[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY AND LABORATORY OF NUCLEAR SCIENCE, MASSACHUSETTS INSTITUTE OF TECHNOLOGY, CAMBRIDGE, MASSACHUSETTS]

The Physical Chemistry of Protein Solutions. XII. The Effects of Temperature and Hydroxide Ion on the Binding of Small Anions to Human Serum Albumin[1,2]

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The binding of chloride, iodide, and thiocyanate ion to isoionic human serum albumin has been measured with silver–silver salt electrodes at 0 and 25°. Constants have been determined for the 27 most active sites; and the corresponding free energies, enthalpies, and entropies have been calculated. The binding of iodide and chloride ions at 25° at higher pH values has been measured. The results indicate that very strong binding is due to a decrease in enthalpy and is lessened by a decrease in entropy, but that weaker binding may be largely due to an entropy increase. They indicate that there is one very active site, which may be the single α-amino group or may be a stronger base, four less active sites which appear to be four of the 16 imidazoles, and that the next 22 sites are stronger bases. The results are compared with the earlier measurements.

Some years ago, one of us stated the problems of the binding of small ions to proteins as, "How many? How tightly? Where? Why?"[3] For chloride, iodide, and thiocyanate ions with human serum albumin, this paper gives more precise answers to the first two, gives the first answer to the third, and makes a little progress toward the answer to the last question.

Experimental

Materials.—Conductivity water distilled from alkaline permanganate in the Kraus-type still of this laboratory was used throughout. Analytical grades (Mallinckrodt) of sodium chloride, sodium iodide, and sodium thiocyanate were used without further purification to prepare stock solutions of each salt. The other solutions were prepared from the stock solutions by weight dilution. The concentration of the sodium chloride stock solution was determined by evaporation to dryness, those of sodium iodide and sodium thiocyanate were determined by weighing the precipitated silver salt. The sodium hydroxide stock solution was made from Acculute standard volumetric solutions. The concentration was determined by titration with potassium acid phthalate with phenolphthalein as indicator. All stock solutions were stored in a 2° room.

Albumin.—Our supply of bovine mercaptalbumin was practically used up, so we have used human serum albumin. The Protein Foundation, through the courtesy of Dr. R. L. Pennell, presented us with some "Fraction V. reworked" prepared partly from fresh blood and partly from newly outdated blood by the standard Cohn alcohol fractionation method. We received it freeze-dried; we deionized it as described below and freeze-dried it again. It was stored at −30° both before and after deionization. The albumin was analyzed electrophoretically and in the ultracentrifuge by Dr. Karl Schmid of Boston University Medical School. At pH 8.6 there was 1% of an electrophoretic component moving faster than albumin and 1.5% of a component moving slower, presumably both α-globulins. In the ultracentrifuge 12% had a coefficient of 6.6 instead of 4.4, presumably a dimer. Compared to crystallized albumin it has the disadvantage of the 2.5% of globulins, but the advantage that no surfactant has been added in crystallization.

This albumin contains about 2 moles of fatty acid/mole of albumin as analyzed by the method of Dole.[4] We have tried several methods of removing it without otherwise altering the albumin. Mixed bed ion-exchange columns, gel filtration with Sephadex G25, raising the pH to 7.2, or treating with concentrated sodium thiocyanate before electrodialysis through ion-exchanger membranes showed little or no change. Acetone extraction at −30° removed only one-fifth of the fatty acid. A column of Rohm and Haas macroreticular anion exchanger in the hydroxyl form reduced the fatty acid to albumin ratio almost half but retained about half the albumin in a small-batch treatment. Acidification to pH 3.5 followed by neutralization in a column of Dowex 1 in the hydroxyl form removed almost half the fatty acid. Acidification to pH 2.9, passing through a Dowex-1 column followed by a column of Dow Et 561, a low capacity cation exchanger useful in removing many polar organics, in the hydrogen form was only slightly more successful. A commercial "fatty-acid-free" albumin from Mann Co. was practically free of fatty acid but so altered that its binding capacity was considerably reduced. We therefore used in our studies the original material with 2 moles of fatty acid as the most reproducible albumin available.

Deionization by Electrodialysis.—The idea of deionization by electrodialysis through a two-membrane stack of ion-exchanger membranes came from a paper of Katz and Ellinger.[5] Our apparatus was designed after the commercial multimembrane stack apparatus for desalination of water, and resembles closely the apparatus in which Duecker and Haller prepared water of the lowest conductivity yet attained,[6] which became known to us, however, only after our apparatus was constructed and in use.

The parts of the apparatus are shown in Fig. 1. It is made of three blocks of Lucite. The central block is 5.5 mm. thick with a cross section of 115 × 36 mm. with rounded corners. It has a central slot 95 × 11 mm. with semicircular ends. A 2-mm. i.d. tube is connected to this slot through each end. The side blocks are 140 × 70 × 19 mm. The inside of each has a groove 2 mm. deep to hold a membrane and a gasket, and an 8-mm. deeper groove to correspond to the slot in the central block. At each end of the latter is a 2-mm. i.d. tube leading through the back. Each side block has a platinum electrode 85 × 10 × 0.3 mm. in the smaller groove, about 1.5 mm. from the bottom of the larger groove. The cell also contains a cation and an anion-exchanger membrane, each 0.6 mm. thick, of the same size as the central block and two Teflon washers, 0.7 mm. thick, of the same size and with slots corresponding to those in the central block. The cell is assembled with the ion-exchanger membranes next to the central block and the Teflon washers between the membranes and the outer blocks. It is held tightly together with six metal screws with wing nuts through matching holes in the outer blocks. The distance between the electrodes is 11 mm., of which 5.5 mm. is the solution compartment, each membrane is 0.6 mm., and the conducting path in each of the outer compartments is 2.2 mm. The power supply is Model F-6005-A of Precise Measurements, Inc. It is connected so that the cathode is next to the cation-exchanger membrane.

The cell is operated in a 2° cold room. Conductivity water flowing upward through the electrode compartments at about 1 l./hr. serves to cool the cell and to remove electrolytes and gas bubbles. A 2.4% protein solution is flowed through the central compartment at a rate of about 10 ml./hr., regulated by a Sigmamotor pump. Thus, the retention time of the protein in the cell is about 0.5 hr. When the voltage across the two electrodes is 450 v., the current is about 20 ma. and the temperature of the protein solution at the outlet is 10°. The pH of the deionized solution at 25° is 4.99.

Apparatus.—The cell used for measuring the electromotive force is pictured in Fig. 2. It was constructed by Dr. Y. Victor Wu in this laboratory and copied in part from that used by Tan-

(1) Taken in part from the Ph.D. Thesis of W. T. Yap, Department of Chemistry, Massachusetts Institute of Technology, 1964. This work was supported in part by a research grant from the National Heart Institute of the National Institutes of Health, U. S. Public Health Service, and in part by the Atomic Energy Commission under Contract AT(30-1)-905.

(2) Paper XI; G. Scatchard and J. Pigliacampi, *J. Am. Chem. Soc.*, **84**, 127 (1962).

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

(4) V. J. Dole, *J. Clin. Invest.*, **35**, 150 (1956).

(5) S. Katz and F. Ellinger, *Biochemistry*, **2**, 406 (1963).

(6) H. C. Duecker and N. Haller, *J. Res. Natl. Bur. Std.*, **64A**, 527 (1960); *J. Phys. Chem.*, **66**, 225 (1962).

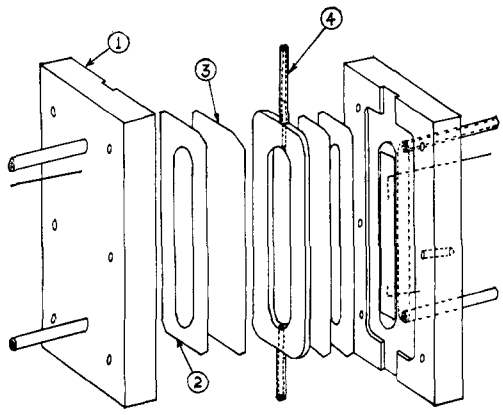

Fig. 1.—Electrodialysis cell: (1) outer Lucite block containing electrode, (2) Teflon gasket, (3) ion-exchanger membrane, (4) middle Lucite block.

ford for pH measurements.[7] The water-jacketed cell is connected through a three-way stopcock to a Y-tube, one arm of which is connected to the water-jacketed calomel electrode cell (a microcondenser) and the other through a stopcock to a reservoir of saturated potassium chloride. The calomel electrode cell is also filled with saturated potassium chloride in which is inserted the calomel electrode (Beckman 39270); this cell is made air-tight by a Rubber-stopper-no-air at the top.

The silver–silver salt electrodes were of the thermal-electrolytic type.[8] A spiral of platinum wire (0.016-in. diameter) was sealed to a 3-mm. glass tube about 6.5 cm. long. Silver was plated onto the platinum from a freshly boiled solution about 1 *m* in potassium cyanide and 0.25 *m* in silver nitrate for 2 hr. at a current density of about 20 ma./cm.^{2}. The silver electrode was washed thoroughly in conductivity water with stirring for about 24 hr. It was then dipped in a thick paste of silver oxide so that the paste filled the spaces of the spiral. It was heated in a furnace to 470° for about 0.5 hr. and then cooled slowly. A portion of the silver was converted to the silver salt by electrolysis in a solution of hydrochloric acid, hydriodic acid, or sodium thiocyanate acidified with nitric acid or with thiocyanic acid freshly prepared with a cation exchanger in the hydrogen form. The current density was about 4 ma./cm.^{2}. Nitrogen gas was bubbled through the solution during the salt formation. The electrode was covered with collodion by dipping in a collodion solution, was dried in nitrogen, and was stored in a solution of the sodium salt at least overnight. The electrodes were protected from light as much as possible.

The whole apparatus was placed in a grounded metal box with a screen door. At 25° water from a thermostat at that temperature was circulated through the water jackets around the two electrodes. The water was also grounded. The 0° measurements were made in a room at that temperature. The electromotive force was measured with a vibrating reed electrometer Model 31 of Applied Physics Corp. and recorded on a multiple range Brown Model 39 recorder.

The pH was measured with a Model TTT1a titrator of Radiometer, Copenhagen, with Radiometer shielded glass electrode Type G222B and calomel electrode Type K 130.

Procedure.—The silver–silver salt electrodes were equilibrated with 5.5 ml. of the solution of the same concentrations as that in the cell and at the temperature of the cell for about 2 hr. with magnetic stirring. They were transferred to the cell, which was then sealed with Parafilm. Before each measurement, 4 or 5 drops of the solution were flowed through the three-way stopcock and then about 20 drops of saturated potassium chloride solution was flowed to ensure a sharp junction at the top of the stopcock.

The electromotive force was always compared with that of a solution of such a concentration that the difference ΔE was very small. The molality of the unbound chloride ion (X^-) was determined by the relation

$$\Delta E = E - E' = -\frac{RT}{\mathfrak{F}} \ln \frac{(X^-)\gamma_-}{(X^-)'\gamma_-'} \qquad (1)$$

(7) C. Tanford, "Electrochemistry in Biology and Medicine," T. Shedlovsky, Ed., John Wiley and Sons, Inc., New York, N. Y., 1955, p. 248.

(8) D. J. G. Ives and G. J. Janz, "Reference Electrodes," Academic Press, New York, N. Y., 1961, Chapter 4.

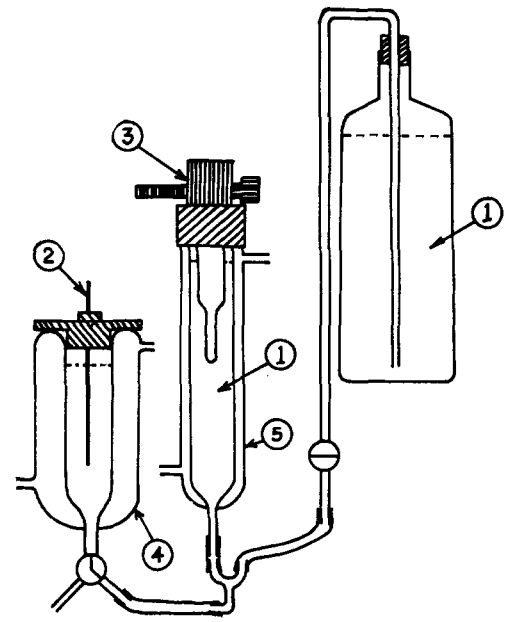

Fig. 2.—Electromotive force cell: (1) saturated KCl, (2) Ag,AgX electrode, (3) calomel electrode, (4) water-jacketed cell, (5) microcondenser.

in which the primed quantities refer to the solution without protein and the corresponding unprimed ones to that with protein.

TABLE I

BINDING OF SMALL ANIONS TO ISOIONIC HUMAN SERUM ALBUMIN AT 0°

Salt	$-\log m_3$	$-\log m_2$	$-\log (X^-)$	$\bar{\nu}_X$, exptl.	$\bar{\nu}_X$, calcd.
NaCl	3.882	3.544	4.009	0.12	0.15
	3.381	3.546	3.516	.39	.41
	3.348	3.517	3.477	.38	.38
	2.889	3.533	2.988	.90	.91
	2.885	3.567	2.979	.93	.92
	2.372	3.530	2.434	1.91	1.95
	1.947	3.523	1.987	3.34	3.41
NaI	4.409	3.539	5.269	0.12	0.11
	4.390	3.550	5.193	.12	.13
	3.858	3.542	4.623	.40	.39
	3.858	3.542	4.620	.40	.39
	3.361	3.546	3.932	1.12	1.07
	2.669	3.550	2.909	3.22	3.21
	2.111	3.546	2.212	5.66	5.93
	1.636	3.543	1.687	8.86	9.17
NaSCN	3.964	3.556	5.370	0.38	0.37
	3.681	3.559	4.844	.70	.73
	3.680	3.563	4.849	.71	.72
	3.233	3.550	3.914	1.64	1.67
	3.229	3.551	3.887	1.64	1.62
	2.380	3.575	2.548	4.95	4.98

The use of this equation depends on the assumptions that the only effect of the protein is the change in $(X^-)\gamma_-$, that γ_- is a function only of the ionic strength, I, and the same function as $\gamma_\pm$, and that the ionic strength in isoionic or more alkaline solutions is equal to the molality of sodium ion. The first of these assumptions is the same as that made in interpreting pH as $-\log a_{H^+}$.

We have used the following equations for the mean activity coefficients

$$\log \gamma_\pm^{NaCl} = -0.5090\sqrt{I}/(1 + 1.55\sqrt{I}) + 0.01090I + 0.00921I^2 \qquad (2)$$

$$\log \gamma_\pm^{NaI} = -0.5090\sqrt{I}/(1 + 1.55\sqrt{I}) + 0.05866I + 0.00921I^2 \qquad (3)$$

$$\log \gamma_\pm^{NaSCN} = -0.5085\sqrt{I}/(1 + 1.5\sqrt{I}) + 0.052I \qquad (4)$$

The average binding of X^- per mole of albumin is

$$\bar{\nu}_X = (m_3 - (X^-))/m_2 \quad (5)$$

in which m_2 is the molality of albumin, and m_3 that of the sodium salt.

The results for isoionic albumin at 0° are given in Table I, those at 25° in Table II, and those for higher pH at 25° in Table III. Tables I and II show the concentrations of salt, protein, and free anion $-\log m_3$, $-\log m_2$, $-\log (X^-)$, $\bar{\nu}_X$ exptl., and $\bar{\nu}_X$ calcd. Table III gives also the concentration of NaOH as $-\log m_7$, the pH, and $\bar{\nu}_{OH} = -\bar{\nu}_H$.

TABLE II

BINDING OF SMALL ANIONS TO ISOIONIC HUMAN SERUM ALBUMIN AT 25°

Salt	$-\log m_3$	$-\log m_2$	$-\log (X^-)$	$\bar{\nu}_X$, exptl.	$\bar{\nu}_X$, calcd.
NaCl	3.403	3.539	3.506	0.29	0.30
	3.377	3.516	3.482	.30	.32
	3.348	3.517	3.459	.33	.33
	2.904	3.534	2.991	.78	.76
	2.899	3.592	2.973	.77	.79
	2.413	3.531	2.473	1.70	1.67
	2.413	3.531	2.471	1.65	1.69
	1.927	3.567	1.960	3.21	3.35
NaI	4.408	3.559	4.900	0.10	0.11
	3.940	3.546	4.469	.28	.27
	3.870	3.551	4.326	.31	.36
	3.840	3.553	4.324	.35	.36
	3.361	3.546	3.807	.98	.84
	2.705	3.573	2.907	2.74	2.73
	2.695	3.528	2.902	2.58	2.79
	2.128	3.556	2.227	5.47	5.39
	2.128	3.556	2.225	5.36	5.44
	1.652	3.555	1.702	8.81	8.68
NaSCN	3.938	3.581	4.806	0.38	0.38
	3.924	3.577	4.799	0.39	0.38
	3.415	3.565	3.978	1.03	1.05
	2.742	3.577	2.970	2.80	2.79
	2.383	3.576	2.522	4.29	4.28

TABLE III

BINDING OF SMALL ANIONS TO HUMAN SERUM ALBUMIN IN BASIC SOLUTIONS AT 25°

$-\log m_3$	$-\log m_7$	$-\log m_2$	pH	$\bar{\nu}_{OH}$	$\bar{\nu}_X$, exptl.	$\bar{\nu}_X$, calcd.
Albumin (2), NaI (3), NaOH (7)						
2.702	3.155	3.530	5.45	2.37	2.23	2.32
2.701	2.928	3.569	5.70	4.37	2.06	2.04
2.700	2.725	3.528	6.09	6.35	1.69	1.76
2.710	2.551	3.537	6.68	9.69	1.16	1.31
2.710	2.444	3.541	7.02	12.52	1.05	1.01
2.713	2.349	3.542	7.55	15.59	0.74	0.75
2.722	2.106	3.550	9.38	27.74	.28	.29
3.193	2.631	3.531	6.21	7.95	.62	.71
3.304	2.615	3.561	6.48	8.83	.46	.53
Albumin (2), NaCl (3), NaOH (7)						
2.410	2.947	3.529	5.52	3.82	1.15	1.20
2.422	2.521	3.530	6.65	10.20	0.68	0.61
2.423	2.413	3.543	7.15	13.47	.41	.43

Discussion

The binding of a small anion to a protein may be expressed as

$$\bar{\nu}_H = \sum_i \bar{\nu}_{Xi} = \sum_i n_i K_{Xi} \alpha_X / (1 + K_{Xi} \alpha_X) \quad (6)$$

in which $\bar{\nu}_{Xi}$ is the average number of X^- ions bound at the ith type binding sites, n_i is the total number of sites of this type, K_{Xi} is the binding constant, and

$$\alpha_X = (X^-) \gamma_X e^{-2w\bar{Z}_p Z_X} \quad (7)$$

if $2w\bar{Z}_p Z_X RT$ is the electrostatic work of bringing an ion with charge Z_X to the surface of a protein molecule with average charge $\bar{Z}_p$.

If the charge on the protein molecule is distributed uniformly over the surface of a sphere with radius b before and after the addition and if uncombined small ions can approach the center of the protein molecule to a distance a, which is not smaller than b, the Debye theory gives

$$w = (e^2/2DkT)[1/b - \kappa/(1 + \kappa a)] \quad (8)$$

in which e is the protonic charge, D is the dielectric constant, k is Boltzmann's constant, T is the absolute temperature, and κ has its usual meaning in the Debye theory. Following the custom of this laboratory, we assume that the value of w with b = 30 Å. and a = 32.5 Å. may be used for albumin molecules to give

$$2w/2.303 = 0.1005 - 0.977\sqrt{I}/(1 + 10.53\sqrt{I}) \quad \text{at } 0° \quad (9)$$

$$2w/2.303 = 0.1034 - 1.017\sqrt{I}/(1 + 10.66\sqrt{I}) \quad \text{at } 25° \quad (10)$$

Scatchard, Scheinberg, and Armstrong[9] determined the n and k values by plotting $\bar{\nu}_X/\alpha_X$ *vs.* $\bar{\nu}_X$. Scatchard, Coleman, and Shen[10] and Scatchard, Wu, and Shen[11] determined them by superposing a plot of log $y/(1 + y)$ *vs.* log y, in which y is a generalized working variable, upon a plot of log $\bar{\nu}_X$ *vs.* log α_X. We have preferred a modification of the first method which gives lines of smaller relative slope in the range of extrapolation. We plot log $\bar{\nu}_X/\alpha_X$ *vs.* $\bar{\nu}_X$. The intercept at $\bar{\nu}_X = 0$ is

$$\log (\sum_i n_i K_{Xi}) = \log (n_1 K_{X1}) + \log (\sum_i n_i K_{Xi}/n_1 K_{X1}) \quad (11)$$

and the asymptotic slope is

$$-0.4343(\sum_i n_i K_{Xi}^2)/(\sum_i n_i K_{Xi})^2 = -0.4343(\sum_i n_i K_{Xi}^2/n_1 K_{X1}^2)/n_1(\sum_i n_i K_{Xi}/n_1 K_{X1})^2 \quad (12)$$

These plots are shown in Fig. 3.

Values of n_1 and K_{X1} are obtained by assuming that $\sum_i n_i K_{Xi}/n_1 K_{X1}$ and $\sum_i n_i K_{Xi}^2/n_1 K_{X1}^2$ are very nearly unity, and that n_1 is an integer. Repetition on $(\bar{\nu}_X - \bar{\nu}_{X1})$ gives n_2 and K_{X2}, and on $(\bar{\nu}_X - \bar{\nu}_{X1} - \bar{\nu}_{X2})$ gives n_3 and K_{X3}, etc. Refinement of these values is obtained by iteration using $\bar{\nu}_X$ minus all the $\bar{\nu}_{Xi}$ values except the one for which the parameters are desired.

We found three types of binding sites with n_1 = 1, n_2 = 4, n_3 = 22 with K_{X2} much smaller than K_{X1} and K_{X3} much smaller than K_{X2}. Our measurements were not carried to values of $\bar{\nu}_X$ large enough to determine n_3 and K_{X3} with any precision. The product $n_3 K_{X3}$ is better known but it may well represent more than one type of site. We chose n_3 = 22 to make $(n_1 + n_2 + n_3)$ the same as that used in the measurements with bovine mercaptalbumin.

(9) G. Scatchard, I. H. Scheinberg, and S. H. Armstrong, *J. Am. Chem. Soc.*, **72**, 535, 540 (1950).

(10) G. Scatchard, J. S. Coleman, and A. L. Shen, *ibid.*, **79**, 12 (1957).

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

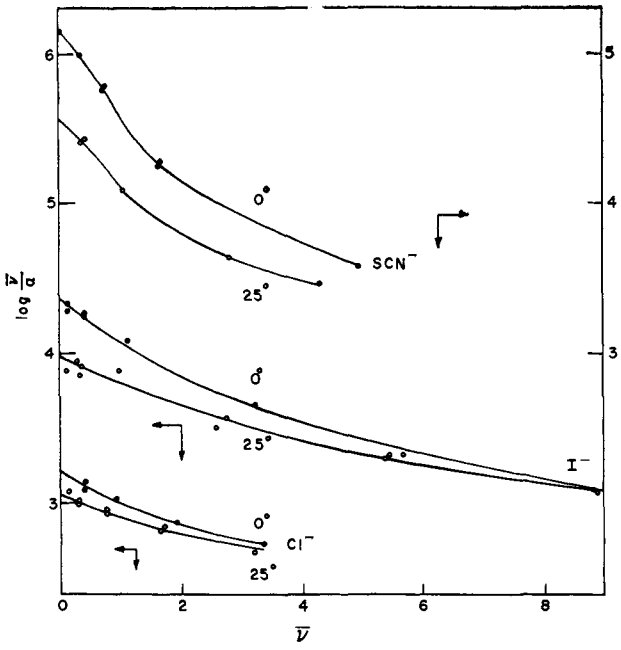

Fig. 3.—Plots of $\log \bar{\nu}/\alpha$ *vs.* $\bar{\nu}$: filled circles 0°, open circles 25°. Curves are calculated values.

From our values of the constants at 0° and at 25°, we have computed the corresponding values of the free energy, the enthalpy, and the entropy. Table IV shows the values of n_i, K_{Xi} at 0° and 25°, $-\Delta G_i°$ at 0° and 25°, $-\Delta H_i°$, and $\Delta S_i°$.

TABLE IV

THERMODYNAMIC FUNCTIONS OF BINDING OF SMALL ANIONS TO HUMAN SERUM ALBUMIN

	Salt	—log K_{Xi}— 0°	25°	$-\Delta G_{Xi}°$, —kcal./mole— 0°	25°	$-\Delta H_{Xi}°$, kcal./mole	$\Delta S_{Xi}°$, e.u.
$n_1 = 1$	NaCl	3.054	2.857	3.82	3.91	2.96	3.18
	NaI	4.257	3.789	5.34	5.18	7.03	−6.19
	NaSCN	5.113	4.527	6.40	6.19	8.79	−8.75
$n_2 = 4$	NaCl	1.869	1.785	2.34	2.44	1.26	3.96
	NaI	3.041	2.824	3.82	3.86	3.26	2.03
	NaSCN	3.331	2.894	4.17	3.95	6.56	−8.73
$n_3 = 22$	NaCl	0.996	0.996	1.25	1.36	0.00	4.56
	NaI	1.556	1.556	1.95	2.13	.00	7.13
	NaSCN	1.589	1.568	1.99	2.14	.31	6.15

The quickest way to survey these results is to examine the relative values of $-\Delta G_i°$ and $-\Delta H_i°$. When $-\Delta G_i°$ is very large, for thiocyanate on the first and second type sites, $-\Delta H_i°$ is even larger and $\Delta S_i°$ is negative so that it hinders the binding. On the other hand, the $\Delta H_i°$ never becomes positive although it is zero for the chloride and iodide ions on the third type sites and is very small for the thiocyanate ion on these sites. Figure 4 shows the average values $-\Delta\bar{G}$ and $-\Delta\bar{H}$ as functions of $\bar{\nu}$. It shows clearly that $-\Delta\bar{H}$ is always much larger than $-\Delta\bar{G}$ for thiocyanate ion and always much smaller for chloride ion, but that for iodide ion $-\Delta\bar{H}$ is larger than $-\Delta\bar{G}$ for the first molecules bound and becomes smaller when $\bar{\nu}$ becomes large.

On the addition of alkali the binding of the anions is markedly decreased, as is shown in Fig. 5. The major part of this decrease is caused by the increased negative charge on the protein caused by the removal of protons, as shown by the upper broken curve. There is a further effect, however, of the removal of protons

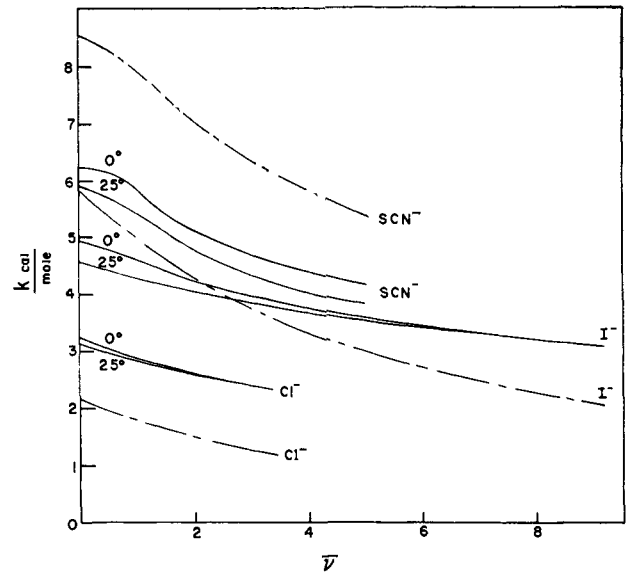

Fig. 4.—Average free energy and enthalpy of binding: full lines $\Delta\bar{G}$, broken lines $\Delta\bar{H}$.

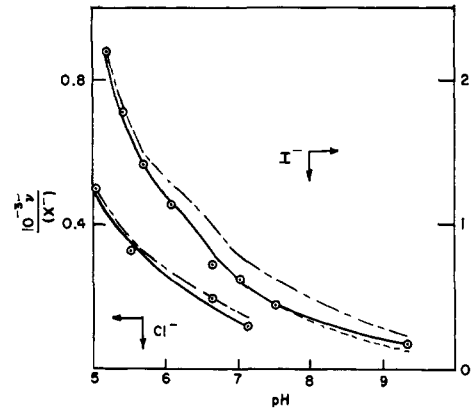

Fig. 5.—Binding ratio, $\bar{\nu}/(X^-)$ *vs.* pH: broken line, calculated for electrostatic effect only; full line, calculated for electrostatic effect and $pK_{H2} = 6.1$; dotted line calculated for electrostatic effect and $pK_{H2} = 6.1$, $pK_{H1} = 8.0$.

from active sites, for the anions appear to be bound only to the acid form. If there are n_i sites of type i, of which $\bar{\nu}_{Xi}$ have bound anions $\equiv NH^+X^-$ or $\equiv NHX$, $\bar{\nu}_{0i}$ are free acid sites $\equiv NH^+$ and $\bar{\nu}_{Bi}$ are in the basic form $\equiv N$

$$n_i = \bar{\nu}_{0i} + \bar{\nu}_{Xi} + \bar{\nu}_{Bi} = \bar{\nu}_{0i}(1 + K_{Xi}\alpha + K_{Bi}/\alpha_H)$$

and

$$\bar{\nu}_{Xi} = n_i K_{Xi}\alpha_X/(1 + K_{Xi}\alpha_X + K_{Bi}/\alpha_H) \quad (13)$$

The full curve, which fits the measurements within the apparent experimental error, represents the binding with $K_{H2} = 10^{-6.1}$ or $pK_{H2} = 6.1$. This constant corresponds to the imidazole range and indicates that the type two sites are four of the 16 imidazole groups. It gives no indication, of course, as to why the other 12 imidazole groups have little tendency to bind anions. The type one site and the type three sites are definitely not imidazoles. The lower, dotted, curve corresponds to $pK_{H1} = 8.0$, which would correspond to the type one site being the single α-amino group. It fits our measurement within the apparent error, though not as well as the assumption that $pK_{H1} > 8$.

Comparison with Earlier Measurements.—Scatchard, Scheinberg, and Armstrong (SSA)[9] expressed the binding of thiocyanate and chloride ions to crystallized human serum albumin by 10 active groups and 30 more with a constant $^1/_{30}$ as large. Scatchard, Coleman, and Shen (SCS)[10] expressed the binding of iodide and chloride ions to bovine mercaptalbumin at 25° by 1 very active group, 8 more with a constant $^1/_{24}$ as large, and 18 more with a constant $^1/_{30}$ that of the second type. Scatchard, Wu, and Shen (SWS)[11] expressed the binding of thiocyanate, trichloroacetate, and fluoride ion to bovine mercaptalbumin at 25° according to the same relations. Our present results (SY), which have put more emphasis on measurements in the more dilute solutions, are incompatible with eight groups of type two. They do not extend to large enough binding to determine the number of groups of type three, so we have chosen to consider a total of 27 groups to be consistent with the earlier expressions. On the other hand these earlier results are expressed very nearly as well, or just as well, with the 1-4-22 scheme as with the 1-8-18 scheme, provided that the condition that ratios of the constants be the same for all anions is given up. This permits a comparison of the different measurements by listing the three constants in Table V. The story is completed by

TABLE V

COMPARISON OF BINDING CONSTANTS

Anion	Albumin	Authors	Range $\bar{\nu}$	log K_1	log K_2	log K_3
Cl^-	CHSA	SSA	0.1–7.5	2.477	1.699	0.778
	BMA	SCS	0.6–9.2	3.380	2.301	0.845
	HSA	SY	0.3–3.3	2.857	1.785	0.996
I^-	BMA	SCS	1.3–18.3	3.966	2.978	1.342
	HSA	SY	0.1–8.8	3.789	2.824	1.550
CNS^-	CHSA	SSA	0.2–28.2	3.869	3.146	1.699
	BMA	SWS	0.4–20.7	4.342	3.447	1.806
	HSA	SY	0.4–4.3	4.527	2.849	1.568
F^-	BMA	SWS	0.5–7.6	3.556	2.505	1.000
$(TCA)^-$	BMA	SWS	0.6–30.8	5.079	4.602	2.431

including the results of SWS for fluoride and trichloroacetate. The values of the constants must be regarded with caution, however, particularly when the binding is already large in the most dilute solution. For example, the measurements of SCS and SY for I^- check beautifully through the range of overlap, but the measurements of the latter in more dilute solutions lead to smaller values of K_{11} and K_{12}, and therefore larger values of K_{13}, so that the equation from their results does not agree with the measurements of SCS in more concentrated solutions. Again the measurements of SWS on CNS^- give about 15% more binding than those of SY except for the most dilute solution. This leads to a smaller value of K_{X1} and larger values of K_{X2} and K_{X3} for the former. The measurements of SSA agree with those of SWS for $\bar{\nu}_X$ larger than two, but fall low in more dilute solutions. For Cl^-, on the other hand, the measurements of SCS are always larger than those of SY and the ratio increases from 1.15 to 1.40 as the concentration decreases. Those of SSA are always smaller than those of SY, and the ratio diminishes with decreasing concentration from 0.97 to 0.80. Although the values of the constants may be somewhat misleading, the values of $\bar{\nu}_X$ as a function of α_X in the experimental range are readily computed from eq. 6. The corresponding values of (X^-) and m_3 may be computed by a rapidly converging iteration.

We believe that this comparison shows that with respect to small anion binding there is little or no difference between bovine and human serum albumin, between total albumin and mercaptalbumin, or between reworked and crystallized albumin. The low results of SSA with chloride ion, particularly in dilute solutions, are probably due to the fact that their albumin was not deionized. Their low results with dilute thiocyanate solutions may be due to the fact that the electrodialysis through cellophane was less efficient than deionization with an ion-exchange column or membranes. There seems to be no simple explanation for the facts that the earlier results with chloride ion and with thiocyanate show larger binding than ours, but the intermediate results with iodide ion, which are the most consistent in each series, are essentially identical in the region of overlap.