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.

CAMBRIDGE 39, MASS. RECEIVED JANUARY 20, 1949

(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.

* Editorial Board 1943–.

† Harvard University M.D. 1943; Society of Fellows 1947–.

‡ 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.

(1b) Welch Fellow in Internal Medicine of National Research Council at the time this work was done. Present address, The Presbyterian Hospital of the City of Chicago, Chicago, Illinois.

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

Distribution Method.—Albumin was used without first dialyzing it against distilled water. This made the correction due to the conductance of the impurities in the protein about three times as large as in the chloride studies where the protein was dialyzed before use. The thiocyanate distribution studies were made before those on chloride, and before the advantage of dialysis of the protein was realized.

Electromotive Force Method.—Consistent results of the type obtained with silver–silver chloride electrodes were never obtained with silver–silver thiocyanate electrodes in the presence of albumin. Very frequently the measured voltage across the electrodes was of the opposite sign to that which should result if thiocyanate were being bound by albumin. Such a result might be caused by the albumin–thiocyanate complex binding silver ions and dissolving the solid silver thiocyanate from the electrode. Because of this possibility experiments were performed with silver–silver thiocyanate electrodes which had been coated with collodion to prevent albumin from reaching the electrode surface. This is similar to a technique used by Joseph for reactive amalgams.[2] When this was done consistent readings were more often obtained in albumin–thiocyanate solutions. Not infrequently, however, even with collodion, the sign of the observed voltage was opposite to that expected. Usually it was necessary to select another pair of electrodes when this occurred. Only rarely in these instances could a hole be found in the collodion coating of the electrodes.

Two hours were allowed for collodion-coated electrodes to come to equilibrium with the solutions in the cell before the liquid junctions were made. The constancy of the e. m. f. on refilling the cell, as well as measurements made on protein-free thiocyanate solutions, indicated that diffusion equilibrium across the collodion membrane had been reached. All measurements (except two) were made at least in duplicate for each pair of electrodes, and with at least two different pairs of electrodes. Zero potentials were about 0.1 to 0.3 millivolt, which were higher than with the silver–silver chloride electrodes. It was possible to obtain duplicate readings with two pairs of electrodes which checked each other about as closely as the duplicate fillings of any one pair. This was the criterion for accepting measurements. However, it should be pointed out that using collodion-coated silver–silver thiocyanate electrodes in albumin solutions was more difficult, and subject to more erratic results, than using uncoated silver–silver chloride electrodes.

To show that the use of collodion-coated electrodes should yield the same e. m. f. values as uncoated electrodes would for the same solutions[2] we may consider what changes of state can be assumed to take place in each instance. We shall use the notations of the previous paper.[1c] Let

Subscripts 1, 2 and 3 refer to water, albumin and salt, respectively.
n be the number of moles of free component.
g_1 be the number of kilograms of water.
m be the stoichiometric concentration 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).
(The designation II,′ then, refers to the protein free solution in equilibrium across a collodion membrane with Solution II.)
$\bar{\nu}$ be the average number of small ions bound to each albumin molecule.

For uncoated electrodes the cell is

Ag, AgSCN, Solution II, Sat. KCl, Solution I, AgSCN, Ag

If we assume that the changes of state occurring at the two liquid junctions may be neglected then the passage of unit, infinitesimal quantity of electricity will result in the following

$$\begin{array}{l} \mathrm{Ag(s) + SCN^-(in\ II) = AgSCN(s) + e^-} \\ \mathrm{AgSCN(s) + e^- = Ag(s) + SCN^-(in\ I)} \\ \hline \mathrm{SCN^-(in\ II) = SCN^-(in\ I)} \end{array}$$

The e. m. f. produced, E, is then, assuming that $\gamma = \gamma'$,

$$E = RT/F \ln (\mathrm{SCN^-})'/(\mathrm{SCN^-}) \qquad (1)$$

For the collodion-coated electrodes the cell is

Ag, AgSCN Solution II′, Collodion Membrane, Solution II, Sat. KCl, Solution I, AgSCN, Ag

If we pass unit, infinitesimal quantity of electricity through this cell and neglect changes of state at the two liquid junctions, we may consider the following to occur.

$$\begin{array}{l} \mathrm{Ag(s) + SCN^-\ (in\ II')} = \mathrm{AgSCN(s) + e^-} \\ t_+\mathrm{Na^+\ (in\ II')} = t_+\mathrm{Na^+\ (in\ II)} \\ (1 - t_+)\mathrm{SCN^-\ (in\ II)} = (1 - t_+)\mathrm{SCN^-\ (in\ II')} \\ \mathrm{AgSCN(s) + e^-} = \mathrm{Ag(s) + SCN^-\ (in\ I)} \\ \hline t_+\mathrm{Na^+SCN^-\ (in\ II') + SCN^-\ (in\ II)} = t_+\mathrm{Na^+SCN^-\ (in\ II) + SCN^-\ (in\ I)} \end{array}$$

where t_+ is the transference number of Na^+.

Since equilibrium is established across the collodion membrane before the measurements are made, the transfer of t_+NaSCN from II′ to II will involve no net change in free energy, and so the e. m. f. of the cell, E, is the same as in the cell with uncoated electrodes and will be given by equation 1.

An experimental confirmation of this was made by filling the cell with two protein-free sodium thiocyanate solutions the concentrations of which were 0.1144 and 0.1350 molal. The e. m. f. of the cell was measured using two different pairs of collodion-coated electrodes. Each member of each pair was used in both the 0.1144 and 0.1350 molal solutions. The mean net e. m. f. of five determinations with the first pair of electrodes was 4.08 millivolts and the average of the absolute value of

(2) N. R. Joseph, *J. Biol. Chem.*, **126**, 389 (1938).

the deviation of each determination from the mean was 0.09 millivolt. For the second pair these figures were 4.45 millivolts and 0.16 millivolt. The calculated value is 4.23 millivolts.

Other experimental details were the same as those described in the previous paper.

Materials.—Human serum albumin was generously given to us by Dr. W. L. Hughes, Jr. The distribution experiments, and a very few of the e. m. f. experiments, were done with lot 179-5x, which had been crystallized five times with the aid of chloroform.[3] Most of the e. m. f. experiments were done with lot decanol 10, which had been crystallized three times with the aid of decanol.[3] The latter preparation was always electrodialyzed against distilled water, to remove small ions, before use.

Sodium Thiocyanate.—Merck Reagent material was dissolved in conductivity water[1c] and the solution analyzed gravimetrically for thiocyanate by precipitation of silver thiocyanate.[4]

Silver-Silver Thiocyanate Electrodes.—Six platinum wire electrodes, 0.5 mm. in diameter, were plated with silver in a 1% solution of $KAg(CN)_2$ for three hours at 1-2 milliamperes.[1c,5] After overnight washing in running distilled water they were plated, as the anodes, in 0.1 molar sodium thiocyanate at a current of 0.5–2.0 milliamperes. The usual duration of plating with sodium thiocyanate was two hours, and plating for as long as four hours did not seem any more advantageous. At this point the electrodes were sometimes brown and sometimes white. They were more likely to be brown when the lower current densities were used in coating with silver thiocyanate.

The electrodes were air-dried, at room temperature, for about ten minutes and then dipped into the collodion solution so that the entire electrode and about half of the glass mounting were submerged. After about thirty seconds the electrode was removed and allowed to dry at room temperature. Sometimes a second collodion coat was put on after the first had dried six minutes though no striking difference in behavior resulted. After drying for one to three hours a thread was tied tightly around the collodion on the glass mounting of the electrode to prevent solution reaching the electrode over the edge of the collodion. The electrodes were stored in sodium thiocyanate solutions of approximately the same concentration as those in which they were to be used.

Collodion.—Nitrocellulose (Howe and French 35/40 Series B 2643) was dried for two hours at 110°. Twenty-four and one-half cc. of anhydrous ethylene glycol (Eastman Kodak Co.) was added to about 8.3 g. of the nitrocellulose in a bottle, and the nitrocellulose was coated as thoroughly as possible by shaking. A mixture of 75 cc. of anhydrous ether and 214 cc. of absolute alcohol was added to this, and the mixture shaken until all the nitrocellulose had dissolved.

Other materials were the same as in the previous paper.[1c]

Results

Table I lists the results of the distribution experiments, and Table II those of the e. m. f. experiments. As in the chloride experiments the range of thiocyanate concentrations over which experiments could be made was limited. In the distribution studies, at high concentrations, the fraction of the total thiocyanate bound was small so the relative difference between (SCN^-) and $(SCN^-)'$ was also small. Thus, with $(SCN^-) = 0.71$ molal and $m_2 = 9.3 \times 10^{-4}$ molal, only about 5% of the thiocyanate inside the membrane was bound, whereas with $(SCN^-) = 0.0008$ molal and $m_2 = 7.1 \times 10^{-4}$ molal, about 73% of the thiocyanate in the membrane was bound. The resulting probable error in $\bar{\nu}$ is negligible at low concentrations but is about 11% of $\bar{\nu}$ at 0.7 molal (SCN^-). On the other hand, at low concentrations of thiocyanate the impurities present in the protein made the relative accuracy of the determination of $(SCN^-)'$ poorer. The correction for the conductance due to these protein impurities corresponded to about 1.8×10^{-4} molal sodium thiocyanate, when the protein concentration was about 7×10^{-4} molal, or to about 1.1 in $\bar{\nu}$, and was nearly independent of (SCN^-).

TABLE I

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

$m_3 \times 10^3$	$(SCN^-) \times 10^3$	$m_2 = n_2/g_1 \times 10^4$	$\bar{\nu}$	$\frac{\bar{\nu}e^2w\bar{\nu}}{\gamma(SCN^-)}$
2.62	0.80	7.07	2.70	5443
7.75	4.08	6.70	5.73	3204
11.6	6.79	7.19	7.13	2686
49.4	32.6	12.9	14.20	1645
44.3	34.9	6.70	14.70	1713
47.0	35.9	7.56	15.17	1784
46.2	40.0	3.52	18.13	2461
51.7	40.2	7.60	15.75	1671
46.1	40.2	3.43	17.52	2251
74.2	62.1	6.82	18.57	1414
83.3	70.2	6.83	19.84	1415
112.0	96.5	7.08	22.71	1221
146.0	130.0	6.60	24.85	1103
179.0	160.0	8.00	25.27	879
300	278	7.42	29.76	635
618	584	8.42	41.34	559
744	708	9.26	41.24	429

In using the e. m. f. method it was possible to obtain satisfactory readings with concentrations of free thiocyanate as low as 2×10^{-5} molal. This is in contrast to the behavior of the silver–silver chloride electrodes which were not collodion coated, and which behaved erratically in solutions more dilute than about $(Cl^-) = 5 \times 10^{-4}$ molal.

Four attempts to make measurements on solutions more concentrated than those listed in Table II gave erratic results. Most of the voltages measured were of opposite sign to that expected, although the total thiocyanate concentrations in these 4 solutions only covered the range from 0.23 to 0.45 molal.

In the range of concentrations where measurements were made the average deviation of the e. m. f. of each pair of electrodes from the mean e. m. f. of the two or three pairs used was 0.3 millivolt. The magnitude of the deviation tended to vary inversely with the thiocyanate concentration. 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

(3) E. J. Cohn, W. L. Hughes, Jr., and J. H. Weare, THIS JOURNAL, **69**, 1753 (1947).

(4) H. H. Willard and N. H. Furman, "Elementary Quantitative Analysis," 2nd ed., D. Van Nostrand Co., Inc., New York, N. Y., 1935, p. 298.

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

TABLE II

EFFECT OF ALBUMIN ON ELECTROMOTIVE FORCE OF SILVER–SILVER THIOCYANATE ELECTRODES IN SODIUM THIOCYANATE SOLUTIONS

	m_1 × 10^3	(SCN^-) × 10^3	m_2 × 10^4	Mean net e. m. f.,[a] millivolts	$\bar{\nu}$	$\Delta\bar{\nu}$	$\frac{\bar{\nu}e^{2w\bar{\nu}}}{\gamma\,(SCN)^-}$	Temp., °C.
				A				
	0.1057	0.0214	4.76	41.04	0.18	0.00	8,862	24.8
	.4886	.0879	5.14	44.37	.78	.01	10,592	24.5
C	.1014	.0893	0.15	3.27	.80	.16	11,085	26.8
B	.1033	.0907	.16	2.88	.81	.21	10,762	24.6
A	.1033	.0926	.15	2.27	.72	.13	9,194	27.0
	1.094	.257	5.05	37.01	1.66	.03	8,965	24.1
	5.082	2.438	4.98	18.69	5.30	.09	4,903	23.3
	9.775	6.471	4.98	10.65	6.64	.31	2,554	23.5
	10.24	7.706	2.64	7.48	9.59	.62	4,020	25.9
	10.10	7.89	2.54	6.52	8.72	.26	3,598	26.3
	23.10	17.77	5.09	6.72	10.46	.02	1,895	24.4
	36.67	31.19	4.48	4.17	12.53	1.60	1,437	24.2
	40.34	31.77	7.22	6.07[b]	11.8	..	1,237	26.0
	42.67	32.50	5.05	7.32	20.3	0.57	4,169	22.7
	41.72	36.86	3.51	3.11	13.86	.16	1,466	23.2
	59.40	54.38	4.20	2.26	11.95	.71	667	23.9
	127.51	118.66	4.85	1.87	18.26	1.33	625	25.2
	145.12	140.38	3.00	0.91	15.82	2.75	381	24.7
	158.18	144.06	5.00	2.38	28.21	0.19	1,369	23.4
				B				
	10.21	6.424	5.21	10.43	7.28	0.42	3,037	2.5
	9.775	6.428	4.98	9.74	6.72	.18	2,611	4.5
	10.10	7.96	2.54	5.77[b]	8.45	..	3,278	2.1
	20.51	15.91	5.19	6.03	8.86	.32	1,612	3.3
	23.10	17.67	5.09	6.49	10.66	.60	1,984	4.2
	154.49	144.47	3.63	1.57	27.58	.67	1,279	4.4
	219.05	211.94	3.64	0.78	19.56	1.21	332	4.3

[a] E. m. f. with Solutions I and II minus zero potential. [b] One pair only.

value of $\bar{\nu}$ calculated from the measurements of the two or three pairs used.

Reversibility.—We consider that the reversibility of the combination of chloride ion with albumin is established by the repeated preparation of salt-poor albumin from plasma, which contains about 0.1 molar chloride.[3] However, it is desirable to prove the reversibility of the combination for the more tightly bound thiocyanate ion. A solution of 54.05×10^{-4} molal sodium thiocyanate and 7.76×10^{-4} molal albumin, for which $\bar{\nu}$ was 4.8, was diluted fifty-fold one hour after preparation to give Solution A (Table II). A solution of 51.88×10^{-4} molal sodium thiocyanate and 7.83×10^{-4} molal albumin, for which $\bar{\nu}$ was 4.7, was diluted fifty-fold after standing about forty-eight hours at 2° to give Solution B (Table II). Solution C (Table II) was prepared by diluting a sodium thiocyanate solution before the addition of albumin so that the albumin was never exposed to the more concentrated solution. The measured e. m. f.'s corresponded to $\bar{\nu}$'s of 0.7_2, 0.8_1 and 0.8_0 for A, B and C, respectively. These measurements show that the thiocyanate ion is not bound irreversibly.

Discussion

There is evidence[6,7] that each positively charged nitrogen of a protein can combine with a small anion. It is probable that, just as for the combination with hydrogen ion, these groups can be divided into a few classes such that the association constants are very nearly the same for every group within a class. The constants are so much smaller than those for the hydrogen ion, however, that only a few of the groups react under conditions which permit a quantitative study.

Our measurements with chloride ion and isoionic human serum albumin can be represented by about 11 groups per molecule of albumin with a single intrinsic association constant, or by 10 groups with one constant and 30 groups with a much smaller constant. In either case the effective association constants vary with the charge on the molecule in accordance with the Debye theory,[1c,8] It is only the measurements with acid albumin which require two sets of groups.

(6) G. E. Perlmann, *J. Biol. Chem.*, **137**, 707 (1941).

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

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

In the simpler case a plot of $\bar{\nu}e^{2w z_A^2 \bar{\nu}}/\gamma_A$ (A) against $\bar{\nu}$ gives a straight line with intercepts on the two axes $k^\circ n$ and n, if n is the number of groups on a protein molecule capable of reacting with small ions, A, and k° is the intrinsic constant for the reaction at a single group, $\bar{\nu}$ is the average number of groups per protein molecule which have reacted when the concentration of the free small ions is (A) and their activity coefficient γ_A, and kTw is the work of charging the neutral protein molecule with a unit charge. The equations for w and γ, and the values of the parameters used are given in the previous paper.[1c]

Our measurements with albumin and thiocyanate ion do not give such a simple curve. If there are i classes of groups, each of which can be described by a single constant, the intercepts are $\Sigma_i n_i k_i^\circ$ and $\Sigma_i n_i$, and the asymptotic slopes are $-\Sigma_i n_i k_i^{\circ 2}/\Sigma_i n_i k_i^\circ$ and $-\Sigma_i n_i/\Sigma_i n_i/k_i^\circ$, but the lines are no longer straight and it is much more difficult to determine the intercepts or the asymptotes.

Figure 1 shows this plot for albumin and thiocyanate. The filled circles are the e. m. f. results at room temperature, the squares are the e. m. f. results at about 4° and the open circles are the distribution results at 5°. It is possible to extrapolate to the left-hand side rather surely to give the first intercept and asymptote, but extrapolation to the right is much less certain. Then by successive approximation of the entire curve k_a°, n_a and $k_b^\circ n_b$ can be determined with fair accuracy, and we see that $n_a + n_b$ cannot be much less than 40, so n_b cannot be much less than 30 if n_a is about 10. It is not certain, however, that n_b is not larger than the value we have chosen. The curve corresponds to two classes of groups with $n_a = 10, k_a^\circ = 1000, n_b = 30, k_b^\circ = 25$ or

$$\bar{\nu} = \frac{10{,}000\ \gamma(\mathrm{SCN}^-)e^{-2w\bar{\nu}}}{1 + 1000\ \gamma(\mathrm{SCN}^-)e^{-2w\bar{\nu}}} + \frac{750\ \gamma(\mathrm{SCN}^-)e^{-2w\bar{\nu}}}{1 + 25\ \gamma(\mathrm{SCN}^-)e^{-2w\bar{\nu}}} \quad (2)$$

In Fig. 2 the same results and curve are plotted as $\bar{\nu}$ against $-\log(\mathrm{SCN}^-)$. This plot gives a better balanced picture than Fig. 1 of the agreement between the different kinds of measurements and between measurements and theory.

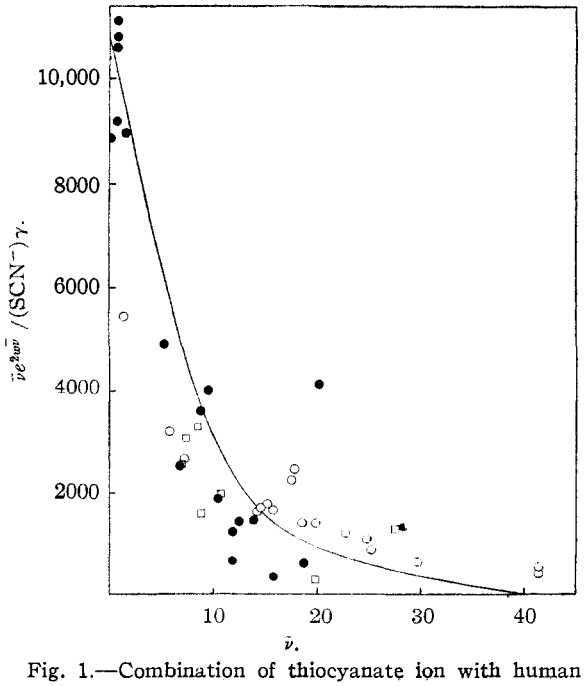

Fig. 1.—Combination of thiocyanate ion with human serum albumin: ●, e. m. f. measurements at ~25°; □, e. m. f. measurements at ~4°; O, distribution measurements at 5°.

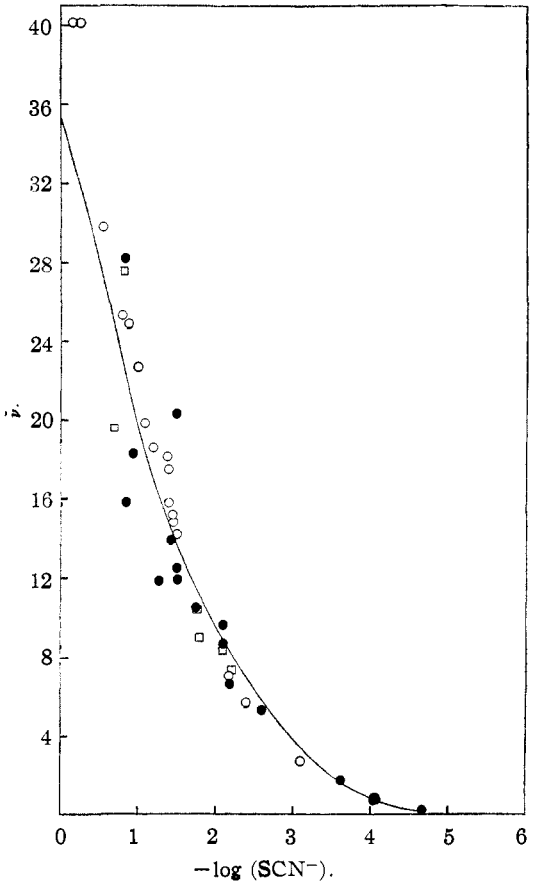

Fig. 2.—Combination of thiocyanate ion with human serum albumin; ●, $\bar{\nu}$ from e. m. f. at ~25°; □, $\bar{\nu}$ from e. m. f. at ~4°; O, $\bar{\nu}$ from distribution at 5°.

Figure 2 shows that there is no significant difference betwen the e. m. f. results at room temperature (filled circles) and those at about 4° (squares). So we conclude that the heat of the reaction is very nearly zero. The same result was found for the albumin–chloride reaction.[1c]

Figure 2 also shows that the distribution results agree well with the e. m. f. results and certainly do not indicate a larger value of $\bar{\nu}$. This confirms the conclusion drawn from the albumin-chloride study that sodium ion is not bound appreciably to serum albumin

The Effect of Charge.—In the previous paper[1c] we showed that adding hydrogen ion to serum albumin increased its reactions with chloride ion, and that the binding can be calculated from the assumptions that the same two sets or classes of groups are reacting with chloride as with thiocyanate, that the ratio of the constants for the two sets of groups is the same, and that the interactions between combined ions can be calculated from the Debye theory of electrostatic interaction of ions. We reserved discussion, however, until the evidence for the thiocyanate interaction was presented in this paper. The addition of 78 hydrogen ions made the chloride binding greater than the thiocyanate binding with isoionic albumin. It is also possible to account for the binding of thiocyanate ion in serum with these same equations and the additional assumption that the binding with serum proteins other than albumin is negligible.[9] In serum the *p*H is 7.4 and the chloride ion concentration is about 0.1 *M*. So the average charge on the albumin molecule is about −25, which reduces the interaction with thiocyanate considerably. The competition of a large excess of chloride ion reduces it still more so that the apparent constants for thiocyanate are reduced from 1000 and 25 to 78 and 3.9, respectively.

Figure 3 shows the number of chloride ions and of thiocyanate ions bound as a function of the hydrogen ions bound when the ionic strength is 0.15 molal and the protein concentration is negligibly small, calculated on the assumption that the only effect of the charges is the electrostatic one. A negative number of hydrogen ions is, of course, the same positive number of hydroxyl ions. If the hydroxyl ions compete for the same groups as the other anions, the curves will be reduced even more on the right-hand side.

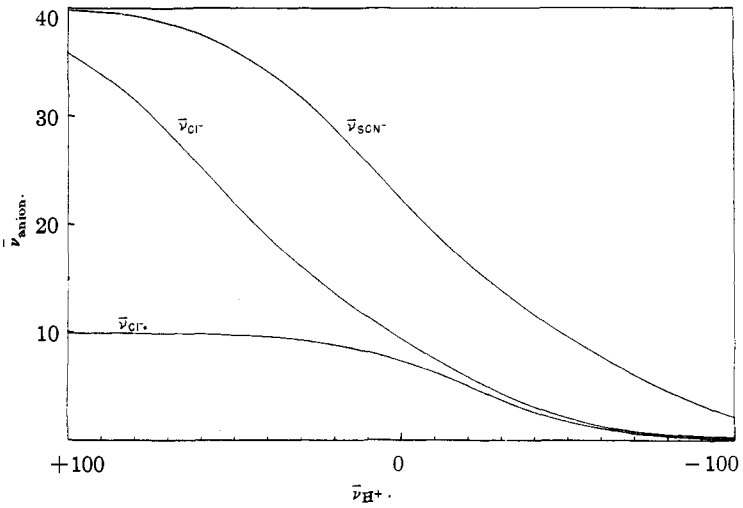

Fig. 3.—Effect of charge on binding.

The lower curve shows the binding of chloride ion with the 10 more active groups, the middle curve shows the binding of chloride ion with all 40 groups, and the top curve shows the binding of thiocyanate ion with the 40 groups. It may be obtained from the middle curve by displacement to the right by 51.11 hydrogen ions. Together these curves show practically the whole range. The bottom curve also shows practically the whole range, so the corresponding curve for thiocyanate is omitted. It can also be obtained by a horizontal displacement of 51.11 hydrogen ions.

These curves correspond to constants for chloride ion the same as those for thiocyanate ion for a protein molecule with 51.11 fewer hydrogen ions. At higher ionic strengths the difference in hydrogen ions must be greater to give equality in the constants. At lower ionic strengths it must be less, until in the limit of zero ionic strength the difference is only 13.13.

Comparison with Other Ions.—With these assumptions the interaction of human serum albumin with chloride and thiocyanate ions is represented by an analytical expression with only one parameter characteristic of the small ion. It is important to know whether other ions show the same relationship. Hydroxyl ion interacts with classes of groups with a single constant for each class, but these classes are apparently not the same as for chloride and thiocyanate ion. It does not appear to react much more readily with a few proteins such as albumin than it does with many others like serum globulin or lactoglobulin. However, the reaction is probably the displacement of a proton rather than the addition of the hydroxyl ions, so differences are to be expected.

Most of the other studies of combination have been made upon bovine serum albumin, and we are not sure how closely this resembles human serum albumin. Most of them suffer from the disadvantages of buffer solutions. There is an unknown amount of competition from the buffer anion, and an unknown association or dissociation of hydrogen ions, which also leads to an unknown electrostatic effect. The results of Scatchard, Batchelder and Brown,[10] when plotted on a curve like Fig. 3, indicate a smaller value for the first constant for the interaction of albumin with chloride, k° = 31, but the scatter of the points is larger than the difference from 44 because of the difficulties of measuring the small fraction bound in 0.15 *M* salt. Klotz and Urquhart[11] give a k of 27 determined from the competition of chloride ion with methyl orange, essentially by determin-

(9) I. H. Scheinberg and H. J. Kowalski, *J. Clin. Investigation*, in press.

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

(11) I. M. Klotz and J. M. Urquhart, *J. Phys. and Colloid Chem.*, **53**, 100 (1949).

ing for methyl orange the ratio k'/k of equation 8 of reference (1c). Their k is for concentrations in 0.1 M NaCl at pH 6.6, or with -6 hydrogen ions, and should correspond to $k^\circ\gamma e^{-2w^6}$, which is 21 for human serum albumin. The difference between 21 and 27 may well be within the error of their measurements. It is worth noting that these competition measurements give k directly, while the distribution measurements themselves give most clearly the product kn.

For the measurements of Klotz, Walker and Pivan[12] of the binding of methyl orange with bovine serum albumin in 0.1 M phosphate buffer at pH 5.7, we have made no correction for electrostatic action. These measurements determine $k' = k/(1 + k_B a_B)$, in which the subscript B refers to the phosphate ion. The measurements of Klotz and Urquhart[11] indicate that under these conditions $k_B a_B$ is so much larger than unity, that the correction would be very small if the primary phosphate were bound, and would be negative if it were the secondary phosphate ion. With $k_a = 6 \times 10^3$ and the values of n_a, n_b and k_b/k_a used above, we fit the measurements with $\bar{\nu}$ less than 8 with an average absolute error of 0.2 in $\bar{\nu}$. The agreement at 8.84 is only fair (8.02), and that at 15.3 is very poor (11.7). If the measurements in these two more concentrated solutions are correct, $k_b n_b$ differs less from $k_a n_a$ for bovine albumin and methyl orange than it does for human albumin and the smaller ions.

The measurements of Teresi and Luck[13] on m- and p-nitrophenols and bovine albumin are fitted almost within the experimental error with the two classes, but results in concentrated solutions indicate the same trend as those with methyl orange. Those with o-nitrophenol agree not too badly with 5 groups in the first class and 15 in the second, or just half the number for the others, but they agree a little better with a single class of six groups as found by the authors.

The measurements of Karush and Sonenberg[14] with bovine albumin and decyl sulfate in 0.025 M phosphate at pH 6.1 agree fairly well with five groups in the first class and ten in the second.

Maintaining the ratio of constants while altering the number of groups probably has little significance. These results do indicate, however, that when the interaction is largely polar the first forty groups do fall approximately into the same two classes as for the chloride and thiocyanate ions, and that when there is a large non-electrostatic action with non-polar groups, as in o-nitrophenol or decyl sulfate, some of the groups are protected so that they become much less reactive.

The authors are grateful to Ernestine L. Rolls for valuable assistance in carrying out the experiments described in this paper.

Summary

The combination of thiocyanate ions with human serum albumin has been studied by two methods. In one the distribution of sodium thiocyanate across a cellophane membrane, with albumin on one side, was measured by means of conductance. In the second method, the effect of albumin on the electromotive force of a thiocyanate concentration cell was determined by the use of collodion-coated silver–silver thiocyanate electrodes. The range of concentrations of free thiocyanate included in the measurements extended from 2×10^{-5} to 0.7 molal.

These experiments indicate that as many as 40 molecules of thiocyanate ion can be bound to each albumin molecule. The results are well described by assuming that each albumin molecule possesses 10 groups or points with an intrinsic association constant of 1000, and 30 groups with a constant of 25. It is also necessary to take into account the effect of electrostatic interaction among the groups.

Measurements made at 4° and at room temperature show no detectable heat of reaction.

Thiocyanate ion and chloride ion appear to react with the same two classes of groups in human serum albumin and to have the same ratio of intrinsic constants k_b°/k_a°. A review of studies with other ions indicates approximately the same grouping unless there are large active non-polar radicals which appear to protect some of the groups in albumin and change them to a much less reactive class.

These results suggest that the 100 positively charged nitrogen atoms of human serum albumin may behave, in binding anions, like a small number of classes of groups with a single intrinsic association constant for each class toward a given anion.

CAMBRIDGE 39, MASSACHUSETTS

RECEIVED AUGUST 2, 1949

(12) I. M. Klotz, F. M. Walker and R. B. Pivan, THIS JOURNAL, **68**, 1486 (1946).

(13) J. D. Teresi and J. M. Luck, *J. Biol. Chem.*, **173**, 653 (1948).

(14) F. Karush and M. Sonenberg, THIS JOURNAL, **71**, 1369 (1949).

We are unable to confirm the statement of these authors that their measurements cannot be described by the equation

$$\bar{\nu} = nk(\mathrm{A})e^{-2w\bar{\nu}} / (1 + k(\mathrm{A})e^{-2w\bar{\nu}})$$

With $n = 14$, the value they choose for their Gaussian distribution of log k, the agreement is just as good for this equation as for the Gaussian distribution. However, the value of w needed is five times that calculated from electrostatic theory with the assumption that there is no competition from the phosphate ions. So we do confirm these authors in their more important conclusion that their results cannot be explained by the electrostatic theory and a single association constant.