Discussion

The data given in the experimental part show that the pyrolysis of 1 : 3 borazine–methanol adduct at 100-120° gives the volatile ammonia–trimethoxyborane and not the suggested CH_3OBNH. Assuming complete dissociation in the gas phase the observed molecular weight of the complex would be 60.46, one-half that for $H_3NB(OCH_3)_3$. The observed value of 59–61 supports this assumption. The isolation of the trimer $B_3(OCH_3)_3N_3H_3$ by vacuum sublimation at 55° makes it appear improbable that there was present any stable monomer CH_3OBNH which was not detected by reason of its being retained by the solids in the bomb tube. The existence of any such monomer stable with regard to polymerization at room temperature appears to be highly doubtful.

Pyrolysis of the borazine–methanol adduct evidently proceeds by at least two paths, one of which probably is represented by eq. 1 and another of which may well be an intermolecular polymerization of the borazine–methanol adduct to liberate H_2 and some methanol and to form a polymer $B_xN_xH_y(OCH_3)_y$. Of course, intramolecular reaction to give CH_3OH is not precluded and the data are obscured by the fact that the trimethoxyborazine produced during the pyrolysis is itself changed into a non-volatile residue, methanol and a little ammonia–trimethoxyborane when subjected alone to similar pyrolysis.

Methanol and Pyrex react to give methyl borate.[12] However, a blank, as described in the Experimental part, indicated that no significant amount of methyl borate was produced in this fashion under the pyrolysis conditions.

The borazine–methanol adduct was practically unattacked by excess liquid methanol, whereas borazine underwent partial ethanolysis to $B(OC_2H_5)_3$ and NH_3 at temperatures as low as −30°. An attempt to displace the methanol from the borazine–methanol adduct by ethanol at 25° resulted in partial ethanolysis of the adduct. At 0° ethanol was observed to have no effect on the borazine–methanol adduct.

These observations might tempt one to speculate as to the relative reactivity of methanol and ethanol toward borazine–alcohol adducts. However, the adducts, especially $B_3N_3H_6\cdot3CH_3OH$, appear to be only slightly soluble in their corresponding alcohols. Therefore the observed differences in behavior may well be due to differences in solubility, and the apparent stability of $B_3N_3H_6\cdot3CH_3OH$ may arise from the slowness of a heterogeneous phase reaction occurring at the surface of the adduct.

Some preliminary experiments support this suggestion. A sample of $B_3N_3H_6\cdot3CH_3OH$ was partially dissolved in benzene. When methanol was added to the resulting solid–solution mixture, the remaining solid rapidly dissolved with evolution of a gas from the solid surface, although such evolution did not occur when the adduct initially dissolved in the benzene. This suggests that further studies of the reaction of borazines with alcohols should be carried out in neutral solvent. Also, to prepare $B_3N_3H_6\cdot3C_2H_5OH$ and other kinds of related adducts one should consider the reaction of the desired borazine and alcohol in solvents in which the adduct formed will be relatively insoluble. If no such solvent can be found, then the addition of dilute solutions of the alcohol to solutions of the borazine may be considered.

The authors wish to express their appreciation to Dr. C. J. Barr and Mr. W. J. Meikle of the Olin–Mathieson Corporation for their generosity in making an infrared spectrograph available for use; and to Mr. R. Buxton for the determination and interpretation of the infrared spectrum of ammonia–trimethoxyborane.

We are appreciative of the generous assistance of the Research Corporation which supported this work by a Frederick G. Cottrell Grant.

ST. LOUIS, MISSOURI

(12) R. F. Porter, *J. Phys. Chem.*, **61**, 1260 (1957).

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

Hydrogen Ion Equilibria and the Interaction of Cu^{II} and Co^{II} with Bovine Serum Albumin

BY HIRA LAL*

RECEIVED JULY 31, 1958

The interaction of Cu^{II} and Co^{II} with bovine serum albumin has been followed by titration and distribution dialysis studies. It has been shown that Co^{II} is bound to bovine albumin through its imidazole sites. The existence of compound sites through which the first two cupric ions are bound to bovine albumin has been confirmed; a log k^0 value of 6.8 ± 0.3 for the intrinsic affinity of these sites for Cu^{II} has been obtained. It has been shown that the subsequent binding of cupric copper with bovine albumin occurs 1:1 with the imidazole sites. Evidence has been presented that cupric copper is partially hydrolyzed and bound as such to the protein in the neutral *p*H region.

Previous studies in acetate buffer medium of *p*H 6.5 have indicated that bovine albumin contains a class of two "compound" sites through which the first two cupric ions are bound to the protein molecule.[1] The nature of these sites, adduced essentially from electrophoretic studies, is such as to cause a release of two protons per metal ion bound.[1,2] If these sites do exist, a direct evidence for their presence should be obtainable from titration studies. Studies in buffer solutions have also revealed that Co^{II} is bound to the carboxyl

* Indian Institute of Technology, Worli, Bombay 18.

(1) M. S. N. Rao and H. Lal, THIS JOURNAL, **80**, 3226 (1958).

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

sites of bovine albumin.[1-3] These results are indeed in conflict with the known affinity of Co^{II} for imidazole.[4] It was therefore felt that a study of the interaction of these metallic ions with bovine albumin in an unbuffered medium should be of considerable interest. It may be remarked that titration studies should help in defining the broad nature of these interactions in much the same manner as electrophoretic studies do in buffer solutions. If, in addition, distribution dialysis data were also available, a quantitative interpretation of the data in terms of intrinsic constants should be possible. These studies which in their general features correspond to those of Gurd and Goodman[5] for the interaction of Zn^{II} with human serum albumin are described in the present paper.

Experimental

*p*H measurements were made with a glass electrode in conjunction with a precision potentiometer and an electrometer null-detector.[4] The over-all accuracy of measurements was expected to be within 0.01 *p*H unit. Unless otherwise stated, all measurements were made in an air-conditioned room at 22.5 ± 0.5°.

Bovine albumin was an Armour product, Lot No. 23883. A stock solution of the protein was de-ionized and estimated for its protein content by drying to constant weight at 105–110°. Stock solutions of copper nitrate[6] and cobalt nitrate were standardized by appropriate methods. Analytical grade reagents were used throughout. All measurements were made in a nitrate medium of 0.15 ionic strength.

The experimental procedure consisted in titrating a known weight of bovine albumin in 20 ml. of solution 0.15 *M* with respect to sodum nitrate, against standard sodium hydroxide (or nitric acid) containing excess sodium nitrate to give 0.15 ionic strength. The protein concentrations were 0.8–1.1% and 1.8–2.2% for studies involving Cu^{II} and Co^{II}, respectively. The molar ratio r of total metal to total protein was varied from experiment to experiment.

For distribution dialysis studies, 10 ml. of suitable mixture of protein, metal ion and sodium hydroxide in 0.15 *M* sodium nitrate was dialyzed against 10 ml. of 0.15 *M* sodium nitrate. With continuous shaking and polystyrene beads to assist stirring of protein solution, equilibrium may be expected to reach in 24 hr. A period of 60 hr. was however allowed to ensure complete equilibration. After equilibrium dialysis, the protein solution was examined for its *p*H, metal ion and protein concentrations. The protein-free solution was estimated for its metal ion concentration alone. Distribution dialysis studies were made in an air-conditioned room maintained at 16.0 ± 1.0°; in view of the fact that the binding of metal ions by bovine albumin is only slightly influenced by temperature,[3,7] the binding data at 22.5° may be expected to be essentially the same as at 16°. *p*H measurements were however made at 22.5°. Cu^{II} and Co^{II} were estimated as described previously.[1,3] The protein concentration was determined from its absorption at 280 mμ.[8]

A molecular weight of 65000 was assumed for bovine albumin.

Results and Discussion

The titration data for bovine albumin are presented in Fig. 1A, the continuous curve being the calculated curve constructed from the dissociation of each class of functional groups as given by[9]

$$\log \frac{n_B}{n_A} = pH - pK^0 + 0.868\,\omega z_p \qquad (1)$$

where n_A and n_B are the number of functional groups of a given class in the acid and basic form, respectively, K^0 the intrinsic dissociation constant, ω the Debye–Hückel function assumed 0.028 at 0.15 ionic strength and z_p the charge on the protein molecule. Following Gurd and Goodman,[5] it has been assumed that 12 nitrate ions are bound to the isoionic protein at $\mu = 0.15$ and the protein charge, z_p, under any given conditions is given by

$$z_p = -12 - 0.6B \qquad (2)$$

where B is the number of equivalents of base added per mole protein. Using pK^0 values as reported by Tanford, Swanson and Shore,[9] the calculated curve fits the experimental data to within ± 0.2 equivalent.

Interaction of Cu^{II} with Bovine Albumin.—The fact that the electrophoretic mobility of Cu^{II}–BSA system in acetate buffer of *p*H 6.5 and ionic strength 0.20 passes through a minimum at $\bar{\nu} \simeq 2$ has been attributed by Rao and Lal[1] to the interaction process involving compound sites in such a manner as to cause a release of two protons per metal ion bound. This conclusion finds further support from titration studies. Thus, at $r_{Cu} = 2.0$, (Fig 1B) somewhat more than four equivalents of alkali

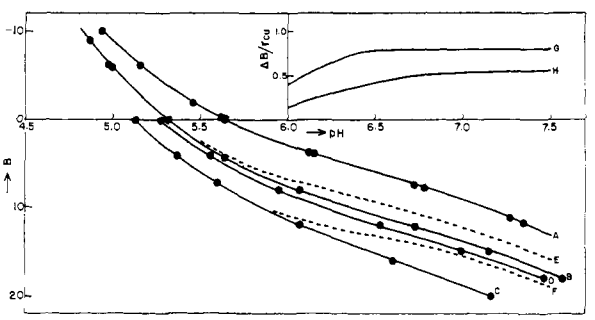

Fig. 1.—Titration of bovine serum albumin in the presence of cupric copper r_{Cu}: (A) 0.0; (B) 2.0; (C) 6.0; (D) 2.0; $r_{Hg} = 0.7$. For E, F, G and H, see text.

are required to titrate the protein to a given *p*H. A further proton release of the same order is obtained in presence of an additional four moles of Cu^{II}, *i.e.*, at $r_{Cu} = 6.0$ (Fig. 1C). The broad nature of the interaction process is therefore obvious: the first two cupric ions are bound to bovine albumin through some special sites so that two protons are released per metal ion bound and, subsequently, a simple 1:1 interaction occurs, presumably through imidazole sites, to cause a release of one proton per metal ion bound.

It has also been shown from electrophoretic studies that the first two cupric ions are bound to bovine albumin through the special type of sites described above and that the 0.7 sulfhydryl group of total albumin is not involved in the initial interaction process.[1] These conclusions are also confirmed by the present studies. Thus, whereas the interaction of the first two cupric ions causes a

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

(4) R. Mathur and H. Lal, *J. Phys. Chem.*, in press.

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

(6) A known excess of nitric acid was added to the stock solution of copper nitrate to prevent precipitation of metal hydroxides on long standing.

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

(8) The extinction at 280 mμ was observed to increase by approximately 2% per cupric ion bound to the protein and must be taken into account for a correct evaluation of protein concentration. The corresponding effect in the system: Co^{II}–BSA was only of a very minor order.

(9) C. Tanford, S. A. Swanson and W. S. Shore. THIS JOURNAL, **77**, 6414 (1955).

release of ~4 protons (Fig. 1B), the presence of an additional 0.7 mole Hg^{II} depresses the titration curve by approximately 0.8 equivalent of alkali only (Fig. 1D). The titration of bovine albumin at $r_{Hg} = 0.7$ leads to much the same result. It may be concluded, therefore, that Cu^{II} and Hg^{II} do not compete with each other for occupying the same sites and, consequently, the interaction of Cu^{II} with the sulfhydryl site can occur only after two cupric ions are bound to the protein molecule.[10,11]

It may be worthwhile to bear in mind that Cu^{II} is also an acid and, as such, may compete with the protein to react with OH^- ions. In the absence of bovine albumin, the metal ion is precipitated as hydroxide in the neighborhood of *p*H 5.5.[12] Whereas no such precipitation is noticeable in the presence of protein, the possibility of a partial hydrolysis of the metal ion in the neutral *p*H region cannot be ruled out. This particular aspect of the problem assumes a special significance if we consider the displacement of Fig. 1B from Fig. 1A. Such a comparison reveals that at $r_{Cu} = 2.0$, a maximum of about five additional equivalents of alkali are required to titrate the protein to a given *p*H in the *p*H range 6–6.5. Clearly, a maximum of only four protons are expected to be released from the protein. As such, there is strong reason to believe that the cupric ion is being hydrolyzed and bound as such to the protein molecule. A similar situation obtains if we consider the titration curve at $r_{Cu} = 6.0$ (Fig 1C) and compare it with Fig. 1B bearing in mind that the additional four cupric ions are bound 1:1 with the imidazole sites.[13] For a quantitative study of this aspect, it is necessary to construct the expected titration curves at r_{Cu}. For $r_{Cu} = 2.0$, it is assumed that the interaction occurs through a class of sites involving two ligand groups, L_1 and L_2, in suitable juxtaposition to form a compound site and that one of the ligand groups, say L_1, is an imidazole group and L_2 is such that its pK^0 is much larger than 7 so that it would contribute one proton per metal ion bound over the entire *p*H range studied. The association of the metal with nitrate ion being insignificant, the species present in solution is largely Cu^{++}, so that the protein charge is given by

$$z_p = -22 + 0.6(2\bar{\nu} - B) \quad (3)$$

where $\bar{\nu}$ is the number of metal ions bound per mole protein. $\bar{\nu}$ may be assumed to be equal to r_{Cu}, an assumption essentially justifiable in the present case. For a calculation of the dissociation of imidazolium ions from eq. 1, n_B equals $16 - \bar{\nu} - n_A$. The resulting titration curve at $r_{Cu} = 2.0$ (Fig. 1E) differs markedly from the experimental curve (Fig. 1B). The difference between the experimental and the calculated curve divided by r_{Cu} (*i.e.*, $\Delta B/r_{Cu}$) is plotted in Fig. 1G as a function of *p*H. The calculated curve at $r_{Cu} = 6.0$ (Fig. 1F), assuming that the additional four cupric ions are bound 1:1 with the imidazole sites,[12] shows a similar trend; $\Delta B/r_{Cu}$ for this system is plotted in Fig. 1H as a function of *p*H. Taking into account the probable error involved in assuming $\bar{\nu} = r$, it may be thus inferred that 0.8 ± 0.3 hydroxyl ions are bound to the cupric ion in the significant *p*H region 6.5–7.5. As practically all the metal present is bound (see Tables I and II), it is reasonable to assume that the partially hydrolyzed metal ion is bound as such to the protein molecule. It appears to us that the state of solution of metal ions in the presence of reactive ligand groups needs to be more closely investigated than has been the case hitherto.

TABLE I

Cu^{II}–BSA

I

B	*p*H	$\bar{\nu}$	*p*(*A*)	log k^0
2.1	5.66	1.5	5.12	6.56
− 4.0	5.15	1.7	4.75	7.07
− 8.0	4.99	1.0	4.92	6.69
−12.0	4.83	1.2	4.70	7.00
−19.5	4.56	1.3	4.39	7.03

TABLE II

11.9	6.05	3.6	4.27	4.00
12.4	6.23	5.5	4.48	4.40
17.0	6.48	5.4	4.19	4.12
16.0	6.33	7.4	4.17	4.28
21.4	6.62	8.8	4.09	4.08

TABLE III

Co^{II}–BSA

4.2	6.00	0.5	3.42	2.52
11.6	6.61	1.3	3.57	2.47
8.0	6.26	1.7	3.14	2.53
13.3	6.65	2.0	3.20	2.29
12.9	6.47	5.1	2.07	1.91

It may be concluded with reasonable certainty that the first two cupric ions are bound to bovine albumin through compound sites. The nature of the ligand groups forming this class of sites is still largely speculative. There is reason to believe, however, that one of the ligand groups, L_1, is an imidazole group. Comparing Fig. 1B with Fig. 1A, we find that, at $r_{Cu} = 2.0$ approximately five protons are released at *p*H 6–6.5 but that, as the *p*H is increased, this number decreases to approximately four at *p*H 7.5. The extent of this decrease and the *p*H region in which it occurs is consistent with one of the ligand groups being an imidazole

(10) The following experiments in acetate buffer solutions (*p*H 6.50, ionic strength 0.20) are of particular interest, inasmuch as they throw some light on the sequence of events as Cu^{II} concentration is increased. Ten ml. of 1% bovine albumin containing $r_{Cu} = 10$ was dialyzed at 0° against 500 ml. of buffer solution, fresh buffer being used every day, over a period of several days. After ten days, the protein solution was analyzed and found to contain 2.5 Cu^{II} ions per protein molecule. A further dialysis for five days did not reduce this metal:protein ratio to any significant extent. In a corresponding experiment in which an additional 0.7 mole of Hg^{II} was also present per protein molecule, it was found that 2.0 Cu^{II} ions were firmly bound to bovine albumin. It would appear, therefore, that the interaction of Cu^{II} with the 0.7 sulfhydryl group of total albumin occurs immediately after two copper ions are bound to the protein molecule and precedes the 1:1 binding of Cu^{II} by the imidazole sites. These results are in accord with, or at least do not contradict, the hitherto known facts on the reactivity of the sulfhydryl site of bovine albumin with Cu^{II}.[11] For the construction of expected titration curve at $r_{Cu} = 6.0$ (Fig. 1F), the participation of the sulfhydryl site has been taken into account. It has, however, been ignored in the evaluation of intrinsic constants listed in Table II; any possible error thus introduced would be insignificant.

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

(12) H. T. S. Britton, "Hydrogen Ions," Vol. II, Chapman Hall Ltd., London, p. 57.

(13) See, however, ref. 10.

group. The ligand group, L_2, may then be assumed to be a group such as an ϵ-amino, phenolic OH or peptide >NH group which would contribute one proton per metal ion bound in the significant pH region.

If the above picture of the interaction process is correct, it is indeed reasonable to treat the two compound sites and the fourteen simple imidazole groups as two independent set of sites.[14] For an evaluation of the distribution dialysis data in terms of the intrinsic affinity constant k^0 we then have[1,5]

$$\log k^0 = \log \bar{\nu} - \log n_A - p\mathrm{H} + 0.868\omega z_p + p(A) + pK^0 \quad (4)$$

where n_A is the number of functional groups of a given class in the acid form, A the concentration of free metal ion at equilibrium and pK^0 refers to the imidazole sites and is assumed 6.90.[9] The distribution dialysis data in the lower binding region ($\bar{\nu} < 2$) is presented in Table I. For an evaluation of intrinsic constant, n_A was put as being $2 - \bar{\nu}$. A value of log $k^0 = 6.8 \pm 0.3$ is thus obtained.[15] It may be remarked that in a previous study of the interaction of Cu^{II} with bovine albumin in acetate buffer of pH 6.5, a log k^0 value of 4.86 was obtained.[1] In this evaluation, however, the entire binding data were interpreted on the basis of a 1:1 interaction, *i.e.*, n_A was calculated from eq. 1 by putting $n_B = 16 - \bar{\nu} - n_A$. A re-evaluation on the basis of the two compound sites as being an independent set of sites gives log $k^0 \simeq 6.0$.[15,16]

It may be stressed that the log k^0 value of 6.8 ± 0.3 for the interaction of Cu^{II} with the compound sites of the type described above is only a preliminary value and is dependent on one of the ligand groups forming the compound site being an imidazole group. The avidity with which Cu^{II} reacts with this class of sites in preference to the sulfhydryl group of total albumin may not be inconsistent with this value. These intrinsic constants, however, appear to be inconsistent with the postulation of Rao and Lal[1] that the compound sites involve the imidazole group and neighboring peptide > NH group.[17] There is indeed no reason why only two peptide > NH groups may be so favorably situated. It is not improbable that two independent functional groups—one of them an imidazole group—in suitably rigid configuration to form a chelate through Cu^{II} but not through Co^{II} may form this class of sites. It may be of interest to note that the titration of Cu^{II}–BSA solutions in the presence of approximately 8 M urea does not indicate any basic change in the nature of the interaction. Apparently, any unfolding of the protein molecule caused by urea does not affect the region in which these compound sites may be situated.

The distribution dialysis data for the interaction of Cu^{II} with bovine albumin in the higher binding region ($\bar{\nu}$ = 4–9) are presented in Table II. As the interaction process involves a 1:1 interaction with imidazole sites except for the first two metal ions bound, the intrinsic constant can be evaluated from eq. 4 by inserting log $(\bar{\nu} - 2)$ in place of log $\bar{\nu}$ and evaluating n_A appropriately from eq. 1. The intrinsic constant thus obtained has a value of log $k^0 = 4.2 \pm 0.2$ and agrees well with the first association constant for the interaction of imidazole[18] or 4-methylimidazole[19] with cupric copper. It may therefore be concluded, in conformity with the conclusions arrived at by Rao and Lal,[1] that once the two compound sites are occupied the interaction of Cu^{II} occurs 1:1 with the imidazole sites of bovine albumin.[12]

Interaction of Co^{II} with Bovine Albumin.—The data for the titration of bovine albumin in the presence of Co^{II} are presented in Fig. 2. It is

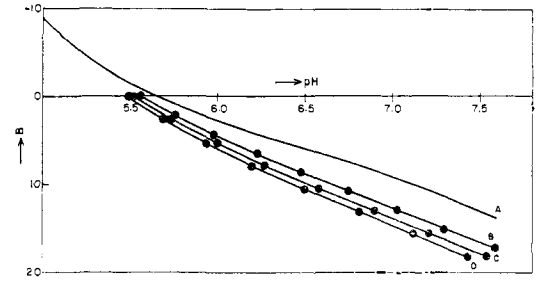

Fig. 2.—Titration of bovine serum albumin in the presence of cobaltous cobalt. r_{Co}: (A) 0.0; (B) 4.0; (C) 8.0; (D) 12.0.

apparent that the initial interaction process in the pH region around 6.5 is competitive and involves a 1:1 interaction with the imidazole sites. Compound sites of the type encountered in Cu^{II}–BSA systems do not appear to be involved in interaction with Co^{II}. The intrinsic constants calculated from the distribution dialysis data support the above conclusions (Table III). Thus, the log k^0 value of 2.5 agrees well with the first association constant for the interaction of imidazole with Co^{II}.[4] With increasing concentration of Co^{II}, log k^0 tends to decrease, presumably, due to non-competitive interaction with the carboxyl sites. This is to some extent re-

(14) The titration data presented in this paper and the electrophoretic data in buffer solutions lead one to conclude that a competition between the compound sites and simple imidazole sites of bovine albumin for interaction with Cu^{II} favors the former class of sites to the exclusion of the latter. As such, an unambiguous evaluation of intrinsic constant for the association of the compound sites with Cu^{II} entails a treatment based on these two sites being an independent set of sites.

(15) Reliable values of the intrinsic constant may be expected to be obtained from the binding data in the pH region 4.5–5, in which hydrogen ions are able to compete successfully with Cu^{II} to occupy this class of sites. At higher pH values, the metal ion being practically completely bound to the protein, a precise evaluation of log k^0 depends largely on a precise evaluation of free metal ion concentration in the extreme region of applicability of the colorimetric method of estimation used. Furthermore, under these conditions of pH, it is doubtful if n_A would equal $2 - \nu$, as assumed.

(16) As the interaction of Zn^{II} and Cd^{II} with bovine albumin involves a class of two compound sites described by Rao and Lal,[1] it is not unlikely that the intrinsic constants listed in Table II of ref. 1 may also have to be upgraded to log $k^0 \sim 5$. This value of log k^0 may not be inconsistent with the assumption of an imidazole group and a carboxyl group forming this class of compound sites.

(17) Dr. I. M. Klotz has pointed out (private communication) that the evidence of intrinsic constants alone is not enough justification for or against any given class of sites unless corroborative evidence is forthcoming. Inasmuch as the evaluated intrinsic constants depend largely on the postulated nature of the interaction process, we are in agreement with this view. On the other hand, where electrophoretic and titration data may indeed point to a reasonably accurate picture of the interaction process, the evaluated intrinsic constants may be regarded as reasonably accurate.

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

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

flected in the titration curves (Fig. 2). Thus, whereas at $r_{Co} = 4$, the interaction process appears to be competitive, the proton release occasioned by the addition of further Co^{II} is of a small order (compare Figs. 1C and D with Fig. 1B). Clearly, a very large proportion of the added metal ion must be free. Under the circumstances, the extent of the uptake of hydroxyl ion by Co^{II} cannot be evaluated for, unlike Cu^{II}–BSA, we can no longer assume that practically all the added metal ion is bound to the protein. As in the absence of bovine albumin the precipitation of cobalt hydroxide occurs at *p*H $\sim$ 8,[13,20,21] it is unlikely that hydroxyl ion would be bound by Co^{II} in the presence of the protein in the *p*H range investigated. Furthermore, the affinity of bovine albumin for Co^{II} being relatively weak, the data in the higher binding region are likely to be unreliable; as such, no definite conclusions can be drawn for the interaction process in the higher binding region. It is not unlikely, however, that whereas the first few Co^{II} ions are bound 1:1 with the imidazole sites of bovine albumin in the neutral *p*H region, non-competitive interaction with the free carboxyl sites may play an increasingly predominant role in the higher binding region.

Buffered and Unbuffered Systems.—It may be appropriate at this stage to discuss and compare the results obtained in the unbuffered nitrate medium with those obtained by Rao and Lal[1] in the acetate buffer medium of *p*H 6.5. The two studies, as discussed above, yield essentially the same picture of the interaction process for the system: Cu^{II}–BSA. The interaction of Co^{II} with bovine albumin in the acetate buffer has, however, been attributed to the free carboxyl sites of the protein molecule[1-3]; a similar postulation of the interaction process has been made by Fiess.[22] The results presented above show, however, that Co^{II} is bound 1:1 with the imidazole sites of bovine albumin in the unbuffered nitrate medium. An explanation of this anomaly may possibly lie in the nature of the ionic species involved in the buffered acetate medium. It may be noted that the nature of the interaction process, competitive or otherwise, in the buffered medium has been derived essentially from electrophoretic studies. If the only metallic species involved were the $CoAc^+$ ions, as assumed, the electrophoretic data cannot be explained except on the basis of a non-competitive interaction involving carboxyl sites. If, however, a large proportion of the bound metal were to consist of the species Co^{++}, the same electrophoretic data would not be inconsistent with a competitive interaction through imidazole sites. It would thus appear that the relative affinities of Co^{++} and $CoAc^+$ for bovine albumin may not be the same and, consequently, that there may be a disproportionation between the metallic species in the protein phase and the electrolyte medium. If this disproportioning were such as to account for the mobility data on the basis of a 1:1 interaction with imidazole sites, a re-evaluation of the binding data gives $\log k^0 = 2.3 \pm 0.2$, in essential agreement with the intrinsic constants listed in Table III.

There is indeed some evidence that the association of metallic ions with buffer anions decreases their over-all affinity for bovine albumin. A comparison of the relevant binding data under comparable conditions leads one to suspect that, in the lower binding region at least, the binding of cobalt is somewhat weaker in the buffered medium than in the unbuffered nitrate medium. The data in the higher binding region are probably not sufficiently precise to warrant such a comparison. A study of the system: Cu^{II}–NBSA throws some further light on this subject. It may be noted that the binding data listed in Table I at *p*H values around 4.8 differ markedly from those reported by Klotz and Fiess[7] in an acetate buffer of *p*H 4.8. Thus, at *p*H 4.83 and $\bar{\nu} = 1.2$ we have the equilibrium concentration of free copper as 2.0×10^{-5} *M;* this is markedly lower than the corresponding value of 1.0×10^{-4} *M* obtained from the data of Klotz and Fiess in the acetate buffer of *p*H 4.80. Again, the free copper concentration at equilibrium at $\bar{\nu} = 1.0$ in an acetate buffer medium of *p*H 4.93 was found from an independent experiment to be 7.2×10^{-5} *M;* this is markedly larger than the value of 1.2×10^{-5} *M* in the nitrate medium of *p*H 4.99 (Table I). These differences are indeed much larger than could possibly be attributed to electrostatic factors alone.

A knowledge of the nature and extent of various possible ionic species bound to the protein molecule is indeed essential for an interpretation of electrophoretic data. The electrophoretic measurements are thus subject to errors of interpretation within the limitations imposed by our present knowledge of the state of metallic ions in solution. These uncertainties are minimized in titration studies wherein appreciable errors in the assumed state of metallic species may not be expected to influence the titration data to any significant extent. Thus, the nature of the interaction process as revealed by titration studies is likely to be more reliable than that adduced from electrophoretic measurements. This is not to say that electrophoresis cannot, within certain limitations, be used effectively for the study of metal protein interactions; its usefulness as a powerful tool for these studies has indeed been demonstrated already. It is recommended that, wherever possible, both electrophoresis and titration methods be used as complimentary techniques for the elucidation of metal–protein interactions.

POONA 8, INDIA

(20) K. H. Gayer and A. B. Garrett, THIS JOURNAL, **72**, 3921 (1950).
(21) K. H. Gayer and L. Wootner, *ibid.*, **74**, 1436 (1952).
(22) H. A. Fiess, *ibid.*, **74**, 3539 (1952).