[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, MASSACHUSETTS INSTITUTE OF TECHNOLOGY, AND THE DEPARTMENT OF PHYSICAL CHEMISTRY, HARVARD MEDICAL SCHOOL]

Preparation and Properties of Serum and Plasma Proteins. VI. Osmotic Equilibria in Solutions of Serum Albumin and Sodium Chloride[1,2,3]

BY GEORGE SCATCHARD, ALAN C. BATCHELDER[4] AND ALEXANDER BROWN[5]

Introduction

This study was undertaken because the most important of all osmotic pressures is that of serum albumin under physiological conditions, which is the reason for the efficacy of human serum albumin and plasma in the treatment of shock. Since the difference in osmotic behavior between bovine and human serum albumin was no greater than that between different specimens of either, the less precious bovine albumin was used in this study. Some parts of this and the succeeding papers which have direct application to this problem are reported elsewhere.[6,7] The more fundamental scientific interest lies in measurements for the first time of salt distribution and osmotic pressure over a large range of *p*H as well as of protein concentration and salt concentration in solutions uncomplicated by the presence of buffers other than the albumin. They permit the calculation of the effects of changes in protein concentration on the activity coefficients of the salt and of the protein as functions of the *p*H and the salt concentration.

Throughout this paper the notation employed is the same as that of the preceding paper.[7b] Italicized references to equations refer to the preceding paper and references in Roman type refer to equations in the present paper.

Experimental

The osmometer, a modification of the one described by Oakley,[8] is illustrated diagrammatically in Fig. 1. The test-tube-shaped collodion membrane, B, containing the protein solution, has a diameter of 1.0 cm. and a length of approximately 11.0 cm. The concentric glass vessel, C, surrounding the membrane has internal dimensions of 1.3 × 13.0 cm., chosen so that the volumes of the solution inside and outside the membrane are approximately equal. The membrane is snugly fitted over a short piece of soft rubber tubing on the end of the capillary which leads through the rubber stopper from the upper vessel, A, and is fastened securely with three or four tightly tied loops of string. The internal diameter of this short length of capillary is 1.0 mm, chosen to permit introduction of a long hypodermic needle for filling the membrane after the two parts of the osmometer have been assembled. The lower end of the capillary bore is flared to facilitate removal of any air bubbles remaining after the membrane is filled. The dimensions of vessel A are 1.5 × 12.5 cm.

The capillary tubing leading to the two stopcocks and to the ground glass joint has a bore diameter of 1.0 mm. A flexible connection between the two parts of the apparatus is provided by the short length of rubber tubing above stopcock D, which is securely fastened on with wire. It is essential that stopcock D, in particular, be well ground and free of any leaks.

The toluene manometer, H, illustrated in Fig. 1, is constructed of 0.5–0.7 mm. bore capillary tubing. The length of the long arm on the manometers we use is 60 cm. This length is determined solely by the depth of the glass walled thermostat available and considerations of the mechanical strength of the final assembly. Evaporation is reduced and the system is protected from accidental contamination by the rubber tubing connecting the open end of the manometer to the top of vessel A. The salt solution–toluene interface is located in the bulb, F, which has an internal diameter of 1.0 cm. Preliminary experiments indicated that a diameter appreciably less than this led to significant irregularities in the pressure readings because of sticking at the salt solution–toluene interface.

The manometer tubing is calibrated for variations in bore diameter by measurement of the capillary rise of toluene. A short length of capillary tubing and the manometer tubing are suspended vertically in a deep cylinder filled with toluene. Using the toluene meniscus in the short, stationary piece of tubing as a reference point, the variation in the capillary rise in the manometer tubing is measured at intervals throughout its length with a cathetometer. The resulting corrections have been applied to all pressure readings.

We use the mercury manometer, J, also illustrated in Fig. 1, for the measurement of pressures greater than those that can be measured with the toluene manometer. It is connected in series with the short toluene manometer, G, which is constructed of 0.5–0.7-mm. glass capillary tubing.

(1) This work has been carried out under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

(2) Much of this work is taken from the thesis submitted by Alexander Brown in partial fulfillment of the requirements of the Ph.D. degree from the Massachusetts Institute of Technology. This paper is given the number II in the series "Physical Chemistry of Protein Solutions," from that laboratory.

(3) Presented in part at the Buffalo meeting of the American Chemical Society, September 10, 1942.

(4) Present address: Hercules Powder Company, Wilmington, Delaware.

(5) Present address: Carbide and Carbon Chemicals Corporation, South Charleston, West Virginia.

(6) G. Scatchard, A. C. Batchelder and A. Brown, *J. Clin. Inv.*, **23**, 458 (1944).

(7) (a) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publishing Corp., New York, N. Y., 1943, pp. 392–395. (b) G. Scatchard, THIS JOURNAL, in press.

(8) H. B. Oakley, *Trans. Faraday Soc.*, **31**, 136 (1935).

The toluene and mercury are separated by an air space in the ground glass joint and the inverted U of capillary tubing. The two arms of the mercury manometer are uncalibrated 7-mm. outside diameter glass tubing and the U is heavy-walled rubber tubing. Chance leakage at the upper ground glass joint on G is eliminated by fitting a sleeve of rubber tubing over the joint and sealing the joint with glycerol. A suitable shield in the water-bath is necessary to prevent any motion of the mercury manometer. We use a rectangular plate glass box, open at top and bottom, in which the mercury manometers are suspended.

For the measurement of very low pressures, it is frequently more convenient to use simply the short calibrated toluene manometer G in place of the longer toluene manometer H.

When the osmometers are filled for use, the protein solution is contained inside the membrane B and extends through the short capillary into A to a level just below the side arm. The salt solution is outside the membrane and extends through the ground glass joint into F and through the stopcock D into A, to a level of one or two cm. above the side arm. Dilution of the membrane contents by the salt solution in A is effectively prevented by the underlying layer of protein solution and the short length of capillary tubing leading to the membrane.

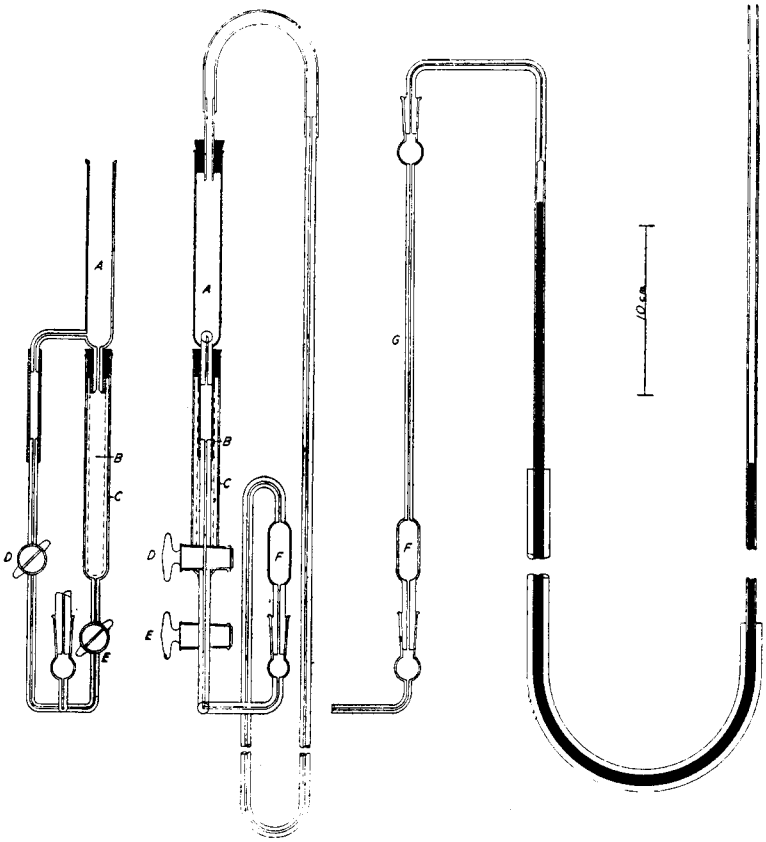

FIG. 1.—Osmometer.

After the apparatus is assembled, it is placed, with both stopcocks closed, in the water-bath to a level one or two cm. below the top of A and allowed to stand overnight to approach equilibrium. By manipulation of stopcocks D and E the level of the manometer liquid is adjusted to correspond to the expected pressure without releasing the tension on the membrane and vessel C is then connected to the manometer for measurement of the pressure. In the case of the toluene manometer, H, measurements with a cathetometer of the toluene level at intervals of three or four hours serve to follow the approach to equilibrium pressure. In the case of measurements of higher pressures, the mercury manometer usually can be set to within a few millimeters of the pressure by observing the motion of the toluene level in G immediately after stopcock E is opened. Measurement of the two mercury levels and the toluene level in G then serves to follow the approach to equilibrium pressure. Several approaches to equilibrium pressure were obtained in many of the present experiments in the same manner. In all cases, however, the pressures given in the tables are those of the final approach to equilibrium before the solutions were analyzed since minor variations in consecutive readings usually resulted from the attendant dilution or concentration of the protein solution.

At the conclusion of the experiment, the levels of the manometer liquids corresponding to zero pressure are measured. With manometer H, stopcock E is closed and D opened and the resulting toluene level measured. The pressure, in millimeters of mercury at 0°, is equal to

$$0.06333(T_O - T_E) - 0.074(S_O - S_E) + 0.0105(I_O - I_E) + \alpha + \beta$$

where T, S and I are the levels of the toluene in the manometer, the salt solution–air interface in A, and the salt solution–toluene interface in F, respectively, at the equilibrium position or at zero pressure as denoted by subscripts E or O. α is the correction for variations in capillary rise in the manometer and β is a correction equal to the distance in millimeters from the boundary between the protein and salt solutions in A to the middle of the membrane multiplied by the density relative to that of mercury of the inner solution minus that of the outer solution. The equilibrium pressure varies linearly from the top to the bottom of the membrane due to this density difference, and there is a corresponding difference in concentration. Since it is the average concentration which

is measured, the average pressure also is taken. The numerical factors are the appropriate density ratios for conversion to millimeters of mercury at 0°.

In the case of high pressure measurements, the mercury manometer is detached from the short toluene manometer and the short arm is raised so that the mercury levels are at the same position in the tubes that they occupied in the two arms during the pressure measurement. In the absence of horizontal motion of the two arms during this adjustment the subsequent difference in the two levels includes corrections for any differences in capillary rise in the two arms and for parallax arising from adjustment of the cathetometer. The level of the toluene in G with stopcock D open is measured. The pressure in millimeters of mercury at 0° is equal to

$$0.9955(\Delta Hg_E - \Delta Hg_O) + 0.06333(T_O - T_E) + \beta$$

where T and ΔHg are the toluene level and difference in mercury levels at zero pressure and at the measured pressure as indicated by subscripts O and E. Corrections for changes in levels of the interfaces in F and A are negligible.

The *p*H of the inside solution was measured before and after the experiment with a MacInnes type glass electrode which was calibrated with buffer solutions whose *p*H had been determined with a hydrogen electrode. After the pressure measurement the albumin and chloride concentrations were determined by evaporating an aliquot of the solution to constant weight at 105°. The protein was then removed by digestion on the steam-bath with concentrated nitric acid in the presence of an excess silver with thiocyanate in the presence of ferric alum indicator, except in experiments 52–72. In experiments 52–54 an aliquot of the solution was evaporated to dryness and the chloride extracted from the residuum with hot water. The chloride was then titrated with dilute silver nitrate solution, using a differential potentiometric end-point. The determination of chloride in experiments 61 and 62 was the same as that employed in the earlier experiments with the exception that the excess silver was titrated with dilute sodium chloride solution and the end-point was determined potentiometrically. The protein concentrations in experiments 61 through 72 were determined by micro-Kjeldahl nitrogen analysis, using a factor of 6.23 to convert grams of nitrogen to grams of protein. The protein concentrations are given as grams of isoelectric protein and were calculated from the preceding analyses and the titration curve of albumin in 0.15 M sodium chloride.

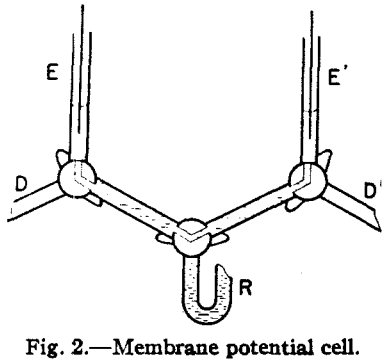

Fig. 2.—Membrane potential cell.

Since the outer solution contains no buffer, the membrane potential was measured as the potential of the cell

Ag(s), AgCl(s), solution B, KCl (sat.), solution A, AgCl(s), Ag(s)

This corresponds to cells I and II of the previous paper (page 2315) if cell III is Ag(s), AgCl(s), solution A, membrane, solution B, AgCl(s), Ag(s). Since the potential of this latter cell is zero, the potential of the first is equal to that of the cell IV which is the membrane potential

IV Hg(l), Hg_2Cl_2(s), KCl (sat.), solution A, membrane, solution B, KCl (sat.), Hg_2Cl_2(s), Hg(l)

The cell, which is illustrated diagrammatically in Fig. 2, consists of three 120° three-way stopcocks, with a bore diameter of 2 mm. The bottom stopcock gives connections to the saturated potassium chloride reservoir through R. Each of the others connects to an electrode-vessel, E or E′, and to the drain through D or D′. In use each electrode-vessel was rinsed and flushed through the drain, then the stopcock was flushed with potassium chloride. Finally the liquid junction was formed at the top of the stopcock by connecting the electrode-vessel to the potassium chloride arm. The silver–silver chloride electrodes were similar to those described by Brown.[9] Each pair of electrodes was tested immediately before use by measurement of the cell Ag, AgCl, 0.15 M NaCl, AgCl, Ag and any pair having a voltage difference greater than 0.07 mv. was rejected. A given set of electrodes could be used in several solutions before replating was necessary. The measurements were made at room temperature with a Leeds and Northrup Type K potentiometer and Type R galvanometer. The values are corrected to 25°. In the first measurements, the membrane potential was not determined. Then it was determined with a saturated potassium nitrate bridge for fear that diffusion from saturated potassium chloride would alter the potential. This fear was found groundless and the last measurements were made with a saturated potassium chloride bridge.

The albumin used was from lot 4-5-6 prepared by Dr. W. L. Hughes by the method developed by Cohn and Hughes.[10] It was separated from normal bovine plasma and three times recrystallized. It contained less than 0.1% carbohydrate by the orcinol test and was homogeneous electrophoretically at *p*H 7.5. Its isoelectric point was 5.17 when salt free and 5.37 in 0.15 M sodium chloride.

All other reagents were C. P. grade. The solu-

(9) A. S. Brown, THIS JOURNAL, **56**, 646 (1934).

(10) E. J. Cohn and W. L. Hughes, not yet published.

TABLE I

No.	w_2	w_2'	pH	z_2	$-z_2m_2$	m_2	m_2'	$-b_{22}\times 10^4$	$-\beta_{33}$	$-\beta_{23}$	$-\beta_{23}+(\beta_{33})_e$	E_m	$E_m/(w_2-w_2')$	P	$B_{22}\times 10^5$	β_{22}	$\beta_{22}-(\beta_{22})_e$
38	60.31	0.21	4.22	27.9	−0.0245	0.1587	0.1530	6.10	1.061	77.1	35.5	+1.70	0.0283	17.55	148	−1659	−1559
61	9.63	.00	4.23	27.0	− .0032	.1516	.1495	14.43	1.107	182.4				2.65	279	734	827
54	57.79	.15	4.24	26.5	− .0224	.1620	.1528	10.15	1.040	128.3	91.8	+0.64	.0111	16.97	167	− 481	− 395
46	60.05	.10	4.29	24.3	− .0211	.1596	.1530	7.06	1.055	89.2	57.9	+0.52	.0087	18.13	213	− 863	− 788
49	58.85	.05	4.36	21.5	− .0184	.1566	.1517	5.41	1.074	68.4	43.3	+0.55	.0093	17.09	144	− 877	− 816
30	59.03	.06	4.52	16.1	− .0139	.1549	.1507	4.68	1.086	59.2	44.9	+0.05	.0008	17.46	177	− 397	− 362
39	57.23	.05	4.79	8.9	− .0075	.1524	.1494	3.48	1.102	44.0	39.6	+0.82	.0143	17.82	285	294	305
29	57.42	.06	4.98	5.3	− .0040	.1523	.1488	4.06	1.103	51.3	49.7	+0.91[a]	.0158[a]	18.93	402	682	686
18	27.28	.00	5.28	1.2	− .0005	.1506	.1489	4.19	1.114	53.0				8.35	521	945	945
17	56.20	.04	5.29	1.0	− .0008	.1506	.1477	3.45	1.114	43.6				19.33	506	851	851
20	8.95	.00	5.30	1.0	− .0001	.1500	.1488	8.94	1.119	113.0				2.51	521	1761	1761
21	75.03	.16	5.35	0.3	− .0003	.1521	.1482	3.47	1.103	43.9				28.52	560	933	933
19	17.69	.15	5.42	− 1.1	+ .0002	.1498	.1488	3.82	1.120	48.3				5.07	441	796	796
28	57.71	.18	6.19	− 6.5	.0052	.1512	.1482	3.48	1.111	44.0	41.6	+0.41[a]	.0072[a]	21.48	679	957	963
27	56.17	.42	6.64	− 9.2	.0076	.1518	.1491	3.21	1.106	40.6	35.8	+0.32[a]	.0057[a]	21.40	764	911	923
41	16.76	.02	7.00	−11.7	.0029	.1514	.1513	0.42	1.109	5.3		+0.42	.0251	5.19	934	839	859
22	50.15	.07	7.06	−12.2	.0090	.1537	.1501	4.73	1.093	59.8	51.5	−0.03[a]	− .0006[a]	19.27	868	1014	1035
23	29.46	.12	7.06	−12.2	.0053	.1520	.1503	3.82	1.105	48.3		+0.62[a]	.0212[a]	9.90	875	912	933
35	95.95	.18	7.28	−13.9	.0194	.1555	.1501	3.70	1.082	46.8	36.2	−1.32	− .0118	50.65	1013	963	989
25	66.88	.31	7.28	−13.9	.0137	.1543	.1501	4.15	1.090	52.5	41.8	−0.19[a]	− .0029[a]	29.45	968	942	970
62	8.71	.00	7.43	−15.1	.0035	.1526	.1508	13.66	1.101	172.7				2.93	293	2141	2175
36	59.72	.09	7.45	−15.3	.0133	.1534	.1501	3.66	1.095	46.3	33.2	−0.25	− .0042	25.15	959	740	773
26	59.76	.09	7.54	−16.1	.0141	.1554	.1514	4.37	1.082	55.2	41.0	−0.52[a]	− .0087[a]	25.24	966	758	793
37	58.64	.16	7.61	−16.6	.0142	.1540	.1514	2.91	1.092	36.8	21.5	−0.57	− .0098	24.24	930	502	540
50	57.33	.14	7.97	−20.5	.0171	.1541	.1522	2.17	1.091	27.4	4.1	−1.28	− .0224	24.34	1023	111	169
34	57.11	.14	8.15	−22.9	.0190	.1542	.1511	3.56	1.090	45.0	15.9	−1.44	− .0253	24.46	1052	− 78	− 4
53	66.73	.78	4.16	24.1	− .02336	.06615	.04274	6.63	2.273	846.1				22.87	436		
45	61.30	.00	5.41	− 0.3	.00024	.05175	.04871	9.87	2.761	126.5		−1.63	− .0266	22.30	583	1250	1250
44	55.95	.13	7.55	−16.1	.01310	.05444	.05122	10.93	2.655	140.0	86.3	−3.45	− .0618	29.59	1744	601	867
52	55.18	.05	4.19	22.7	− .0236	.1072	.1024	8.31	1.516	105.2	59.3	+1.15	+ .0209	16.54	216	−1460	−1302
33	58.50	.16	5.37	0.0	.0000	.1004	.0977	4.68	1.605	59.4		−0.56	− .0096	20.12	489	868	868
31	56.10	.12	7.27	−13.7	.0115	.1035	.1004	5.43	1.563	68.9	51.4	−1.15[a]	− .0205[a]	24.04	1071	838	900
51	56.33	.09	4.26	25.6	− .0209	.2087	.2017	6.06	0.811	76.5	52.0	+0.78	+ .0139	16.89	214	− 608	− 566
43	58.08	.04	5.39	0.1	.0001	.2030	.1994	3.08	.834	38.9		−0.15	− .0026	20.51	548	924	924
32	55.92	.21	7.23	−13.5	.0112	.2034	.2008	2.32	.833	29.3	22.2	−0.66[a]	− .0107[a]	21.61	797	748	761

[a] With potassium nitrate bridge.

tions were made up from an approximately 12% stock solution in distilled water by addition of calculated amounts of distilled water, 1.5 M sodium chloride solution, and 0.1 N sodium hydroxide or hydrochloric acid, the latter reagents being added slowly and with sufficient stirring to minimize any possible effects of local excesses.

Experimental Results

The experimental results for bovine albumin No. 4-5-6 in sodium chloride solutions are given in Table I. The albumin concentration inside the membrane is given in column 2, and that outside in column 3. The pH values in column 4 were measured on an aliquot of the inside solution at the conclusion of the pressure measurement. The values of z_2 in column 5 were read from the titration curve of bovine albumin No. 4-5-6 in 0.15 M sodium chloride and at a protein concentration of 55–65 g. per kg. of water, obtained from pH measurements on these solutions prior to the pressure measurements. The difference between the sodium and chloride ion concentrations inside the membrane, $m_{Na^+} - m_{Cl^-} = -z_2m_2$, and the average of these concentrations $(m_{Na^+} + m_{Cl^-})/2$, given in columns 6 and 7, were calculated from the measured chloride ion concentrations inside the membrane and the values for z_2 and w_2; in column 8 is given m_2' the salt concentration in the solution outside the membrane. The osmotic pressure, in millimeters of mercury, is given in column 15. B_2, b_{23}, β_{22}, β_{23} and β_{33} are defined by *equations 30, 21, 10, 11* and *12*, respectively.[10a]

The membrane potential E_m, in millivolts, and

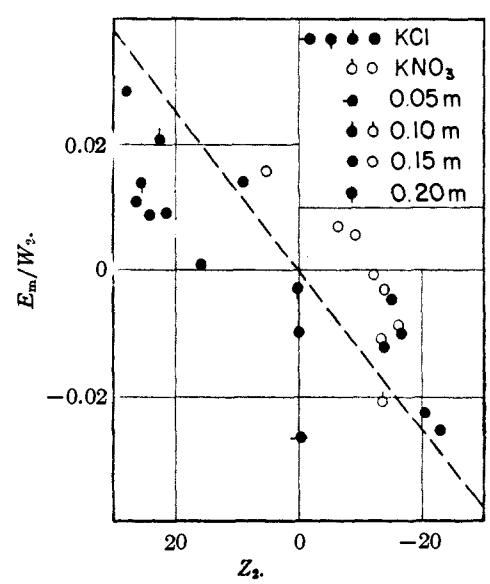

Fig. 3.—Membrane potential.

(10a) When w_2' differs from zero, $w_2 - w_2'$ is substituted for w_2 and $w_2^2 - w_2'^2 = (w_2 - w_2')(w_2 + w_2')$ for w_2^2 in these equations and in those which follow.

the ratio of E_m/w_2 are also listed, and E_m/w_2 is plotted against z_2 in Fig. 3. The broken line in this figure represents E_m/w_2 for ideal solutions with the same values of $z_2/\overline{W}_2$ and with $m_3 = 0.15$. The membrane potential with a potassium nitrate bridge is definitely more positive than that with a potassium chloride bridge.

Protein-Salt Interaction

The distribution of salt across the membrane at *p*H 5.37 and 7.0 is illustrated in Fig. 4, in which $\ln m_3/m_3'$ is plotted against w_2. It is estimated that, in the case of 0.15 *M* solutions, individual analyses have an uncertainty of approximately 0.25%. Since each point in the figure depends upon two analyses, the experimental uncertainty is somewhat less than 0.5%. Within the limits of experimental error, the distribution may be represented by the equation

$$\ln m_3/m_3' = -b_{23}w_2 \qquad (1)$$

with b_{23} independent of the protein concentration. It is dependent upon the salt concentration and upon the valence of the protein. The line in Fig. 4 is drawn to fit the two higher points at *p*H 5.37. It corresponds to a value of -3.43×10^{-4} for b_{23}.

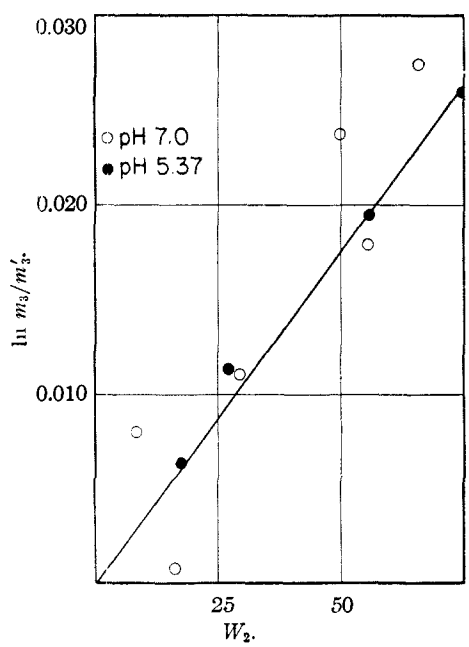

Fig. 4.—Distribution of sodium chloride.

The variation of b_{23} with z_2 and with the salt concentration is shown in Fig. 5. Only those experiments with $w_2 > 50$ g. per kg. of water are illustrated. From *equation 27*, we may determine $\beta_{23} = \mathrm{d} \ln \gamma_2/\mathrm{d}m_3 = 2\,\mathrm{d} \ln \gamma_3/\mathrm{d}m_2$ as

$$\beta^0_{23} = \overline{W}_2 b_{23} (2 + \beta^0_{33} m^0_3) \qquad (2)$$

for the contribution of $\overline{V}^0_3/V_m{}^0$ is less than our experimental error. β_{33} was calculated from the osmotic coefficient at 25° determined from the

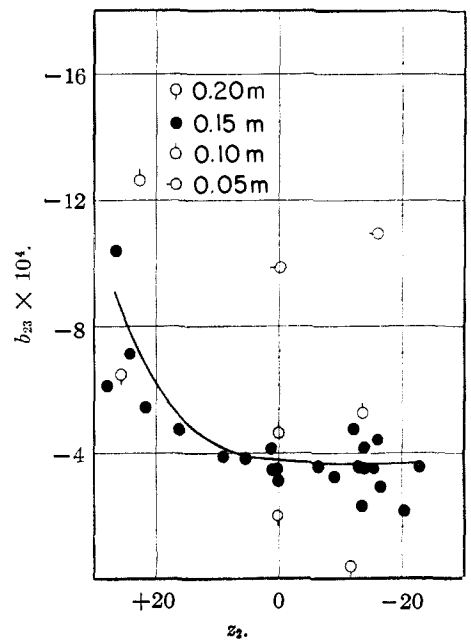

Fig. 5.—Distribution of sodium chloride.

freezing point measurements of Scatchard and Prentiss[11] and the heat of dilution measurements of Gulbransen and Robinson,[12] which give

$$\beta_3/2 = \ln \gamma_3 = \frac{-1.172\sqrt{m_3}}{1 + 1.55\sqrt{m_3}} + 0.0251 m_3 + 0.0212\, m_3^2$$

for sodium chloride at 25° from $m_3 = 0$ to 1. β_{23} is plotted as filled circles in Fig. 6 as a function of m_3 for $z_2 = 24$, 0 and -16, and in Fig. 7 as a function of z_2 for $m_3 = 0.15$. It is evident that the behavior of the isoionic protein and that with a valence of -16 are very similar.

There must be an electrostatic effect, however, which varies with the valence. At such large ionic strengths, it does not depend upon the valence alone, and the part of β^0_{23} which is to be attributed to electrostatic action is somewhat arbitrary. We shall take it to be given by the Debye-Hückel expression for a spherical ion with valence z_2 and a radius of 30 Å., or with a value of a in sodium chloride solutions of 32.5 Å. A 30 Å. sphere has the same volume as the ellipsoid of revolution with major axis 150 Å. and minor axis 38 Å. which appears to represent the albumin molecule.[13] At 25°

$$(\ln \gamma_k)_e = -\frac{1.17\, z_k^2\sqrt{I/2}}{1 + \kappa a} = -\frac{1.17\, z_k^2\sqrt{I/2}}{1 + 0.328a\sqrt{I/2}} \qquad (3)$$

if a is in Ångströms and I is the ional concentration in moles per kilogram of water, or twice the ionic strength.[14] Since the ions of sodium

(11) G. Scatchard and S. S. Prentiss, THIS JOURNAL, **55**, 4355 (1933); **56**, 2314 (1934).

(12) E. A. Gulbransen and A. L. Robinson, *ibid.*, **56**, 2637 (1934).

(13) E. J. Cohn, *Trans. Coll. Physicians Phila.*, [4] **10**, 149 (1942).

(14) J. G. Kirkwood, *J. Chem. Phys.*, **2**, 351 (1934).

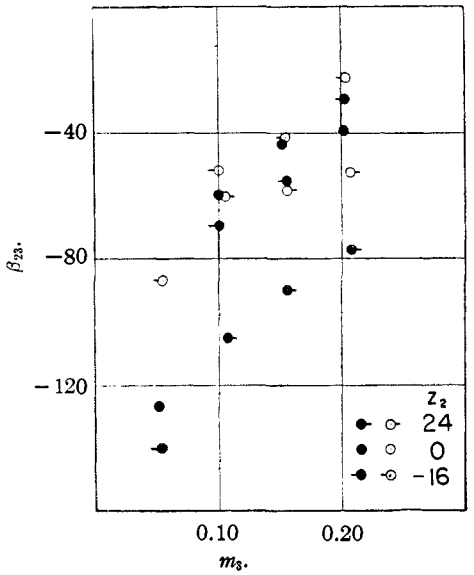

Fig. 6.—Protein–salt interaction.

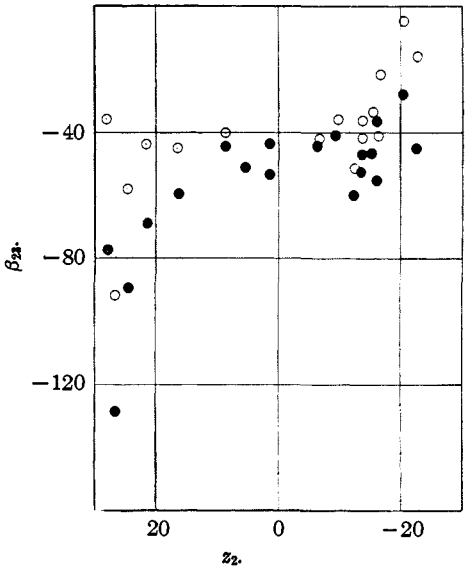

Fig. 7.—Protein–salt interaction.

chloride are both univalent

$$(\beta^0_{23})_e = \frac{2(\mathrm{d}\ln\gamma_3)_e}{\mathrm{d}m_2} = -\frac{1.17z_2^2}{\sqrt{m_3}(1+0.328a\sqrt{m_3})^2} \qquad (4)^{14a}$$

The open circles in Figs. 6 and 7 are $\beta^0_{23} - (\beta^0_{23})_e$.

This simple electrostatic effect accounts for a large part of β^0_{23} when $z_2 = -26$ or $+24$, but not when $z_2 = 0$. The remainders $\beta_{23} - (\beta_{23})_e$ are roughly proportional to $1/m_3$ for each value of z_2. This would be accounted for if there were a tight chemical binding of a few ions of the salt and but little interaction other than that represented in $(\beta_{23})_e$ with those not bound.[15a] Then the number bound to each albumin molecule would be $[\beta^0_{23} - (\beta^0_{23})_e]m_3$ which should be independent of the salt concentration. The values so obtained are given in Table I for the points in Fig. 7. This indicates a compound with 6 or 7 ions when z_2 is $+24$ or 0, and with 3 or 4 ions when z_2 is -16. This cannot be taken as proof of the correctness of our calculation of the electrostatic contribution, for $\beta^0_{23}m_3$, also listed in Table I, is also nearly independent of m_3. If we ignored the electrostatic effect, we would say that there is a compound with 6 or 7 ions when z_2 is -16 or 0, and one with about 12 ions when z_2 is $+24$. We can say that β_{23} for isoionic albumin can be explained by a compound with 6 or 7 molecules of one of the ions of sodium chloride, and that the number bound probably increases as the valence becomes more positive. The facts that the membrane potential, with potassium chloride bridge, is more negative than the ideal curve and that the apparent compound formation, as well as the deficiency of the membrane potential, increases as the valence becomes more positive both indicate that the chloride ion is combined more strongly than the sodium ion, which may not be combined at all. The ultra filtration experiments of Luck and his collaborators[16] show much more complex formation with large organic anions.

(14a) It would have been more exact to use the mean square valence, $\bar{z}_2^2$, which is greater than z_2^2 by the amount $-\mathrm{d}z_2/2.3\ \mathrm{d}p\mathrm{H}$.[15] From our titration curve at 0.15 M sodium chloride, we obtain the following values for $\bar{z}_2^2 - z_2^2$.

z_2	30	20	10	0	−10	−20	−30
$\bar{z}_2^2 - z_2^2$	22	15	9	5	4	4	5

We have not measured the values at other salt concentrations, which increase with increasing salt concentration. The difference is small relative to the uncertainty of our other assumptions.

(15) Cohn and Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publishing Co., New York, N. Y., 1943, pp. 71, 462.

(15a) The combination of chloride ion with neutral protein was noted by W. Pauli and T. Oryng, *Biochem. Z.*, **70**, 368 (1915).

Molecular Weight of Albumin

The relation of the osmotic pressure to the albumin concentration at pH 7.0 and 5.37 when $m_3 = 0.15$ is shown in Fig. 8, in which P/w_2, the ratio of the osmotic pressure in mm. at 0° to the albumin concentration in grams per kg. of water, is plotted against w_2. This relation may be expressed, by *equations 30* and *32*

$$P = A_2w_2(1 + B_2w_2) \qquad (5)$$

$$A = RT/V^0_{\mathrm{m}}\overline{W}_2 \qquad (6)$$

in which R and T have their usual significance, $\overline{W}_2$ is the molecular weight of the albumin, V^0_{m} is the volume of the solution containing one kilogram of water when w_2 is zero. The value of B_2 is independent of the protein concentration in the concentration range of these experiments within the accuracy of our measurements.[16a] Since the pH of the individual experiments deviate slightly

(16) P. D. Boyer, G. A. Ballou and J. M. Luck, to be published.

(16a) Later measurements upon more concentrated solutions indicate a positive curvature, which would make our values of B determined at $w_3 = 60$ too large perhaps by as much as 10×10^{-4}. This would make the values of β_{23} about 140 too positive. The effect seems to be nearly independent of the valence.

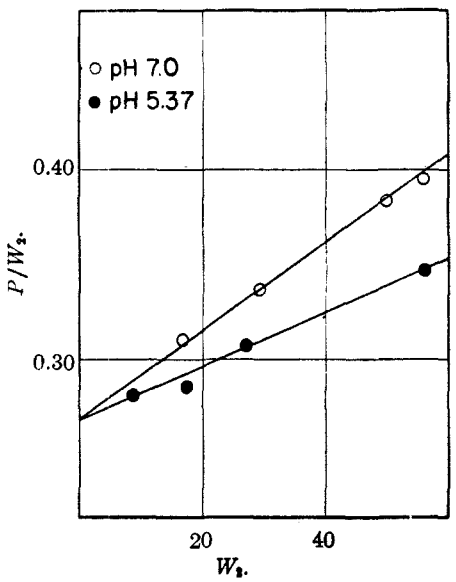

Fig. 8.—Osmotic pressure.

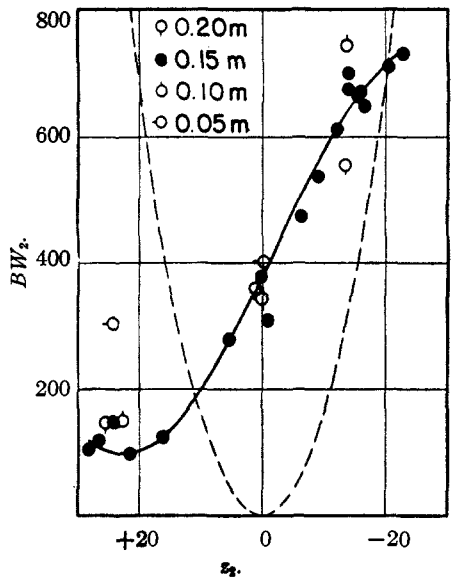

Fig. 9.—Osmotic pressure.

from 7.0 or 5.37, the P/w_2 ratios were corrected for this difference before plotting by using the fact that B_2 is a linear function of z_2 within this pH range.

The intercept of the curves upon the P/w_2 axis in Fig. 8 determines the value of A. We have employed the value of 0.268 calculated by least squares from the eight points below $w_2 = 60$. This corresponds to a value for the molecular weight of 69,000. Adair and Robinson[17] have reported a preliminary value of 70,000 from osmotic pressure measurements. Our results may also be compared with the value of 68,000 from sedimentation and diffusion measurements by Oncley[13] on this same bovine albumin preparation.

The relation between B and the valence of the albumin, z_2, and the salt concentration is illustrated in Fig. 9, presenting the measurements at albumin concentrations greater than 50 g. per kg. H_2O. The points were calculated from the pressure and concentration by equation (1), using the value of 0.268 for A_2. The solid curve is drawn to fit the experimental points for 0.15 M sodium chloride. The measurements on solutions at sodium chloride concentrations other than 0.15 M are indicated by open circles suitably indexed.

For ideal solutions, the osmotic pressure and valence of the protein are related by the equation

$$P = \frac{RT}{V^0_m}\frac{w_2}{\overline{W}_2}\left[1 + \frac{w_2 z_2^2}{4m_3^0\overline{W}_2}\right] \qquad (7)$$

where $RT/V^0_m w_2$ equals A_2 (equation (5)) and $z_2^2/4m_3\overline{W}_2$ corresponds to B_2. In Fig. 10 we have plotted as a broken line $z_2^2/4m_3\overline{W}_2$ against z_2 for $m_3 = 0.15$. For any other value of m_3, the ordinates of this dotted curve should be multiplied by $0.15/m_3^0$. The difference between this and the experimental curve is due to the departure from ideality of the solutions. For the solutions containing 0.15 M NaCl, the experimental curve may be described from $-z_2 = -9$ to $+17$ (pH = 4.9 to 7.1) by the linear equation

$$B_2 = (56.4 - 2.82\, z_2) \times 10^{-4} \qquad (8)$$

Since the curve for ideal solutions is parabolic, the deviations from ideality within this range must obey a quadratic equation in z. It is remarkable that over such a wide range in pH, in which the number and distribution of the charges on the protein molecule vary widely, such a simple expression would be found to obtain.

One outstanding difference between the experimental and ideal curves is in the location of the minimum. The ideal curve is centered on $z_2 = 0$ at pH = 5.37, which is the pH, determined by measurements with a hydrogen electrode, of electrodialyzed solutions of this bovine albumin preparation after the addition of 0.15 mole of sodium chloride per kg. of water. The same solution before addition of sodium chloride had a pH of 5.18. The minimum in the experimental curve is located at $z_2 = 22$, corresponding to a pH of 4.55. Thus, because of the deviations from the behavior of ideal solutions, the pH of minimum osmotic pressure of a protein may lie at an appreciable distance from the isoionic point of the protein, introducing inherent uncertainties into the use of this minimum as a practical method for determining the isoionic point.

The protein–protein interaction is related to B_2 by *equation 34* as

$$2B\overline{W}_2 = \frac{z_2^2}{2m_3^0} + \beta_{22} - \left(\frac{dm_3}{dm_2} - \frac{dm_3'}{dm_2}\right)^2\left(\frac{2}{m_3^0} + \beta_{33}\right) \qquad (9)$$

An approximate value for the electrostatic contribution to β_{22}, using the same model as that used to calculate the contribution to β_{23}, may be determined from the electrical potential at the surface

(17) G. S. Adair and M. E. Robinson, *Biochem. J.*, **24**, 1864 (1930).

of an ion with closest distance of approach a_{22} for some of the ions and a smaller distance a_{23} for other ions.[18] In our case $\tau = a = 32.5$ Å. and $\sigma = 2b = 60$ Å.

Application of the Güntelberg-Müller charging process to equation 23 of that paper, corrected for some typographical errors, and rearranging gives, for our case

$$(\beta_2)_e = \frac{-\frac{\epsilon^2 z_2^2 \kappa'}{2DkT}\left[1 + \frac{\kappa - \kappa'}{\kappa + \kappa'} e^{-2\kappa'(a_{22}-a_{23})}\right]}{1 + \kappa' a_{23} - (1 - \kappa' a_{23})\frac{\kappa - \kappa'}{\kappa + \kappa'} e^{-2\kappa'(a_{22}-a_{23})}} -$$

$$\left(\frac{\mu_2}{\mu_3}\right)\frac{\kappa'}{\kappa + \kappa'} e^{-\kappa'(a_{22}-a_{23})}$$

$$\left[\frac{1 + \kappa a_{23}}{(1 + \kappa' a_{33}) - (1 - \kappa' a_{33})\frac{\kappa - \kappa'}{\kappa + \kappa'} e^{-2\kappa'(a_{22}-a_{23})}} - \right.$$

$$\left.\frac{1 + \kappa a_{22}}{(1 + \kappa' a_{23}) - (1 - \kappa' a_{23})\frac{\kappa - \kappa'}{\kappa + \kappa'} e^{-2\kappa'(a_{22}-a_{23})}}\right] \quad (10)$$

$$\kappa' = \rho\sqrt{\mu_2} \quad (11)$$

$$\kappa = \kappa'\sqrt{1 + \mu_2/\mu_3} \quad (12)$$

$\mu_2 = z_2^2 m_2/2$ and $\mu_3 = \Sigma_i \nu_{3i} z_i^2 m_3/2$

Then

$$(\beta_{22}^1)_e = -\frac{\epsilon^2 z_2^4}{2DkT}\frac{\rho}{4\sqrt{\mu_3^0}}\frac{e^{-2\kappa(a_{22}-a_{23})}}{(1 + \kappa a_{23})^2} -$$

$$\frac{\rho^2 z_2^2}{2} e^{-\kappa(a_{22}-a_{23})}\left[\frac{(a_{23}^2 - a_{22}a_{33})}{(1 + \kappa a_{23})(1 + \kappa a_{33})}\right] \quad (13)[19]$$

If $r_2 = a_{22}/2$ and $r_3 = a_{33}/2$, $r_2 + r_3 = a_{23}$ and the second term of equation 13 may be written

$$(\beta_{22}^{00})_e^r = -\frac{z_2^2}{2\mu_3^0} e^{-\kappa(r_2-r_3)^2}\left[\frac{\kappa^2(r_2 - r_3)^2}{[1 + \kappa(r_2 + r_3)](1 + \kappa 2r_3)}\right] \quad (14)$$

$$= -\frac{z_2^2 \rho^2 (r_2 - r_3)^2}{2}\frac{e^{-\kappa(r_2-r_3)}}{[1 + \kappa(r_2 + r_3)](1 + \kappa 2r_3)} \quad (15)$$

Equation 10 leads to the same value of β_{23}^0 as equation 3, and permits the calculation of β_{23} at finite values of m_2.

In Fig. 10, β_{22}^0 is plotted against m_3 for $z_2 = -16$, 0 and 24, and in Fig. 11, β_{22} is plotted against z_2 for $m_3 = 0.15$ and $w_2 > 50$. In both figures the filled circles are the values of β_{22}^0 determined from equation 9, and the open circles are $\beta_{22}^0 - (\beta_{22}^0)_e$, with the latter determined from equation 13. At the values of z_2 and m_2 at which we worked, the first term in equation 13 may be neglected. In the limit $m_3 = 0$, the second term is equal to

$$(\beta_{22}^0)_e^r = -\frac{z_2^2 \rho^2 (r_2 - r_3)^2}{4} \quad (16)$$

but it decreases rapidly with increasing m_3, both because of the denominator and of the exponential. The correction for electrostatic effect brings the results at valence -16 in closer agreement with the isoionic albumin, but the correction is much smaller than the change with valence for positive values of z_2.

(18) G. Scatchard, *Physik. Z.*, **33**, 22 (1932).

(19) If we consider average values as for equation 4, we use $\overline{z_1^2}$ for the first term and $\overline{z_2^2}$ for the second.

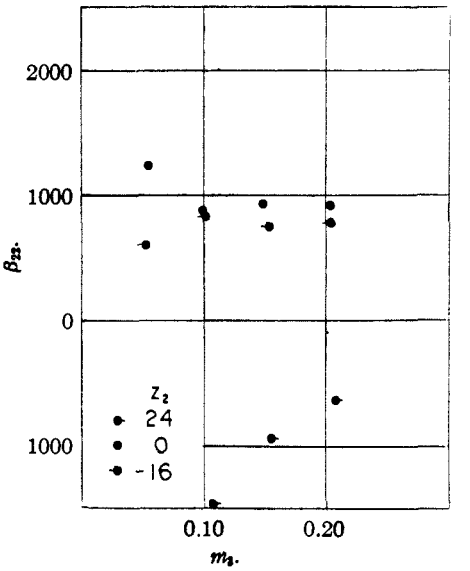

Fig. 10.—Protein-protein interaction.

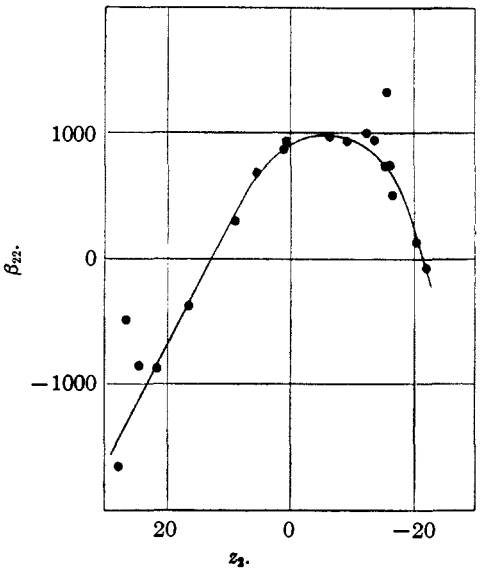

Fig. 11.—Protein-protein interaction.

Osmotic Pressure in the Absence of Salt

Table II gives the results of several measurements of the osmotic pressure of electrodialyzed isoelectric bovine albumin in the absence of salt.

TABLE II

OSMOTIC PRESSURES OF SALT FREE ISOIONIC ALBUMIN

Number	w_2	P	$P/0.268w_2$	$B \times 10^4$	β_{22}
69	19.71	7.44	1.409	207	2860
68	46.05	12.97	1.051	11	153
67	63.25	19.62	1.157	25	342
72	19.56	4.26	.813	−96	−1320
71	40.88	8.14	.743	−63	− 867
70	60.81	11.31	.694	−50	− 684

Because of the large errors that can be introduced into measurements under these conditions by traces of acid or alkali, extreme precautions were found to be necessary. In experiments 67–69, the membranes and osmometers were exhaustively washed before use with conductivity water that had been collected under nitrogen, the albumin solutions were made up in the same solvent, and contact of the solutions with the air was kept to a minimum. During these measurements the pressure increased at a steady rate of approximately 0.2 mm. of toluene per hour and the solutions became more alkaline by 0.2–0.3 pH unit, as noted in Table II.[20] Since it seemed probable that these results could be attributed largely to the solution of alkali from the glass, conductivity water in equilibrium with air at 25° was used in experiments 70–72 in order to decrease the pH change by the presence of the dissolved CO_2. These pressures showed a greater stability (increases of 0.00, 0.06 and 0.10 mm. of toluene per hour, respectively) and the pH readings remained essentially unchanged. This extreme sensitivity of the osmotic pressures of salt-free isoelectric protein solutions to traces of acid or alkali has been noted previously by Adair, and it is also to be expected from theoretical considerations.

Although the initial change of the activity coefficient of isoionic albumin in the absence of salt must be proportional to the square root of its concentration because the average of the square of its valence is about five, the large value of a reduces the electrostatic term very rapidly. Since water is the only diffusible component, the electrostatic contribution to the osmotic coefficient can be calculated as for a simple salt. It varies from -0.014 to -0.016 as w_2 varies from 20 to 60, and is therefore entirely negligible compared with the error of these measurements.

The two techniques yield different signs for B and β_{22}, with a very wide scatter in dilute solutions for either technique. So we have not tried to utilize these results in the interpretation of our measurements in salt solutions.

Discussion

The value of β_{22} for isoionic albumin, about 1000, is very large. If we were to invoke hydration to account for it, we would have to assume 500 kilos of water of hydration per mole of albumin, or 7 g. of water per gram of albumin. The magnitude of β_{22} does correspond to the large size of the albumin molecule, however. According to the simple theory of non-electrolyte solutions[21] in

$$\beta_{22} = \frac{-2A_{12}}{1000\,RT}\overline{V}_2^2$$

in which R and T have their usual significance, $\overline{V}_2$ is the partial molal volume of the albumin, and A_{12} is the energy of transfer of unit volume of water from pure water to an infinite volume of liquid albumin. If the specific volume of albumin is 0.75, A_{12} is -0.08 cal./cc. This may be compared to the $+550$ cal./cc. of transfer from liquid water to a vacuum. The experimental value of β_{22} is also very small compared to the value computed from the equation for the effect of volume of the solute on the entropy of mixing which Huggins obtains by generalizing his calculations for long chain polymers,[22] which is 160,000.

The albumin–albumin interaction is thus huge if viewed from the point of view of moles, but tiny from the point of view of grams. For practical purposes we are usually interested in an intermediate quantity, the change of the excess chemical potential per mole, or of the logarithm of the activity coefficient, when unit weight of the material is added, which is $\beta_{22}/\overline{W}_2$. It is this quantity which would determine the effect of unit weight of albumin upon the solubility of another protein which differed from it only in being slightly soluble. For isoionic albumin $\beta_{22}/\overline{W}_2$ is 0.01, so that the solubility of this hypothetical protein would be reduced one per cent. for each gram of albumin per kilo of water.

When we consider the action of sodium chloride on this hypothetical protein, we use $\beta_{23}/\overline{W}_3$ and find that in 0.15 M sodium chloride the rate of solubility increase corresponds to doubling for each gram of sodium chloride per liter. In more dilute salt solutions the effect would be even greater. When we consider the effect of albumin on a salt which differs from sodium chloride only in having a solubility of 0.15 molal, we use $\beta_{23}/2\overline{W}_2$ to find that the solubility is increased only 0.03% for each gram of albumin per liter. For any small solubility the effect would be the same as though each gram of albumin removed a tenth of a millimole of chloride ion from solution.

Summary

The osmotic pressure and the distribution of sodium chloride across a membrane impermeable to albumin have been measured for aqueous solutions of crystallized bovine serum albumin and sodium chloride at 25°, with albumin concentration varied from one to six per cent., salt concentration from 0.05 to 0.20 molal, and pH from 4.2 to 8.2.

In this range the logarithm of the ratio of the

(20) The pH of isoelectric albumin is about 0.3 unit higher in 0.01 M sodium chloride than in water. So the change is probably not all true increase in alkalinity.

(21) C. Scatchard, *Chem. Rev.*, **8**, 321 (1931).

(22) M. L. Huggins, THIS JOURNAL, **64**, 1712 (1942). His equation (2) may be written

$$\ln \gamma_2 = -\ln(1 + R_2 r_2) + \frac{(R_2 - 1)R_2 r_2}{1 + R_2 r_2} - \frac{\overline{V}_1 R_2 A_{12}}{RT(1 + R_2 r_2)^2}(2R_2 r_2 - R_2^2 r_1^2)$$

if r_1 is the ratio of the number of moles of solute to number of moles of solvent, or $0.018\,m_2$ for aqueous solutions, R_2 is the ratio of the partial volumes $\overline{V}_2/\overline{V}_1$. This gives

$$\beta_{22}^2 = \frac{\overline{W}_1}{10{,}000}\left\{[R_2^2 - 2R_2] - \frac{2\overline{V}_1 R_2^2 A_{12}}{RT}\right\}$$

salt concentrations on the two sides of the membrane was found to be proportional to the albumin concentration, and approximately inversely proportional to the salt concentration. Such a relation might result from a tight binding of a few, about six, salt ions to each albumin molecule.

The membrane potentials and other independent measurements indicate that it is the chloride ion which is bound.

The osmotic pressure was found to be a quadratic function of the albumin concentration. The first term corresponds to a molecular weight of 69,000. From the second term, the salt distribution and the valence of the albumin, the effect of albumin on its own activity coefficient was calculated. This varies from a large positive value for neutral albumin to a larger negative value for albumin in acid solutions. Some of the implications are discussed.

CAMBRIDGE, MASS. RECEIVED MAY 27, 1946

[CONTRIBUTION FROM THE DIVISION OF CHEMISTRY, COLLEGE OF AGRICULTURE, UNIVERSITY OF CALIFORNIA]

Polarographic Determination of Cupric Glycinate and Cupric Alaninate Complex Ions

BY R. M. KEEFER

Many investigators[1] have studied the complex ions formed between cupric ion and glycinate (G^-) or alaninate (A^-) ion. Boorsook and Thimann[1] concluded from spectrophotometric and electrometric data that the complex ions formed in basic solutions were CuG_2 and CuA_3^-. Gould and Vosburgh[2] using the method of continuous variation determined from spectrophotometric data that with equal molal quantities of cupric ion and glycinate ion CuG^+ was formed and that with glycinate ion more than twice the concentration of cupric ion that CuG_2 was formed. Riley and co-workers[3,4] used a cupric ion concentration cell and state that CuG_3^- and CuA_3^- are the main complex ions formed. Only the last investigation gave values for the dissociation constants of the complex ions. This investigation was undertaken using the dropping mercury electrode to determine the formula of the complex ions formed[5] and their dissociation constants.

Experimental

Glycine and alanine were purified by recrystallization. Potassium nitrate and potassium hydroxide were of Merck reagent quality. Stock solutions of potassium glycinate or potassium alaninate were prepared from the amino acids and potassium hydroxide using boiled distilled water. Potassium nitrate was used as a supporting electrolyte. At low glycinate or alaninate concentrations the electrode reaction was irreversible if the solutions were prepared using equivalent amounts of amino acid and potassium hydroxide. To obtain values at low glycinate or alaninate concentrations runs were made with solutions containing less than the equivalent amount of potassium hydroxide. The pH of all solutions was determined using a Coleman Model 3D pH Electrometer. To minimize errors in calculating glycinate or alaninate concentrations from the pH the pK_2 for glycine and alanine was determined at the same ionic strength using carbonate free sodium hydroxide.

All solutions to be analyzed by the dropping mercury electrode were made up to 5×10^{-4} M cupric nitrate. Sufficient potassium nitrate was added to keep the ionic strength constant at $\mu = 1.0$ or 0.1. Methyl red (0.003%) and brom cresol green (0.002%) were used as a maximum suppressor. The solutions were prepared and analyzed at 25.00 ± 0.05°. The solutions were analyzed using a Fisher Elecdropode modified as follows: (1) A cell was constructed[6] so that oxygen could be eliminated by bubbling nitrogen through the solution. (2) Since the potential of the quiet electrode varied during the electrolysis, the potential of the dropping electrode was checked at every voltage against a saturated calomel electrode using a Leeds and Northrup student's potentiometer. The currents were corrected by subtracting the current due to the supporting electrolyte. The reversibility of the electrode reaction was tested for each analysis by plotting log $i/(i_d - i)$ against $E_{d.e.}$. The analysis was discarded unless a straight line[5] was obtained with $\Delta E/\Delta \log i/i_d - i = 0.035 \pm 0.002$. 5×10^{-4} M cupric nitrate in 0.1 M potassium nitrate solutions gave a slope of 0.035 (calcd. = 0.0296). The value of $E_{1/2}$ was obtained from the plot of $E_{d.e.}$ *vs.* log $i/(i_d - i)$ at log $i/(i_d - i) = 0$. $E_{1/2}$ values could be duplicated, in the solutions up to 0.1 M, to ±1 mv. At higher glycinate ion concentrations the slope tended to increase till at 1 M glycinate ion the slope was 0.039 ± 0.003. The $E_{1/2}$ values at 1 M glycinate were reproducible to ±5 mv.

Discussion

The reduction to a metallic state (amalgam) of a complex ion of copper may be represented by

$$CuX_p^{(a-pb)+} + ne + Hg \rightleftarrows Cu(Hg) + X^{-b} \quad (1)$$

(1) Summary in Boorsook and Thimann, *J. Biol. Chem.*, **98**, 671 (1932).

(2) Gould and Vosburgh, THIS JOURNAL, **64**, 1630 (1942).

(3) Riley and Gallafent, *J. Chem. Soc.*, 2029 (1931).

(4) Ferrel, Ridgion and Riley, *ibid.*, 1440 (1934).

(5) Lingane, *Chem. Rev.*, **29**, 1 (1941).

(6) Kolthoff and Lingane, "Polarography," Interscience Publishers, Inc., New York, N. Y., 1941, p. 245.