[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, MASSACHUSETTS INSTITUTE OF TECHNOLOGY]

Physical Chemistry of Protein Solutions. IV. The Combination of Human Serum Albumin with Chloride Ion[1]

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The interaction of proteins with small ions and molecules has become increasingly important to the chemist and the physician. Combinations of proteins with small anions and cations,[4-8] sulfonamides,[9,10] dyes,[11] alkyl sulfates,[12] fatty acids[13] and aromatic compounds[14] have recently been described. The present work was undertaken in an attempt to clarify the general principles involved in protein-small ion interaction. For these purposes human serum albumin and sodium chloride were selected as a relatively simple system to study and one of special importance because the natural environment of the serum proteins contains much sodium chloride. A previous investigation of the osmotic pressure of serum albumin in solutions of sodium chloride[8] and of the effect of salts on the isoionic points of proteins[15] showed that albumin in 0.15 molar sodium chloride binds several chloride ions but probably no sodium ion.

In the present work, we calculate from the experimental measurements, with the aid of simplifying assumptions, the average number of chloride ions bound to each albumin molecule in solutions of varied composition of sodium chloride and albumin. These results, and further assumptions, permit us to determine the number of groups in an albumin molecule which combine with chloride ion, and the intrinsic association constant for this binding.

Experimental

Two methods were used. The first was the determination of the distribution of sodium chloride across a cellophane membrane on one side of which albumin was present. This is essentially the method used by Klotz[11] in similar studies, with the modifications that the concentration of the small ion, in this case chloride, in the albumin-free solution, was measured by conductance, and no buffer was used. The second method made use of the electromotive force developed in a concentration cell both half-cells of which contained the same concentration of sodium chloride, and one of which also contained albumin.

Distribution Method.—Pyrex test-tubes, 200 × 25 mm., were used as dialysis vessels. Into each was placed a cellophane sac, tied at both ends, and containing a known amount of isoionic albumin and a Pyrex glass bead 15 mm. in diameter. Rubber gloves, washed in conductivity water, were used in filling and tying the membranes to avoid contaminating the solution with salt from the skin. The test-tube also contained known amounts of sodium chloride and conductivity water. All solutions were made by weight and the amounts of albumin, sodium chloride and water present were known to 1, 0.1 and 0.02%, respectively. Blank measurements, some without salt and some without protein, were also made.

After filling, each test-tube was stoppered tightly and place in a holder which turned it end over end every eight seconds in a room maintained at 5.0 ± 0.3°. The Pyrex bead, rolling from one end of the cellophane sac to the other stirred its contents and the motion of the sac stirred the outside solution. After twenty-seven hours the volume of solution inside each membrane was measured roughly (to 3%), and the conductivity of the solution outside each membrane was determined. The conductances were determined in a Washburn cell[16] immersed in an oil-bath thermostated at 9.68 ± 0.01°. Measurements were made at 1000 cycles using a bridge designed by Jones,[17] a General Radio Beat Frequency Oscillator, Type 913-B, and a General Radio Cathode Ray Null Detector, Type 707-A. The accuracy of the conductance measurements was 0.1%. A calibration curve giving concentration as a function of conductance was used, and the measured conductances were corrected for the conductance of the blank with protein but no salt. To minimize the latter correction the protein was dialyzed for two days against conductivity water before it was used in an experiment.

The average number of chloride ions bound to each albumin molecule, a quantity we will call $\bar{\nu}$,

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(1) The products of Plasma fractional employed in this work were developed from blood which was collected by the American Red Cross, by the Department of Physical Chemistry, Harvard Medical School, Boston, Massachusetts, under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and Harvard University.

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(4) J. Steinhardt, C. H. Fugitt and M. Harris, *J. Research Nat. Bur. Standards*, **28**, 201 (1942).

(5) I. M. Klotz and H. G. Curme, THIS JOURNAL, **70**, 939 (1948).

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

(7) E. J. Cohn and B. A. Koechlin, presented before Division of Biological Chemistry, American Chemical Society, September, 1947.

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

(9) B. D. Davis, *J. Clin. Investigation*, **22**, 753 (1943).

(10) I. M. Klotz and F. M. Walker, THIS JOURNAL, **70**, 943 (1948).

(11) I. M. Klotz, F. M. Walker and R. B. Pivan, *ibid.*, **68**, 1486 (1946).

(12) F. W. Putnam and H. Neurath, *ibid.*, **66**, 1992 (1944).

(13) B. D. Davis and R. J. Dubos, *Arch. Biochem.*, **11**, 201 (1946).

(14) J. D. Teresi and J. M. Luck, *J. Biol. Chem.*, **174**, 653 (1948).

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

(16) E. W. Washburn, THIS JOURNAL, **38**, 2431 (1916).

(17) G. Jones and R. C. Josephs, *ibid.*, **50**, 1049 (1928).

was calculated as follows. Let

g_1 = kilograms of water
n_2 = moles of albumin
n_3 = moles of sodium chloride
n_3^0 = total number of moles of sodium chloride in test tube
( ) denote concentration of unbound ion in moles per kilogram of water
n_{B+} and n_{B-} = total number of moles of sodium and chloride, respectively, bound to albumin
Primed quantities refer to the solution outside the cellophane sac
Unprimed quantities refer to the solution inside the cellophane sac

Define

$$\Delta n = n_3^0 - [g_1 + g_1'](n_3'/g_1')$$

Then, inside the membrane

$$(Na^+) = \frac{n_3^0 - n_3' - n_{B+}}{g_1} = \frac{\Delta n + n_3' \cdot \frac{g_1}{g_1'} - n_{B+}}{g_1}$$

$$(Cl^-) = \frac{n_3^0 - n_3' - n_{B-}}{g_1} = \frac{\Delta n + n_3' \cdot \frac{g_1}{g_1'} - n_{B-}}{g_1}$$

and, outside the membrane

$$(Na^+)' = (Cl^-)' = \frac{n_3'}{g_1'}$$

Assuming that the activity coefficients of the uncombined sodium and chloride ions are the same inside the membrane as outside, the Donnan equilibrium becomes

$$\frac{\left[\Delta n + n_3' \cdot \frac{g_1}{g_1'} - n_{B+}\right]}{g_1} \frac{\left[\Delta n + n_3' \cdot \frac{g_1}{g_1'} - n_{B-}\right]}{g_1} = \left[\frac{n_3'}{g_1'}\right]^2 \quad (1)$$

If equal amounts of sodium and chloride are bound, then $n_{B+} = n_{B-} = n_B$ and equation 1 reduces to

$$n_B = \Delta n$$

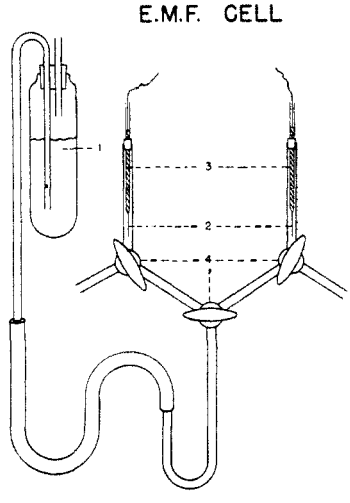

Fig. 1.—E. m. f. cell: 1, saturated potassium chloride reservoir; 2, Ag–AgCl electrode; 3, 3-mm. soft glass mounting, mercury filled, for electrode; 4, three-way, 120°, 4-mm. bore stopcocks (Corning no. 7450).

If unequal amounts of sodium and chloride are bound, then equation 1 may be reduced to

$$n_{B+} + n_{B-} = \frac{2\Delta n\left[1 + \frac{\Delta n\, g_1'}{2n_3'\, g_1}\right] + \frac{n_{B+}\, n_{B-}\, g_1'}{n_3'\, g_1}}{\left[1 + \frac{\Delta n\, g_1'}{n_3'\, g_1}\right]} \quad (2)$$

We shall assume that no sodium ion is bound,[5,12] *i. e.*, $n_{B+} = 0$, and then the second term of equation 2 drops out and we may calculate the number of moles of bound chloride, n_{B-}, since all quantities on the right side of equation 2 are known.

$\bar{\nu}$ may then be calculated since $\bar{\nu} = n_{B-}/n_2$.

E. m. f. Method.—For each experiment two solutions, designated by I and II, were made, by weight, with concentrations of sodium chloride, in moles per kilogram of water, differing by less than 0.1%. Solution II also contained albumin in a concentration of about 7×10^{-4} mole per kilogram of water.

Figure 1 is a diagram of the cell used.[8] After rinsing, each vertical limb of the cell was filled with portions of the same solution of sodium chloride having a concentration of salt close to that in solutions I and II. Each half of the salt bridge was swept out with saturated potassium chloride from the reservoir, and a silver–silver chloride electrode placed in each half-cell. One-hole rubber stoppers, carrying the glass mounting of the electrode, were held pressed to the top of each limb of the cell by rubber bands, making a virtually airtight seal. The electrodes were allowed to come to equilibrium with the solution for an hour or more. Then the liquid junctions were made by turning the stopcocks so that each of the half-cells was connected to the salt bridge, and the e. m. f. was read to 0.01 mv. This reading was the zero potential for the particular pair of electrodes. The cell was rinsed and refilled until successive fillings gave zero readings agreeing within 0.05 mv. Finally one-half of the cell was filled with solution I and the other with solution II, and the e. m. f. measured in the same manner. Readings were taken as soon as the liquid junctions were made and at minute intervals for six to ten minutes thereafter. The cell was drained, rinsed, refilled and the measurements repeated. No measurements were accepted unless at least two different pairs of electrodes gave results which agreed about as closely as the duplicate measurements with either pair.

The e. m. f. was measured using a Leeds and Northrup type K No. 7551 potentiometer and a Rubicon 6-volt box type galvanometer. For solutions more dilute than 0.001 molar the external resistance was so high that a Leeds and Northrup No. 7673 thermionic amplifier was inserted in the circuit. The majority of the experiments were made at room temperature, but measurements on four solutions were made at 5°, in an attempt to estimate the temperature coefficient of the reaction.

By analogy with the notations used for the distribution method, let

m_2 = the stoichiometric concentration of albumin in moles per kilogram of water
m_3 = the stoichiometric concentration of sodium chloride in moles per kilogram of water
( ) denote concentration of unbound ion in moles per kilogram of water
γ = the activity coefficient of unbound ion
Primed quantities refer to the albumin free solution (solution I)
Unprimed quantities refer to the solution containing albumin (solution II)

Then the voltage, E, of the cell is

$$E = \frac{RT}{F} \ln \frac{(Cl^-)' \gamma'}{(Cl^-) \gamma} \qquad (3)$$

with the electrode in solution II positive. R is the gas constant; T, the absolute temperature; and F, the faraday. We assume that $\gamma' = \gamma$. This is equivalent to saying that γ' and γ are functions only of the stoichiometric concentration of sodium chloride, which was the same, within 0.1%, in solutions I and II, and that the effect of the albumin on γ is negligible.

From equation 3 the ratio of free chloride in the two solutions, $(Cl^-)'/(Cl^-)$, may be calculated. Then, since $(Cl^-)' = m_3'$

$$(Cl^-) = \frac{m_3'}{(Cl^-)'/(Cl^-)}$$

and

$$\bar{\nu} = \frac{m_3 - (Cl^-)}{m_2}$$

Materials.—Human serum albumin was generously supplied by Dr. W. L. Hughes, Jr. All experiments were performed with material from lot 179-5x which was five times recrystallized with the aid of chloroform.[18] This material contained approximately one mole of long chain fatty acid per mole of albumin and small amounts of electrolytes, and was used without further purification. When dissolved in conductivity water a 1% solution had a pH of 4.92, agreeing with the value of 4.9 given in reference 18. This albumin is somewhat less stable than that crystallized with the aid of decanol[18] as indicated by decreased heat stability, and ultracentrifugal analyses which indicate there is a small amount of component of higher molecular weight than albumin.[19] The latter is probably due to a small portion of the albumin which is denatured or aggregated, and very probably accounts for the average molecular weight, determined by osmotic pressure measurements, of 75,000[20] for this preparation, instead of 69,000. Since we are interested in the characteristics of albumin of 69,000 molecular weight, and since the higher molecular weight component probably consists of aggregates of material of 69,000 molecular weight, we assumed a value of 69,000 in our calculations.

Stock solutions of albumin were made in conductivity water, the concentration determined, by dry weight, to 0.3%, and weighed aliquots used in making the solutions for each experiment. The pH of the protein solutions used in the experiments varied from 4.9 in the absence of salt to 5.2 in the presence of 0.6 m sodium chloride.

Sodium Chloride.—Mallinckrodt analytical reagent grade material was dried at 140° for two hours and used as a primary standard.

Potassium Chloride.—Merck, reagent grade material was used.

Cellophane membranes were made from sausage casing (Visking) of 19 mm. diameter. Equal lengths were cut, weighed, tied at one end, washed overnight in several changes of distilled water and then in conductivity water. When tied at both ends each bag was about 175 mm. in length.

Conductivity water was prepared from distilled water in a Kraus type still using sodium hydroxide and potassium permanganate, and stored in Jena glass bottles. The specific conductance of the water in each bottle was determined before use and the water was rejected if this was greater than 1×10^{-6} reciprocal ohm.

Silver–Silver Chloride Electrodes.—Two-cm. lengths of platinum wire, 0.5 mm. in diameter, were sealed into 3 mm. soft glass tubing. Following the method of Brown[21] the platinum wire was plated with silver, and then chloridized. Electrodes were stored in solutions of approximately the same concentration as those in which they were to be used.

Results

Table I lists the results of the distribution experiments, and Table II those of the e. m. f. experiments.

The range of chloride concentration over which measurements could be made was limited in both methods. In the distribution studies, at high chloride concentrations the fraction of the total chloride bound was small so that the relative difference between $(Cl^-)'$ and (Cl^-) was very small. With $(Cl^-) = 0.14$ molar, and $m_2 = 0.001$ molar, for example, only about 4% of the total chloride inside the membrane was bound, whereas with the same albumin concentration and $(Cl^-) = 0.001$ molar about 39% of the chloride within the membrane was bound. The resulting probable error in $\bar{\nu}$ is negligible at low concentrations but is about 40% of $\bar{\nu}$ at 0.3 m sodium chloride. On the other hand, as the chloride concentration decreased the impurities present in the protein and conductivity water made the percentage accuracy of the determination of $(Cl^-)'$ poorer. The correction for the conductance of the protein impurities corresponds to about 4×10^{-5} molar $(Cl^-)'$, or to about 0.4 in $\bar{\nu}$ and is nearly independent of (Cl^-).

TABLE I

DISTRIBUTION OF SODIUM CHLORIDE BETWEEN WATER AND ALBUMIN SOLUTION AT 5°

$m_3 \times 10^3$	$(Cl^-) \times 10^3$	$m_2 = m_2/g_1 \times 10^3$	$\bar{\nu}$
2.33	1.44	1.02	0.9
5.39	3.67	1.12	1.6
10.36	7.67	1.07	2.7
64.97	59.89	1.08	5.0
142.9	137.35	1.04	5.8
149.4	142.50	1.14	6.5
197.3	188.42	1.21	7.9
389.6	380.57	1.05	9.3

Using the e. m. f. method the voltage produced in the solution containing about 0.1 molar chloride and 0.0007 molar albumin was so low as to be only about ten times the reproducibility of individual readings. In solutions more dilute than about 0.0005 molar chloride the electrodes be-

(18) E. J. Cohn, W. L. Hughes, Jr., and J. H. Weare, THIS JOURNAL, **69**, 1753 (1947).
(19) Personal communication from W. L. Hughes, Jr.
(20) Personal communication from J. Weeks and A. Gee.
(21) A. S. Brown, THIS JOURNAL, **56**, 646 (1934).

TABLE II

EFFECT OF ALBUMIN ON ELECTROMOTIVE FORCE OF SILVER-SILVER CHLORIDE ELECTRODES IN SODIUM CHLORIDE SOLUTIONS

$m_3 \times 10^3$	$(Cl^-) \times 10^3$	$m_2 \times 10^4$	Mean net e. m. f.,[a] millivolts	$\bar{\nu}$	$\Delta\bar{\nu}$	$\frac{\bar{\nu}e^2w\bar{z}}{\gamma(Cl^-)}$	Temp. °C.
			A				
0.42	0.35	7.62	4.28	0.09	0.02	253	24
.75	.59	7.62	7.90	.26	.05	477	27
1.09	.76	7.57	9.30	.44	.02	640	25
1.09	.78	7.66	8.45	.39	.02	555	27
1.09	.81	7.54	7.32	.37	.02	505	26
3.17	2.57	6.87	5.42	.87	.01	410	27
6.29	5.16	6.87	5.09	1.7	.01	432	27
6.23	5.23	6.87	4.51	1.4	.03	368	29
12.50	10.7	6.87	4.10	2.6	.08	367	30
12.54	10.8	6.87	3.68	2.5	.17	334	28
21.06	18.07	7.47	3.93	4.0	.03	291	24
32.29	28.66	7.75	3.01	4.7	.04	381	25
50.50	46.04	7.52	2.40	6.0	.10	250	24
71.21	65.8	7.77	2.06	7.0	.23	220	25
99.49	94.4	7.78	1.37	6.6	.33	130	24
			B				
9.6	8.07	6.50	4.19	2.4	.29	...	4.5
10.8	9.70	5.83	2.65	2.0	.12	...	4.5
10.8	9.60	5.83	3.07	2.1	..[b]	...	27
50.5	46.9	7.52	1.84	4.8	..[b]	...	4.5
57.8	54.1	5.61	1.55	6.5	.63	...	4.7
57.8	53.6	5.61	1.57	7.5	1.37	...	25

[a] E. m. f. with solutions I and II minus zero potential. [b] Only one pair of electrodes used.

haved erratically. In Table II the column headed $\Delta\bar{\nu}$ lists the average deviation of $\bar{\nu}$, calculated from the e. m. f. measurements of each electrode pair, from the mean $\bar{\nu}$ calculated from the measurements of both pairs.

Discussion

If each of n groups on a protein molecule, P, reacts with a small ion, A, with the same intrinsic constant, k_A°, but each reaction is influenced electrostatically by the other ions which have reacted, the equilibrium may be expressed by the equations

$$\bar{\nu}_A = \frac{nk_A a_A}{1 + k_A a_A} \quad (4)$$

$$k_A = k_A^\circ e^{-2w\bar{z}_P z_A} \quad (5)$$

provided that n is not too small and w is not too large. $\bar{\nu}_A$ is the average number of A ions combined with one molecule of P when the activity of A ions is $a_A = (A)\gamma_A$, $\bar{z}_P$ is the average valence of the protein, z_A the valence of the A ion, and $2w\bar{z}_P z_A kT$ the electrostatic work of bringing the ion to the surface of a protein molecule with average charge.

If the total charge on the protein molecule is distributed symmetrically over the surface of a sphere of radius b both before and after the addition of an A ion and if uncombined small ions can approach the protein molecule to a distance a, not smaller than b, the Debye theory gives

$$w = \frac{\epsilon^2}{2DkT}\left[\frac{1}{b} - \frac{\kappa}{1 + \kappa a}\right] \quad (6)$$

if ϵ is the protonic charge, D the dielectric constant of the solvent, k is Boltzmann's constant, T the absolute temperature and κ has its usual significance in the Debye theory.

This relation has been derived simply[22] for the special case of reaction with an isoionic protein for which $\bar{z}_P = \bar{\nu}_A z_A$. In the general case the electrostatic work of bringing a charge z_A to a molecule with charge $(z_P - z_A)$ is

$$kTw[z_P^2 - (z_P - z_A)^2] = 2kTw[z_P z_A - z_A^2/2]$$

When

$$\nu_A = \nu_A(1 + 1/n), \text{ then } z_P = \bar{z}_P + \bar{\nu}_A z_A/n$$

and the work is

$$2kTw[\bar{z}_P z_A + z_A^2(\nu_A/n - 1/2)] \text{ or approximately } 2kTw\bar{z}_P z_A$$

If there are several small ions competing for the reactive groups in the protein, equation 4 must be replaced by

$$\bar{\nu}_A = \frac{nk'_A a_A}{1 + k'_A a_A} \quad (7)$$

and

$$k'_A = \frac{k_A}{1 + k_B a_B + k_D a_D + \ldots.} \quad (8)$$

If there are several classes of groups on the protein, such that n_a groups have the intrinsic constants k°_{Aa}, k°_{Ba}, etc., n_b groups have the intrinsic constants k°_{Ab}, k°_{Bb} etc., equation 7 must be replaced by

$$\bar{\nu}_A = \bar{\nu}_{Aa} + \bar{\nu}_{Ab} + \ldots = \frac{n_a k'_{Aa} a_A}{1 + k'_{Aa} a_A} + \frac{n_b k'_{Ab} a_A}{1 + k'_{Ab} a_A} + \ldots \quad (9)$$

and equation 8 by

$$k'_{Aa} = \frac{k_{Aa}}{1 + k_{Ba} a_B + k_{Da} a_D + \ldots} \quad (10)$$

Each of the k's is given by an equation 5.

For each kind of group we may combine equations 4 and 5 and rearrange, to give

$$\frac{\bar{\nu}_{Aa} e^{2w\bar{z}_P z_A}}{(A)\gamma_A} = k_{Aa}(n_a - \bar{\nu}_{Aa}) \quad (11)$$

If $\bar{\nu}_{Aa}e^{2w\bar{z}_P z_A}/(A)\gamma_A$ is plotted against $\bar{\nu}_{Aa}$, equation 11 gives a straight line. Its intercepts on the axes are $k^\circ_{Aa}n_a$ and n_a. Even if the curve is not linear because of an error in calculating w or in the assumptions, the intercepts will be the same. If there is more than one class of groups, the curve for $\bar{\nu}_A e^{2w\bar{z}_P z_A}/(A)\gamma_A$ will not be linear. The intercepts will be $n_a k^\circ_{Aa} + n_b k^\circ_{Ab} + \ldots$ and $n_a + n_b + \ldots$ but these intercepts may be very difficult to determine.

For serum albumin and sodium chloride we have taken $b = 30$ Å. and $a = 32.5$ Å.[8] With

(22) G. Scatchard, *Ann. N. Y. Acad. Sci.*, **51**, 660 (1949).

the values of the constants of the Debye theory used in this laboratory[23,24] we obtain

$$w/2.303 = 0.0517 - 0.5085\sqrt{\mu}/(1 + 10.663\sqrt{\mu}) \quad \text{at } 25° \quad (12)$$

$$= 0.0505 - 0.492\sqrt{\mu}/(1 + 10.56\sqrt{\mu}) \quad \text{at } 5° \quad (13)$$

in which μ is the ionic strength. We have calculated the ionic strength as the stoichiometric concentration of sodium chloride.

For the activity coefficient of the chloride ion, we have used the approximate expression

$$-\log \gamma = 0.5\sqrt{\mu}/(1 + 2\sqrt{\mu}) \quad (14)$$

which corresponds to the Debye–Hückel theory for an a of 6.1 Å.

Isoionic Albumin.—Figure 2 shows a plot of the e. m. f. results at 25° corresponding to equation 11. The straight broken line corresponds to $n = 11$, $k_A^\circ = 44$. The full curve corresponds to two classes of groups $n_a = 10$, $k_{Aa}^\circ = 44$ and $n_b = 30$, $k_{Ab}^\circ = 1.1$.

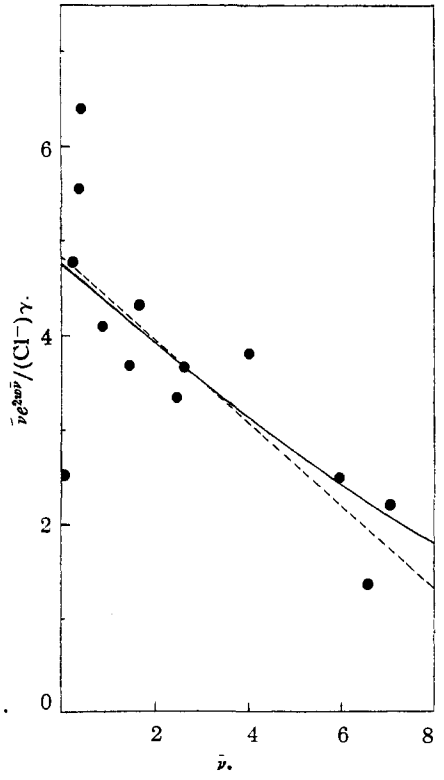

Fig. 2.—Combination of chloride ion with human serum albumin, e. m. f. ~25°.

Figure 3 shows both the e. m. f. results and the distribution results as $\bar{\nu}$ *vs.* $-\log (Cl^-)$. The filled circles are the e. m. f. results at 25°, the squares are the e. m. f. results at 5° and the open circles are the distribution results at 5°. The curves are the theoretical curves for 25° with the constants given above. The broken line again corresponds to 11 groups with the equation

$$\bar{\nu} = \frac{484\ (Cl)\gamma e^{-2w\bar{\nu}}}{1 + 44\ (Cl)\ \gamma e^{-2w\bar{\nu}}} \quad (15)$$

and the full line to two classes of groups with the equation

$$\bar{\nu} = \frac{440\ (Cl)\gamma e^{-2w\bar{\nu}}}{1 + 44\ (Cl)\ \gamma e^{-2w\bar{\nu}}} + \frac{33\ (Cl)\gamma e^{-2w\bar{\nu}}}{1 + 1.1\ (Cl)\ \gamma e^{-2w\bar{\nu}}} \quad (16)$$

Figure 3 gives a better balanced picture than Fig. 2 of the agreement between measurements and theory.

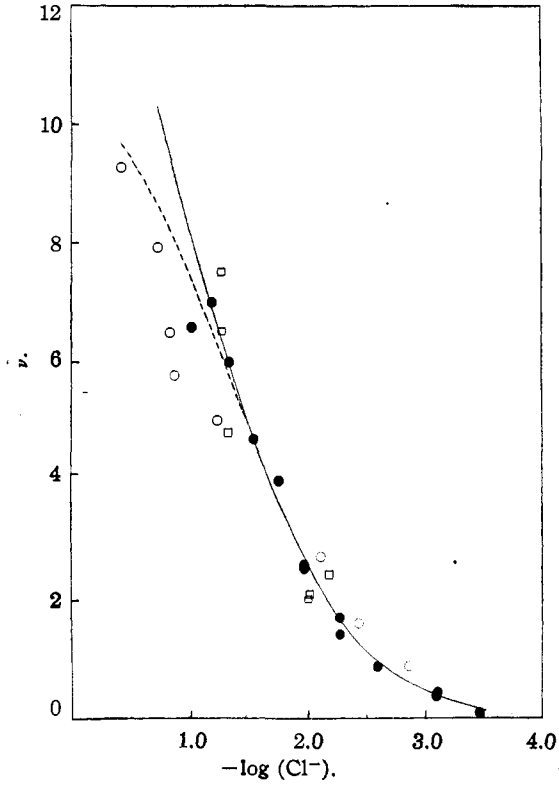

Fig. 3.—Combination of chloride ion with human serum albumin: ●, $\bar{\nu}$ from e. m. f. at ~25°; □, $\bar{\nu}$ from e. m. f. at ~4.5°; O, $\bar{\nu}$ from distribution at 5°.

Our measurements with isoionic albumin cannot distinguish between equation 15 and equation 16. However, measurements with acid albumin indicate that many more than 11 chloride ions are bound to each albumin molecule, and agree well with equation 16 which was determined by the assumption that n_b, like n_a, and k_b°/k_a° are the same for chloride ion as for thiocyanate ion.[25]

Binding of Sodium Ion.—The curves of Fig. 3 fit the open circles of the distribution measurements satisfactorily. Certainly, the open circles do not indicate more combination than the full circles of the e. m. f. Since the e. m. f. measures only the binding of the chloride ion and the dis-

(23) G. Scatchard and L. F. Epstein, *Chem. Revs.*, **30**, 21 (1942).
(24) G. Scatchard, THIS JOURNAL, **65**, 1249 (1943).
(25) G. Scatchard, I. H. Scheinberg and S. H. Armstrong, Jr., *ibid.*, **72**, 540 (1950).

tribution measures the sum of the chloride and sodium ions bound, this agreement shows that not more than a very small amount of sodium ion is bound.

Temperature Variation.—The curve calculated at 25° seems to fit the measurements at 5° as well as those at the higher temperature. Calculations of the change in enthalpy by the relation

$$\Delta H = \frac{2.3\, RT_1T_2}{T_2 - T_1} \log \frac{K_2}{K_1}$$

for the four points gives 430 ± 540 cal. per mole of chloride ion bound. We believe that our measurements prove that the enthalpy change is very small and indicate that it is positive. Klotz and Urquhart[26] found $\Delta H = -2100$ cal./mole of methyl orange and $\Delta H = -2000$ cal./mole of azosulfathiazole.

Acid Albumin.—To test the effect of charge on the binding of chloride ion by human serum albumin, we measured the e. m. f. of a solution of 3.02×10^{-4} molal albumin and 0.1494 molal total chloride ion with pH = 3.2, in which the number of bound hydrogen ions was 78 per molecule more than in isoionic albumin, with silver–silver chloride electrodes against a solution containing the same amount of sodium chloride and hydrochloric acid, but no albumin, which had pH = 1.8.[27] The average net e. m. f. of 9 determinations with 4 different electrode pairs was 1.74 millivolts. This corresponded to a $\bar{\nu}$ of 31 ± 4.

The exponentials in equation 16 must now be replaced by $e^{2w(78 - \bar{\nu})}$. Solution by successive approximations gives $\bar{\nu} = 30.5$, which agrees with the measured 31 ± 4 much better than the accuracy of either the experiments or the assumption that k_b/k_a is the same for chloride ion as for thiocyanate ion. The significance of these results will be discussed later.[25]

The authors are grateful to Harriet Anderson Campbell for her valuable assistance in carrying out the experiments described in this paper.

Summary

The combination of chloride ion with human serum albumin has been investigated by two methods. One is the measurement by conductance of the distribution of sodium chloride across a cellophane membrane with albumin on one side. The other utilizes the electromotive force developed in a concentration cell with silver–silver chloride electrodes both half-cells of which contain sodium chloride, and one of which contains albumin.

The results are well described by the law of mass action if account is taken of the electrostatic interaction of combined ions. The measurements with isoionic albumin may be accounted for with 11 groups per albumin molecule with an intrinsic association constant of 44, or with 10 groups with an intrinsic constant of 44 and 30 others with a constant of 1.1. Measurements at a pH of 3.2 show that many more than 11 chloride ions are bound to each albumin molecule.

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(26) I. M. Klotz and J. M. Urquhart, THIS JOURNAL, **71**, 847 (1949).

(27) These solutions were kindly prepared for us by Dr. Charles Tanford of the Harvard Medical School who measured the titration curves of the human serum albumin, to be published shortly. During the readings a white precipitate appeared at the liquid junction. It was almost certainly acid albumin precipitated by the concentrated potassium chloride, and had no apparent effect on the e. m. f.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, MASSACHUSETTS INSTITUTE OF TECHNOLOGY]

Physical Chemistry of Protein Solutions. V. The Combination of Human Serum Albumin with Thiocyanate Ion[1a]

BY GEORGE SCATCHARD,* I. HERBERT SCHEINBERG,† AND S. HOWARD ARMSTRONG, JR.‡[1b]

It has been shown in the preceding paper[1c] that chloride ions combine with human serum albumin in a manner which is well described by the law of mass action. Thiocyanate ions combine with albumin more tightly and in greater number than chloride ions, and the investigation of this combination was undertaken to extend the information available for understanding the nature of protein–small ion interaction.

Experimental

Two methods were used. The first was the determination of the distribution of sodium thiocyanate across a cellophane membrane on one side of which albumin was present. In the second method the effect of albumin on the electromotive force of a thiocyanate concentration cell was measured. Both methods and their attendant calculations were described in detail in the preceding paper,[1c] and only the modifications introduced in the present study will be mentioned here.

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‡ Harvard University M.D. 1937; Society of Fellows 1940–1943.

(1a) The products of Plasma fractionation employed in this work were developed from blood which was collected by the American Red Cross, by the Department of Physical Chemistry, Harvard Medical School, Boston, Massachusetts, under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and Harvard University.

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(1c) G. Scatchard, I. H. Scheinberg and S. H. Armstrong, Jr., THIS JOURNAL, **72**, 535 (1950).