tegration of mm./g. *vs.* log P/P_0 was rather unsatisfactory at the lower values of P/P_0. However, upon noting that θ, the exponent in the Freundlich sorption isotherm, was approximately 0.7 it developed that quite satisfactory evaluations in this region could be made with a plot of $[\text{mm./g.}/(P/P_0)^{0.7}]$ *vs.* $[(P/P_0)^{0.7}]$. This follows from the fact that an integral of the form $\int x\, d(\ln p)$ is equal to $1/\theta \int x/p^\theta \cdot d(p^\theta)$. Moreover, if $x = kp^\theta$, *i.e.*, if the sorption can be represented by the Freundlich isotherm in the region of low relative pressures, then the plot of (x/p^θ) *vs.* p^θ will give straight line extrapolations to zero relative pressure.

Using data for various polar gases on EA at 25.0° and setting M_2 = 42,000 the following ΔF_h values were obtained in kcal./mole of protein: EtCl = 38, H_2O = 66, EtOH = 79 and MeOH = 118. It is noteworthy that these values follow a definite trend with respect to the stress factor, DV^0, employed in the empirical treatment. The values for DV^0 in (cc.) (unit area)/(mole) were: EtCl = 1680, H_2O = 6060, EtOH = 7440 and MeOH = 7520. However, again, the departure of MeOH from the linear trend of the other adsorbates was disturbing. It tends to emphasize certain omissions in the present treatment which do not account for the specific geometry of adsorbate–R group arrangements.

LOS ANGELES, CAL.

[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF NORTHWESTERN UNIVERSITY]

The Binding of Organic Ions by Proteins. Volume Changes

BY ROBERT M. ROSENBERG[1,2] AND IRVING M. KLOTZ

RECEIVED NOVEMBER 3, 1954

The volume change in the reaction between bovine serum albumin and sodium dodecyl sulfate has been determined from pycnometric measurements of the apparent molal volumes of bovine serum albumin, sodium dodecyl sulfate and the complex between them. For solutions in which approximately 13 dodecyl sulfate anions are bound per mole of albumin, the volume change for bound anion is 6.7 ± 1.4 ml. The results have been compared with predictions based on a simple electrostatic theory, and possible explanations for the deviations have been given.

Introduction

There has been much discussion in the literature[3–6] of the factors which determine the strength of binding of anions to serum albumin. Since previous measurements of the thermodynamic quantities, ΔF and ΔS of binding,[7] have suggested the importance of electrostatic effects in the binding of anions, it was considered worthwhile to determine yet another thermodynamic property, the volume change of the reaction, which should also reflect the contribution of electrostatic factors.

The reaction studied was the binding of sodium dodecyl sulfate by bovine serum albumin. The free energy and entropy changes in this reaction had been determined previously by Karush and Sonenberg.[8] The number of dodecyl sulfate ions bound is sufficient to produce a measurable volume change.

The formation of the complex may be represented by the equation

$$P + rA \rightleftharpoons PA_r \qquad (1)$$

where P represents free protein, A free anion and PA_r the complex containing r bound anions. The volume change for this reaction was determined by measurement of the densities of solutions of protein, small ion, and mixtures of the two, and calculation of the apparent molal volumes of the respective species by standard methods.[9] The volume change may be computed from the expression

$$\Delta V = \phi(V)_{PA_r} - \phi(V)_P - r\phi(V)_A \qquad (2)$$

where $\phi(V)$ represents the apparent molal volume.

Experimental

Materials.—Crystallized bovine serum albumin was purchased from Armour and Company. The moisture content, determined by heating to constant weight at 100° over phosphorus pentoxide in an Abderhalden drying pistol, was 3.0%. The dry protein was found to contain 0.74% sodium chloride.[10]

The sodium dodecyl sulfate was a specially purified sample generously supplied by the Fine Chemicals Division of E. I. du Pont de Nemours and Company.

Toluene used in the thermoregulator was of C.P. grade and was further purified by shaking it over mercury overnight and by allowing it to stand over mercury one week.

Sodium hydroxide was J. T. Baker Analyzed grade.

Distilled water from the laboratory tap was further redistilled from alkaline permanganate in an all-Pyrex apparatus before use in solutions for the density measurements.

Methods.—The densities of solutions of bovine serum albumin, sodium dodecyl sulfate and mixtures of the two were measured by a differential pycnometric method. The pycnometers (of about 60-ml. volume) were constructed by Dr. C. E. Moser for an investigation of the apparent molal volumes of amino acids and related compounds. The measurements, with minor modifications, were carried out by the method of Gucker and Moser.[11,12]

The differential method provides a precise value for the difference in density $d - d_0$ between solution (d) and solvent (d_0), as required for calculation of the apparent molal

(1) Atomic Energy Commission, Predoctoral Fellow in the Physical Sciences, 1949–1950; H. E. Russell Fellow of Trinity College, 1947–1949.

(2) Taken from part of a dissertation submitted by Robert M. Rosenberg to the Graduate School of Northwestern University in partial fulfillment of the requirements for the Ph.D. degree.

(3) B. D. Davis, *Am. Scientist*, **34**, 611 (1946).

(4) J. M. Luck, *Discs. Faraday Soc.*, **6**, 44 (1949).

(5) I. M. Klotz, *Cold Spring Harbor Symposia on Quantitative Biology*, **14**, 97 (1949).

(6) F. Karush, THIS JOURNAL, **72**, 2705 (1950).

(7) I. M. Klotz and J. M. Urquhart, *ibid.*, **71**, 847 (1949).

(8) F. Karush and M. Sonenberg, *ibid.*, **71**, 1369 (1949).

(9) F. T. Gucker, Jr., *J. Phys. Chem.*, **38**, 307 (1934).

(10) We are indebted to Miss Janet Ayers for the determinations of moisture and chloride contents.

(11) F. T. Gucker, F. W. Gage and C. E. Moser, THIS JOURNAL, **60**, 2582 (1938).

(12) C. E. Moser, Ph.D. Dissertation, Northwestern University, 1939.

TABLE I

APPARENT MOLAL VOLUMES OF SERUM ALBUMIN, DODECYL SULFATE AND THEIR COMPLEX

Soln. no.	Weight (grams) Bovine albumin	NaCl	Sodium dodecyl sulfate	H_2O	pH	$c \times 10^4$, moles/l.	$(d - d_0) \times 10^3$	$\phi(V_2)$	r
1			0.1683	199.65		29.13[a]	0.155	235.9[d]	
							0.150	237.6[d]	
2			.1701	199.67		29.43[a]	0.154	236.8[d]	
							0.151	237.8[d]	
3	11.902	0.089		1035.7	5.3	1.647	3.078	50,830	
							3.102	50,680	
4	44.97[b]	.334		1012.2	7.4	6.191	11.977	50,485	
							11.973	50,490	
5	6.440[b]	.0477	.3580	154.34	7.7	5.814	11.604	53,735	13.36
							11.603	53,735	13.36
6	6.440[b]	.0477	.3672	144.97	7.7	5.805	11.583	53,840	13.70
							11.578	53,845	13.70
7	6.444[b]	.0477		155.03	7.4	5.804	11.230	50,480	
							11.231	50,480	
8	4.290[b]	.0318		146.39	7.4	4.127	7.985	50,485	
							7.990	50,470	
9	2.142[b]	.0159		148.01	7.4	2.060	4.001	50,410	
							3.998	50,425	
10	4.1264[b,c]	.0306	.2197	153.24	7.7	3.794	7.569	53,580	12.79
							7.573	53,570	12.79

[a] This concentration is safely below the critical micelle concentration of 6×10^{-3} M.[16] [b] The weights given are those of the component, sodium proteinate, computed from the weight of protein and added sodium hydroxide. [c] The density of this solution was measured immediately afer mixing. [d] The mean value of these four measurements is 237.0 ml.

volume, without any need for a determination of the absolute value of the density to the same precision.

The constant temperature bath was set at 25.00° as measured with a Bureau of Standards thermometer. The temperature was kept constant as observed with a Beckmann thermometer, within 0.002°, with a toluene thermoregulator[13] which actuated a heater through a commercial regulator containing a 2021 gas-filled tetrode and a mercury switch. This variation in temperature corresponds to a variation in volume, for sixty ml. of water, of $\pm 1 \times 10^{-5}$ ml.

The pycnometers were weighed to 0.1 mg. on a Chainomatic balance by the method of transposition. All weighings were reduced to vacuum. The pycnometers were cleaned with a 1% solution of detergent and thoroughly rinsed with distilled water. They were filled by suction and emptied and dried by a stream of tank nitrogen passed through two calcium chloride drying towers.

Solutions, whose densities were to be measured, were made up by weight and the molar concentration calculated from the composition by weight and the measured density.

In measurements of the densities of solutions containing both albumin and dodecyl sulfate, the solutions with one exception were first allowed to come to equilibrium at 25° for four hours. This was deemed sufficient, since Karush and Sonenberg[8] reported that equilibrium was attained in four hours in dialysis experiments at room temperatures. Since anionic detergents form precipitates with albumin at pH's at or below the isoelectric point,[14] the albumin stock solution was brought to a pH near 7 by the addition of a known quantity of dilute sodium hydroxide. This stock solution was used to make up dilutions of serum albumin and also of the protein–detergent mixtures.

Results

The experimental results are collected in Table I. The molar concentration was calculated from the composition by weight and the density by means of the equation

$$c = \frac{1000 w_2 d}{M_2 w} \qquad (3)$$

where c is the concentration in moles per liter, d is the density of solution in grams per ml., M_2 is the molecular weight of solute, w is the total weight of solution and w_2 the weight of solute. The density is obtained by adding the density of water to the density difference obtained from the pycnometric measurements. The density of water was taken to be 0.997075 g./ml. at 25°.[15,16]

In solutions containing both albumin and dodecyl sulfate, all of the detergent anion is assumed to be in the form of the complex, in view of the observations of Karush,[17] that in unbuffered solutions at pH's near 7, dodecyl sulfate is essentially completely bound, up to a mole ratio of detergent to albumin of fifteen. Therefore, the molar concentration of complex is taken as equal to the concentration of albumin, and r, the number of ions bound per mole of albumin, is taken as equal to the mole ratio of total detergent to total protein in the solution. The molecular weight of albumin is taken as 69,000.[18]

In solutions to which sodium hydroxide has been added, the protein component may be taken as sodium proteinate, and the molecular weight as well as the actual weight of component present in a given solution have been computed from the known amounts of protein and sodium hydroxide. The molecular weight of the complex is then the molecular weight of sodium albuminate plus r times the molecular weight of sodium dodecyl sulfate.

(13) R. L. Weber, "Temperature Measurement and Control," Blakiston and Co., Philadelphia, Pa., 1941, p. 189.

(14) F. W. Putnam and H. Neurath, THIS JOURNAL, **66**, 692 (1944).

(15) L. W. Tilton and J. K. Taylor, *J. Research Natl. Bur. Standards*, **18**, 205 (1937).

(16) M. L. Corrin and W. D. Harkins, THIS JOURNAL, **69**, 683 (1947).

(17) F. Karush, personal communication.

(18) J. L. Oncley, G. Scatchard and A. Brown, *J. Phys. Colloid Chem.*, **51**, 184 (1947).

The apparent molal volume of sodium dodecyl sulfate can be calculated from the concentration and observed density difference by the equation[9]

$$\phi(V_2) = \frac{1000(d_0 - d)}{cd_0} + \frac{M_2}{d_0} \quad (4)$$

In the case of the protein solutions, a correction must be made for the sodium chloride present, since the density difference is referred to pure water. Assuming no interaction between the sodium chloride and protein, a reasonable assumption at a chloride concentration of 6×10^{-3} M or less,[19] the apparent molal volume of the protein component is given by the equation (derived in the Appendix)

$$\phi(V_2) = \frac{1000(d_0 - d)}{c_2 d_0} + \frac{M_2}{d_0} + \frac{w_3 M_2}{w_2 M_3}\left[\frac{M_3}{d_0} - \phi(V_3)\right] \quad (5)$$

where subscript 2 refers to the protein component and subscript 3 refers to the sodium chloride. For the solutions considered here, the correction amounted to more than 0.7% of the apparent molal volume of the protein component. $\phi(V_3)$ for sodium chloride was taken to be the value at infinite dilution given by Harned and Owen.[20]

The volume change on formation of the complex was calculated from equation 2. The value of $\phi(V_2)$ for dodecyl sulfate may be taken as the average of the four experimental values 237.0 ± 0.8, where 0.8 is the standard deviation. Three values of $\phi(V_2)$ for the complex can be obtained from the results for solutions 5, 6 and 10. The value used for albumin is the average of the results for solutions 4, 7 and 8. The resultant volume changes for complex formation are shown in Table II. For each bound anion, ΔV is 6.7 ± 1.4 ml., where 1.4 is the standard deviation.

TABLE II

VOLUME CHANGE IN FORMATION OF COMPLEX

Soln.	V(ml.)	$\Delta V/r$, ml./mole anion
5	89	6.7
6	116	8.5
10	64	5.0
		Av. 6.7 ± 1.4

Discussion

Specific Volume of Serum Albumin.—Although the experiments were carried out primarily to obtain the change in volume upon addition of anion to protein, some of the results can be used to compute the partial specific volume of bovine serum albumin itself. It should be noted first that the apparent molal volume of bovine serum albumin shows no significant dependence on concentration over the range examined (Table I). Similar behavior has been reported by Oncley, Scatchard and Brown[18] for human albumin, and Dayhoff, Perlmann and MacInnes[21] for bovine albumin. Under these circumstances the partial specific volume $\bar{v}_2$ may be computed directly from the expression

$$\bar{v}_2 = \phi(V_2)/M_2 \quad (6)$$

Using the results for solution 3, near the isoionic point, we obtain a value of 0.7355 for $\bar{v}_2$ for bovine albumin. This is in reasonable agreement with the values 0.734 and 0.7343 for bovine albumin[21,22] and 0.733 for human albumin[18] reported in the literature. The value for solution 3 is inherently less precise than those for the more concentrated solutions, but it is the only one available for the isoionic pH.

Change of Volume with pH.—Although the experiments were not designed for measuring pH effects, one measurement on an albumin solution alone was made at pH 5.3, in addition to the detailed experiments at pH 7. It is possible, therefore, to make a rough estimate of the volume change for the neutralization of protein by base, *i.e.*, for the reaction

$$P + n\text{NaOH} = PNa_n + nH_2O \quad (7)$$

This computation, in contrast to that for the volume change in complex formation, cannot give a very precise result with our data, since it depends on weighings of two separate protein solutions with attendant errors, particularly in moisture equilibration.

For reaction (7) the volume change is

$$\Delta V = \phi(V)_{PNa_n} + n\phi(V)_{H_2O} - \phi(V)_P - n\phi(V)_{NaOH} \quad (8)$$

For solution 4, n = 14.3. Combining the information in Table I with published values for the apparent molal volumes of NaOH[20] and of H_2O[15] we obtain +79 ml. for the neutralization of 14.3 protons or 5.5 ml. per proton. This value differs substantially from that of Weber and Nachmannsohn,[23] obtained by direct dilatometric measurements, of 16.8 ml. per mole of base added to isoionic horse serum albumin. However, if we use the value of 0.734 for the isoionic protein, the volume change rises to +11 ml. per mole of H^+ neutralized. For reliable measurements of pH effects it would be necessary, therefore, to re-design the experiment to eliminate separate weighings of two protein stock solutions at different pH's.

Volume Change in Binding.—The volume change expected for a purely electrostatic interaction may be calculated in a manner comparable to the calculation by Klotz and Fiess[24] of ΔS_{elec} for the binding of Cu^{++} by albumin. If the free energy change for the binding process is entirely due to electrostatic forces, then ΔF_{elec} is a function of the charges, z, and the radii, $\mathbf{r}$, of the protein and small ion, and the dielectric constant D of the medium

$$\Delta F_{elec} = \frac{\varphi(\mathbf{r}, z)}{D} \quad (9)$$

All the parameters except D are grouped together in $\varphi(\mathbf{r}, z)$. If we assume that the radii and charges are independent of pressure, we may calculate ΔV_{elec} from the thermodynamic relation

$$\Delta V_{elec} = \left(\frac{\partial \Delta F_{elec}}{\partial P}\right)_T = -\frac{\varphi(\mathbf{r}, z)}{D^2}\left(\frac{\partial D}{\partial P}\right)_T \quad (10)$$

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

(20) H. S. Harned and B. B. Owen, "The Physical Chemistry of Electrolytic Solutions," Reinhold Publ. Corp., New York, N. Y., 1943, p. 253.

(21) M. O. Dayhoff, G. E. Perlmann and D. A. MacInnes, THIS JOURNAL, **74**, 2515 (1952).

(22) J. F. Taylor, *Federation Proc.*, **9**, 237 (1950).

(23) H. M. Weber and D. Nachmannsohn, *Biochem. Z.*, **204**, 215 (1929).

(24) I. M. Klotz and H. A. Fiess, *J. Phys. Colloid Chem.*, **55**, 101 (1951).

$$= -\frac{\varphi(\mathbf{r},z)}{D}\left(\frac{\partial \ln D}{\partial P}\right)_T$$
$$= -\Delta F_{\text{elec}}\left(\frac{\partial \ln D}{\partial P}\right)_T$$

For the free energy of binding of dodecyl sulfate by bovine albumin at 27°, Karush and Sonenberg[8] have found $\Delta F^0 = -7{,}220$ cal./mole detergent bound. Data for the variation of the dielectric constant of water with pressure have been obtained by Kyropoulos,[25] and the value given for $(\partial \ln D/\partial P)_T$ is 6.02×10^{-11} cm.^{2}/dyne. Substituting in equation 10 we obtain

$$\Delta V_{\text{elec}} = -(-7220)(4.18 \times 10^7 \text{ ergs/cal.})(6.02 \times 10^{-11})$$
$$= 18.2 \text{ ml./mole bound anion}$$

Before considering possible reasons for the deviation between the observed ΔV of 6.7 ml. and that calculated from purely electrostatic grounds, we should estimate the probable uncertainty in the experimental value.

Ordinary weighing errors of 0.2 mg. in determinations of the density difference, $d - d_0$, would result in an uncertainty of ±1 ml. in $\phi(V_2)$ for a $(3 \times 10^{-3}\ M)$ dodecyl sulfate solution and of ±5 ml. for the protein $(6 \times 10^{-4}\ M)$ solutions. For the protein solutions, the most important source of uncertainty, however, lies in the concentration. Although the actual weighing of the protein in solutions 3–8 and 10 is precise to about one part in four thousand, the dry weight is at best significant to 0.1%. A consideration of each of the terms in equation 5 shows that an error of 0.1% in protein concentration, added to the indeterminacy of one part in seventy in weight of sodium chloride mixed with the original protein leads to an uncertainty of ±25 ml. in $\phi(V_2)$. Thus the absolute value of $\phi(V_2)$ for a protein solution has a total uncertainty of ±30 ml.

Nevertheless, the volume change for the binding of dodecyl sulfate anions is more precise than $2 \times (\pm 30)$ ml. Solutions 5, 6, 7, 8 and 9 were all prepared by dilution of solution 4, either with water or with a solution of dodecyl sulfate, so that errors in protein concentration tend to cancel. Reference to equation 5 shows that in the first term a concentration uncertainty is in the same direction for solution of protein or of protein-anion complex and hence disappears when ΔV is calculated (from equation 2). The second term is slightly different for the complex than for the protein alone since the molecular weight of the former is approximately 3000 greater; thus an error of about 3 ml. may be introduced. The third term, due to sodium chloride in the protein, is the same for all solutions made up from the same stock protein solution. The uncertainty in molal volume of dodecyl sulfate is ±1 ml.; hence ±13 ml. error may be introduced into ΔV for 13 bound ions. The weight of added dodecyl sulfate should not be in error by more than 0.1% so that the uncertainty from this source should not exceed ±3 ml. Summation of the possible errors from all of these sources, together with weighing errors in $d - d_0$ mentioned in the preceding paragraph leads to a total uncertainty of about ±29 ml. for ΔV in equation 2 or a maximum of about ±2.2 ml. per dodecyl sulfate anion bound.

(25) S. Kyropoulos, *Z. Physik*, **40**, 507 (1926).

The difference between the experimental value of 6.7 ml. and the electrostatic one of 18 ml. is thus a very real one. It may be due primarily to the contribution to the volume change of the non-electrostatic forces in the interaction; the binding of the twelve-carbon organic portion of the anion by the protein may produce a volume contraction. A small contribution may arise also from the uptake of hydrogen ions concurrently. In the *p*H region near 7.5 such protons would go on histidine or α-amino groups. The data of Weber[26] indicate substantial volume contractions for the removal of a proton from water by a basic nitrogen.

The most uncertain factor, however, is the protein, itself. There exists much evidence that human serum albumin undergoes configurational changes[27] in the region of *p*H 7. These changes make available new sites for binding of ions and neutral molecules. If these sites are highly polar, as seems likely, unfolding or swelling of the protein could "freeze" a large number of water molecules and thereby produce a volume contraction. Evidence for such a configurational change in bovine serum albumin in the presence of sodium dodecyl sulfate is provided by the results of Pallansch and Briggs,[28] who found a new electrophoretic component and an increased number of binding sites when more than ten dodecyl sulfate anions are bound by bovine serum albumin. The observed ΔV might then reflect the net result of release of water molecules upon binding of anions at charged sites, balanced by the uptake of water molecules associated with the swelling caused by the charge placed on the protein by the bound anions. It seems probable, then, that the expected increase in volume due to neutralization of charge occurs, but the magnitude of the volume increase due to this effect alone cannot be determined because the binding of anions also induces configurational changes in the protein.

Appendix

The apparent molal volume of component 2 (protein) in a three component system may be defined by the equation

$$\phi(V_2) = \frac{V - [n_1\bar{v}_1^\circ + n_3\phi(V_3)]}{n_2}$$

$\phi(V_3)$ is the apparent molal volume (in ml.) of component 3 (sodium chloride) in the absence of the protein, and $\bar{v}_1^\circ$ is the molal volume of pure solvent. By the following steps the equation may be transformed into one that is more convenient for calculation. We note first that

$$\frac{V}{n_2} = \frac{1000}{c_2}$$

and

$$\frac{n_1\bar{v}_1^\circ}{n_2} = \frac{1000}{d_0 m_2}$$

where c_2 is the concentration of component 2 in moles per liter of solution and m_2 the concentration in moles per kilogram of solvent. But

$$c_2 = \frac{1000 n_2 d}{n_1 M_1 + n_2 M_2 + n_3 M_3}$$

(26) H. H. Weber, *Biochem. Z.*, **218**, 1 (1930).

(27) I. M. Klotz, R. K. Burkard and J. M. Urquhart, THIS JOURNAL, **74**, 202 (1952).

(28) M. J. Pallansch and D. R. Briggs, *ibid.*, **76**, 1396 (1954).

and

$$\frac{1}{c_2} = \frac{1}{m_2 d} + \frac{M_2}{1000d} + \frac{n_3 M_3}{1000 n_2 d}$$

Therefore

$$\frac{1}{m_2} = \frac{d}{c_2} - \frac{M_2}{1000} - \frac{n_3 M_3}{1000 n_2}$$

and

$$\frac{n_1 \bar{v}_1^\circ}{n_2} = \frac{1000}{d_0}\left[\frac{d}{c_2} - \frac{M_2}{1000} - \frac{n_3 M_3}{1000 n_2}\right]$$

Thus

$$\phi(V_2) = \frac{1000}{c_2} - \frac{1000d}{c_2 d_0} + \frac{M_2}{d_0} + \frac{n_3 M_3}{n_2 d_0} - \frac{n_3}{n_2}\phi(V_3)$$

$$= \frac{1000}{c_2}\left(\frac{d_0 - d}{d_0}\right) + \frac{M_2}{d_0} + \frac{n_3}{n_2}\left[\frac{M_3}{d_0} - \phi(V_3)\right]$$

EVANSTON, ILLINOIS

[CONTRIBUTION FROM OAK RIDGE NATIONAL LABORATORY AND THE LABORATORY OF BIOCHEMISTRY, NATIONAL CANCER INSTITUTE, NATIONAL INSTITUTES OF HEALTH, PUBLIC HEALTH SERVICE]

New Methods for the Calculation of Association Constants of Complex Ion Systems[1]

BY JOHN Z. HEARON AND JAMES B. GILBERT

RECEIVED JULY 30, 1954

Extensions of and additions to existing methods of computing association constants are presented. New methods, with special reference to data from potentiometric titration, are described in detail for the cases of one, two or three complexes. These methods give a calculated value for some, or all, of the constants for each point on the titration curve. A general equation for the concentration of free ligand is derived and the limits of applicability discussed. A new method for computing the concentration of free ligand and $\bar{n}$ from the known constants is discussed.

I. Introduction

In practice it is usually possible to determine directly, or to compute from observed quantities, the concentrations of only a few of the relevant chemical species in a complex ion system. Previous investigations[2-5] of the computation of association constants for such systems have produced no clear discussion of the number of known relations in such a system and no attempt to exploit the mathematical properties of these relations for the purpose of computing association constants in a more rigorous manner. The present paper is presented with that aim. In particular, there is considered the determination of association constants for an ampholyte–metal ion system on the basis of data from potentiometric titration. While we have in mind specifically the association of peptides and amino acids with metal ions[6] the formulation is fairly general. This work had been completed[1] when several relevant reports appeared (especially ref. 4, 5) and the relation of this investigation to certain others will be discussed.

II. Some General Considerations

Consider the equilibria

$$\varphi + M\varphi_{k-1} \rightleftarrows M\varphi_k,\ k = 1, 2, \ldots, \alpha \qquad (1)$$

where M, the central ion or molecule, is of valence $+p$, the ligand φ of valence $-m$, and the valence of the kth complex, $M\varphi_k$, is $p-km$. It is assumed that the ligand φ is the completely ionized form of φH_n and is formed in the nth of the equilibria

$$\varphi H_{n-k+1} \rightleftarrows \varphi H_{n-k} + H^+,\ k = 1, 2, \ldots, n \qquad (2)$$

Proton-bearing complexes (*e.g.*, $(\varphi H_j)M$, $j > 0$) and polynuclear complexes (*e.g.*, $M_j\varphi_k$, $j > 1$) are excluded from consideration. This limitation of the analysis to follow is discussed later. With this understanding, then, the "totality conditions" or material balances are

$$c = \sum_{k=0}^{n} [\varphi H_k] + \sum_{k=0}^{\alpha} k[M\varphi_k] \qquad (3)$$

$$b = \sum_{k=0}^{\alpha} [M\varphi_k] \qquad (4)$$

where c and b are the total concentration of ligand and metal. The condition of electroneutrality is

$$S + \sum_{k=0}^{n} (k - m)[\varphi H_k] + \sum_{k=0}^{\alpha} (p - mk)[M\varphi_k] = 0 \qquad (5)$$

where $S = \Sigma \nu_i [I_i]$, and ν_i and $[I_i]$ are the valence and concentration of the ith ion which contains neither M nor φ. We assume S to be known, for it contains $[H^+]$ (which we assume to be directly determined), $[OH^-]$ (which is determined by $[H^+]$ and the ionization constant of water), the anions of the metal salt and any strong acids and the cations of any strong bases added to the system. In practice [M], the concentration of free metal ion often can be determined experimentally and there is a method[3] based predominantly on this fact. We are interested here in the situation in which $[H^+]$, or pH, is the experimentally determined quantity.[7] Under these conditions there are $(n + \alpha + 2)$ concentrations ($[\varphi H_k]$, $k = 0, 1, \ldots, n$ and $[M\varphi_k]$, $k = 0, 1, \ldots, \alpha$) yet to be determined. If the ionization constants of φH_n are known, the corresponding mass action expressions for (2) furnish n relations and these with (3), (4) and (5) provide $(n + 3)$ relations. Now it happens that, although

(1) Presented, in part, at the 119th Meeting of the American Chemical Society at Cleveland, Ohio, Abstracts of papers, p. 39c, April, 1951.

(2) J. Bjerrum, "Metal Ammine Formation in Aqueous Solution," P. Haase and Son, Copenhagen, 1941.

(3) I. Leden, *Z. physik. Chem.*, **A188**, 160 (1941).

(4) S. Fronaeus, *Acta Chem. Scand.*, **4**, 72 (1950).

(5) J. C. Sullivan and J. C. Hindman, THIS JOURNAL, **74**, 6091 (1952).

(6) J. B. Gilbert, M. C. Otey and J. Z. Hearon, *ibid.*, **77**, 2599 (1955).

(7) It is of course assumed that b and c are known and the situation referred to here corresponds to the titration of a solution of the ampholyte and metal salt with strong base, the titration of the ampholyte with the hydroxide of M, the titration of an acid solution of M with the ampholyte, etc., with a determination of $[H^+]$ or pH after each addition.