[CONTRIBUTION FROM THE PHYSICAL CHEMISTRY DIVISION, NATIONAL CHEMICAL LABORATORY]

Metal Protein Interactions in Buffer Solutions.[1] I. An Electrophoretic Study of the Interaction of Copper, Zinc, Cadmium and Cobalt Ions with Native and Modified Bovine Serum Albumins

BY HIRA LAL AND M. S. NARASINGA RAO[2]

RECEIVED DECEMBER 10, 1956

General concepts governing the interpretation of metal-protein interactions in buffer solutions have been outlined. It has been shown that the binding data should be supplemented by mobility measurements if the interactions are to be treated as a competition between the metal and hydrogen ions for combining with a given set of sites on the protein molecule. The existing data for the binding of cupric and cobaltous ions by bovine serum albumin in an acetate buffer of *p*H 6.5 and ionic strength 0.20, together with the corresponding mobility data reported in the present communication, have been analyzed. The intrinsic constant for the association of cobaltous ion with the carboxyl groups of serum albumin remained constant over the entire binding range, and had a low value characteristic of metal-acetate complexes. The constant for the association of cupric ion with the imidazole groups of serum albumin, however, tended to decrease with the number of bound metal ions, due, possibly, to an increased interaction, in the higher binding region, with the free carboxyl groups of the protein.

The interaction of zinc ion with human serum albumin has been studied by Gurd and Goodman[3] in unbuffered solutions. These authors have shown that the binding data could be interpreted in terms of a competition between the metal and hydrogen ions for reaction with the same sites, *viz.*, the imidazole groups of the protein molecule. The difficulties associated with the measurement and interpretation of such a competition in buffered solutions have been pointed out by Scatchard.[4] Most measurements of the interaction of small ions with proteins have however been made in buffered solutions. It is therefore of interest to attempt an interpretation of binding data obtained in buffer solutions in terms of a competition phenomenon.

A competition between metal and hydrogen ions entails that the metal ions can combine only with the basic (unprotonated) functional groups. The uptake of metal ion will however shift the hydrogen ion equilibria in the direction of the basic (unprotonated) form of the functional groups. The hydrogen ion release, thus occasioned, results, in unbuffered systems, in a decrease in *p*H.[3] For metal–protein interactions in buffer solutions, however, the relevant quantities may be derived from the electrophoretic mobility data provided allowance is made, as in unbuffered systems, for the binding of anions by the protein.

The electrophoretic behavior of metal–protein systems has not, as far as we are aware, been reported so far.[5] Such a study of the interaction of cupric, zinc, cadmium and cobaltous ions with bovine serum albumin in an acetate buffer of *p*H 6.5, ionic strength 0.20 is presented in the present communication.

Experimental

Native Bovine Serum Albumin (NBSA).—Crystallized bovine serum albumin was an Armour product, Lot No. M 66909. A polarographic examination did not indicate any detectable amount of metallic impurities. It was electrophoretically homogeneous in an acetate buffer of *p*H 6.5 and ionic strength 0.20, and had a mobility of -3.12×10^{-5} cm.^{2}/volt/sec., corresponding to a net charge of approximately -16 (electron units) calculated on the basis of a 0.2×10^{-5} cm.^{2}/volt/sec. change in mobility per unit change in charge.[6]

Esterified Bovine Serum Albumin (EBSA).—Serum albumin was esterified according to the method of Fraenkel-Conrat and Olcott.[7] The ester was analyzed for its methoxy groups[8] and showed that all the free carboxyl groups of the albumin molecule had been esterified. The material was electrophoretically homogeneous and had a mobility of 6.02×10^{-5} cm.^{2}/volt/sec. (acetate buffer, *p*H 6.5, ionic strength 0.20) corresponding to a net charge of +30 units. It would appear from the mobility of EBSA as compared to that of NBSA that only 46 carboxyl groups of the albumin molecule had been esterified as against the value of 100 obtained from titration data.[9] This discrepancy has been attributed by Saroff, *et al.*,[10] to a splitting of the esterified derivative into two nearly equal halves. It is our opinion, however, that the discrepancy may possibly be due to an increased uptake of buffer anions by the highly positively charged esterified derivative (see Discussion).

Acetylated Bovine Serum Albumin (ABSA).—Serum albumin was acetylated by the method of Fraenkel-Conrat, *et al.*,[11] using acetic anhydride as the acetylating agent. Acetic anhydride is specific for the free α- and ϵ-amino groups.[12] It may be assumed, therefore, that other functional groups, such as the imidazolium and guanidinium, are not affected. The acetylated derivative was analyzed for its free amino groups by the Van Slyke nitrogen estimation as modified by Baddiley, *et al.*,[13] indicating 83% acetylation of the free amino groups of the albumin molecule.

The acetylated derivative was found to be electrophoretically inhomogeneous in an acetate buffer of *p*H 6.5 and ionic strength 0.20 (Fig. 2A). Two components were observed: a slow one, constituting about 60% of the total, had a mobility of -7.60×10^{-5} cm.^{2}/volt/sec., and a fast one, constituting the rest, had a mobility of -8.60×10^{-5} cm.^{2}/volt/sec. The mobility computed from the first moment of the gradient curve had a value of -7.87×10^{-5} cm.^{2}/volt/sec., corresponding to a net charge of about -39 units.

Solutions.—All studies, unless otherwise stated, were made in an acetate buffer of *p*H 6.5 and ionic strength 0.20. 1% protein solutions were used. Solutions of the protein

(1) A note on the interaction of the cobaltous ion with bovine serum albumins was published in THIS JOURNAL, **76**, 4867 (1954).

(2) Chemistry Department, Clark University, Worcester 10, Mass.

(3) F. R. N. Gurd and DeWitt S. Goodman, THIS JOURNAL, **74**, 670 (1952).

(4) G. Scatchard, *American Scientist*, **40**, No. 1, 61 (1949).

(5) See, however, ref. 1.

(6) L. G. Longsworth and C. F. Jacobsen, *J. Phys. Colloid Chem.*, **53**, 126 (1949).

(7) H. Fraenkel-Conrat and H. S. Olcott, *J. Biol. Chem.*, **161**, 259 (1945).

(8) J. B. Niederl and V. Niederl, "Micromethods of Quantitative Organic Analysis," New York, 1942.

(9) C. Tanford, S. A. Swanson and W. S. Shore, THIS JOURNAL, **77**, 6414 (1955); see also C. Tanford, *ibid.*, **72**, 441 (1950).

(10) H. A. Saroff, N. R. Rosenthal, E. R. Adamic, N. Hages and H. A. Scheraga, *J. Biol. Chem.*, **205**, 255 (1953).

(11) H. Fraenkel-Conrat, R. S. Bean and H. Lineweaver, *ibid.*, **177**, 38 (1949).

(12) F. W. Putnam in "The Proteins," Vol. I, part B, ed. H. Neurath and K. Bailey, Academic Press, Inc., New York, N. Y., 1953, p. 904.

(13) J. Baddiley, R. A. Kekwick and E. M. Thain, *Nature*, **170**, 968 (1952).

were prepared by direct weighing, corrections being made for its moisture content.[14]

Electrophoresis.—Mobility measurements were made at 0.5° in a Tiselius electrophoresis apparatus (Perkin-Elmer Model 38[15]) using Longsworth's scanning system. A 2-ml. cell was used. The boundaries were formed between the protein and buffer solutions to which suitable additions of metal ion, corresponding to the metal distribution in equilibrium-dialyzed systems,[1,16-18] had previously been made. Standard procedure for the formation of boundaries was employed. After thermostating the cell assembly for three hours, the boundaries were brought into view and their positions photographed. The positions of the boundaries were also photographed at regular intervals during the electrophoresis run. The runs were usually made from 1-3 hours depending on the nature of the protein. Mobilities were calculated from the displacements in the descending boundary. Only those runs in which the boundary displacement was a linear function of time (at constant current) were used for the evaluation of mobilities.

The current passing through the cell was estimated to within ±0.1% from the potential difference developed across a standard resistance placed in series with the cell. The conductivity of protein solutions was measured at 0° with a Leeds and Northrup conductivity bridge using a conductivity cell of 0.5-ml. capacity (cell constant 12.6). The boundary displacements were measured from the schlieren patterns with a travelling microscope. In the systems involving non-homogeneous proteins, such as the acetylated serum albumin, the schlieren patterns were enlarged and the boundary displacements measured from the first moment of the entire gradient curve.

Analar cupric chloride and cupric acetate, and Merck zinc sulfate, zinc acetate, cadmium acetate and cobalt chloride were used as the source of metal ions.

Results

Metal-NBSA Systems.—The mobility increment $\Delta\mu$ ($= \mu_{PAi} - \mu_P$) is plotted in Fig. 1A as a function of $\bar{\nu}$, the number of metal ions bound per protein molecule. It may be noticed that the slope of the curves represented in Fig. 1A is positive for the system Co-NBSA and decreases in the order: Co-NBSA → Cd-NBSA → Zn-NBSA. The corresponding slope for the system Cu-NBSA, however, shows a negative slope—a result which is contrary to what should be expected from the uptake of a metal ion by the protein. As the metal uptake increases, however, the mobility of the protein is affected in a manner depending upon the nature of the metal ion bound. Thus the mobility of serum albumin tends toward a steady value as more and more cobalt ions are bound. This is in contrast to the behavior of Cd-NBSA and Zn-NBSA systems in which the mobility increases more and more as the value of $\bar{\nu}$ is increased, the effect being more pronounced in the former than in the latter. The mobility of the system Cu-NBSA passes through a minimum at $\bar{\nu} \simeq 2$.

Metal-ABSA Systems.—The $\Delta\mu$ *vs.* $\bar{\nu}$ curves for the metal-ABSA systems are shown in Fig. 1B.

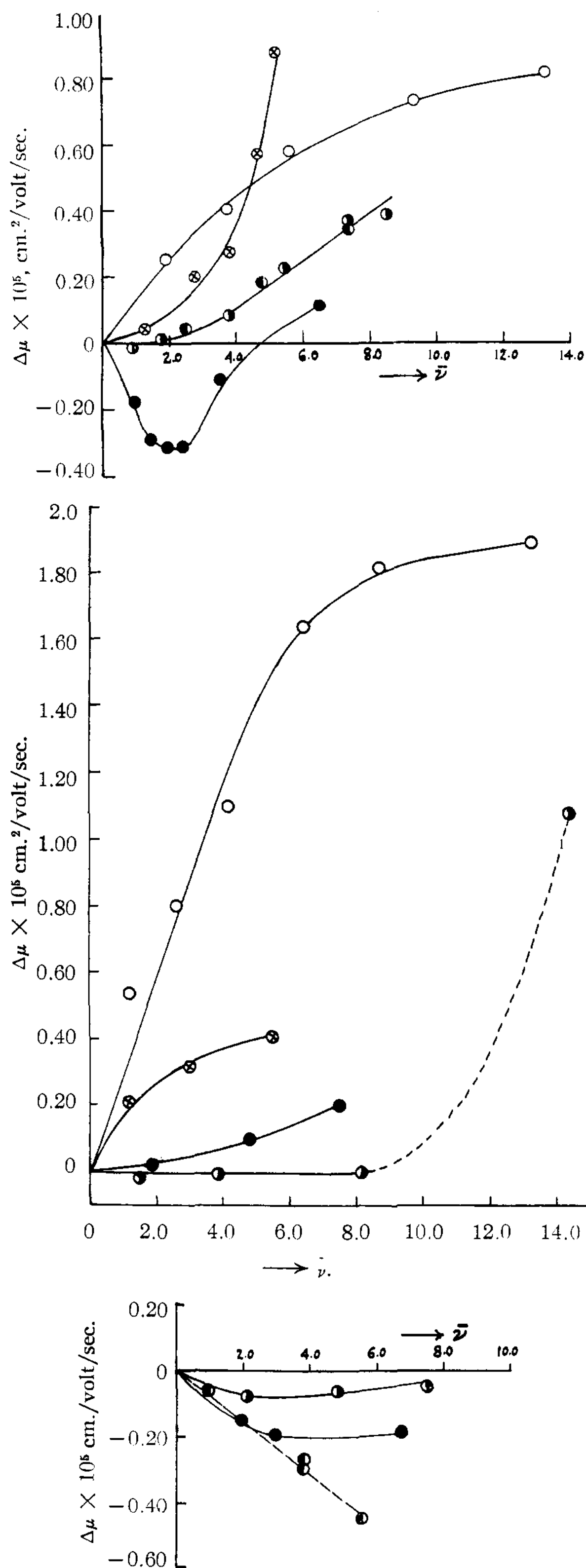

Fig. 1.—The effect of the binding of metal ions on the electrophoretic mobility of bovine serum albumins (0°, acetate buffer, *p*H 6.5, ionic strength 0.20): A, NBSA; B, ABSA; C, EBSA; —⊙—, Co^{++} as $CoCl_2$; —⊗—, Cd^{++} as $Cd(CH_3COO)_2$; —◑—, Zn^{++} as $Zn(CH_3COO)_2$; —◐—, Zn^{++} as $ZnSO_4$; —●— Cu^{++} as $Cu(CH_3COO)_2$.

(14) Stock solutions containing 2% protein in the acetate buffer medium were stored in a cold room maintained at 0°. The solutions containing EBSA, however, tended to hydrolyze on prolonged storage. This tendency toward hydrolysis was suppressed by storing the stock solution in a deep freeze.

(15) D. H. Moore and J. U. White, *Rev. Sci. Instruments*, **19**, 700 (1948).

(16) M. S. N. Rao, Ph.D. thesis, Poona University, 1956.

(17) I. M. Klotz and H. A. Fiess, *J. Phys. Colloid Chem.*, **55**, 102 (1951).

(18) In line with the molecular weight of NBSA assumed for the titration data of ref. 9, the binding data of ref. 1, 16 and 17 were re-valuated on the basis of a molecular weight of 65000 for NBSA and 66000 for ABSA and EBSA.

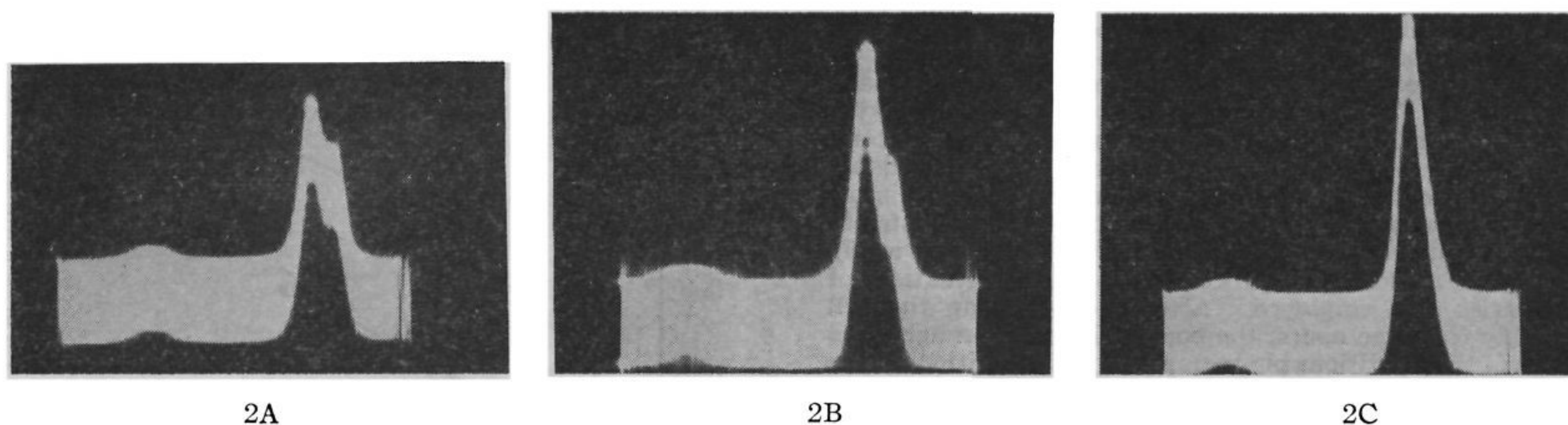

Fig. 2.—Schlieren patterns of ABSA at (A) $\bar{\nu} = 0$, (B) $\bar{\nu} = 2$, (C) $\bar{\nu} = 8$ (0°, acetate buffer, pH 6.5, ionic strength 0.20)

It may be noticed that the mobility increment is relatively much more for ABSA than for NBSA in so far as the uptake of cobalt and cadmium ions is concerned. The binding of zinc and copper ions is, however, characterized by a relatively negligible effect on the mobility of the acetylated albumin.

In the course of the electrophoretic investigation of metal–ABSA systems, it was observed that whereas the uptake of cobalt and cadmium ions did not affect the shape of the schlieren pattern of the protein, that of copper and zinc ions tended to make the protein electrophoretically homogeneous. The phenomenon is illustrated in Figs. 2A, B and C for the system Cu–ABSA. It was found that the protein became electrophoretically homogeneous with the uptake of about 8 and 15 copper and zinc ions, respectively.

Metal–EBSA Systems.—The effect of the binding of zinc and copper ions by the esterified albumin on its mobility is illustrated in Fig. 1C. As the protein has a high positive charge, it is imperative that the metal be added only as the metal acetate. The addition of zinc as zinc sulfate caused a considerable decrease in the mobility of the protein indicating that sulfate ions are bound by the esterified serum albumin. The addition of zinc as zinc acetate caused only a slight decrease in the mobility. The binding data obtained with zinc sulfate are, however, identical with those obtained with zinc acetate.[16] It may be noticed from Fig. 1C that the mobility of EBSA is slightly reduced by the uptake of copper and zinc ions. Cadmium and cobalt have no appreciable effect on the mobility of this protein; these ions are, however, *bound little* by EBSA.[1,16]

Discussion

Following Klotz and Fiess[17] we shall assume that, in the acetate buffer of pH 6.5 and ionic strength 0.20, the metal ions exist largely as the MAc^+ complex ion and that this complex ion participates in the binding process. This assumption shall be tacitly adhered to in the following discussion. If the binding process were to involve the simple addition of MAc^+ ions, the charge on the protein should be a linear function of $\bar{\nu}$. Consequently, the slope of $\Delta\mu$ *vs.* $\bar{\nu}$ curves should be of the order of 0.2×10^{-5} cm.^{2}/volt/sec. An examination of Fig. 1, however, reveals that this simple state of affairs does not in fact exist and that the behavior of the metal–protein systems investigated depends in a rather complex manner on the nature of the metal ion and of the protein. It seems to us, therefore, that a proper evaluation of the electrophoretic mobility data must entail a definition of (i) the functional groups of the serum albumin molecule in terms of their equilibria in the acetate buffer of pH 6.5 and ionic strength 0.20, and (ii) the extent to which these equilibria are influenced by the uptake of MAc^+ ions.

Equilibria in Serum Albumin Solutions

Equilibria Involving Hydrogen Ions.—The dissociation of functional groups in a given set of sites may be represented by the equilibrium equation[9]

$$\frac{n_A}{n_B} = (H^+)k^0_H e^{-2\omega z^0_p} \qquad (1)$$

where n_A and n_B are the number of a given set of functional groups in the acid and basic forms, respectively, (H^+) the activity of hydrogen ions, k^0_H the proton affinity constant for the functional group, z^0_p the charge on the protein molecule and ω the familiar function of ionic strength. The hydrogen ion equilibria in bovine serum albumin solutions have been investigated by Tanford, Swanson and Shore.[9] It may be concluded from their data that the guanidinium, sulfhydryl, phenolic, α- and ϵ-amino groups, in view of their high proton affinities, are present in the acid form at pH 6.5, and are not available for binding metal ions. We are therefore interested only in the hydrogen ion equilibria involving imidazole and carboxyl groups of the protein molecule. It follows from eq. 1 that, of the 100 carboxyl groups of the protein molecule, 99.2 are dissociated in NBSA and 97.8 in ABSA. Similarly it may be shown that, of the 16 imidazole groups in the albumin molecule, 10 exist in the neutral (basic) form in EBSA, 2.5 in NBSA and only 0.9 in ABSA. The intrinsic proton affinity constant was assumed to be $\log k^0_H = 4.05$ for the carboxyl groups, and $\log k^0_H = 6.90$ for the imidazole groups; the function ω was given a value of 0.024,

TABLE I

CHARGE BALANCE IN BOVINE SERUM ALBUMIN SOLUTIONS

(pH 6.5, ionic strength 0.20, 25°)

	Guanidinium	α- + ε-amino	n_{HIm}	n_c (1) + (2) + (3)	n_{COO^-}	(4) − (5)	z^0_p	n_{Ac} (6) − (7)	k^0_{Ac}
	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
NBSA	22	58	13.5	93.5	99.2	− 5.7	−16	10.3	1.33
ABSA	22	9.9	15.1	47.0	97.8	−50.8	−39	(−11.8)	..
EBSA	22	58	6.0	86.0	0	+86	+30	56	2.21
NBSA (pH 4.71)	22	58	16	96	82	+14	0	14	1.71

being 80% of the calculated value in a medium of ionic strength 0.20.[9]

Binding of Acetate Ions.—The interaction of proteins with anions may be assumed to occur at the charged cationic functional groups of the proteins. The equilibrium may be represented by the equation

$$\frac{n_{Ac}}{n_c - n_{Ac}} = (Ac^-)k^0_{Ac}e^{2\omega z^0_p} \qquad (2)$$

where n_c is the total number of charged cationic groups, n_{Ac} the number of acetate ions bound per protein molecule, (Ac^-) the activity of free acetate ions at equilibrium (assumed 0.20) and k^0_{Ac} the intrinsic association constant for the binding of the acetate ion by the protein.

No quantitative work on the binding of acetate ions by serum albumin has been reported so far. A reasonably accurate evaluation of this interaction may, however, be made from considerations of electroneutrality. The charge balance in serum albumin solutions in the acetate buffer indicates that if the albumin molecule were not to bind the acetate ion it should have a net charge of +86, −5.7 and −50.8 for EBSA, NBSA and ABSA, respectively (see Table I, column 6). These values differ markedly from those obtained from mobility measurements. It is therefore reasonable to assume that the acetate ion is bound to the extent of 56 and 10.3 acetate ions per mole of EBSA and NBSA, respectively (Table I, column 8). With the values of n_c, n_{Ac} and z^0_p given in the table, and with $\omega = 0.024$, we may evaluate k^0_{Ac} from eq. 2. These values are also given in the table (column 9).

A fair estimate of the ability of bovine serum albumin to bind the acetate ion may also be obtained from the results of Longsworth and Jacobsen[6] who have reported the isoelectric pH of bovine serum albumin in 0.1 acetate solution to be 4.71. From the charge balance at pH 4.71, the number of acetate ions bound per mole of protein comes to 14 (Table I). The value of k^0_{Ac} as calculated from eq. 2 will then be 1.71, in good agreement with the values given in column 9 (Table I).

In what follows we have assumed that the intrinsic association constant governing the binding of acetate ion by bovine serum albumin has a value of 1.33 for NBSA and 2.21 for EBSA. As we are interested essentially in the change in acetate binding consequent on the uptake of metal ions by the protein, the dual value used for k^0_{Ac} may not be particularly objectional.

The ability of the acetylated derivative to bind the acetate ion may be shown to be practically nil. We are thus led to expect this protein to have a charge of −50.8 as against the value of −39 obtained from mobility measurements. The binding of acetate ions, even if it were to occur, will tend to make the discrepancy even larger. This protein is however electrophoretically non-homogeneous for reasons which are not at present completely understood. The charge on the acetylated derivative has therefore been assumed to be −39.

Equilibria in Metal–Protein Systems

The equilibrium conditions in bovine serum albumin solutions in an acetate buffer of pH 6.5 and ionic strength 0.20 have been discussed above. We shall now examine how these equilibria may be expected to be influenced by the uptake of metal ions. A tacit assumption has been made in that the metal uptake, as stated earlier, involves the singly charged MAc^+ complex ion. The binding of $\bar{\nu}$ metal ions will then increase the charge on the protein by $\bar{\nu}$ units. This will tend to favor the uptake of the negatively charged acetate ion and the release of bound hydrogen ion.

The effect of metal uptake on the acetate ion–protein equilibria may be evaluated from eq. 2, the factor z^0_p being now, by definition, replaced by $(z^0_p + \bar{\nu})$. With k^0_{Ac} and n_c as given in the table, the value of n_{Ac} may be evaluated as a function of $\bar{\nu}$.

The effect of metal uptake on the dissociation of the imidazole and carboxyl groups will depend on the functional group of the protein molecule to which the metal ion may be bound. If the combination occurs with the basic imidazole groups of the protein molecule, the dissociation of the imidazole and carboxyl groups will be governed by

$$\frac{n_{HIm}}{16 - \bar{\nu} - n_{HIm}} = (H^+)k^0_{HIm}e^{-2\omega(z^0_p + \bar{\nu})} \qquad (3a)$$

and

$$\frac{n_{COOH}}{100 - n_{COOH}} = (H^+)k^0_{COOH}e^{-2\omega(z^0_p + \bar{\nu})} \qquad (4a)$$

respectively. If, however, the metal uptake occurs at the basic carboxyl groups, we have the corresponding equations

$$\frac{n_{HIm}}{16 - n_{HIm}} = (H^+)k^0_{HIm}e^{-2\omega(z^0_p + \bar{\nu})} \qquad (3b)$$

and

$$\frac{n_{COOH}}{100 - \nu - n_{COOH}} = (H^+)k^0_{COOH}e^{-2\omega(z^0_p + \bar{\nu})} \qquad (4b)$$

In the above equations, n_{HIm} represents the number of acid imidazole groups and n_{COOH} that of the acid carboxyl groups per protein molecule, k^0_{HIm} the intrinsic proton affinity constant for the imidazole group and k^0_{COOH} that for the carboxyl group.

The net increment, Δz_p, in the charge on the protein molecule consequent on the binding of metal ions may be represented as

$$\Delta z_p = \bar{\nu} - \Delta n_{Ac} + \Delta n_{HIm} + \Delta n_{COOH} \qquad (5)$$

The expected net increment in charge on the protein molecule, thus calculated, is plotted in Fig. 3 as a function of the number of metal ions, $\bar{\nu}$, bound per protein molecule. It may be immediately noticed from Fig. 3 that the electrophoretic mobility data may give useful information as to the nature of the functional group of the protein molecule with which a metal ion may react and the manner in which this interaction may be influenced by successive additions of metal ion.

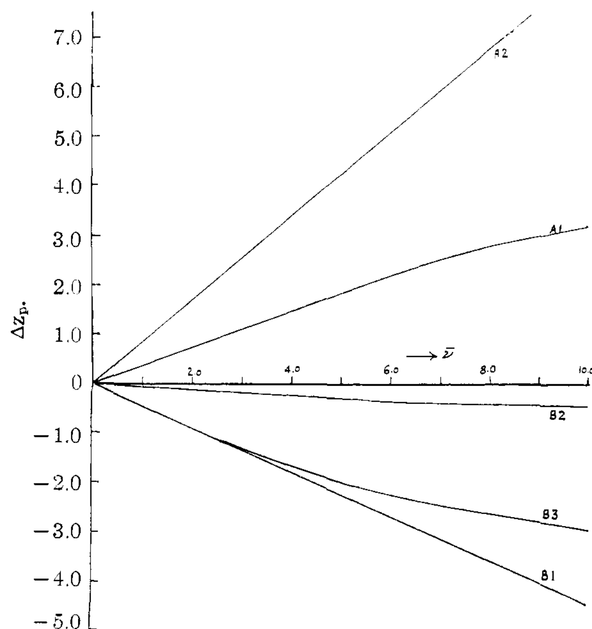

Fig. 3.—Calculated Δz_p *vs.* $\bar{\nu}$ curves, assuming interaction at: (A) carboxyl sites; (B) imidazole sites (25°, acetate buffer, *p*H 6.5, ionic strength 0.20); A1, B1, NBSA; A2, B2, ABSA; B3, EBSA.

A comparison of the experimental mobility data (Fig. 1A, B and C) with Fig. 3 reveals the following interesting features of the interaction process.

(i) The electrophoretic mobility data point distinctly to the conclusion that the cobaltous ion is bound essentially at the free carboxyl groups of the albumin molecule, thus supporting the conclusions derived earlier from equilibrium dialysis and optical absorption studies.[1]

(ii) The electrophoretic mobility data for the systems: copper–NBSA and copper–ABSA are characterized by the binding of the metal ions at the imidazole groups followed by an increasing participation of the carboxyl groups of the protein molecule in the interaction process after a few metal ions are bound by the protein. This participation of the carboxyl groups in the interaction process appears to increase in the direction: copper → zinc → cadmium, so much so that the mobility behavior of systems involving cadmium, especially of the system cadmium–ABSA, resembles that of the corresponding systems involving cobalt ions.

Calculation of Intrinsic Association Constants.—The calculations made in the preceding section have been shown to give useful qualitative information as to the nature of the interaction of bovine serum albumin with metal ions. These calculations, however, merely give the trend in which the equilibrium may be expected to move depending on whether the interaction involves the imidazole or the carboxyl groups of the albumin molecule. For a quantitative determination of intrinsic constants, however, we must analyze the equilibrium state as experimentally obtained. As there is little doubt that the interaction of cobaltous ion involves only the free carboxyl groups of the protein molecule, we shall in this section deal only with the equilibria involving bovine serum albumin and its derivatives on the one hand and copper, zinc and cadmium ions on the other. If the metal ion is bound at the basic imidazole groups, we have the equilibrium equation

$$\frac{\bar{\nu}}{16 - \bar{\nu} - n_{HIm}} = (A)k^0_{MIm}e^{-2wz_p} \qquad (6)$$

For the corresponding equilibrium between imidazole groups and hydrogen ion we have

$$\frac{n_{HIm}}{16 - \bar{\nu} - n_{HIm}} = (H^+)k^0_{HIm}e^{-2wz_p} \qquad (7)$$

In the above equations, (A) represents the activity of free metal ions in equilibrium with the metal–protein complex, z_p the charge on the metal–protein complex, and k^0_{MIm} an intrinsic constant representing the association of the metal ion with the imidazole group of the protein molecule. Combining eq. 6 and 7, we have

$$k^0_{MIm} = k^0_{HIm} \times \frac{\bar{\nu}}{n_{HIm}} \times \frac{(H^+)}{(A)} \qquad (8)$$

Assuming that interaction process involves the binding of MAc^+ ion at the imidazole sites of the albumin molecule and that all the imidazole sites are equivalent, k^0_{MIm} may be expected to be independent of $\bar{\nu}$, and have a value in reasonably good agreement with the first association constant of the metal ion with imidazole alone.

In eq. 8, $\bar{\nu}$ and (A) are known from separate equilibrium dialysis data[1,16,17] and k^0_{HIm} from the titration data of Tanford, Swanson and Shore referred to earlier.[9] The buffer medium of *p*H 6.5 fixes the value of (H+). For an evaluation of k^0_{MIm}, we thus require to know the value of n_{HIm} at equilibrium at each stage of interaction process. The values of n_{HIm} at $\bar{\nu} = 0$ for the three protein solutions have already been listed (Table I). The increment, Δn_{HIm}, associated with the reaction of metal ion with the imidazole groups of the protein molecule may be evaluated from eq. 5. In eq. 5, Δz_p is

known from the electrophoretic mobility data; the values of n_{Ac}[19] and n_{COOH} (and hence Δn_{Ac} and Δn_{COOH}) at each stage of the interaction process may be evaluated from the following equations, respectively.

$$\frac{n_{Ac}}{n_c - n_{Ac}} = (Ac^-)k^0_{Ac}e^{2\omega z_p} \quad (9)$$

$$\frac{n_{COOH}}{100 - n_{COOH}} = (H^+)k^0_{COOH}e^{-2\omega z_p} \quad (10)$$

It would thus seem that the data for the binding of metal ions by bovine serum albumin together with the corresponding electrophoretic mobility data may be employed usefully to follow the course of the interaction process.

Data of Klotz and Fiess.—Klotz and Fiess[17] have studied the binding of cupric ion by bovine serum albumin at 25° in an acetate buffer of *p*H 6.5, ionic strength 0.20. We shall examine their data in detail in order to illustrate the concepts on which the binding data in buffer solutions may be analyzed. Taking electrostatic interaction into account, it can be shown that, for a set of n equivalent sites[20]

$$\frac{\bar{\nu}}{A} e^{-2\omega\Delta z_p} = kn - k\bar{\nu} \quad (11)$$

where k is an intrinsic association constant uncorrected for the charge on the protein molecule. It is obvious from the electrophoretic mobility data presented in this paper that Δz_p (and hence the electrostatic interaction correction factor) is a complex function of $\bar{\nu}$. A plot of $(\bar{\nu}/A)e^{-2\omega\Delta z_p}$ *vs.* $\bar{\nu}$ should, however, give a straight line with the intercepts kn and n. Such a plot for the binding data obtained by Klotz and Fiess combined with the corresponding electrostatic interaction correction, as experimentally obtained from the mobility data presented in this paper, is given in Fig. 4.

It is immediately obvious from Fig. 4 that even when electrostatic interaction is taken into account the binding of copper ions by bovine serum albumin cannot be explained on a single value of n equivalent sites. The intercept kn $(= k_1$, the first association constant) can however be determined easily, and has a value of 3.8×10^5. For an evaluation of k, we require to know the value of n, the number of sites available for reaction with the first metal ion.

In view of the shape of the curve in Fig. 4, it is rather difficult to evaluate the intercept n by extrapolation. Fiess and Klotz[21] have indeed pointed out that this intercept may have a value of 20–100. The extrapolated value for n, even if extrapolation could be made precisely, should not however be used for the evaluation of the intrinsic association constant. Assuming that the interaction involves

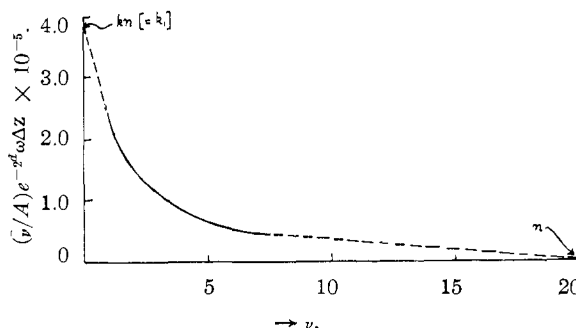

Fig. 4.—The interaction of cupric ion with NBSA (from the data of Klotz and Fiess): 25°, acetate buffer, *p*H 6.5, ionic strength 0.20.

the imidazole groups, it is reasonable to expect that it is only the unprotonated imidazole groups which are available for reaction with metal ions, and furthermore, that their value, as shown in the preceding sections, increases with $\bar{\nu}$. n is therefore dependent on the number of metal ions bound to the imidazole sites of the protein molecule. We may thus define

$$n = 16 - n_{HIm} = n^0[1 + f\bar{\nu}] \quad (12)$$

where n^0 is the number of basic imidazole groups present on the protein molecule at $\bar{\nu} = 0$, and f is a function governing the rate of change of n with $\bar{\nu}$. Substituting for n in eq. 11, we then have

$$\frac{\bar{\nu}}{A} e^{-2\omega\Delta z_p} = kn^0 - [1 - n^0 f]k\bar{\nu} \quad (13)$$

It is obvious from eq. 13 that the intercept on the ordinate in Fig. 4 represents kn^0. With a value of 2.5 for n ($n^0_{HIm} = 13.5$ at $\bar{\nu} = 0$, see Table I, column 3), and correcting for electrostatic effect due to the charge on the protein, the intrinsic association constant can be evaluated. A value of log k^0 = 4.86 was thus obtained.[22]

Assuming that the copper ion is bound at the imidazole sites of the albumin molecule, the in-

(19) As the metal uptake has been assumed to occur at the imidazole groups, the value for n_c, the number of charged cationic centers on the protein molecule, may be expected to depend on $\bar{\nu}$. Assuming that an imidazole group whether covered by metal ion or by hydrogen ion acts as a cationic center, the increase, Δn_c, in the number of cationic centers over the values given in Table I (column 4) may be expected to be $\bar{\nu} + \Delta n_{HIm}$. For systems involving NBSA, Δn_c was assumed to be zero as the uptake of a metal ion may be expected to cause a nearly equivalent proton release from the imidazole sites of NBSA (assumptions concerning the value of n_c are not critical in their effect on the final value of n_{HIm}). For systems involving EBSA, however, the values given for n_c are critical and must be accurately known; in these systems, Δn_c was assumed to be 0.6 $\bar{\nu}$ as, on an average, 0.4 proton may be expected to be released for every metal ion bound to EBSA. These estimates, necessarily approximate, were based on eq. 3a.

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

(21) H. A. Fiess and I. M. Klotz, THIS JOURNAL, **74**, 887 (1952).

(22) The intrinsic constant (log k^0_{CuIm} = 4.86) is much larger than the first association constant for the interaction of cupric ion with imidazole alone (log k_1 = 4.3[23] at 25°). In this connection it is of interest to note that the value of the intrinsic constant for the association of zinc ion with human serum albumin (ref. 3, log k^0_{ZnIm} = 2.82 at 0°) needs to be revised in view of the recent titration data of Tanford, Swanson and Shore (ref. 9). Assuming that the intrinsic proton affinity constants of the imidazole groups in NBSA and HSA are identical (see Discussion in ref. 9), it is obvious in view of eq. 5 in ref. 3 that the intrinsic association constant for the interaction of zinc ion with HSA as reported by Gurd and Goodman should be upgraded by 0.8 logarithmic units to give log k^0_{ZnIm} = 3.62. A comparison of this revised value with the first association constant for the interaction of zinc ion with imidazole alone (log k_1 = 2.8[23] at 0°) reveals a similar trend to the one observed in the corresponding systems involving cupric ion.

(23) J. T. Edsall, G. Felsenfeld, D. S. Goodman and F. R. N. Gurd, THIS JOURNAL, **76**, 3054 (1954).

trinsic association constant was evaluated as a function of $\bar{\nu}$ (eq. 8). The relevant log k^0 *vs.* $\bar{\nu}$ plot is given in Fig. 5A. It may be noticed that k^0 decreases with $\bar{\nu}$—a fact which may be attributed, among others, to an increasing participation of the free carboxyl groups of the albumin in the interaction process as more and more metal ions are bound by the protein. The matter shall be discussed further in a later communication.

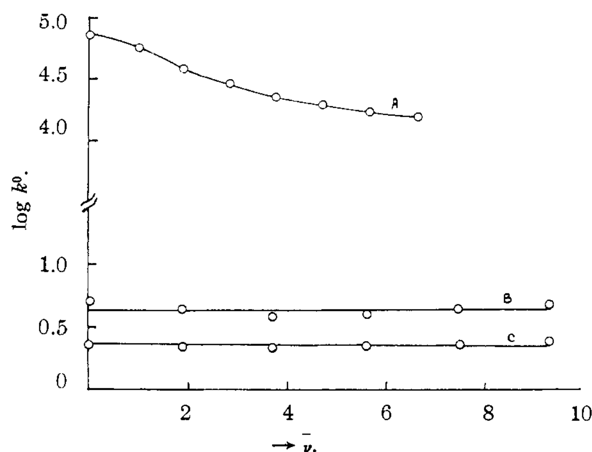

Fig. 5.—log k^0 *vs.* $\bar{\nu}$ curves: A, system copper–NBSA (from the binding data of Klotz and Fiess); B, system cobalt–NBSA (Rao and Lal); C, system cobalt–ABSA (Rao and Lal).

The Data of Rao and Lal.—The interaction of native and modified bovine serum albumins with the cobaltous ion in an acetate buffer of *p*H 6.5, ionic strength 0.20, has been studied by Rao and Lal.[1] There is conclusive evidence to show that the cobaltous ion is bound at the three carboxyl groups of the albumin molecule. Assuming that all the 100 carboxyl groups are in the basic form (see Table I, column 5), the value of the intrinsic association constant, k^0, evaluated from the first association constant, k_1, was found to be 5.1 for NBSA and 2.3 for ABSA. The intrinsic association constant remained independent of the number of cobaltous ions bound by the proteins (Figs. 5B and C). The low value of the intrinsic association constant together with the fact that it is independent of $\bar{\nu}$ point conclusively to the interaction of the cobaltous ion with the free carboxyl groups of the serum albumin molecule.

In the present work, we have outlined the general concepts governing the interpretation of metal–protein interactions in buffer solutions. A full treatment of our data for the binding of metal ions by bovine serum albumins and its modified derivatives shall be presented in a separate communication.

Acknowledgment.—The authors are grateful to Dr. A. B. Biswas for helpful discussions.

POONA 8, INDIA