[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF NORTHWESTERN UNIVERSITY]

Mediation by Metals of the Binding of Small Molecules by Proteins: Effect of Hydrolytic Equilibria of the Metal

BY THOMAS R. HUGHES AND IRVING M. KLOTZ

RECEIVED DECEMBER 5, 1955

The extent of formation of the ternary complex protein–Zn–small molecule (I) has been measured by the equilibrium dialysis technique over the *p*H range 6–8. These data, together with measurements of the extent of binding of zinc ion, M, and of small molecule, A, alone, permit the computation of k_{MA}, the intrinsic binding constant for attachment of A to M on the protein, as a function of *p*H. There is no change in k_{MA} from *p*H 6 to 7, but at *p*H 8.29, the constant drops sharply because of interference of hydroxyl ions with the ternary complex I. Photometric titrations show that similar competitive effects of OH^- ions are obtained with ternary complexes having Ni^{++} or Cd^{++} in place of Zn^{++}. These results, together with earlier studies of corresponding complexes with Hg^{++} and Cu^{++}, indicate that hydroxylation plays an important role in determining whether a protein-bound metal ion can interact further with a small molecule.

Introduction

It has been shown previously[1] that small organic molecules not ordinarily bound to proteins form strong complexes if suitable metal ions are added. A variety of experiments have demonstrated that the metal acts as a bridge between protein and small molecule, so that a ternary complex Protein–Metal–Dye (I) is produced. Effective mediators include Hg^{++}, Cu^{++}, Ag^+, Ni^{++}, Zn^{++}, Co^{++} and Mn^{++}.

A comparison of mediating effectiveness of these ions on a qualitative basis shows a general correlation with their chelate stability constants, in agreement with expectations for a metal-bridge complex. On a quantitative basis, however, the formation constants for the ternary complexes with mercury or copper are not nearly as large as one would expect. These discrepancies could be attributed to the strong affinity of these metal ions for hydroxyl ion at the *p*H's (5–6) used in previous studies, the metal–hydroxo complexes being less capable of combining with the small organic molecule.

At the *p*H's previously used (5 to 6) only Hg^{++} or Cu^{++} (of the metals examined) could be in hydroxo complexes. One would expect this complication to disappear if more acid solutions were studied. However, a test of this prediction cannot be made conveniently because the organic molecule below *p*H 5 takes up protons which block coördination positions for the metal ion, and hence interpretation of the analyses becomes very unwieldy. On the other hand, if the anomalous behavior of mercury and copper is really due to competing hydroxo complexes, one might expect similar behavior to be induced in the ternary complex of zinc above *p*H 7, since in more basic solutions hydrolytic equilibria for this metal ion also become significant.[2,3] Detailed quantitative measurements with zinc have been extended, therefore, to the *p*H range 6 to 8. In addition, qualitative spectroscopic observations have been made with zinc, nickel and cadmium ions, respectively, up to much higher *p*H's (>11). All of these new experiments confirm the importance of the formation of hydroxo complexes in the displacement of small organic molecules from the metal-mediated protein complex.

Experimental

Reagents.—A sample of pure pyridine-2-azo-*p*-dimethylaniline was kindly supplied by Dr. E. V. Brown.[4] Stock solutions of zinc ion were prepared by the addition of nitric acid to a weighed quantity of reagent grade metal, followed by neutralization with sodium hydroxide and dilution to a concentration of about 0.05 *M*. Other metals were used in the form of reagent grade salts: $CuCl_2 \cdot 2H_2O$, $Ni(NO_3)_2 \cdot 6H_2O$, $CdCl_2$. All other inorganic chemicals were also of reagent grade. Crystallized bovine serum albumin, from which the acetylated protein was prepared, was purchased from Armour and Co.

Acetylated bovine serum albumin was prepared according to the procedure of Fraenkel-Conrat, Bean and Lineweaver.[5] Analyses for the ε-amino groups showed that 58.3 ± 1.2% of these groups had been acetylated.

Early in the course of these studies, it was found that the metal content of ordinary distilled water is sufficient to produce a detectable amount of protein–metal–dye complex. All of the water used was redistilled therefore in an all-glass apparatus.

All other chemicals, except Eriochrome Black T, were reagent grade materials. The dye was a sample from the National Aniline Division of Allied Chemical and Dye Corporation.

Absorption Spectra.—The absorption of light was measured with the Beckman spectrophotometer, model DU, at approximately 25°. One-cm. cells were used. Extinction coefficients, ε, were calculated from the equation

$$\epsilon = \frac{1}{cd} \log_{10} (I_0/I)$$

where I_0 is the intensity of the light emerging from the solvent, I the intensity of the light emerging from the solution, c the molar concentration of the solute and d the thickness of the absorption cell in centimeters.

Dialysis Measurements.—The extent of binding of pyridine-2-azo-*p*-dimethylaniline and of zinc by protein was determined by the equilibrium dialysis technique.[1] The dialysis bags were first soaked in water in a beaker on a steam-bath and then in distilled water for several days to remove as much of the metal and sulfur contaminants as possible. Each bag was also soaked for several hours in 0.15 *M* sodium nitrate adjusted to the *p*H of the binding run. For tubes containing protein, each bag was filled with 10 ml. of 0.5% protein solution. In runs with zinc, metal ion was placed inside the bag with the protein. Ten ml. of dye solution of a known concentration was used outside the bag. The tubes were shaken in an ice-bath at 0° for approximately 24 hours, and the solutions external to the bag analyzed for ligand concentration. The extent of binding

(1) I. M. Klotz and W.-C. Loh Ming, THIS JOURNAL, **76**, 805 (1954).

(2) J. Bjerrum, "Metal Ammine Formation in Aqueous Solutions," P. Haase and Son, Copenhagen, 1941, p. 75.

(3) S. Chaberek, Jr., R. C. Courtney and A. E. Martell, THIS JOURNAL, **74**, 5057 (1952).

(4) R. W. Faessinger and E. V. Brown, THIS JOURNAL, **73**, 4606 (1951).

(5) H. Fraenkel-Conrat, R. S. Bean and H. Lineweaver, *J. Biol. Chem.*, **177**, 385 (1945); see also "Amino Acids and Proteins," edited by D. M. Greenberg, Charles C. Thomas, Springfield, Ill., 1951, p. 547.

by the bag itself as a function of unbound dye concentration was measured by means of a set of tubes in which protein was omitted from inside the bag and replaced by 10 ml. of solvent.

In tubes containing zinc, optical densities of the external solution were measured at 470 and 538 mμ (the absorption maxima of free dye and of dye–zinc complex, respectively). From these data the concentrations of free dye and of zinc–dye complex were computed. The distribution so obtained refers, however, to 25°; the fraction of dye which would be free at 0°, the temperature of the dialyses, was calculated from the temperature dependence of the equilibrium constant for the formation of the 1:1 zinc complex.[1,18] To the number of moles of free dye and zinc–dye found in the solution was added the moles of dye bound by the bag. In the case of the protein-containing tubes, this sum subtracted from the total dye initially put in the tube leads to the amount of dye bound by the protein.

In dialysis studies of the binary dye–protein complex, in the absence of metal, optical densities were still read at two wave lengths because of indications that minute metal ion impurities were introduced by the casing. With two density readings an estimate of the amount of dye–metal complex could be made. Up to 2% of the dye was in the form of a metal complex at *p*H 8.2 and as high as 8% at *p*H 7.1 and 6.1.

Zinc Analyses.—The Versenate titration method, as modified by Diehl, Goetz and Hach,[6] was used for the determination of zinc. Ethylenediaminetetraacetic acid (0.003 *M*) containing $MgCl_2$ was titrated against the zinc solution, with Eriochrome Black T as indicator. The end-point was determined photometrically with a Coleman spectrophotometer, set at 640 mμ. The precision of this method is illustrated by the case of triplicate titration of a standard zinc sample of 3.7×10^{-6} mole in which agreement to within 0.4% was obtained.

Some difficulty was encountered when this procedure was used to determine zinc concentrations in tubes that did not contain protein. Breaks in the titration curve were less sharp. Furthermore more zinc was found by titration than had actually been added so that "negative" bag binding was obtained. Evidently some metal impurities are leached from the bag and interfere with the titration. Fortunately, zinc ion is bound so strongly to acetylated bovine serum albumin that the uncertain bag-binding corrections have only a small effect on the calculation of the amount of metal bound by the protein.

Metal Impurities in Dialysis Bags.—A small portion of Visking Nojax sausage casing was oxidized, ashed and then redissolved in approximately 0.03 *M* HNO_3. When a portion of this solution was tested with *o*-phenanthroline and hydroxylamine hydrochloride, the typical orange color of the ferrous–phenanthroline complex appeared.[7,8] Its intensity corresponded to 2×10^{-8} mole of iron per inch of casing. Another portion of this solution was tested for copper by the colorimetric biquinoline method[9]; this metal was present to the extent of 1.8×10^{-8} mole per inch of casing. If each of these metals were completely leached from the (eight inches of) casing in a bag into the 20 ml. of solution used in a dialysis experiment, they would produce a concentration of 0.5 p.p.m. of each. Such a concentration of iron would not interfere with the Versenate titration for zinc, but the copper present would be above the innocuous limit.[6]

Photometric Titrations.—Qualitative measurements of the effect of *p*H on several ternary complexes were made by the following procedure. Samples of protein, dye and metal ion were mixed in 0.15 *M* sodium nitrate solution, the *p*H was taken and the optical density measured. A few hundredths of a cc. of 0.2 *M* sodium hydroxide was added from a microburet, the *p*H taken again and the new optical density measured. This cycle was repeated until the highest *p*H desired was attained. Corrections in optical density were made for the dilution effect of added base. The optical densities at each of several wave lengths were then plotted as a function of *p*H to show up trends and sharp breaks.

(6) H. Diehl, C. A. Goetz and C. C. Hach, *J. Am. Water Works Assoc.*, **42**, 40 (1950).

(7) F. C. Hummel and H. H. Willard, *Ind. Eng. Chem., Anal. Ed.*, **10**, 13 (1938).

(8) W. B. Fortune and M. G. Mellon, *ibid.*, **10**, 60 (1938).

(9) J. Hoste, *Anal. Chim. Acta*, **4**, 23 (1950).

Results and Discussion

Quantitative Treatment of Ternary Complexes.—In a system containing protein, P, metal ion, M, and organic molecule, A, binding of A by P may occur directly or through a bridge link with M. A variety of ternary complexes may thus be formed, which may be represented as

$$\begin{array}{ll} (\mathrm{A})_u\text{—P—}(\mathrm{M})_{s-t} & 0 \leq u \leq m \\ \quad\;\;| & 0 \leq s \leq n \\ \quad(\mathrm{MA})_t \quad \mathrm{II} & 0 \leq t \leq s \end{array}$$

if *m* is the number of sites capable of binding A directly and *n* is the number capable of binding M. We assume that all sites included in *m* are equivalent, as are all those grouped in *n*. The concentration of this general species, $PM_{s-t}(MA)_tA_u$, in which *s* metal ions are bound to the protein, *t* organic molecules are bound to the protein through the metal ions and *u* organic molecules are bound directly may be related to the concentrations of free (P), (M) and (A) by the steps[10]

$$\text{(a)}\quad \mathrm{P} + \mathrm{M} \overset{k_{100}}{=\!=} \mathrm{PM};\ \cdots\ \mathrm{PM}_{s-1} + \mathrm{M} \overset{k_{s00}}{=\!=} \mathrm{PM}_s \qquad (1)$$

$$(\mathrm{PM}) = k_{100}(\mathrm{P})(\mathrm{M}) \qquad (2)$$

$$(\mathrm{PM})_s = \left(\prod_{s'=1}^{s \leq n} k_{s'00}\right)(\mathrm{P})(\mathrm{M})^s \qquad (3)$$

$$\text{(b)}\quad \mathrm{PM}_s + \mathrm{A} \overset{k_{s10}}{=\!=} \mathrm{PM}_{s-1}(\mathrm{MA});$$

$$\ldots\ \mathrm{PM}_{s-t+1}(\mathrm{MA})_{t-1} + \mathrm{A} \overset{k_{st0}}{=\!=} \mathrm{PM}_{s-t}(\mathrm{MA})_t \qquad (4)$$

$$\Big(\mathrm{PM}_{s-1}(\mathrm{MA})\Big) = k_{s10}(\mathrm{PM}_s)(\mathrm{A}) \qquad (5)$$

$$\Big(\mathrm{PM}_{s-t}(\mathrm{MA}_t)\Big) = \left(\prod_{t'=1}^{t \leq s} k_{st'0}\right)(\mathrm{PM}_s)(\mathrm{A})^t \qquad (6)$$

$$\text{(c)}\quad \mathrm{PM}_{s-t}(\mathrm{MA})_t + \mathrm{A} \overset{k_{st1}}{=\!=} \mathrm{PM}_{s-t}(\mathrm{MA})_t\mathrm{A};\ \ldots$$

$$\mathrm{PM}_{s-t}(\mathrm{MA})_t\mathrm{A}_{u-1} + \mathrm{A} \overset{k_{stu}}{=\!=} \mathrm{PM}_{s-t}(\mathrm{MA})_t\mathrm{A}_u \qquad (7)$$

$$\Big(\mathrm{PM}_{s-t}(\mathrm{MA})_t\,\mathrm{A}\Big) = k_{st1}\Big(\mathrm{PM}_{s-t}(\mathrm{MA})_t\Big)(\mathrm{A}) \qquad (8)$$

$$\Big(\mathrm{PM}_{s-t}(\mathrm{MA})_t\mathrm{A}_u\Big) = \left(\prod_{u'=1}^{u \leq m} k_{stu'}\right)\Big(\mathrm{PM}_{s-t}(\mathrm{MA}_t)\Big)(\mathrm{A})^u \qquad (9)$$

Combinations of equations 3, 6 and 9 yields

$$(\mathrm{PM}_{s-t}(\mathrm{MA})_t(\mathrm{A})_u) = \left(\prod_{s'=1}^{s \leq n}\prod_{t'=1}^{t \leq s}\prod_{u'=1}^{u \leq m} k_{s'00}k_{st'0}k_{stu'}\right)(\mathrm{P})(\mathrm{M})^s(\mathrm{A})^{t+u} \qquad (10)$$

If we assume that there are no interactions among the binding sites of the protein, then we may adopt an intrinsic constant characteristic of the binding of each ligand to a particular type of site and the whole series of equilibrium constants in equation 10 may be interrelated by statistical factors (for details see ref. 10). Equation 10 can then be replaced by

(10) The *k*'s in the equations shown are most conveniently defined rigorously in terms of concentrations of the protein species but activities of the small ions or molecules. In the present discussion, however, no account will be taken of this distinction. For further details see T. R. Hughes, Ph.D. Dissertation, Northwestern University, 1955.

$$(PM_{s-t}(MA)_t(A)_u) = \frac{n!}{s!(n-s)!}[k_M(M)]^s \frac{s!}{t!(s-t)!}[k_{MA}(A)]^t \frac{m!}{u!(m-u)!}[k_A(A)]^u (P) \quad (11)$$

where k_M represents the intrinsic constant for the binding of M to P, k_{MA} for the binding of A to P through M and k_A for the binding of A directly to P.

Experimentally, the equilibrium dialysis experiments give r_A, the average number of A molecules bound per protein molecule (or per unit weight of protein) or r_M, the average number of M ions bound by each protein molecule. In terms of the concentrations of the contributing complexes, r_A may be represented as

$$r_A = \frac{\sum_{s=0}^{n}\sum_{t=0}^{s}\sum_{u=0}^{m}(t+u)(PM_{s-t}(MA)_tA_u)}{\sum_{s=0}^{n}\sum_{t=0}^{s}\sum_{u=0}^{m}(PM_{s-t}(MA)_tA_u)} \quad (12)$$

Substitution of equation 11 into 12 followed by appropriate summations leads to (see ref. 10)

$$r_A = \frac{nk_M(M)k_{MA}(A)}{1 + k_M(M)[1 + k_{MA}(A)]} + \frac{mk_A(A)}{1 + k_A(A)} \quad (13)$$

from which it follows that

$$\lim_{A \to 0}\left[\frac{r_A}{(A)}\right] = k_{MA}\left[\frac{nk_M(M)}{1 + k_M(M)}\right] + mk_A \quad (14)$$

The factor in brackets on the right-hand side of equation 14 is, however, merely $(r_M)_{A=0}$, *i.e.*, the average number of metal ions bound per protein molecule when no organic molecule A is present, as can be verified by reference to an analysis of simple binary binding.[11] Thus we obtain the equation

$$\lim_{A \to 0}\left[\frac{r_A}{(A)}\right] = k_{MA}(r_M)_{A=0} + mk_A \quad (15)$$

It is from this equation that we can evaluate k_{MA}, the intrinsic constant for the binding of organic molecule A to a metallic site on the protein.

It is evident from equation 15 that we need first data for r_A as a function of (A) in the presence of metal as well as dye. Extrapolation of $r_A/(A)$ to zero concentration of organic molecule yields the sum of the two terms on the right-hand side of equation 15. The second term, a measure of the binding of dye by the protein at sites not occupied by metal ion, is evaluated from a separate set of experiments with protein and organic molecule alone. For such binary binding it is known[11] that

$$\lim_{A \to 0}\left[\frac{r_A}{(A)}\right]_{M=0} = mk_A \quad (16)$$

so that a suitable extrapolation for dye binding in the absence of metal yields the desired second term. Finally, $(r_M)_{A=0}$ is obtained from measurements of metal binding by protein in the absence of any organic molecule. With all three groups of data at hand, k_{MA} can be computed.

Binding of Dye to Protein in the Presence of Zinc.—The data for ternary complex formation at at 0° and ionic strength 0.15 are summarized in Fig. 1 for each of three *p*H's, 6.05, 7.02 and 8.29. To allow comparison with previous studies[1] with other proteins, r_A is expressed as the moles of bound dye per 10^5 grams of acetylated bovine serum albumin. The quantity of zinc in each tube was 1.441×10^{-5} mole, 1.455×10^{-5} mole and 1.444×10^{-5} mole at *p*H's 6.05, 7.02 and 8.29, respectively.

Binding of the dye, pyridine-2-azo-*p*-dimethylaniline, by the dialysis bag was determined at *p*H's 6.0, 6.7 and 7.4 (with zinc concentrations corresponding to those in the protein tubes) and these data were used for bag binding corrections at *p*H's 6.05, 7.02 and 8.29, respectively. There is apparently little change in bag binding between *p*H 6.0 to 6.7 but an increase of about 15% occurs when the *p*H is raised to 7.4. It is possible that a further increase would have been observed at *p*H 8.3, but in the absence of protein, precipitation of zinc hydroxide precluded the use of such a high *p*H in the blank tubes. The binding of the azopyridine by the bag is rather weak, corrections of the *p*H 8.29 ternary data amounting to only 10–14% of the total bound dye. Consequently, even if the bag binding at *p*H 8.29 were 15% higher than at 7.4, the amount of dye bound to protein would be only 1–2% less than the values shown in Fig. 1.

As Fig. 1 shows there is a marked increase in extent of ternary complex formation as the *p*H is increased from the 6.05 to 7.02, but a small drop as the *p*H is raised further to 8.29.

Binding of Dye to Protein in Absence of Zinc.—The data for binary complex formation at 0° and ionic strength 0.15 are summarized in Fig. 2. In these runs, bag binding corrections could be measured at *p*H's within about 0.1 unit of those in the protein tubes since the absence of zinc removed difficulties due to precipitation. Bag binding in the absence of zinc tended to be slightly lower than in its presence.

The binding of dye to (10^5 grams of) protein in the absence of zinc (Fig. 2) tends to be considerably below that observed in the presence of the metal ion. Some difficulty was encountered in measurements at the lowest *p*H (6.0) because of the presence of traces of copper and iron from the dialysis casing. These metals are largely hydroxylated at higher *p*H's, but copper in particular retains a considerable ability to interact with the dye at *p*H 6 and hence can mediate the formation of a ternary complex. Thus the points corresponding to the more dilute solutions in Fig. 2 are noticeably too high, for in dilute solutions the ternary binding due to metal impurities makes a greater relative contribution to the total measured binding. In the run at *p*H 6.00, 10^{-4} *M* citrate was added to the solutions and it apparently did reduce the effect of the copper impurities.

Binding of Zinc to Protein in Absence of Dye.—The extent of binding of zinc ion by (10^5 grams of) acetylated bovine albumin at 0° and 0.15 ionic strength as a function of *p*H is summarized in Fig. 3. For every experiment the total zinc concentration was within 1% of the value in the ternary binding runs. Some difficulty was encountered in blank runs attempting to establish the correction for binding of zinc ion by the dialysis bag, for small

(11) I. M. Klotz in "The Proteins," edited by H. Neurath and K. Bailey, Academic Press, Inc., New York, N. Y., 1953, pp. 727–806.

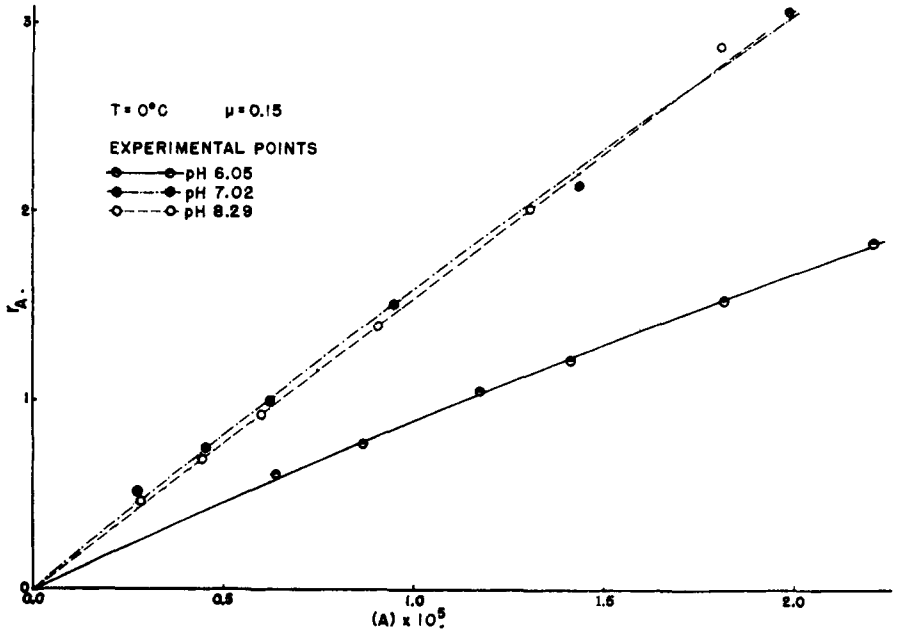

Fig. 1.—Binding of pyridine-2-azo-*p*-dimethylaniline to acetylated bovine serum albumin in presence of zinc. r_A expressed as moles of bound dye per 10^5 grams of protein, (A) is concentration of free dye. Total zinc concentration: *p*H 6.05, 0.721×10^{-3} *M*; *p*H 7.02, 0.728×10^{-3} *M*; *p*H 8.29, 0.722×10^{-3} *M*.

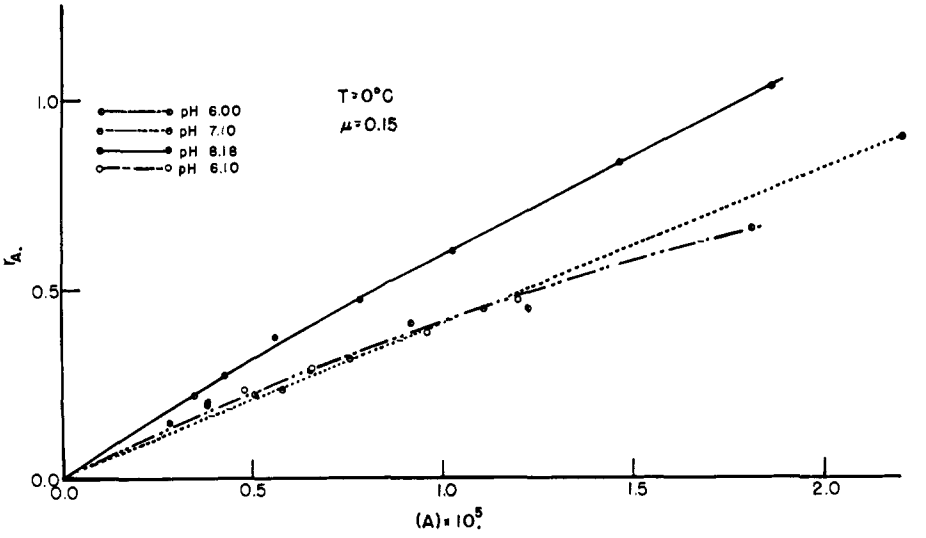

Fig. 2.—Binding of pyridine-2-azo-*p*-dimethylaniline to acetylated bovine serum albumin in absence of zinc.

"negative" binding frequently was observed. These "negative" blanks are probably due to the leaching of copper and iron from the dialysis casing. It is not certain, therefore, that the blank tubes yield valid bag-binding corrections, for the acetylated albumin in the protein tubes would be expected to bind a considerable fraction of the copper and iron impurities in solution. Consequently, two sets of values for r_{Zn} are shown in Fig. 3, in one of which negative bag-binding is used as a correction and in the other of which bag corrections are ignored. The difference between these computations is small (0–2%) at *p*H's 7–8 but somewhat more serious (14%) at *p*H's near 6.

In any event it is obvious that the extent of zinc binding increases appreciably as the *p*H is raised, in general agreement with previously published work[12–16] on bovine and human serum albumin. It is perhaps worth notice also that the number of zinc ions (26) bound at *p*H 8 exceeds the number of imidazole groups (22.7) per 10^5 grams of protein and that there is no indication that zinc binding has reached its maximum at this *p*H.

At *p*H 7, measurements of zinc ion binding by acetylated bovine albumin were also made over a range of metal concentrations and the results (for 10^5 grams protein) are summarized in Fig. 4. A *p*H of 7 was chosen for these measurements because

(12) C. Tanford, THIS JOURNAL, **74**, 211 (1952).
(13) F. R. N. Gurd and D. S. Goodman, *ibid.*, **74**, 670 (1952).
(14) H. A. Saroff and H. J. Mark, *ibid.*, **75**, 1420 (1953).
(15) F. R. N. Gurd, *J. Phys. Chem.*, **58**, 788 (1954).
(16) V. Kacena, *Chem. Listy*, **48**, 7 (1954).

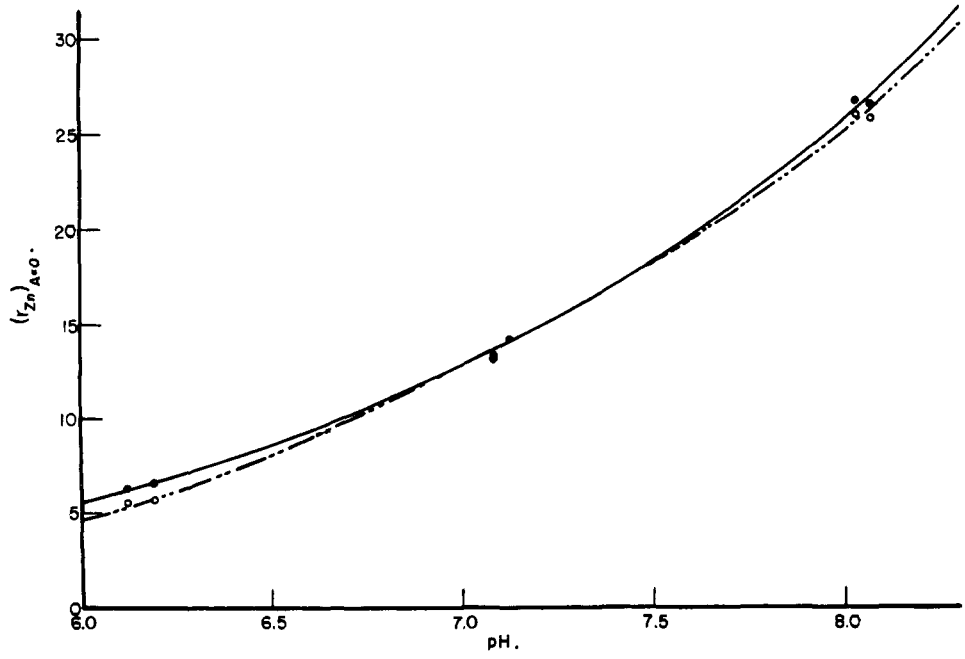

Fig. 3.—Binding of zinc ion to acetylated bovine serum albumin at 0° and 0.15 ionic strength as a function of pH. r_{Zn} expressed as moles bound ion per 10^5 grams of protein: ●, binding by protein corrected for bag binding; O, bag binding corrections taken as zero (see text). Contents of tubes:

pH	6.12	6.19	7.08	7.12	8.03	8.07
Zn, moles × 10^5	1.442	1.448	1.462	1.462	1.454	1.454
Protein grams × 10^2	4.688	4.743	4.425	4.425	4.420	4.420

at pH 6 interference by traces of copper and iron is more serious and at pH 8 hydroxylation of the zinc becomes significant. The absence of buffer led to some difficulty in maintaining a fixed pH, but all the data fall within the range 7.15 ± 0.15. Extrapolation of the function $r_{Zn}/(Zn^{++})$ to zero concentration of zinc ion, making use of an equation analogous to 16 but for protein–zinc complexes, leads to a value of 7.8 (±1.1) × 10^4 for nk_M for acetylated bovine albumin and zinc ion at pH 7.15.

Computation of k_{MA}.—We have thus accumulated all the information necessary for the evaluation, at a series of pH's, of the intrinsic constant for the binding of organic molecule A to a metallic site on the protein. The various terms required in equation 15, plus the computed values of k_{MA}, are assembled in Table I.

TABLE I

COMPUTATION OF INTRINSIC TERNARY ASSOCIATION CONSTANT AT 0°, μ = 0.15[a]

pH	$\lim_{A\to 0}\left[\frac{r_A}{(A)}\right]$ × 10^{-4}	$\lim_{A\to 0}\left[\frac{r_A}{(A)}\right]_{Zn=0}$ × 10^{-4}	$(r_{Zn^{++}})_{A=0}$	k_{MA} × 10^{-3}
6.05	9.6[b,c]	4.9[b]	5.3[b,c]	8.9
7.02	16.8	4.3	12.9	9.7
8.29	15.5	6.9	30.8	2.8

[a] All measurements were in 0.15 M $NaNO_3$. [b] Values of r have been computed for a unit weight of 100,000 g. of protein. [c] The total quantity of zinc ion in each tube was within the interval 1.441 to 1.455 × 10^{-5} mole.

Dependence of k_{MA} on pH.—We may first compare the values of 8.9 × 10^3 and 9.7 × 10^3 at pH's 6.05 and 7.02, respectively. A much greater uncertainty exists in the value at the lower pH for several reasons. The figure of 4.9 for mk_A is probably high because the minute copper and iron impurities in the dialysis casing effect some ternary binding of dye despite the fact that the zinc concentration has been kept at zero. A high value of mk_A would tend to lower k_{MA}. In addition there are uncertainties in $r_{Zn}{}^{++}$ because of bag-binding corrections which are greater, proportionally, when $r_{Zn}{}^{++}$ is smaller. Consequently it seems reasonable to conclude that k_{MA} is substantially constant over the pH range 6 to 7 and that the best value for this intrinsic constant at 0° is 9.7 × 10^3.

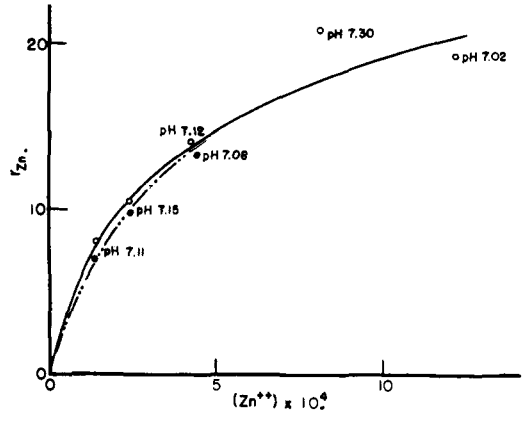

Fig. 4.—Binding of zinc to acetylated bovine serum albumin as a function of free metal ion concentration: 0°, 0.15 ionic strength, pH ≃ 7.1.

Turning to pH 8.29 we find (Table I) a value of 2.8 × 10^3 for the intrinsic constant for the binding of dye to a zinc ion on the protein. This value is

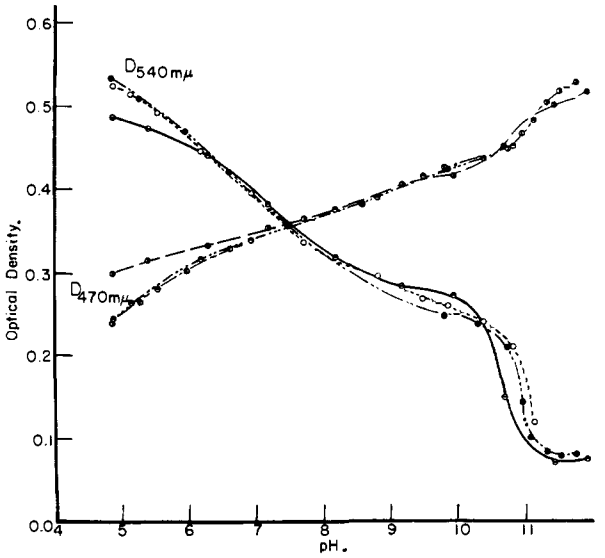

Fig. 5.—Photometric titration of Cu(II), pyridine-2-azo-*p*-methylaniline and acetylated bovine serum albumin; dye concentration 2.38×10^{-5} *M*; (e.t.) refers to equilibrium titration, (r.t.) to rapid titration: ●, and ◑, 1.19×10^{-4} *M* Cu(II) and 0.505% protein (r.t.); ○ and ◓, 1.19×10^{-4} *M* Cu(II) and 0.497% protein (r.t.); ⊖ and ◑, 1.23×10^{-4} *M* Cu(II) and 0.513% protein (e.t.).

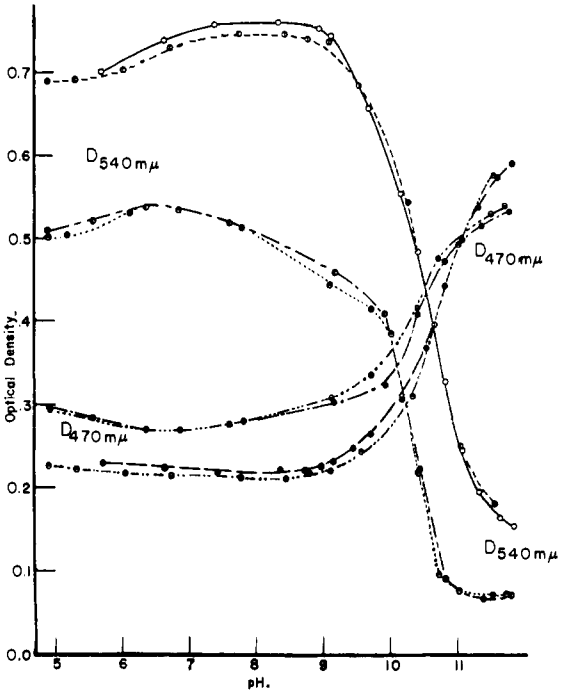

Fig. 6.—Photometric titration of Ni(II), pyridine-2-azo-*p*-dimethylaniline and acetylated bovine serum albumin; dye concentration 2.38×10^{-5} *M*; ○ and ●, 1.40×10^{-3} *M* Ni(II) and 0.502% protein (r.t.); ◑ and ⊖, 1.43×10^{-3} *M* Ni(II) and 0.486% protein (e.t.); ◐ and ⊕, 1.43×10^{-4} *M* Ni(II) and 0.484% protein (e.t.); ◓ and ◒, 1.40×10^{-4} *M* Ni(II) and 0.497% protein (r.t.).

markedly lower than those at lower *p*H's. Since increase in *p*H from 7 to 8 has no effect on the state of the azopyridine molecule and since the rise in *p*H should increase the attraction of the negatively-charged protein for the cationic metal, the drop in binding constant must be a reflection of the competing influence of OH^- ions for coördination positions of the zinc ion. Thus it is evident that the ability of zinc ion (like that of copper and mercury at lower *p*H's) to act as a bridge in ternary complexes with protein and small organic molecule is strongly interfered with by the competing hydroxylation reaction.

Photometric *p*H Titrations of Protein–Metal–Dye Systems.—The preceding dialysis equilibrium experiments thus confirm previous spectrophotometric indications[1] that zinc ions on the protein also become hydroxylated at a *p*H near 8. It becomes apparent, therefore, that semi-quantitative data on the effect of hydroxylation for a number of ions could be obtained with much less effort by a photometric *p*H titration. The absorption maximum of the free dye is at 470 mμ, whereas that of the dye in the ternary protein complex is near 540 mμ. Thus a decrease in optical density at 540 mμ as the *p*H is raised indicates dissociation of the dye from the ternary complex,[17] and should be accompanied by an increase in absorption at 470 mμ due to an increase in concentration of free dye.

Titration curves for pyridine-2-azo-*p*-dimethylaniline with acetylated bovine albumin in the presence of Cu(II), Ni(II), Zn(II) and Cd(II), respectively, are shown in Figs. 5–8.

(17) The extinction coefficient of the binary metal–dye chelate also has a maximum at 540 mμ. However, the ternary association constant is about 30 times greater than the binary one. Hence the optical density at 540 mμ is largely a measure of the ternary complex present.

For all the solutions it should be noted first that there are small differences between readings obtained in a rapid titration as contrasted to a slow, equilibrium titration. In the former procedure, readings were recorded within ten minutes after each addition of base, whereas for constant optical densities in an equilibrium titration almost 100 minutes often were required. The general trends of the curves for each type of titration are essentially the same, nevertheless.

Since the behavior of the 470 mμ band complements the larger changes in the 540 mμ peak, we may focus attention on the latter. With Cu(II) (Fig. 5) the absorption begins to drop even at *p*H 5 and continues gradually downward as the competitive effect of OH^- increases at higher *p*H's. Nickel(II) (Fig. 6) shows first a slight increase in ternary complex formation up to about *p*H 6.5 and then the effect of hydroxylation makes itself evident (particularly at 10^{-4} *M* Ni(II)). The behavior of the 540 mμ band with (10^{-3} *M*) Zn(II) or Cd(II) is similar in that in each case the metal binding of azo dye is relatively weak, so that in the *p*H range of 5–6 some of the cationic form of the dye, with strong absorption at 540 mμ, still is present. Consequently a drop in absorption occurs as the *p*H rises from 5 to 6 and more cationic dye is converted to the neutral form. Above *p*H 6, the absorption rises again as more metal ion is bound to protein and hence more dye goes into the ternary complex. At a *p*H slightly below 8 for zinc and slightly above for Cd, competition with OH^- ion

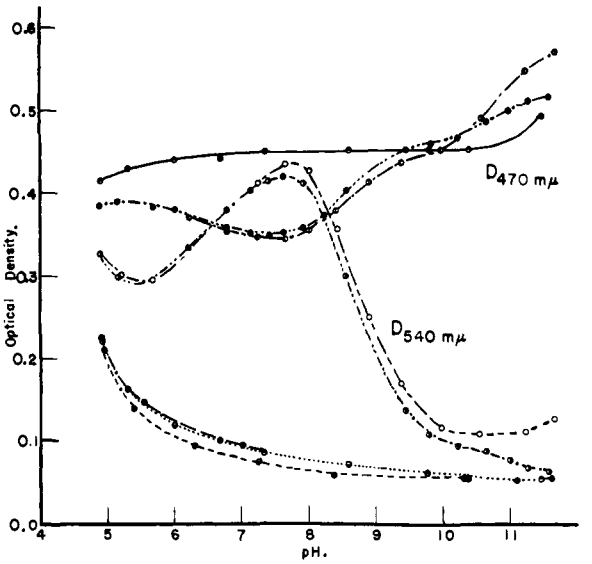

Fig. 7.—Photometric titration of Zn(II), pyridine-2-azo-*p*-dimethylaniline and acetylated bovine serum albumin; dye concentration 2.38×10^{-5} *M*; ● and ◑, 1.38×10^{-3} *M* Zn(II) and 0.485% protein (r.t.); ⊕ and O, 1.38×10^{-3} *M* Zn(II) and 0.521% protein (e.t.); ◓ and ⊖, 1.38×10^{-4} *M* Zn(II) and 0.481% protein (e.t.); ⊕, 1.38×10^{-4} *M* Zn(II) and 0.499% protein (r.t.); ◒, 0.503% protein (r.t.).

makes itself evident and the absorption drops again.

Each of these metals in the state of a ternary complex responds to the presence of increasing hydroxyl ion concentrations. The spectrophotometric titrations, at various metal concentrations, indicate an order of sensitivity to OH^- ion of Cu > (Ni, Zn) > Cd. Further inspection of these titration curves shows in addition a distinction in the behavior of Cu and Ni as contrasted to Zn and Cd. With the former pair, the effect of hydroxylation is first to produce a slow drop in absorption at 540 mμ and then, at *p*H ~10, to lead to a precipitous decrease in absorption. In contrast, with Zn and Cd only one drop in absorption occurs and that is essentially complete by *p*H 10. Particularly surprising is the observation that copper still shows some response to increasing OH^- concentration at *p*H 10.5, long after the completion of the zinc–hydroxyl interaction.

These peculiarities in behavior can be understood if one examines in more detail the possible modes of interaction of the OH^- ion with the metal in the ternary complex (I). In addition to equation 17

$$\mathrm{P - Me\overset{\frown}{Dye} + OH^- = P - Me\text{–}OH + Dye} \quad (17)$$

we must also consider the possibility

$$\mathrm{P - Me\overset{\frown}{Dye} + OH^- = P + HO\text{–}Me\overset{\frown}{Dye}} \quad (18)$$

Which of these reactions will occur depends on the relative affinities of the metal ion for the binding site of the protein as compared to the coördination positions of the azopyridine dye. An examination of appropriate association constants indicates that Cu and Zn are clearly different in their behavior and that Ni is probably similar to Cu whereas Cd parallels Zn.

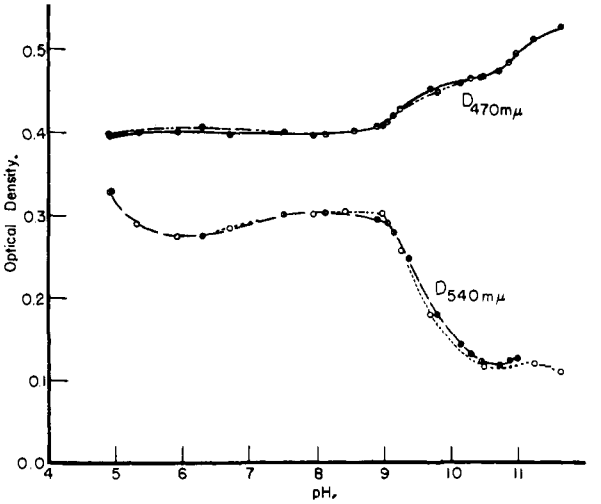

Fig. 8.—Photometric titration of Cd(II), pyridine-2-azo-*p*-dimethylaniline and acetylated bovine serum albumin; dye concentration 2.38×10^{-5} *M*; ⊖ and ⊕, 1.24×10^{-3} *M* Cd(II) and 0.465% protein (r.t.); ● and O, 1.30×10^{-3} *M* Cd(II) and 0.467% protein (e.t.).

The stability constant, K_1, for the formation of a 1:1 azopyridine–Cu(II) chelate is[18] near 1×10^5. If we assume that copper near *p*H 5 is bound at imidazole groups of the protein, the association constant is[19] near 1×10^4; if it is bound at carboxyl groups, the constant is[20] about 2×10^2. In either event, the intrinsic affinity of the Cu(II) for the dye chelate is substantially greater than for the protein site. The first effect of added OH^-, therefore, is probably to displace the metal chelate from the protein, as represented by equation 18. This displacement accounts for the slow drop in absorption at 540 mμ over the *p*H range of 5–10 (Fig. 5). The precipitous drop above *p*H 10 may be accounted for by the subsequent hydrolysis of the copper–dye chelate, which may be represented as

$$\mathrm{HO - Me\overset{\frown}{Dye} + OH^- = Me(OH)_2 + Dye} \quad (19)$$

or may be due to the binding of Cu(II) by ionized tyrosine groups which become available at sufficiently high *p*H. In either event, the net result is the displacement of dye from the metal complex with a concomitant drop in absorption at 540 mμ.

In contrast, with Zn(II), the dialysis experiments described in this paper indicate that the intrinsic constant for binding by protein at *p*H 7 is about 4×10^3 (*i.e.*, $7.8 \times 10^4/20$, assuming approximately twenty sites for zinc binding). The association constant for the 1:1 zinc–dye chelate is[18] in turn 0.229×10^3 at 25° (and slightly larger[10] at 0°). Thus the protein–metal bond is distinctly stronger than that between zinc and dye, and hence reaction 17 probably plays the important role in the interaction of OH^- with the ternary complex. There is, therefore, only one step in which hydroxyl ion affects the metal–dye bond, and hence only one re-

(18) I. M. Klotz and W.-C. Loh Ming, THIS JOURNAL, **75**, 4159 (1953).

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

(20) K. J. Pedersen, *Kgl. Danske Videnskab. Selskab, Math.-fys. Medd.*, **22**, No. 12 (1945).

gion of *p*H in which this interaction produces a drop in absorption at 540 mμ.

Nickel would be expected to behave more nearly like copper than like zinc since its association constant for the 1:1 azopyridine chelate is[18] approximately $2 \times 10,^4$ *i.e.*, much closer to that of copper than of zinc. The affinity of the protein for nickel is less than that for copper, probably by an amount roughly equal to the corresponding drop in association constants for the azopyridine dye. Thus with Ni(II) one would expect reactions 18 and 19 to play the main role in the hydroxylation interaction.

On the other hand, cadmium complexes with nitrogen donors are of the same order of strength as those with zinc. With ethylenediamine, for example, log k_1 is 5.71 for Zn, 5.47 for Cd.[21] Evidently, then, reaction 17 should be the important one for Cd(II). Thus the observed behavior of the four metals (Figs. 5–8) fits what one might expect from their relative affinities for small molecule and protein.[22]

(21) G. A. Carlson, J. P. McReynolds and F. H. Verhoek, THIS JOURNAL, **67**, 1334 (1945).

(22) It is perhaps of interest to note further that Cu and Ni form square-planar complexes whereas Zn and Cd form tetrahedral ones. It has been pointed out by O. K. Rice ["Electronic Structure and Chemical Binding," McGraw-Hill Book Co., Inc., New York, N. Y., 1940] that there is probably more strain in a five-membered chelate ring involving a metal with tetrahedral configuration than in a corresponding square-planar structure. Thus the tetrahedral chelate complexes of Zn and Cd with the dye are readily decomposed by OH^- in accordance with equation 17, while the chelate complexes of square-planar Cu and Ni are preserved at the expense of the rather strong metal–protein bonds, as indicated in equation 18.

Conclusion.—It is now apparent that the interference by OH^- with ternary complex formation previously[1] observed with Hg(II) and Cu(II) also occurs with a variety of other metals, such as Zn(II), Ni(II) and Cd(II). The *p*H at which competition with OH^- becomes evident parallels roughly the tendency of the metals to hydrolyze. The ternary protein–metal–dye complex beings to break up even below *p*H 5 with Hg and below *p*H 6 with Cu, whereas with Ni and Zn, interference starts near *p*H 7 and with Cd even higher. There is every reason to expect, therefore, that similar effects would be found with Ca and Mg at *p*H's around 10 or above. The mechanism by which OH^- interferes with the bridging properties of the metal ion need not be the same in all cases. The small molecule, in these studies a dye, may be displaced from the metal site on the protein, or the metal–dye complex may be displaced as a unit from the protein combination. In either event, however, the small molecule is removed from the neighborhood of the protein molecule. Thus it is evident that hydroxylation plays an important role in establishing the state of the protein-bound metal ion and hence in determining its possible interaction with small molecules.

Acknowledgment.—This investigation was supported in part by a research grant (RG-4134) of the National Institutes of Health, Public Health Service.

EVANSTON, ILLINOIS