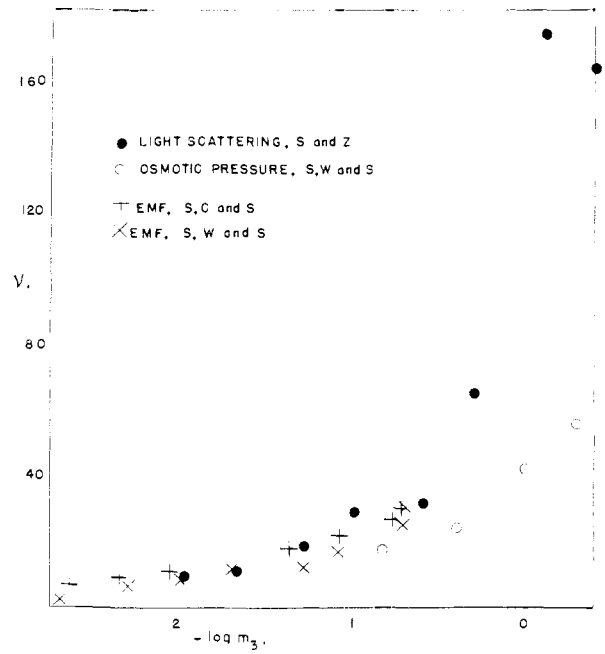

Fig. 2.—Binding of trichloroacetate ion to bovine serum mercaptalbumin.

from osmotic pressures,[8] the values so calculated are absurdly large at the lowest concentrations, and for the others they vary from 1.5 to 8 times those listed in column 8, with an average ratio of 4. It seems much more probable that the variation in the intercept is caused largely by aggregation. Even if the aggregation were constant and known, this method could not yield precise values of ν because a 1% decrease in the intercept corresponds to five ions bound.

(8) J. Pigliacampi, Ph.D. Thesis, M.I.T., 1957.

The values of ν, in column 8, calculated from the slopes, are compared with the electromotive force results of Scatchard, Coleman and Shen[9] and of Scatchard, Wu and Shen[4] and the osmotic pressure results of the latter in Fig. 2. The values of ν^* are omitted to prevent the comparison of ν^* by one method with ν by another. The qualitative comparison is the same for ν^*'s as for ν's. The light scattering results agree well with the electromotive force results in the range of overlap 0.01–0.1 *m* NaTCA, except that the value at 0.1 *M* is high relative to those at other concentrations. The osmotic pressure measurements lead to smaller binding in the dilute solutions and very much smaller binding in the concentrated solution. It seems almost certain that light scattering results in concentrated salt solutions are too high, but it is not so certain that the osmotic pressure results are not too low. The two sets depend upon the same assumptions: that β_{23} is zero, and for ν that β_{22} is zero, or for ν^* that β_{22} is 500 plus an electrostatic part calculable from theory. There is no basis in the present work to choose between the two assumptions.

The results with added acid or base show a surprising tendency for the net charge to remain unchanged at constant salt concentration. This tendency is somewhat obscured by the isoionic result at 0.1 *m* and the value with added base at 0.25 *m*, which both appear to be high. The latter is one of those with very large correction for large particle scattering. This tendency cannot be maintained for large additions of base, and probably is not maintained for large additions of acid.

(9) G. Scatchard, J. S. Coleman and A. L. Shen, THIS JOURNAL, **79**, 12 (1957).

CAMBRIDGE, MASS.

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

Physical Chemistry of Protein Solutions. X. The Binding of Small Anions by Serum Albumin[1]

BY GEORGE SCATCHARD, YING VICTOR WU AND AMY LIN SHEN

RECEIVED MAY 21, 1959

The binding to bovine serum mercaptalbumin of chloride, fluoride, thiocyanate and trichloroacetate ions from solutions of their sodium salts has been studied by measuring the *p*H and the electrical potentials of anion-exchanger electrodes as in earlier work, and in more concentrated salt solutions by measuring the osmotic pressure. The electromotive force measurements indicate that the first twenty-seven anions are bound at the same sites with one site in the first class, eight sites in the second class and eighteen sites in the third class, and the ratios of contants: $K_{1A}^0 = 24K_{2A}^0 = 720K_{3A}^0$, and $K_{1Cl}^0 = 2400$, $K_{1F}^0 = 3600$, $K_{1SCN}^0 = 24{,}000$, $K_{1TCA}^0 = 120{,}000$. The osmotic pressure results indicate that there are many additional sites, perhaps about seventy, with very little specificity among the anions.

This paper describes the continuation of the work of Scatchard, Coleman and Shen[2] giving more precise values for the binding of thiocyanate and trichloroacetate ions, giving results for fluoride ions, and extending the measurements to much higher bindings through the use of osmotic pressure measurements.

(1) Adapted in large part from the Ph.D. thesis of Ying Victor Wu, M.I.T., 1957. This investigation was supported in part by a grant from the Rockefeller Foundation and by a research grant (H-3249) from the National Heart Institute of the National Institutes of Health, U. S. Public Health Service.

(2) G. Scatchard, J. S. Coleman and A. L. Shen, THIS JOURNAL, **79**, 12 (1957).

Apparatus.—The apparatus for measuring the electrical potentials was modified considerably. It contains no cation-exchange membranes, it can be taken apart to equilibrate the membrane with a new solution or to replace the membrane, and it is particularly designed to permit thorough stirring with a very small amount of solution. The cell, shown diagrammatically in Fig. 1, consists of an anion exchanger membrane held between two blocks of plastic which

are held together by four screws with wing-nuts (not shown). A perpendicular groove, 6 mm. long, 2 mm. wide, and 0.8 mm. deep, is milled on the inside surface of each block. Holes, 2 mm. in diameter, are drilled to the two ends of each slot, the lower one is branched and both are continued by 2 mm. Pyrex glass capillaries, 8 mm. o.d. The holes into which the glass tubes fit are drilled square at the ends to avoid dead spaces. The glass tubes are sealed in with Epon Adhesive VI of the Shell Chemical Corporation, heated 2 hr. at 65°. The upper tube (a or b) leads to a small reservoir of 8 mm. o.d. Pyrex tubing, the lower tube (c or d) is tipped with a rubber cap from a medicine dropper for stirring, and the branch leads down to a 120° 3-way capillary stopcock. One arm of the stopcock leads to a calomel electrode in 4.5 *m* KCl and a reservoir of 4.5 *m* KCl, the other arm leads to the drain. The membranes were usually Amberplex A-1 of Rohm and Haas with the edges sealed by pressing gently on a flattened piece of copper tubing through which steam was passing. Scotch electric tape was used to seal the outside of the space between the blocks in order to retard evaporation from the membrane.

For the measurements with trichloroacetate and chloride the cell was made of Rexolite (hardened polystyrene cross-linked with divinylbenzene), the membranes were extra-thick (1.2 mm.) and the two solution contacts were directly opposite each other. For the measurements with thiocyanate and fluoride the cell was made of Lucite (methyl methacrylate), the membranes were regular thickness (0.6 mm.) and the two solution contacts were on opposite sides of the membrane but 4 cm. apart.

The *p*H's were measured with a Beckman Model G *p*H meter, sometimes with the five-way stopcock of the Cambridge Research Model *p*H meter for connection to the salt bridge. Most of the potentials of the ion-exchanger cells were measured with a Leeds and Northrup K-1 or K-2 potentiometer and 2430D galvanometer with a 7673 thermionic amplifier added for higher resistances. A Model 31 Vibrating Reed Electrometer and a Model 39 Multiple Range Recorder of the Applied Physics Corporation were used for the later measurements with high resistances.

The osmotic pressures were measured in a modified Hepp type osmometer with collodion membrane as described by Scatchard, Gee and Weeks[3] in a water-bath thermostated at 25 ± 0.02°.

Materials.—The bovine serum mercaptalbumin was supplied by the Harvard University Department of Biological Chemistry through the courtesy of Professor J. L. Oncley. It had been purified by repeated crystallization of the mercury dimer, and reconstituted and deionized by the ion-exchange method of Dintzis.[4] The fatty-acid-free human serum albumin was supplied by the Department of Health, Education and Welfare, Public Health Service, National Institutes of Health, through the courtesy of Dr. D. S. Goodman. It had been prepared by his method.[5] The albumin plus salt solutions for the electrical measurements were prepared by weight from stock solutions of albumin and of salt. The albumin content of the stock solutions was determined from the dry weight. The most concentrated solutions for osmotic pressure measurements were made by dissolving weighed amounts of freeze-dried albumin in the salt solution. The water content of the albumin was determined by drying separate samples at 105°. The more dilute solutions were made by weight dilution of a more concentrated solution with more of the salt solution used in its preparation. Usually the albumin plus salt was also determined by drying the solutions from the osmometer.

Analytical reagent grade (Mallinckrodt) sodium chloride, sodium thiocyanate and reagent grade (Merck) sodium fluoride were used without further purification. Sodium trichloroacetate was prepared by neutralizing concentrated trichloroacetic acid (Mallinckrodt analytical reagent grade) with 10 *N* carbonate-free sodium hydroxide (Fisher certified reagent grade). The resulting solution was freeze-dried, and the solid sodium trichloroacetate was used to prepare the stock solutions.

The concentrations of the stock solutions of sodium thiocyanate were determined by gravimetric silver thiocyanate analysis. The other stock solutions were prepared by weight from the dry salts. The sodium trichloroacetate solutions were stored at 2° until just before use. The other solutions were prepared from these stock solutions by weight dilution with conductivity water which was redistilled from alkaline permanganate in a Kraus-type still and was collected hot under nitrogen.

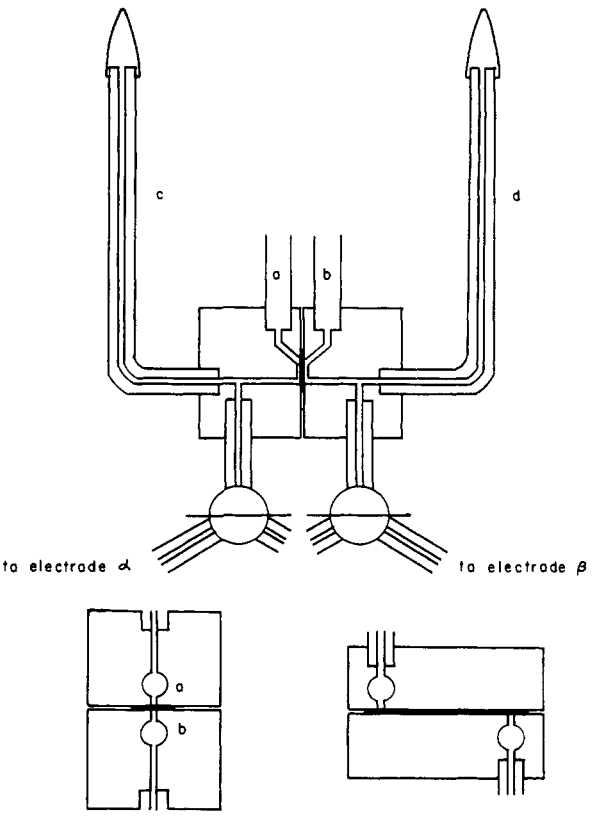

Fig. 1.—Cell for electromotive force measurements.

Electrical Potential Measurements.—Each pair of solutions was studied for a few hours or longer with several refillings of each solution. The solution was drained from the cell and about 15 drops of fresh solution were added. After the solution had been thoroughly mixed by means of the rubber caps about 5 drops were drained through the 3-way stopcock. Then about 10 drops of concentrated KCl were drained through this stopcock so that the bore was filled with KCl solution. Then both stopcocks were turned to give liquid junctions at the top of the plugs. The electrical potential difference between the two calomel electrodes was measured immediately and again after five minutes. Then the stopcocks were closed, the solutions were mixed again and the potential was read as before. If there was much change, the mixing was repeated. If there was little change, the solutions were replaced by fresh samples and the operation repeated. The potentials used were those obtained immediately after adding fresh solution when a change of solutions no longer made an appreciable difference in this potential. The asymmetry potential of the membrane was checked by measurements with the same solution on both sides, and the calomel electrodes were compared directly at least once each day.

Calibration curves without protein were obtained by measuring the potential of 8–10 cells without protein with the concentration ratio of 1 to 2 and concentrations from 0.00005 to 0.2 *m* (to 2 *m* for NaCl) and plotting ΔE *vs.* $\sqrt{m_1 m_2}$.

$$\Delta E = E_{\alpha-\beta} - \frac{RT}{\mathfrak{F}} \ln \frac{m_x \gamma_{\pm}\ (\text{in a})}{m_x \gamma_{\pm}\ (\text{in b})} \qquad (1)$$

The deviations at high concentrations due to the transfer of ions with the same sign charge as the membranes are not important because the measurements with albumin are made at low salt concentrations. The deviations in very dilute salt solutions are important.

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

(4) H. M. Dintzis, Thesis, Harvard University, 1952.

(5) D. S. Goodman, *Science*, **125**, 1296 (1957).

Measurements with protein solutions were made with the same, or nearly the same, total concentration of salt on each side of the membrane. The ratio of the species concentration (X^-) on the side with the protein to the anion concentration on the other side m_3 is calculated from equation 1 with the calibration curves. As discussed in the previous paper[2] this involves the assumption that the ratio of the activity coefficient of the anion to the mean activity coefficient does not change appreciably between the salt concentrations (X^-) and m_3. The binding then is calculated from the equation

$$\nu_x = [m_3 - (X^-)]/m_2 \quad (2)$$

We estimate that the accuracy of our measurements of membrane potential varies from about 0.25 mv. at 0.00008 m to 0.1 mv. at 0.001 m and above, and that the measurements of pH are accurate to 0.02 unit.

The results of the measurements with bovine serum mercaptalbumin are given in Table I and those with fatty-acid-free human serum albumin in Table II. The second column gives $-\log m_3$ and the third gives $-\log m_2$, m_2 and m_3 are the stoichiometric concentrations of protein and of salt. The fourth column gives the pH, the fifth gives w' calculated by equation 8, and the sixth column gives ν_x determined from the measurements with the use of equation 2, the seventh gives ν_{27}, the number of anions bound to the first 27 groups according to equation 7 and the parameters listed for each anion.

TABLE I

THE EFFECT OF BOVINE SERUM MERCAPTALBUMIN ON THE E.M.F. OF ION-EXCHANGER ELECTRODES IN SOLUTIONS OF NaF, NaSCN and NaTCA

Salt	$-\log m_3$	$-\log m_2$	pH	w'	ν_x	ν_{27}
NaSCN	4.101	3.806	5.19	0.0952	0.39	0.40
	3.399	3.816	5.27	.0866	1.66	1.59
	3.101	3.804	5.31	.0814	2.62	2.53
	2.400	3.813	5.48	.0650	5.75	5.96
	2.098	3.816	5.52	.0568	7.32	7.76
	1.401	3.810	5.70	.0386	14.21	12.80
	1.112	3.334	5.68	.0320	16.36	15.33
	0.769	3.213	5.71	.0256	19.04	18.96
	0.738	3.220	5.80	.0252	20.69	19.28
NaF	4.079	3.896	5.20	.0950	0.48	0.22
	3.395	3.915	5.23	.0866	0.81	0.80
	3.077	3.909	5.25	.0804	1.33	1.28
	2.775	3.912	5.28	.0744	1.83	1.92
	2.197	3.932	5.38	.0596	4.03	3.82
	1.778	3.897	5.45	.0482	5.75	5.81
	1.300[a]	3.120	5.41	.0362	5.86	8.30
	1.216	3.525	5.71	.0342	8.86	9.05
	1.003[a]	3.120	5.54	.0299	6.55	10.36
	0.688	3.246	6.35	.0244	7.65	12.88
NaTCA	4.097	3.904	5.12	.0951	0.61	0.59
	3.700	3.899	5.13	.0909	1.54	1.39
	3.399	3.899	5.17	.0866	2.86	2.53
	3.222	3.860	5.30	.0837	3.60	3.30
	3.096	3.808	5.39	.0813	4.13	3.83
	2.700	3.833	5.41	.0726	6.73	6.73
	2.397	3.868	5.42	.0650	8.69	8.99
	2.109	3.871	5.50	.0572	10.91	11.17
	1.397	3.529	5.71	.0385	17.96	17.78
	1.113	3.106	5.76	.0321	21.92	20.44
	0.770	3.106	5.95	.0257	27.47	23.79
	0.738	3.106	5.95	.0252	30.76	24.02

[a] Measured in small Rexolite cell.

Osmotic Pressure Measurements.—In moderately dilute protein solutions the osmotic pressure P may be expressed by the equation

$$PV^0/RT = m_2(1 + Bm_2) = (w_P/\bar{W}_P)(1 + Bw_P/\bar{W}_P) \quad (3)$$

in which V^0 is the volume of solution which contains one kilogram of water, m_2 is the number of moles of protein per kilogram of water, w_P is the weight of protein in grams per kilogram of water, $\bar{W}_P$ is the molecular weight of the protein, and B is the second virial coefficient. It is convenient to define J by the equation

$$J = 10^6 PV^0/RTw_P = (10^6/\bar{W}_P)(1 + Bw_P/\bar{W}_P) \quad (4)$$

A plot of J *vs.* w_P should give a straight line with intercept $10^6/\bar{W}_P$ and slope $10^6 B/\bar{W}_P{}^2$.

TABLE II

THE EFFECT OF FATTY-ACID-FREE HUMAN SERUM ALBUMIN ON THE E.M.F. OF ION-EXCHANGER ELECTRODES IN SOLUTIONS OF NaCl AND NaTCA

Salt	$-\log m_3$	$-\log m_2$	pH	w'	ν_x	ν_{27}
NaCl	4.096	3.863	5.35	0.0951	0.23	0.16
	3.795	3.818	5.45	.0921	.31	.29
	3.494	3.834	5.45	.0881	.59	.51
NaTCA	4.099	3.899	5.41	.0951	.57	.58
	3.799	3.878	5.44	.0921	1.13	1.08
	3.501	3.937	5.52	.0882	2.47	2.21

Since we start with isoionic protein, it is convenient for our purposes to define component 2 as

$$Na_{\nu_x/2}[PCl_{\nu_x}]Cl_{-\nu_x/2} = Na_{\nu_x/2}\,PCl_{\nu_x/2}$$

Then B may be written as

$$B = \nu_x{}^2/4m_3 + \beta_{22}/2 - \left(\frac{dm_3}{dm_2} - \frac{dm_3'}{dm_2}\right)^2\left(\frac{1}{m_3} + \beta_{33}/2\right) \quad (5)$$

in which $\beta_{22} = \partial \ln \gamma_2'/\partial m_2$, $\beta_{33} = 2\partial \ln \gamma_3/\partial m_3$. By the above definition of the protein and the assumption that the species activity coefficient of the salt $g_\pm$ is independent of the protein concentration, the third term of (5) is zero. We assume that the second term is also zero and that all of B is given by the Donnan term for the combined ions. Then

$$\bar{\nu}_x = \sqrt{4m_3(B - \beta_{22}/2)} \quad (6)$$

For each salt concentration four or more albumin concentrations were studied in the range of w_P = 15 to 50. With NaF above 0.05 m, the collodion membranes leaked and measurements were made with 70160-A cellophane membranes (Cenco) or 300 PD cellophane membranes (du Pont), which come to equilibrium at about one-tenth the rate of collodion membranes. Therefore, 24 hr. were allowed to attain equilibrium, only two protein concentrations were studied at each salt concentration, and the precision of the measurements is somewhat less than that with collodion membranes. The maximum concentrations of NaF and of NaCl were fixed by the solubilities, but for NaSCN or NaTCA the limit was set by the action on the membrane or the protein and the measurement with the most concentrated solution is less precise than the others. Both 4 m NaSCN and 2 m NaTCA appear to attack the membrane causing it to leak. In addition the 2 m NaTCA aggregates the albumin, so that the intercept as well as the slope had to be determined in this solution.

The results are given in Tables III and IV. In Table III each salt solution is characterized by the formula and concentration of the salt and by $10^6 V^0/RT$ in parentheses. The first and third columns give the protein concentration w_P in g./kg.

water. The unprimed values are determined by dry weight, the primed values by weight dilution of the most concentrated solution. The second and fourth columns give the osmotic pressure in cm. of decane at 25°.

TABLE III

THE EFFECT OF SALTS ON THE OSMOTIC PRESSURES OF ALBUMIN

w_P	P	w_P	P
	NaCl, 0.1500 m (28.92)		
13.15	6.99	43.45	26.32
19.73	10.66	54.12	34.35
29.16	16.40		
	NaCl, 0.4020 m (29.05)		
14.87	7.628	34.69	19.730
14.62′		34.61′	
24.85	13.364	43.28	25.694
24.44′		44.70′	
	NaCl, 1.000 m (29.38)		
15.24	7.09	35.84	19.32
24.79	12.40	54.05	32.50
	NaCl, 2.000 m (29.96)		
22.53	10.66	19.91	37.10
28.52	14.05	30.90	53.50
	NaCl, 4.016 m (31.22)		
16.86	7.433	37.53	19.503
15.39′		37.08′	
27.91	12.943	48.49	26.147
25.77′		47.63′	
	NaCl, 6.027 m (32.56)		
16.86	6.875	37.73	17.833
15.11′		36.71′	
28.09	12.287	48.40	24.403
26.22′		48.36′	
	NaSCN, 0.1503 m (28.97)		
16.71	8.761	38.46	24.105
27.17	15.840	49.76	33.925
	NaSCN, 0.4128 m (29.27)		
15.34	7.840	35.10	20.519
14.97′		35.28′	
24.95	13.686	43.56	26.934
24.85′			
	NaSCN, 1.000 m (29.94)		
14.52	6.855	33.62	18.555
24.57	12.655	44.17	25.415
	NaSCN, 2.002 m (31.13)		
18.79	8.427	39.09	20.64
27.96	13.934	50.20	28.39
	NaSCN, 4.008 m (33.59)		
15.35′	6.666	32.30′	15.726
16.08′	7.051	48.75′	25.823
	NaTCA, 0.1500 m (29.16)		
15.45′	8.306	34.84′	21.296
25.28′	14.650	44.02′	28.756
	NaTCA, 0.4000 m (29.75)		
15.56′	7.851	37.28′	21.313
26.58′	14.338	48.46′	29.327
	NaTCA, 1.000 m (31.14)		
15.11′	7.659	36.15′	20.237
25.22′	13.338	46.59′	27.593
	NaTCA, 2.000 m (33.72)		
16.39′	5.807	38.71′	15.048
26.99′	10.101	49.99′	21.367
	NaF, 0.0488 m (28.79)		
22.25	11.794	47.11	28.494
34.36	19.553		
	NaF, 0.1503 m (28.79)		
19.44	10.222	49.45	
19.06′		48.40′	30.900
	NaF, 0.4024 m (28.78)		
27.20	15.552	48.97	30.845
26.39′		48.03′	
	NaF, 0.9238 m (28.78)		
25.38	13.902	51.22	32.015
24.39′		50.33′	

TABLE IV

OSMOTIC PRESSURE VALUES OF ANION BINDING

m_3	B_2	ν_x	ν_x^*
	NaCl		
0.150	368.5	14.87	8.65
.4020	319.0	22.65	10.54
.4020′	317.4	22.59	10.41
1.000	279.5	33.43	10.86
2.000	272.1	46.66	13.30
4.016	269.0	65.74	17.47
4.016′	299.6	69.37	28.23
6.027′	222.7	73.28	
	NaSCN		
0.1503	514.6	17.59	12.92
.4128	391.0	25.41	15.26
.4128′	391.0	25.41	15.26
1.000	319.0	35.72	16.61
2.002	293.3	48.44	18.61
4.008′	348.9	74.79	39.82
	NaTCA		
0.1500′	519.8	17.66	13.03
0.4000′	358.8	23.96	13.19
1.000′	453.5	42.59	28.53
2.000′	392.9	56.06	33.81
	NaF		
0.0488	298.3	7.63	3.50
.1503	421.0	15.91	10.40
.4024	434.6	26.45	17.24
.9238	398.1	38.35	23.39

In Table IV the first column gives the salt concentration, the second gives a weighted average of the slope

$$B_2 = (\Sigma w_P \sqrt{B}/\Sigma w_P)^2$$

Except for the run with 2 m NaTCA the slopes were obtained by assuming that the intercept is 14.1 ($\bar{W}_P$ = 71,000) which is the value obtained by Pigliacampi[6] for this lot of mercaptalbumin. Widely discordant values of B were discarded before averaging. The third and fourth columns give the corresponding values of ν_x or ν_x^* calculated, however, for 69,000 grams of protein. In the third column ν_x is calculated from equation 6 on the assumption that β_{22} is zero, and in the fourth column ν_x^* is calculated on the assumption of

(6) J. Pigliacampi, Ph.D. Thesis, M.I.T., 1957.

Paper VIII[7] that β_{22} is 500 plus an electrostatic interaction term calculated from theory. The electrostatic term is negligible for salt concentrations larger than 0.15 m.

Discussion

As in our previous work,[2] we express the binding of an anion, A, to several classes of sites with the K_{iA}^0 for each of the n_i sites in class i as

$$\nu_A = \Sigma_i \nu_{iA} = \Sigma_i \frac{n_i K_{iA}^0 a_A e^{-2w z_p z_A}}{1 + K_{iA}^0 a_A e^{-2w z_p z_A}} = \Sigma_i \frac{n_i K_{iA}^0 \alpha_A}{1 + K_{iA}^0 \alpha_A} \quad (7)$$

which defines α_A as $a_A e^{-2w z_p Z_A}$, and

$$w' = 2w/2.303 = 0.1034 - 1.017\sqrt{I/2}/(1 + 10.663\sqrt{I/2}) \quad (8)$$

which corresponds to the Debye theory for a protein molecule with radius 30 Å. and small ions with radius 2.5 Å. in water at 25° if the charge on the protein has spherical symmetry before and after the binding of an anion.

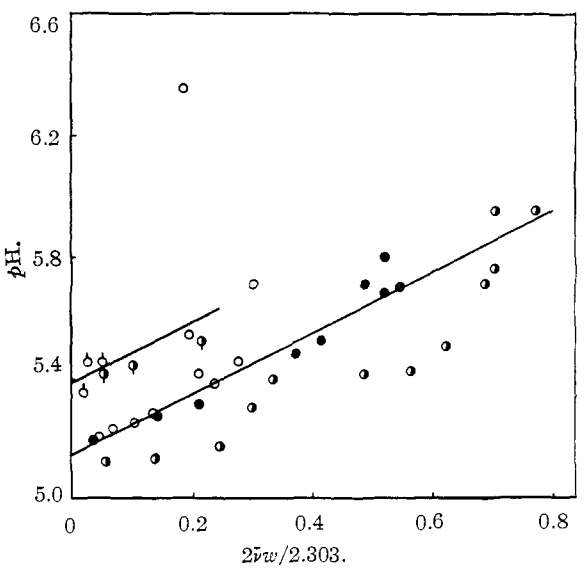

Fig. 2.—Effect of trichloroacetate ion on pH of serum albumin: ◑, NaCl + HSA; ◑, NaTCA + HSA; ○, NaF + BMA; ●, NaSCN + BMA; ◑, NaTCA + BMA.

Scatchard, Coleman and Shen[2] found that the binding of chloride and iodide ions to bovine mercaptalbumin in dilute solution could be explained by binding to three classes of sites with one site in the first class, eight in the second and eighteen in the third and with $K_1 = 24K_2 = 720K_3$. For chloride K_1 is 2400, and for iodide it is 9200. Their preliminary results with thiocyanate and trichloroacetate ions indicated that these ions might be bound to the same sites with the same ratios of the constants and with $K_1 = 46{,}000$. We have repeated the measurements with thiocyanate and trichloroacetate ions and have extended them to fluoride ion. Comparison of the last two columns of Table I shows very good agreement with the findings for chloride and iodide of one site in the first class, eight in the second and eighteen in the third, with $K_1 = 24K_2 = 720K_3$. The agreement covers the whole concentration range for thiocyanate with $K_1 = 24{,}000$, ten times the value for chloride. For trichloroacetate, the agreement with $K_1 = 120{,}000$ is limited to concentrations below 4×10^{-2} m salt. At higher concentrations there is evidence of binding to more than the first twenty-seven sites. For fluoride the agreement with $K_1 = 3600$ is limited to salt concentrations below 10^{-2} m. The electromotive force measurements at higher concentrations indicated binding much less than calculated, and the binding even appears to decrease with increasing salt concentration. The measurements at 0.05 and 0.1 m were repeated with the Rexolite cell of Fig. 1 and thick membranes but with the same results. On the other hand, the change in pH indicates much more binding than calculated. The osmotic pressure results lie between the other two.

A comparison of the last two columns of Table II shows excellent agreement for the binding of chloride and of trichloroacetate in very dilute solutions to the "fatty-acid free" human serum albumin of Goodman.

The pH of the protein–salt solutions is given in the 4th column of Tables I and II and is plotted against $2\nu w/2.303$ in Fig. 2. The lines are drawn with unit slope. The results with thiocyanate fall close to the curve with intercept 5.15, which is the same as that previously found.[2] Those with dilute solutions of fluoride also agree, but the more concentrated solutions show very much larger pH changes. With trichloroacetate the deviations from the line vary from +0.1 to −0.3 pH unit. Most of them are negative, and the average is −0.13 unit.

The measurements with "fatty-acid-free" human serum albumin are in too dilute solutions to tell much about the slope. The line is drawn to fit the average of the sodium chloride values. The intercept is 5.38, which is the expected value for human serum albumin. Again the values for trichloroacetate are below the curve.

The values of ν calculated from the osmotic pressure measurements are shown in Fig. 3. It should be noted again that, except for sodium chloride, the value in the most concentrated solution is less certain than the others. These values involve no assumption concerning single ion activities, but they do involve assumptions as to the effect of albumin on its own activity coefficient and on that of the salt.

The values of ν for sodium trichloroacetate from the osmotic pressure and e.m.f. measurements of this paper, the e.m.f. measurements of Scatchard, Coleman and Shen[2] and the light scattering measurements of Scatchard and Zaromb[8] are shown in Fig. 2 of the latter paper.

The values of ν at 0.15 m from osmotic pressures are compared in Table V with those calculated for the first twenty-seven sites and with those interpolated from the e.m.f. measurements and for chloride[1] and trichloroacetate[8] from light scattering. For the osmotic pressure and the light scattering, ν^* is also given. For NaCl and NaF the values of ν from osmotic pressure or light scatter-

(7) G. Scatchard and J. Bregman, THIS JOURNAL, **81**, 6095 (1959).

(8) G. Scatchard and S. Zaromb, *ibid.*, **81**, 6100 (1959).

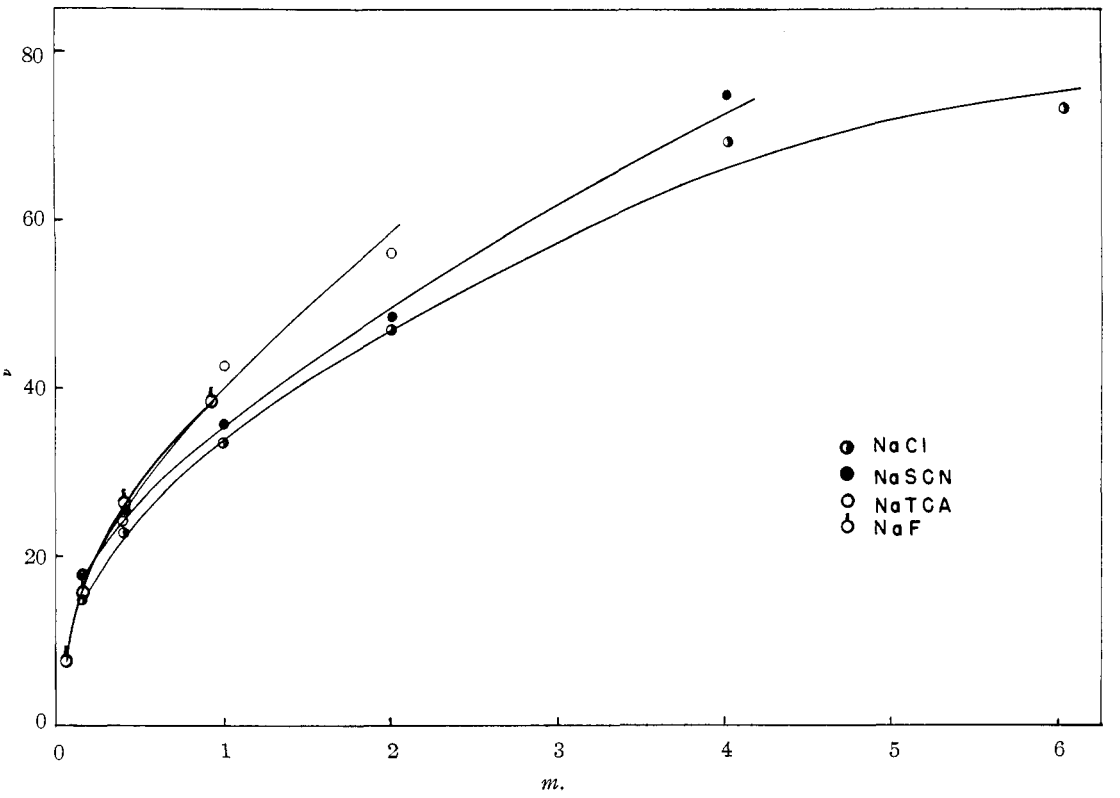

Fig. 3.—Binding of anions to bovine serum mercaptalbumin from osmotic pressures.

ing (NaCl) are much larger than those calculated or measured by electromotive force, while the values of ν^* are smaller. For thiocyanate, ν agrees well but ν^* is small, while for trichloroacetate both osmotic pressure results are very low, while from light scattering ν is a little high and ν^* agrees well.

TABLE V

BINDING AT 0.15 *m* SALT

	Cl^-	F^-	SCN^-	TCA^-
ν_{27}	10.8	11.8	18.2	23.1
ν from e.m.f.	..	7.2	18.6	26.4
ν from osmotic pressure	14.9	15.9	17.6	17.7
ν from light scattering	13.1	..	..	29
ν^* from osmotic pressure	8.9	10.4	12.9	13.0
ν^* from light scattering	5.7	..	..	27

Table IV shows that our measurements with concentrated NaCl are not compatible with the assumption upon which the computation of ν^* is based, that there is a constant non-electrostatic contribution to β_{22} of 500, for this assumption leads to negative values of ν^*. The binding would decrease with increasing concentration if this part of β_{22} were the same at 4 and 6 *m* and greater than 150. Since the 500 was obtained from dilute solutions of NaCl, there is even more doubt about its use with other salts. We will therefore consider for concentrated solutions only the values of ν, which are based on the assumption that in these solutions β_{22} is zero. We must not forget, however, that this assumption is only approximate.

The values of ν do indicate that the limit to the binding is not much less than the hundred ionizable nitrogen groups. If all the sites beyond the most active twenty-seven had the same affinity for an anion, the constants, uncorrected for activity coefficients, would be about $^1/_2$ for trichloroacetate (about 2 from light scattering), $^1/_3$ for thiocyanate and $^1/_4$ for chloride, which are the reciprocals of the concentrations at which ν is about $62 = 27 + 70/2$, since $1/k = m$ when $\nu = n/2$. This indicates that the specificity is much less than for the first twenty-seven sites. The errors in our assumptions are very probably small enough to warrant both of these qualitative conclusions. We are still left with the problem of what the active sites are and what makes them so much more active in albumin than in the globulins.

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