temperature which depended on the diluent with which the sample was in equilibrium. The *p*H-dependence of the transition temperature was accounted for with the aid of a simplified model for the fibrin network. This model consisted of exactly similar, partially helical chains between cross-links. Each helix was assumed to be stabilized by two hydrogen bonds between ionizable side-chain groups, *viz.* one tyrosyl-carboxylate ion and one tyrosyl-histidine bond. The helical portions are disrupted during the transition. The *p*H-dependence of the transition temperature for the helix-random coil transformation was related to the ionization parameters of the side-chain groups. The values of the parameters chosen for the description of this model are consistent with theoretical expectations and with the results from experiments on proteins in aqueous salt and urea solutions.

Introduction

In recent years theories have been proposed to account for the stability of the native conformations of protein molecules and for the denaturation accompanying the loss of the stabilizing influences. The interactions of primary concern here are the hydrogen bonds between ionizable side-chain groups. Theoretical considerations[4] have shown how these interactions can account for the *p*H-dependence of reversible denaturation. The purpose of this paper is to provide experimental data for testing the applicability of the theory by determining the extent to which the thermal transition in the fibrous protein fibrin[5] can be described by a simple model and the theory cited above.[4] This study was carried on concurrently with similar studies of ribonuclease[6,7] and insulin.[6]

The point of view taken here is that the transition in fibrous proteins, observed as a shrinkage in the unconstrained fiber[5,8,9] or the development of a retractive force in a sample of fixed length, is similar to transformations observed in the case of other high polymers[10,11] in passing from a highly ordered microcrystalline form to a more random arrangement of chains in an amorphous form as the temperature of the sample is raised. The validity of this point of view in the case of collagen has been shown by recent work of Flory and coworkers.[12-17] Many other workers have made use of this point of view in experimental and theoretical studies of transitions in proteins and polypeptides.[11,18-28]

The related studies in this Laboratory on ribonuclease[6,7] and insulin[6] were carried out with proteins whose covalent structures are known[29-31] and whose properties may therefore be more easily interpreted in terms of molecular structure than in the case of collagen or fibrin. However, the prior treatment of these materials which allows them to be handled by the methods used here requires the introduction of cross-linkages which are foreign to the native structure, and it has not yet

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