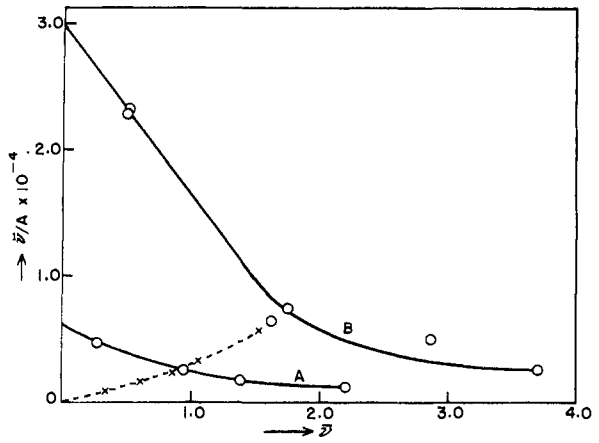


Fig. 3.—The interaction of Zn^{II} and Cd^{II} with bovine albumin: (A) the binding of Cd^{II} in the presence of 5.0×10^{-4} *M* Zn^{II}; (B) the binding of Zn^{II} in the presence of 5.0×10^{-4} *M* Cd^{II}. (The broken curve shows the progressive displacement of Cd^{II} by Zn^{II}).

sites more reactive than imidazole: either the 0.7 sulfhydryl group of total albumin or a chelate site formed by an imidazole group and a neighboring carboxyl group. Klotz and co-workers[12] have shown that the 375 mμ absorption peak characteristic of the interaction of Cu^{II} with the sulfhydryl group of bovine albumin is depressed by Zn^{II} and Cd^{II}, more so by Cd^{II} than by Zn^{II} and, consequently, we may assume that the ability of Cd^{II} to react with the sulfhydryl group of bovine albumin is somewhat more than, or at least equal to, that of Zn^{II}. We should expect therefore that Cd^{II} would be able to compete successfully or even displace Zn^{II} from combination with bovine albumin. We have, however, shown above that even when (approximately) two Zn^{II} ions are bound to bovine albumin, Cd^{II} ions are not able to displace them from combination, and that, in fact, log k_1 for the interaction of Cd^{II} with bovine albumin is reduced to 3.78: assuming a 1:1 interaction with imidazole sites, we thus have log $k^0_{CdI_m} \simeq 3.0$,[13] a value which is in good agreement with the first association constant of Cd^{II} with free imidazole.[12]

The evidence outlined above suggests that bovine albumin molecule contains approximately two especially reactive sites—presumably compound sites involving an imidazole and a neighboring carboxyl group—to which Zn^{II} are bound in preference to Cd^{II} and, furthermore, that if these two sites are occupied by Zn^{II} ions, the intrinsic association of Cd^{II} to bovine albumin corresponds to that expected for a 1:1 combination with imidazole sites. A fuller discussion of the interaction of metal ions with bovine serum albumin as evidenced from electrophoretic, polarographic and equilibrium dialysis studies is presented in the succeeding communication.[5]

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

(12) I. M. Klotz, J. M. Urquhart and H. A. Fiess, THIS JOURNAL, **74**, 5537 (1952); see also: I. M. Klotz, J. M. Urquhart, T. A. Klotz and J. Ayers, *ibid.*, **77**, 1919 (1955).

(13) For the evaluation of intrinsic constant, k^0, *see* ref. 2.

POONA 8, INDIA

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

Metal Protein Interactions in Buffer Solutions. Part III. Interaction of Cu^{II}, Zn^{II}, Cd^{II}, Co^{II} (and Ni^{II}) with Native and Modified Bovine Serum Albumins

BY M. S. NARSINGA RAO[1] AND HIRA LAL

RECEIVED DECEMBER 2, 1957

Quantitative binding data for the interaction of metallic ions with bovine serum albumin have been interpreted in the light of electrophoretic data. It is concluded that the interaction of Cu^{II}, Zn^{II} and Cd^{II} with bovine albumin, in the initial stages, occurs through 2–3 compound sites involving, presumably, an imidazole site and a neighboring peptide N or peptide O. Peptide N appears to be involved in interactions with Cu^{II} and peptide O with Zn^{II} and Cd^{II}. Co^{II} (and presumably Ni^{II}) are, however, bound to free carboxyl sites of bovine albumin. A probable explanation of the distinctive feature of these interactions, as also the effect of acetylation and esterification of bovine albumin, has been put forward. It is further concluded that interactions involving Cu^{II}, Zn^{II} and Cd^{II}, in the higher binding region, are governed by a competition between imidazole and carboxyl sites to interact 1:1 with the metal ions and may be accompanied by configurational changes in the protein molecule.

Gurd and Goodman[2] have concluded from their studies on the interaction of Zn^{II} with human serum albumin (HSA) in the neutral *p*H region that the metal ion is bound 1:1 with the imidazole sites of the protein molecule; the evaluated intrinsic association constant (log $k^0 = 2.82$) agreed well with the first association constant for the Zn^{II}–imidazole system.[3] This agreement is, however, fortuitous in that the intrinsic association constant, as pointed out earlier,[4] must be upgraded to give log $k^0 = 3.62$. If we further note that the affinity of Zn^{II} for 4-methylimidazole[5] (in which the methyl group is substituted at the same position as the histidyl side chain) is somewhat lower than that for unsubstituted imidazole, we are led to conclude that Zn^{II} is bound to serum albumin much more firmly than suggested from a combination with the imidazole sites of the protein molecule. A reconsideration of the polarographic data of Tanford[6] for

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

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

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

(4) H. Lal and M. S. N. Rao, *ibid.*, **79**, 3050 (1957).

(5) Y. Nozaki, F. R. N. Gurd, R. F. Chen and J. T. Edsall, *ibid.*, **79**, 2123 (1957).

(6) C. Tanford, *ibid.*, **74**, 211 (1952).

the interaction of Cu^{II}, Zn^{II} and Cd^{II} with NBSA, and an evaluation of intrinsic constants from the data of Klotz and Fiess[7,4] for the interaction of Cu^{II} with bovine serum albumin at *p*H 6.5, leads to much the same conclusion.

The electrophoretic behavior of metal–bovine albumin systems, described earlier,[4] further reflects the complexity of the interaction phenomena. Electrophoretic measurements, however, serve two useful purposes. Firstly, they help define the electrostatic-interaction term. Secondly, they yield invaluable information as to the extent to which interactions may be competitive or otherwise. Thus, for example, an interaction involving the carboxyl sites which are practically completely dissociated at *p*H 6.5 would be non-competitive, *i.e.*, subject to certain electrostatic effects influencing the binding of buffer anions with the protein, the charge on the protein molecule would increase by $\bar{\nu}\, z_M$ (where $\bar{\nu}$ is the average number of metal ions bound to the protein molecule and z_M the charge on metal ion) resulting in a corresponding increase in electrophoretic mobility. If, on the other hand, interaction were to occur at sites such as the imidazole sites, which are only partly dissociated at *p*H 6.5, it would shift the hydrogen ion equilibria involving these sites toward dissociated (unprotonated) imidazole, thus resulting in a release of hydrogen ions from the protein. The extent of this proton release, as will be shown presently, serves to elucidate the nature of the interaction process.

In the present paper, we present equilibrium dialysis data for the interaction of Cu^{II}, Zn^{II}, Cd^{II}, Co^{II} (and Ni^{II}) with bovine serum albumin and its derivatives in an acetate buffer of *p*H 6.50, ionic strength 0.20. These data together with those computed from polarographic measurements[8] are discussed in the light of the electrophoretic behavior of the systems studied.

Experimental

Bovine serum albumin, its acetylated and esterified derivatives (NBSA, ABSA and EBSA, respectively) and metal salts were as described previously.[4] A molecular weight of 65000 was assumed for NBSA and 66000 for ABSA and EBSA. All studies were made in acetate buffer of *p*H 6.50 and ionic strength 0.20.

Optical Absorption.[9,10]—Absorption measurements were made at room temperature with Beckman Quartz spectrophotometer (Model DU) using 1.0 cm. cells. Two per cent. protein solutions were used; the concentration of metal ions was 0.005 *M* for Cu^{II} and 0.05 *M* for Ni^{II}. With the esterified derivative, however, a 1.0% solution was used with a correspondingly decreased concentration of metal ions.[11] Protein solutions without metal ion were used as blanks.

Equilibrium Dialysis.[9,10]—Ten ml. of 1.0% protein solution placed in a Visking sausage tubing was equilibrated against 10 ml. of buffer solution containing varying amounts of metal ion to be studied. Blanks were run wherever necessary. With mechanical shaking, a period of 24 hr. was found sufficient for the attainment of equilibrium. The studies were made at 25.0 ± 0.1° for Cu^{II} and at 30.0 ± 0.1° for Zn^{II} and Cd^{II}. One half per cent. protein solutions were used for binding studies with Cu^{II}.

The equilibrium-dialyzed solutions were estimated for their metal content. The concentration of Cu^{II} was determined by the method of Woelfel[12] using carbon disulfide and diethanolamine as coloring agent. As the presence of the protein did not interfere with the estimation, blank runs were not considered necessary.

Zn^{II} and Cd^{II} were estimated polarographically. As the concentration of metal ions in the equilibrium-dialyzed solutions containing protein cannot be determined polarographically in a simple manner, blank runs also were made. The binding data were calculated from polarographic analyses of equilibrium-dialyzed protein-free solutions of the experimental runs and of the blank solutions. The procedure for polarographic measurements was the same as described previously.[8]

Results and Discussion

The absorption spectra of Cu^{II} and Ni^{II} ions alone, and in the presence of serum albumins, are given in Figs. 1 and 2. The spectra of Fig. 1 are

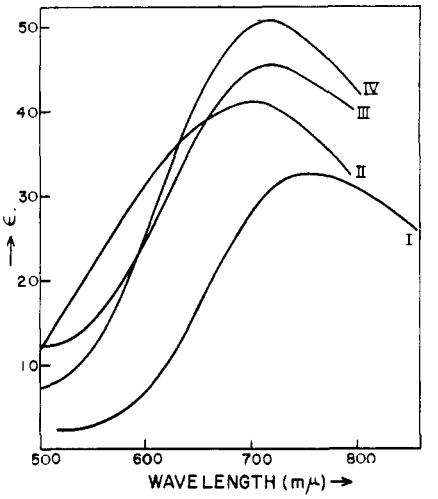


Fig. 1.—Absorption spectra of Cu^{II}–serum albumin complexes (acetate buffer, *p*H 6.50; ionic strength, 0.20): I, Cu^{II}; II, Cu^{II}–EBSA; III, Cu^{II}–NBSA; IV, Cu^{II}–ABSA.

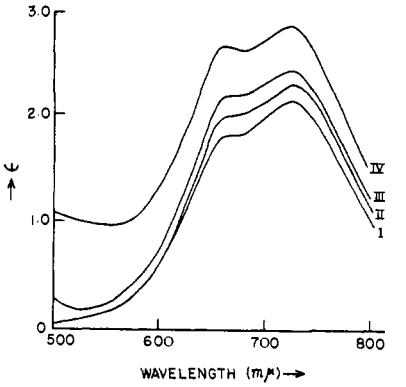


Fig. 2.—Absorption spectra of Ni^{II}–serum albumin complexes (acetate buffer, *p*H 6.50; ionic strength, 0.20): I, Ni^{II}; II, Ni^{II}–EBSA; III, Ni^{II}–NBSA; IV, Ni^{II}–ABSA.

similar to those reported by Klotz and Fiess.[7] The absorption increases in the order: EBSA → NBSA → ABSA, and corresponds to the ability of these proteins to bind Cu^{II} (see Fig. 3). The wave length of maximum absorption is also shifted toward lower wave lengths, the shift being of the same order for the three proteins. This behavior is in

(7) I. M. Klotz and H. A. Fiess, *J. Phys. Chem.*, **55**, 102 (1951); see also H. A. Fiess and I. M. Klotz, THIS JOURNAL, **74**, 887 (1952).

(8) M. S. N. Rao and H. Lal, *ibid.*, **80**, 3222 (1958).

(9) The system Co^{II}–bovine albumin has been described in ref. 10.

(10) M. S. N. Rao and H. Lal, THIS JOURNAL, **76**, 4867 (1954).

(11) Concentrated solutions of EBSA were somewhat turbid.

(12) W. C. Woelfel, *Anal. Chem.*, **20**, 722 (1948).

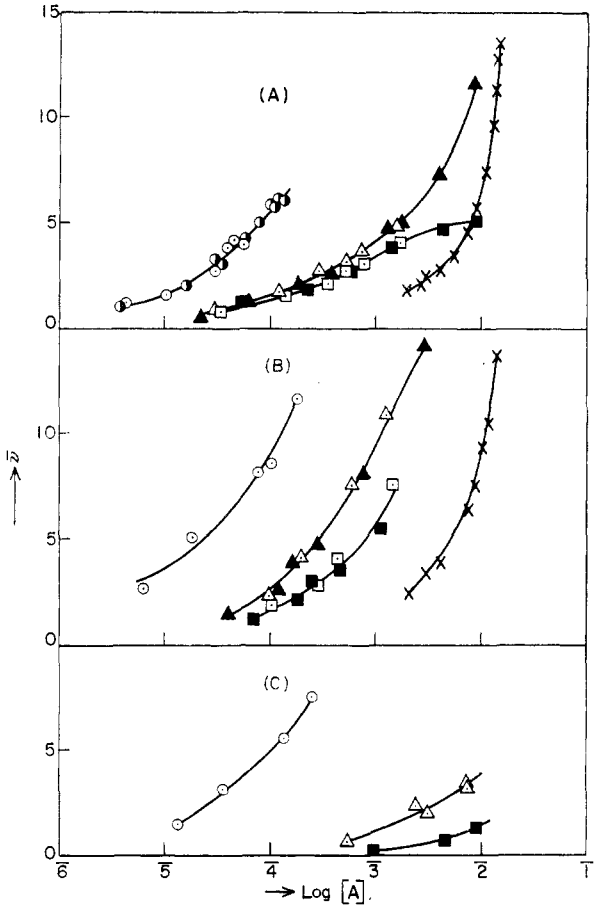


Fig. 3.—The binding of metal ions by bovine serum albumins (acetate buffer, *p*H 6.50; ionic strength, 0.20): (A) NBSA; (B) ABSA; (C) EBSA: ⊙, Cu^{II} (this paper); ◑, Cu^{II} (Klotz and Fiess); ◬, Zn^{II} (this paper); ▲, Zn^{II} (polarographic); ⊡, Cd^{II} (this paper); ■, Cd^{II} (polarographic); ×, Co^{II} (Rao and Lal).

contrast to the observation of Klotz and Fiess[7] who have reported that whereas a 66% esterified serum albumin leads to an increase in absorption of the same order as for the native protein, the absorption peak is shifted to even shorter wave lengths.

The absorption spectra of Cu^{II}–serum albumin systems at *p*H 6.5 suggest, as pointed out by Klotz and Fiess,[7] that binding occurs with N-containing group, presumably the imidazole groups, of the albumin molecule. The absorption spectra of Ni^{II}–serum albumin systems (Fig. 2), however, resemble those of the corresponding systems involving cobaltous ion.[10,13] These systems exhibit only an increased absorption without any noticeable shift in the wave length of maximum absorption. Furthermore, the addition of EBSA has no effect on the absorption spectra of the metal ions. It would thus appear that the binding of Ni^{II} and Co^{II} involves only the free carboxyl groups of the protein molecule, and that M^{II} . . . . N linkage is not favored. It may further be concluded from a comparison of spectra of the two systems that the ability of serum albumin to bind Ni^{II} is of the same order as, or slightly less than, that for Co^{II}.

(13) H. A. Fiess, THIS JOURNAL, **74**, 3539 (1952).

The equilibrium dialysis data for the binding of Cu^{II}, Zn^{II}, Cd^{II} and Co^{II} by bovine serum albumin and its derivatives are presented in Fig. 3 as the familiar $\bar{\nu}$ *vs.* log *A* plots. The data of Klotz and Fiess[7,14] for the system Cu^{II}–NBSA are also given in Fig. 3. The data for the binding of Co^{II} were obtained from the authors' earlier studies.[10,14] The binding data for Zn^{II} and Cd^{II}, as computed from polarographic measurements described in a previous paper,[8] are also presented in Fig. 3 alongside the equilibrium dialysis data. It may be noticed that equilibrium dialysis and polarographic data agree well with each other. An estimate of the binding of Cd^{II} by EBSA (Fig. 3C) was made from polarographic measurements assuming $\alpha = 0.30$[8]; no reliable estimate of the binding of Cd^{II} by EBSA could be made from equilibrium dialysis experiments.[15]

An examination of Fig. 3 reveals that the ability of bovine albumin and its derivatives to bind a given metal ion increases in the order: EBSA → NBSA → ABSA, a conclusion entirely in agreement with the absorption data. Furthermore, the binding of Cu^{II} and Zn^{II} is only slightly affected by esterification of bovine albumin whereas that of Cd^{II} is considerably reduced and that of Co^{II} completely suppressed.[15] The differences between Cd^{II} and Zn^{II} in their interaction with EBSA are rather remarkable. In view of the fact that, under the experimental conditions, the interaction is likely to occur only with the imidazole sites, and further because the intrinsic affinities of Cd^{II} and Zn^{II} for imidazole are practically identical[3,16] (see Table II), the $\bar{\nu}$ *vs.* log *A* curves for these systems should have been nearly superposable. It would thus appear that the mode of binding of Zn^{II} to EBSA is radically different from that of Cd^{II} (and possibly, Co^{II} and Ni^{II}).

The values of free energy change, ΔF_1, accompanying the uptake of the first metal ion by bovine albumin and its derivatives are presented in Table I. These values were calculated from the first association constant $k_1(\Delta F_1 = -RT \ln k_1)$ as extrapolated from $\bar{\nu}/A$ *vs.* $\bar{\nu}$ plots. The ΔF_1 values of −7640 cal./mole and −4140 cal./mole for the systems: Cu^{II}–NBSA and Co^{II}–NBSA, though somewhat large, are of the same order as those reported by Klotz and Fiess[7] and Fiess,[13] respectively (Table I).

TABLE I

	ΔF_1 (cal. per mole)			
	Cu(II) (25°)	Zn(II) (30°)	Cd(II) (30°)	Co(II) (25°)
ABSA	−8290	−6380	−6380	−4330
NBSA	−7640 (−7060[a])	−6380	−6330	−4140 (−3960[b])
EBSA	−6980	−5790	−4500	

[a] Ref. 7. [b] Ref. 13.

The intrinsic association constants, as evaluated from the first association constants according to the procedure outlined in paper I,[4] are listed in

(14) The binding data were reevaluated on the basis of a mol. wt. of 65000 for NBSA.

(15) As in Cd^{II}–EBSA system, equilibrium dialysis revealed little binding of Co^{II} by EBSA. In view of the polarographic evidence for a small binding of Cd^{II} by EBSA, however, it is not unlikely that Co^{II} may also be bound slightly by EBSA.

(16) C. Tanford and W. L. Wagner, THIS JOURNAL, **75**, 434 (1953).

TABLE II

	log k^0			log k_1				
	EBSA 1	ABSA 2	NBSA 3	Acetate 4	Imidazole 5	4-Methyl imidazole 6	Glycyl-glycine GG 7	Diglycyl glycine GGG 8
Cu^{II} 25°	4.78	5.3	4.88 (4.7[a])	2.16[d]	4.33[e]	4.13[g]	5.82[h]	5.30,[h] 5.41[i]
Zn^{II} 30°	3.80	3.82	3.87(3.62,[b] 3.7,[a] 3.1[c])	1.03[d]	2.52[e]	2.41[g]	3.80[i]	3.33[i]
Cd^{II} 30°	2.9	3.82	3.84 (3.6[a])	1.3[d]	2.71[f]	...	3.33[i]	3.30[i]
Co^{II} 25°	..	0.36	0.71					

[a] Ref. 6, NBSA, μ = 0.15, 25°; revised value. [b] Ref. 2, HSA, μ = 0.15, 0°; revised value. [c] Ref. 18, NBSA, μ = 0.05, 26°, *p*H 6.1. [d] Taken from the compilation of Gurd and Wilcox, ref. 21, Table VII, p. 351. [e] Interpolated from ref. 5. [f] Interpolated from ref. 16. [g] Interpolated or extrapolated from ref. 5. [h] Ref. 29. [i] Ref. 26.

Table II. These intrinsic constants were evaluated on the assumption that the first Cu^{II}, Zn^{II} and Cd^{II} was bound 1:1 at the imidazole sites, and the cobaltous ion at the free carboxyl sites of the albumin molecule. The values in brackets under column 3 (Table II) are log k^0 values for the interaction of Cu^{II}, Zn^{II} and Cd^{II} with serum albumin as reported by earlier workers[2,6] and suitably revised by us in the light of the recent titration data of Tanford, Swanson and Shore[17] for bovine albumin. It may be noticed that these revised values obtained in unbuffered systems agree well with those obtained from our data. We have not, however, revised the value of log k^0 = 3.1 reported by Saroff and Mark[18] for the system: Zn^{II}–NBSA in an acetate buffer medium of *p*H 6.1. These authors did not interpret their data on the basis of a competition between metal and hydrogen ions for combining with the imidazole sites of bovine albumin molecule. It is indeed reasonable to expect that a recalculation, in the light of concepts governing the analysis of binding data in buffered systems outlined in paper I,[4] would yield intrinsic constants agreeing reasonably well with those reported by us.

The log k_1 values for the interaction of Cu^{II}, Zn^{II} and Cd^{II} with free imidazole[3,16] and of Cu^{II} and Zn^{II} with 4-methylimidazole[5] are listed in columns 5 and 6, respectively (Table II). It may be noticed that, in general, the intrinsic affinity of bovine albumin for Cu^{II}, Zn^{II} and Cd^{II} is considerably more than that of imidazole or 4-methylimidazole. The only exception seems to be the system: Cd^{II}–EBSA. The intrinsic association constant (log $k^0 \simeq 2.9$) for this system is of the same order as that expected for the corresponding Cd^{II}–imidazole system. We may thus conclude that Cd^{II} is bound to imidazole sites of EBSA in a simple 1:1 fashion.

It now remains to analyse the binding data to give log k^0 *vs.* $\bar{\nu}$ curves. The relevant procedure has been discussed in paper I,[4] the competitive interaction of metal and hydrogen ions with the imidazole sites of bovine albumin being represented by an equation similar to the one used by Gurd and Goodman.[2] Thus

$$k^0_{MIm} = k^0_{HIm} \times \frac{\bar{\nu}}{n_{HIm}} \frac{(H^+)}{(A)} \times e^{2wz_p(z_M - z_H)} \quad (1)$$

where

$$n_{HIM} = n^0_{HIm} + \Delta z_p + \Delta n_{AC} - \Delta n_{COOH} - \bar{\nu} z_M \quad (2)$$

The various terms are as defined in paper I.[4] It is assumed that the metal ion is bound essentially as the MAc^+ ion ($z_M = z_H = 1$; the exponential term in eq. 1 is thus assumed to be unity) in a simple 1:1 interaction with the imidazole sites of the protein molecule. As may be noticed from eq. 2, the quantity $\Delta z_p + \Delta n_{AC} - \Delta n_{COOH} - \bar{\nu} z_M$, may, in the absence of configurational changes in the protein molecule, be equated to $-\Delta H^+$, where ΔH^+ is the number of protons released per $\bar{\nu}$ metal ions bound. Furthermore, if the combination occurs with the imidazole sites, $-\Delta H^+ = \Delta n_{HIm} = n_{HIm} - n^0_{HIm}$, so that the quantity Δn_{HIm} should be negative or zero depending on whether the interaction is competitive or non-competitive, respectively. Δn_{HIm} *vs.* $\bar{\nu}$ plots are given in Fig. 4A, B and C for systems involving NBSA, ABSA and

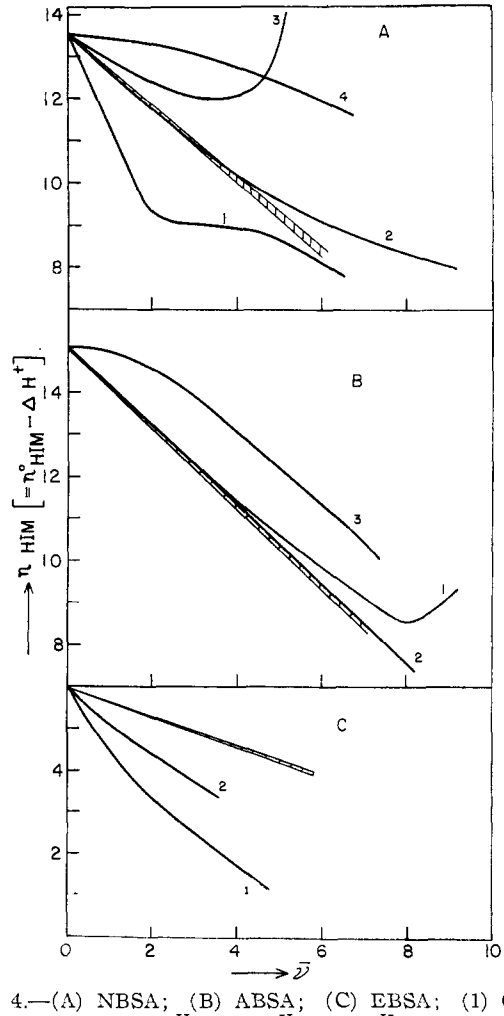


Fig. 4.—(A) NBSA; (B) ABSA; (C) EBSA; (1) Cu^{II}; (2) Zn^{II}; (3) Cd^{II}; (4) Co^{II}.

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

(18) H. A. Saroff and H. J. Mark, *ibid.*, **75**, 1420 (1953).

EBSA, respectively. The shaded curves[19] in Fig. 4 represent the course n_{HIm} *vs.* $\bar{\nu}$ plots should take if 1:1 interaction were to occur with the imidazole sites of the protein molecule. The log k^0 *vs.* $\bar{\nu}$ curves for the systems studied are shown in Fig. 5.

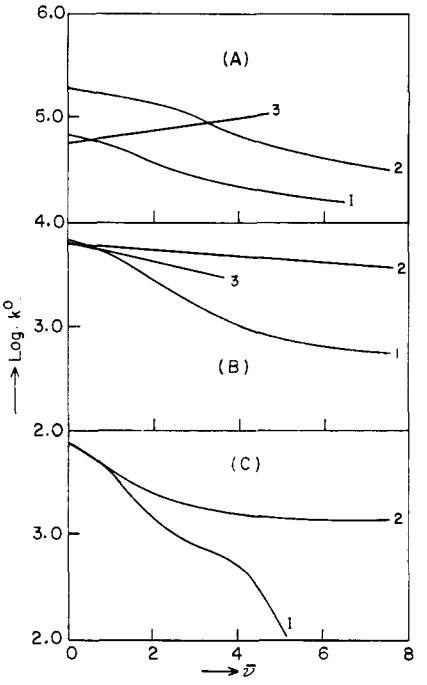


Fig. 5.—Intrinsic constants for 1:1 interaction with imidazole sites: (A) Cu^{II}; (B) Zn^{II}; (C) Cd^{II}; (1) NBSA; (2) ABSA; (3) EBSA.

Sulfhydryl Group of Bovine Albumin

Compound and Simple Sites.—Problems to be discussed in this and later sections may be exemplified by one or two specific systems. Let us first take the system: Zn^{II}–NBSA. It may be noticed from Fig. 4A2 that the uptake of Zn^{II} by NBSA is accompanied by proton release characteristic of 1:1 interaction with imidazole sites up to $\bar{\nu} \simeq 5$. One may expect, therefore, that log k^0 for this system should remain independent of $\bar{\nu}$ up to $\bar{\nu} \simeq 5$ and that it should have a value close to log k_1 for the interaction of Zn^{II} with imidazole or 4-methylimidazole. An examination of Fig. 5B1 reveals, however, that log k^0 falls from a high initial value of 3.87 to a value of approximately 2.7 at $\bar{\nu} \simeq 6$. This latter value of log k^0 is rather close to that expected for a 1:1 interaction with imidazole. It is therefore evident that the first few Zn^{II} ions are bound more firmly to NBSA than would be expected from a combination with imidazole alone, even though proton release accompanying the uptake of zinc ions suggests a simple 1:1 interaction with imidazole sites.

The system Cu^{II}–NBSA is even more revealing. Here, the uptake of the first two cupric ions is accompanied by the release of approximately two protons per metal ion bound (Fig. 4A1). This is indeed a direct consequence of the fact that the electrophoretic mobility of Cu^{II}–NBSA systems passes through a minimum at $\bar{\nu} \simeq 2$.[4] Here again, like Zn^{II}–NBSA, the intrinsic constant falls from an initially high value of log $k^0 = 4.88$ to log $k^0 \simeq 4.1$ at $\bar{\nu} \simeq 6$. This latter value is close to that to be expected for a 1:1 interaction with imidazole alone. We are thus led to conclude that the first few Cu^{II} ions are bound more firmly to NBSA than would be expected from a 1:1 combination with imidazole and that the interaction process in the initial stages is such as to involve a release of two protons per cupric ion bound.

It may be concluded, therefore, that Zn^{II} and Cu^{II} ions are bound initially to sites on bovine albumin which are more reactive than imidazole. One such site may well be the sulfhydryl group of total albumin.[20] As the affinity of this group for metal ions is much more than that of imidazole,[20,21] it is evident that if a metal ion were initially to react with this group, the intrinsic association constant calculated on the basis of an interaction with the imidazole sites alone would tend to be large. The initially large log k^0 values (Table II) together with the fact that they decrease with $\bar{\nu}$ point to such a possibility. In their analysis of the data of Klotz and Fiess[7] for the interaction of Cu^{II} with bovine albumin, Gurd and Wilcox[21] have indeed pointed out that the first cupric ion may possibly be bound to bovine albumin through its sulfhydryl group. There is definite evidence, however, which points to a contrary conclusion. Firstly, the interaction of Cu^{II}, Zn^{II} and Cd^{II} with ABSA, in which the sulfhydryl group is blocked,[22] yields, in general, the same type of log k^0 *vs.* $\bar{\nu}$ curves as those obtained in corresponding systems involving NBSA (Fig. 5). Secondly, the release of two protons per cupric ion bound to NBSA, together with the fact that this behaviour is observed upto $\bar{\nu} \simeq 2$, cannot be explained on the basis of an interaction with sulfhydryl group wherein only one proton may be expected to be released per metal ion bound. Thirdly, it has been shown in paper II[8] that approximately two zinc ions are firmly bound to NBSA and that they cannot be removed from combination by Cd^{II} ions—a fact which cannot be reconciled with the greater ability of Cd^{II} than of Zn^{II} to remove Cu^{II} from combination with the sulfhydryl group of NBSA.[23] Equally inexplicable is the weak affinity of EBSA for Cd^{II} as compared to that for Zn^{II} (see Table II).

Klotz and co-workers[22,23] have shown that the 375 mμ, absorption peak characteristic of the interaction of Cu^{II} with the sulfhydryl group of NBSA does not appear until at least four cupric ions are bound to the protein molecule and that the high absorption peak appears only when approximately ten Cu^{II} ions are bound. It would thus appear that the first cupric ion is bound to sites other than sulfhydryl. As, however, this absorption peak has been attributed to a special configuration involv-

(19) Eq. 7 (ref. 4) was used for this purpose. The spread of the shaded curves arises out of the electrostatic factor which, for a given protein, differs from metal to metal.

(20) See, for example, ref. 21, p. 351.

(21) F. R. N. Gurd and P. E. Wilcox, in "Advances in Protein Chemistry," Vol. XI, ed. M. L. Anson, K. Bailey and J. T. Edsall, Academic Press, Inc., New York, N. Y., 1956, p. 311.

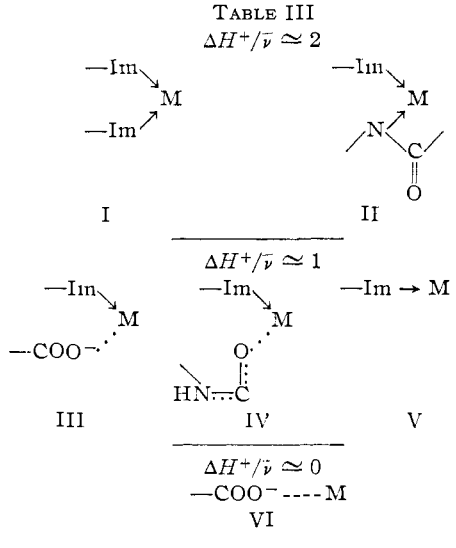
(22) I. M. Klotz, J. M. Urquhart, T. A. Klotz and J. Ayers, THIS JOURNAL, **77**, 1919 (1955).

(23) I. M. Klotz, J. M. Urquhart and H. A. Fiess, *ibid.*, **74**, 5537 (1952).

ing the sulfhydryl group and a neighboring disulfide bridge, it does not necessarily follow that the first cupric ion is not bound at the sulfhydryl site. The authors have carried out a few critical experiments to elucidate this point: it has, we believe, been shown conclusively that the first cupric ion is bound to bovine albumin through sites other than sulfhydryl (see latter Discussion).

If we rule out the sulfhydryl group of bovine albumin as being responsible for the high intrinsic constants observed, we must look for other groups or sets of groups more reactive than imidazole alone. The probable "compound" and simple sites with which metal ions may react at *p*H 6.5 are listed in Table III. It is evident that if the interaction of Cu^{II} with NBSA involves a release of approximately two protons per metal ion bound, the cupric ion must be bound to a compound site involving an imidazole group and a neighboring imidazole group or a neighboring peptide N. Similarly, the binding of the first few (presumably two) zinc ions may occur through sites such as III and IV (Table III) involving an imidazole group and a neighboring carboxyl group or a neighboring peptide O. If we further assume that there may be only a limited number of such compound sites present on bovine albumin, it is easy to explain, at least qualitatively, the initially high intrinsic constants observed in some systems together with the fact that they decrease with $\bar{\nu}$ and tend to approach values characteristic of a simple 1:1 interaction with imidazole sites.

TABLE III



Interactions in the Lower Binding Region

It is clear from the foregoing that evidence for the nature of reactive sites on bovine albumin with which metal ions may combine has to be based on the electrophoretic mobility data as symbolized by Fig. 4 and the calculated intrinsic constants (Table II, Fig. 5). Evidence as to the nature of the interaction process obtained from the mobility data without taking cognizance of intrinsic constants, and *vice versa*, is likely to be misleading.

Systems: Cu^{II}–NBSA, Cu^{II}–EBSA and Zn^{II}–EBSA.—Evidence for the presence on bovine albumin of compound sites such as I and II (Table III) through which the first two cupric ions may be bound to the protein molecule has been presented above. The systems involving EBSA are, however, difficult to interpret due to relatively large uncertainties in the evaluation of Δn_{Ac}.[24] There is, however, ample evidence that the uptake of cupric and zinc ions causes a much larger release of hydrogen ions than that to be expected from a 1:1 interaction with the imidazole sites of EBSA (Fig. 4C). Together with the large intrinsic constants observed (Table II), these results point to a strong probability that the interaction of Cu^{II} and Zn^{II} with EBSA, like that of Cu^{II} with NBSA, occurs through compound sites such as I and II (Table III).

Systems: Cu^{II}–ABSA, Zn–NBSA, Zn^{II}–ABSA, Cd^{II}–NBSA (and Cd^{II}–ABSA).—These systems, with the exception of the system Cd^{II}–ABSA, closely resemble each other in that the relatively large intrinsic constants observed are associated with proton release characteristic of a 1:1 interaction with imidazole sites. As in the system Zn^{II}–NBSA, discussed above, we may therefore postulate the presence of compound sites such as III and IV through which the first few metal ions are bound. As two zinc ions are rather firmly bound to bovine albumin and cannot be displaced from combination by Cd^{II} ions,[8] it is probable that the compound sites responsible for binding Zn^{II} and Cd^{II} may either be the same or have at least one group (forming the compound site) common.

The system Cd^{II}–ABSA is anomalous. It must be remembered that the electrophoretic behavior of systems involving ABSA is easy to evaluate inasmuch as the term Δn_{Ac} is absent. As the term Δn_{COOH} is also negligible, differences between competitive and non-competitive interactions are even more marked than in the corresponding systems involving NBSA. Thus, the electrophoretic mobility of Zn^{II}–ABSA and Cu^{II}–ABSA is practically independent of $\bar{\nu}$[24]—a fact which is reflected in proton release characteristic of 1:1 interaction with imidazole sites (see Figs. 4B1 and 4B2). On the other hand, the mobility of Co^{II}–ABSA increases rapidly with $\bar{\nu}$; we may thus infer that the interaction process is non-competitive. The system Cd^{II}–ABSA resembles closely the corresponding system involving Co^{II} in its electrophoretic mobility behavior.[4] On the other hand, the binding curve for the system Cd^{II}–ABSA is far displaced to the left of the Co^{II}–ABSA system (see Fig. 3B). If, however, we compare the system Cd^{II}–ABSA with Zn^{II}–ABSA, we find that whereas their electrophoretic behavior differs markedly from each other, their binding curves are close together (Fig. 3B). We have indeed no explanation to offer for this anomalous behavior of Cd^{II}–ABSA system. We have, however, preferred to analyze the binding data for this system on the assumption that Cd^{II} is bound to imidazole sites of ABSA; the large log k^0 value obtained (Table II) may then be explained on the same basis as for the corresponding Cd^{II}–NBSA system.

Systems: Cd^{II}–EBSA and Co^{II}–EBSA.—The system Cd^{II}–EBSA already has been discussed

(24) See ref. 4.

above. The high positive charge on the protein together with the fact that the interaction process presumably involves a 1:1 combination with the imidazole sites reduces the affinity of this protein for Cd^{II}. The remarkable differences between the affinities of Cd^{II} and Zn^{II} for EBSA may then be explained on the postulate that whereas Cd^{II} is bound to imidazole sites of EBSA in a simple 1:1 fashion, the interaction of Zn^{II} with EBSA involves compound sites such as I and II (Table III). It is not improbable that Co^{II} (and possibly Ni^{II}) may be bound to EBSA in a manner similar to Cd^{II}.

Systems Co^{II}–NBSA and Co^{II}–ABSA.—Evidence as to the interaction of Co^{II} with the free carboxyl sites of NBSA and ABSA already has been presented elsewhere.[4,10] An examination of Fig. 4A4 reveals a relative absence of proton release accompanying the uptake of Co^{II} ions, thus providing further confirmation to our earlier conclusion that the interaction of Co^{II} with bovine albumin is noncompetitive. If we assume that the compound sites responsible for the strong affinity of bovine albumin for Cu^{II}, Zn^{II} and Cd^{II} are unable to interact with Co^{II} and further that bovine albumin contains no other compound sites to which Co^{II} may be bound, it may be expected, in view of the postulated low affinity of Co^{II} for imidazole sites of EBSA,[15] that carboxyl sites may compete successfully with the imidazole sites of bovine albumin to bind Co^{II}. It may be noted that bovine albumin contains only 2.5 basic imidazole groups[24] (ABSA contains even less) at *p*H 6.5, and unless there are special circumstances such as the presence of compound sites on the protein molecule, the 100 carboxyl sites on the protein molecule may, by virtue of their numerical preponderance, influence the course of the interaction process.

Compound Sites and Intrinsic Association Constants.—We have pointed in the preceding discussion to a strong probability that the presence of compound sites on bovine albumins may account for their interaction with metallic ions in the lower binding region. The presence of an imidazole or a carboxyl group in suitable juxtaposition to an imidazole group to form compound sites I and III is rather unlikely on steric considerations. Even if relatively rigid configurations such as those involving sites I and III were assumed, it is indeed difficult to give a rational explanation of the differences between the various systems discussed above. We shall assume, therefore, that the only compound sites present on bovine albumin are of class II and IV (Table III). The relevant data are summarized in Table IV. We may draw attention to the rather striking differences between EBSA, NBSA and ABSA in their interactions with Cu^{II} and Zn^{II}. Assuming that only sites of class II and IV are involved, we find that Cu^{II} interacts with EBSA and NBSA through the former and with ABSA through the latter class of sites. The interaction of Zn^{II} with EBSA occurs through sites of class II, and with NBSA and ABSA through sites of class IV. Rabin[25] has presented evidence that, in general, peptide O rather than peptide N is involved in the interaction of metal ions with peptides but that the ionization of peptide hydrogen may occur for some copper complexes. We may indeed expect the ability of metal ions, under given conditions, to combine with peptide N to follow the decreasing order $Cu^{II} \rightarrow Zn^{II} \rightarrow Cd^{II}$. This ability of metallic ions to abstract a proton from the peptide >NH group would be further influenced by the charge on the protein molecule; we may thus expect that the tendency of peptide hydrogen to ionize would follow the decreasing order EBSA → NBSA → ABSA. The relatively large differences in the charge on bovine albumin and its derivatives may thus be a determining factor in influencing the participation of the peptide N or peptide O in the interaction process. The differences between metal ions on the one hand and bovine albumin and its derivatives on the other, in so far as the type of sites involved is concerned, may thus be explained on the above basis.

It is clear from the foregoing discussion that many of the unusual characteristics of the metal–protein systems investigated may be attributed to interactions with sites such as II and IV. We shall now investigate whether observed intrinsic constants support such a view. Here, we are faced with the fact that suitable model systems have not been studied so far. Assuming, however, that the affinity of imidazole for metallic ions is of the same order as that of amino group,[20] we suggest that di- and tri-peptides such as glycyl-glycine and diglycyl-glycine, which interact with metal ions through their amino group and peptide N or peptide O,[26–29] may be taken as suitable models for compound sites II and IV. Log k_1 values for the interaction of metal ions with glycyl-glycine and diglycyl-glycine are listed under columns 7 and 8 (Table II), respectively. It may be noticed that wherever sites such as II and IV have been postulated, the observed intrinsic constants compare favorably with the expected values. It must be pointed out, however, that the evidence of intrinsic constants is still largely inconclusive inasmuch as we are unable to distinguish between sites II and IV from the observed intrinsic constants. Much further work with model systems is indeed indicated for an understanding of the role of peptide chain in metal–protein interactions.

TABLE IV

TYPE OF SITES INVOLVED IN THE INTERACTION OF METALLIC IONS WITH BOVINE SERUM ALBUMINS

	Cu^{II}	Zn^{II}	Cd^{II}	Co^{II}
EBSA	II	II	V	V
NBSA	II	IV	IV	VI
ABSA	IV	IV	IV	VI

(25) B. R. Rabin, *Trans. Faraday Soc.*, **52**, 1130 (1956).

(26) W. P. Evans and C. B. Monk, *ibid.*, **51**, 1244 (1955).

(27) I. Greenwald, *J. Phys. Chem.*, **47**, 607 (1943).

(28) H. Dobbie and W. O. Kermack, *Biochem. J.*, **59**, 246 (1955); **59**, 257 (1955).

(29) S. P. Datta and B. R. Rabin, *Trans. Faraday Soc.*, **52**, 1123 (1956).

Interactions in the Higher Binding Region

In general, intrinsic association constants for interaction of Cu^{II}, Zn^{II} and Cd^{II} with bovine albumins decrease with $\bar{\nu}$ and tend to approach values characteristic of 1:1 interaction with imidazole sites. There are, however, three systems, namely, Cu^{II}–EBSA, Zn^{II}–EBSA and Zn^{II}–ABSA, for which log k^0 is practically independent of $\bar{\nu}$ (see Fig. 5). Gurd and Goodman[2] have reported a similar result for the system Zn^{II}–HSA. It may be remarked, however, that log k^0 *vs.* $\bar{\nu}$ curves for systems involving EBSA are subject to considerable errors due to uncertainties in the evaluation of Δn_{Ac}.[24] The data of Gurd and Goodman[2] also need to be revaluated in the light of the titration data reported by Tanford, Swanson and Shore.[17,4] It is not inconceivable, however, that more compound sites of class II and IV (Table III) may be involved in these systems than in others. On the other hand, there is definite evidence that there may be only two sites, presumably, of class II through which NBSA may bind Cu^{II}. It also has been shown that two compound sites, presumably of class IV, are involved in the interaction of NBSA with Zn^{II} and Cd^{II} and that these sites prefer Zn^{II} to Cd^{II}.[8] Subject to these uncertainties, we shall assume, therefore, that only a limited number of compound sites may be available on bovine albumin for interaction with metallic ions. The few compound sites present on bovine albumin having been occupied, it is indeed to be expected that a simple 1:1 interaction may occur with the imidazole sites. Thus, the interaction of Cd^{II} with NBSA the two most reactive sites of which have been occupied by Zn^{II} suggests a simple 1:1 interaction with imidazole sites.[8] An additional factor that has to be taken into account, in the higher binding region at least, is a competition between imidazole and carboxyl sites for 1:1 interaction with the metal ions.[30] The inability of Co^{II} to interact with the imidazole sites of NBSA and ABSA has been attributed, in the preceding discussion, to the carboxyl sites competing successfully with the imidazole sites to bind Co^{II}. In addition to the statistical factors occasioned by the relatively large number of carboxyl as compared to imidazole sites available on the albumin molecule, this competition would be governed by the relative affinities of these sites for metallic ions. It may be expected from the affinities of imidazole and acetate ion for metal ions (Table II) that this competition should favor the carboxyl sites in the following increasing order: $Cu^{II} \rightarrow Zn^{II} \rightarrow Cd^{II} \rightarrow Co^{II}$. Mobility measurements do indeed point to such a tendency.[24,32]

It may be pointed out that the data at large values of $\bar{\nu}$ are subject to considerable errors.[33] Consequently, a certain amount of caution is necessary in the interpretation of data in the higher binding region. There may however be other factors such as configurational changes which may operate in these systems and which cannot be ignored. Thus, for example, the sharp increase in the mobility of Cd^{II}–NBSA at $\bar{\nu} > 3$ is much larger than would be expected from even a non-competitive interaction.[4] This is reflected in proton release occasioned by the uptake of Cd^{II} by NBSA passing through a minimum at $\bar{\nu} \simeq 4$ (Fig. 4A3). This means, in effect, that, in the absence of configurational changes, protons are being added to NBSA molecule when more than four Cd^{II} ions are bound: this is clearly not likely. It is therefore probable that the binding of Cd^{II} tends to change the configuration of NBSA in such a manner as to make it more positively charged. We shall tentatively regard these configurational changes as equivalent to a "folding" of the protein molecule.[35] It is therefore strongly indicated that non-competitive interaction and "folding" may both be operative in determining the electrophoretic mobility of Cd^{II}–NBSA, and to a lesser degree, of Zn^{II}–NBSA and Cu^{II}–NBSA.[36] This "folding" may indeed be responsible for bringing the sulfhydryl group of bovine albumin in suitable juxtaposition with a disulfide bridge to give the 375 mμ absorption peak characteristic of Cu^{II}–NBSA systems at $\bar{\nu} > 4$. The "folding" of NBSA as a result of the uptake of Cd^{II} ions would be expected to render the protein relatively inert; the abrupt decrease in log k^0 at $\bar{\nu} > 4$ (Fig. 5C1) and the fact that the binding curve for this system shows a plateau at $\bar{\nu} \simeq 7$ (see Fig. 3A) lend support to this view. The system Cd^{II}–NBSA appears to be the only one among those studied which shows such a plateau.

The metal–ABSA systems can be classified into two groups—those involving Co^{II} and Cd^{II} form-

(30) Gurd[31] has shown that whereas the interaction of Zn^{II} with HSA occurs essentially through the imidazole sites at 0°, the protein undergoes configurational changes between 25 and 37° resulting in the availability of additional sites for interaction with Zn^{II}. These sites may well be the aspartic and glutamic residues.[21]

(31) F. R. N. Gurd, *J. Phys. Chem.*, **58**, 788 (1954).

(32) It was remarked in ref. 4 that the decrease in log k^0 with $\bar{\nu}$ for the system Cu^{II}–NBSA may be due to an increasing participation of the carboxyl sites in the interaction process. This is indeed not the case. Assuming the intrinsic association constant for the interaction of Cu^{II} with the carboxyl sites of NBSA to be 100, $\bar{\nu}$ can be corrected for binding at the carboxyl sites. This corrected value of $\bar{\nu}$, when inserted in eq. 1, gave log k^0_{CuIm} *vs.* $\bar{\nu}$ curve which was substantially the same as Fig. 5A1 up to $\bar{\nu} \simeq 5$. And yet, it is in the region of $\bar{\nu} = 0 - 5$ that log k^0 decreases sharply with $\bar{\nu}$. Whereas mobility measurements give a better idea of non-competitive interaction at carboxyl sites than the evaluated intrinsic constants, it is strongly indicated that an explanation of the decrease in log k^0 with $\bar{\nu}$ up to $\bar{\nu} \simeq 5$ has to be found in directions other than the participation of carboxyl sites in the interaction process.

(33) Apart from small uncertainties in equilibrium dialysis and mobility measurements, there may be several sources of error in the higher binding region. Firstly, the metal ion may not be bound as the MAc^+ ion when the total metal concentration is relatively large; this applies especially to systems involving Co^{II} and (to a lesser degree) Cd^{II}. Secondly, the evaluation of various terms, especially Δn_{Ac} and Δz_p (eq. 2), from which n_{HIm}, and consequently log k^0, is calculated may not be precise at large values of $\bar{\nu}$. Uncertainties in the evaluation of Δn_{Ac} are rather large in systems involving EBSA.[4] Uncertainties in the evaluation of Δz_p arise from two sources. (1) It has been assumed that the uptake of unit charge by bovine albumin results in a 0.20×10^{-5} cm.²/v./sec. change in mobility[4,34]; this may not be strictly the case for all the systems studied. (2) At large values of $\bar{\nu}$, especially where competitive interactions may occur, the *p*H may decrease slightly due to the fact that the buffering capacity of the acetate buffer is poor at *p*H 6.5. This might give a slight apparent trend in mobility toward positive values; this effect, which is of a minor order in any case, appears at $\bar{\nu} > 4$.

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

(35) There does not appear to be any other reasonable explanation for the relative inertness of NBSA to Cd^{II}, resulting in a plateau in the binding curve (Fig. 3A) and an abrupt decrease in log k^0 at $\bar{\nu} > 4$ (Fig. 5C1).

(36) See Fig. 1A, ref. 4.

ing one group and those involving Zn^{II} and Cu^{II} the other. The systems Co^{II}–ABSA and Cd^{II}–ABSA, as stated above, show a striking resemblance in their mobility behavior. In the initial stages, their mobility behavior is characteristic of non-competitive interactions; as $\bar{\nu}$ is increased, however, the mobility tends to approach steady values[37]—a fact which gives the appearance of competitive interactions being preceded by non-competitive interactions (see Fig. 4B3). This is clearly improbable and suggests that the uptake of Co^{II} (and presumably Cd^{II}) at the carboxyl sites tends to induce configurational changes, presumably of an unfolding type, thus exposing more carboxyl sites on the ABSA molecule.

The differences between Co^{II}–ABSA and Cd^{II}–ABSA systems, on the one hand, and Zn^{II}–ABSA and Cu^{II}–ABSA systems, on the other, are highlighted by the fact that whereas the uptake of approximately eight copper ions or thirteen zinc ions renders the hitherto heterogeneous acetylated derivative electrophoretically homogeneous, the binding of Cd^{II} and Co^{II} ions has no effect.[4] The causes of these differences are obscure but must, directly or indirectly, be related to the differences in sites involved. The systems Cu^{II}–ABSA and Zn^{II}–ABSA, have one feature in common; the electrophoretic mobility remains practically independent of $\bar{\nu}$ up to $\bar{\nu} \simeq 8$ for Cu^{II} and $\bar{\nu} \simeq 13$ for Zn^{II}, suggesting competitive interaction with imidazole sites (Figs. 4B1 and 4B2). This is followed by a rapid increase in mobility toward positive values, which, concurrently with the protein becoming electrophoretically homogeneous, may be attributed to configurational changes, presumably of a folding type, induced by the uptake of approximately 8 Cu^{II} or 13 Zn^{II} ions at the imidazole sites of the acetylated albumin.

Further Evidence That the First Two Cupric Ions Are Not Bound at the Sulfhydryl Site of Bovine Albumin

The system Cu^{II}–NBSA is unique among the systems studied in that it shows a minimum in electrophoretic mobility at $\bar{\nu} \simeq 2$. As discussed above, this minimum cannot be attributed to an interaction with the sulfhydryl site unless configurational changes are also involved. A further electrophoretic investigation of this system has been made by H. Lal. Whereas these studies are still in progress, the data presented below are interesting inasmuch as they give definite evidence that the first two cupric ions are not bound at the sulfhydryl site of albumin.

The bovine albumin used in these studies was an Armour product, Lot No. 20631. It did not give any 375 mμ absorption at $\bar{\nu}_{Cu} = 2.0$; at $\bar{\nu}_{Cu}$ 10, however, the 375 mμ peak ($\epsilon \simeq 140$) was observed. In the presence of Hg^{II} equivalent to the 0.7 sulfhydryl group of bovine albumin, however, the 375 mμ peak characteristic of Cu^{II}–NBSA systems was not observed. These results are in general agreement with the findings of Klotz and co-workers[22,23] and show that Hg^{II} has displaced Cu^{II} from the sulfhydryl site. If, therefore, the sulfhydryl site of bovine albumin be blocked by the addition of an equivalent amount of Hg^{II}, we have a system which will present sites other than sulfhydryl for interaction with Cu.[II]

The relevant mobility data for Cu^{II}–NBSA at the position of the minimum mobility (*i.e.*, $\bar{\nu}_{Cu} = 2.0$), together with those for Hg^{II}–NBSA and Hg^{II}–NBSA-Cu^{II}, are presented in Table V. It may be noticed that the uptake of two cupric ions by NBSA results in a mobility increment of -0.23×10^{-5} cm.²/v./sec. as against a value of -0.31×10^{-5} cm.²/v./sec. obtained with the protein sample studied earlier.[4,38]

As against Cu^{II}–NBSA, the mobility of bovine albumin remains unaffected by the addition of 0.7 mole of Hg^{II} per protein molecule ($r_{Hg} = 0.7$) and increases slightly toward *positive* values at $r_{Hg} = 2.0$. It may be assumed from the strong affinity of Hg^{II} for the acetate ion[20] that Hg^{II}, like Cu^{II}, would be present largely as the $HgAc^+$ ion and bound as such to the protein. The relative absence of any change in the electrophoretic mobility of NBSA consequent on the uptake of Hg^{II} is entirely consistent with the findings of Hughes[39] who has shown that Hg^{II} reacts exclusively with the sulfhydryl group of serum albumin before interaction can occur with any other site.

An examination of Table V reveals that the mobility of Cu^{II}–NBSA at $\bar{\nu}_{Cu} = 2.0$ remains unaffected by the addition of 0.7 mole of Hg^{II}; a result which is indeed to be expected in view of the fact that NBSA suffers no change in mobility by the uptake of 0.7 Hg^{II}. Essentially the same result is obtained at $r_{Hg} = 2.0$. Thus, the mobility of Hg^{II}–NBSA at $r_{Hg} = 2.0$ is -2.97×10^{-5} cm.²/v./sec., whereas it has a value of -3.18×10^{-5} cm.²/v./sec. at $r_{Hg} = 2.0$, $\bar{\nu}_{Cu} = 2.0$. It may therefore be concluded that the addition of two Cu^{II} ions to bovine albumin alone or to bovine albumin containing 0.7 or 2.0 Hg^{II} ions results in a mobility increment of the same order, namely, $-0.22 \pm 0.02 \times 10^{-5}$ cm.²/v./sec. It follows that the uptake of the first two cupric ions by bovine albumin

TABLE V

ELECTROPHORETIC MOBILITY OF METAL–NBSA SYSTEMS AT 0°

(Acetate buffer, pH 6.50, ionic strength 0.20)

r_{Hg}	$\bar{\nu}_{Cu}$	Mobility × 10^5, cm.²/v./sec.
0	0	−3.07
		(−3.12)[a]
0	2.0	−3.30
		(−3.43)[a]
0.7	0	−3.07
0.7	2.0	−3.31
2.0	0	−2.97
2.0	2.0	−3.18

[a] Ref. 4.

(37) This trend toward steady values cannot be explained on the basis of the possible sources of errors referred to in ref. 33.

(38) It is not improbable that bovine albumin may differ somewhat from sample to sample.

(39) W. L. Hughes, in "The Proteins," Vol. II, Part B, ed. H. Neurath and K. Bailey, Academic Press, Inc., New York, N. Y., 1954, p. 663.

must occur at sites other than sulfhydryl and that Cu^{II} and Hg^{II} do not compete, in the early stages of the interaction process at least, for interaction with the same sites on the albumin molecule.

Acknowledgments.—The authors are grateful to Professor J. T. Edsall, Dr. F. R. N. Gurd and Dr. A. B. Biswas for helpful discussions.

POONA 8, INDIA

[CONTRIBUTION FROM THE SCHOOL OF CHEMISTRY OF THE UNIVERSITY OF MINNESOTA]

Reactivity of Sulfhydryl and Disulfide in Proteins. II. Reactive Disulfide as Related to Viscosity and Optical Rotation in Denatured Bovine Serum Albumin

BY I. M. KOLTHOFF, ADA ANASTASI[1] AND B. H. TAN

RECEIVED DECEMBER 23, 1957

Simple methods are described for the amperometric titration with mercuric chloride of disulfide in native and denatured bovine serum albumin (BSA) in the presence of sulfite at a rotated mercury pool electrode. The mercury reacts with the reduced protein in a mole ratio of 1 $HgCl_2$ to 2SH. The total number of disulfide bonds per mole of BSA is 17. Procedures are given for the determination of the equilibrium concentration of disulfide groups in BSA in its reaction with sulfite at *p*H 6. No reactive disulfide is found in the native protein under the specified experimental conditions; in 4 *M* guanidine hydrochloride (GHCl) solution, the maximum value of the number of reacted disulfide groups is 11. The order of magnitude of the equilibrium constant of the reaction has been determined. Breaking these 11 bonds gives rise to an increase of the reduced viscosity from 0.19 to 0.48; rupture of the last 6 disulfide groups has no further effect on the viscosity. In 4 *M* GHCl $[\alpha]_D$ is $-101°$ and this value is not affected by reducing the disulfide groups with sulfite or 2-mercaptoethanol. This observation is in striking contrast to that in 0.2 *M* sodium decyl sulfate as denaturing agent, where rupture of the disulfide bonds gives rise to a large decrease of levorotation. The relation between experimental results and structural characteristics of native and denatured BSA is discussed.

In a recent paper, Markus and Karush[2] related the reactivity of disulfide groups to changes in the specific rotation and reduced viscosity in human serum albumin and bovine γ-globulin, both in the native and denatured state. They used sodium decyl sulfate as denaturing agent and β-mercaptoethylamine as reducing agent.

In the present paper we report on the reactivity of disulfide, with sulfite and with 2-mercaptoethanol (ESH), in bovine serum albumin (BSA), both native and denatured with guanidine hydrochloride (GHCl).

The amount of "reactive" disulfide determined by the sulfite method greatly depends upon experimental conditions, since the reaction with sulfite is an equilibrium reaction.

Denoting the protein disulfide by $P\!\left(\begin{matrix}S\\|\\S\end{matrix}\right)_n$, we can write

$$P\!\left(\begin{matrix}S\\|\\S\end{matrix}\right)_n + mSO_3^{=} \rightleftharpoons P\!\left(\begin{matrix}S^-\\|\\SSO_3^-\end{matrix}\right)_m\!\left(\begin{matrix}-S\\|\\-S\end{matrix}\right)_{n-m} \quad (1)$$

For low molecular weight disulfides (cystine) we had developed in this Laboratory a simple and rapid amperometric titration technique, using a rotated platinum electrode as indicator electrode and silver nitrate as reagent.[3] This titration must be carried out in an ammoniacal medium at *p*H 9, and gives the theoretical disulfide content for low molecular weight compounds. It was applied to the determination of disulfide bonds in BSA. In the native state no reactive disulfide was found, but in an ammoniacal buffer, 4 *M* in GHCl and 0.05 *M* in sodium sulfite, all disulfide groups could be titrated. Dr. Deshmukh, in this Laboratory, found a value of 17 to 18 disulfide bonds per mole, in the present paper an average value of 17 was found, while in an earlier paper[4] an average value of 18 was reported. There is some uncertainty in the literature about the exact disulfide content of BSA. A value of 18.6 is inferred from a statement by Haurowitz.[5] The low value of 14 disulfide and the value of 2 sulfhydryl groups per mole of BSA reported by Tristram[6] cannot be correct. The most accurate work on human serum albumin has been carried out by Brand,[7] who reported 18 disulfide groups.

Using the amperometric argentimetric titration method, an extensive study was made of the reactive disulfide in an ammoniacal denaturation medium at *p*H 9 at varying concentrations of BSA, GHCl and sulfite. The reactive disulfide was found to increase with the time elapsed between each addition of successive volumes of silver nitrate and the measurement of the current. The reason is that the equilibrium in eq. 1 is displaced to the right in the presence of the mercaptide binding agent. Therefore the results have no exact meaning and will not be reported.

The titration with silver nitrate of low molecular weight disulfides gives good results when carried out in an ammoniacal buffer at *p*H 9,[8] but cannot be used at lower *p*H's.

For amperometric titrations of the sulfhydryl formed in the reaction with sulfite (eq. 1) at *p*H lower than 9, we first used mercuric chloride as reagent and the dropping mercury electrode as indicator electrode. This method yielded good and

(1) On leave from S. A. Farmitalia, Milano, Italy.

(2) G. Markus and F. Karush, THIS JOURNAL, **79**, 134 (1957).

(3) I. M. Kolthoff and W. Stricks, *ibid.*, **72**, 1952 (1950).

(4) W. Stricks, I. M. Kolthoff and N. Tanaka, *Anal. Chem.*, **26**, 299 (1954).

(5) F. Haurowitz, "Chemistry and Biology of Proteins," Academic Press, Inc., New York, N. Y., 1950, p. 32.

(6) G. R. Tristram, "The Proteins," edited by H. Neurath and K. Bailey, Academic Press, Inc., New York, N. Y., 1953, Vol. 1A, p. 215.

(7) E. Brand, *Ann. N. Y. Acad. Sci.*, **47**, 187 (1946).

(8) I. M. Kolthoff and W. E. Harris, *Ind. Eng. Chem., Anal. Ed.*, **18**, 161 (1946).