[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF NORTHWESTERN UNIVERSITY]

Mediation by Metals of the Binding of Small Molecules by Proteins[1]

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It has been found that small organic molecules not ordinarily bound to proteins form strong complexes if suitable metals are present. That the metal acts as a bridge has been established by the demonstration that it must be capable of coördinating both with the protein and with the small molecule. Hg^{++}, Cu^{++}, Ag^{+}, Ni^{++}, Zn^{++}, Co^{++} and Mn^{++} are effective mediators, Ca^{++} and Mg^{++} are not. The configuration of the coördination linkages of the metal is not a critical factor. On the other hand, the hydrolytic equilibria of the metal, the structure of the small molecule, and the type of protein are important features. Stability constants for these ternary complexes have been evaluated. Some implications of these observations with respect to metal-catalyzed enzymatic reactions are discussed. The role of metals in the action of hydrolytic enzymes can be very well understood in terms of a mechanism analogous to that applied to simple non-protein systems.

Introduction

The activity of many enzyme systems is markedly dependent on the presence of suitable metal ions. In several cases the function of these ions has been postulated[2-4] to be that of a bridge between substrate and enzyme. A direct demonstration of such a linkage presents a formidable problem, since the complex would be exceedingly unstable. Metal ions, however, form stable complexes with a variety of non-enzymatic proteins also. It may be of interest, therefore, to describe some experiments in which metals act as mediators in the binding of small molecules by proteins which otherwise show little or no affinity for these same molecules. The properties of these artificial substrate–metal–protein complexes may be of some assistance in the interpretation of the behavior of natural enzymatic systems.

Experimental

Reagents.—Samples of pure pyridine-2-azo-*p*-dimethylaniline and the corresponding 4-isomer were kindly supplied by Dr. E. V. Brown[5] of Fordham University. Catecholazobenzenesulfonic acid was a commercial sample purified by Dr. H. A. Fiess essentially according to the reprecipitation method of Witt and Mayer.[6] Catecholazobenzene was prepared by coupling catechol with diazotized aniline according to the procedure of Witt and Mayer[6]; the product after recrystallization from 10% ammonium acetate solution and then from aqueous alcohol melted at 169° (lit.[6] 165°).

All inorganic chemicals were reagent grade. Methods of assay for concentration have been described previously.[7]

Crystallized pepsin, trypsin, ovalbumin, β-lactoglobulin, and bovine serum albumin and bovine γ-globulin were purchased from Armour and Co. The caseins, α- and β-, were samples fractionated previously.[8] Purified calfskin gelatin was an Eastman Kodak sample.

Most of the experiments with pepsin were carried out at *p*H 5, the region of maximum stability of this protein.[9] A few experiments were also performed at *p*H 4 and at *p*H 6. Even at the last *p*H decrease of enzymatic activity at 25° during the 20 hours of a dialysis experiment is probably negligible, since Perlmann[10] reported a loss of only 4% in specific proteolytic activity of pepsin maintained at 37° for 24 hours. Nevertheless, in line with previous reports,[9] considerable quantities of non-protein nitrogen (not precipitated by trichloroacetic acid) were found to diffuse through the dialysis membrane even at *p*H 5. On the basis of absorption of light at 280 mμ, the non-protein component was found to vary usually between 15–20% of the total pepsin added.

Acetylated bovine serum albumin was prepared according to the procedure of Fraenkel-Conrat, Bean and Lineweaver.[11] Analyses for free ε-amino groups showed that 66 ± 5% of these groups had been acetylated.

Absorption Spectra.—The absorption of light was measured with the Beckman spectrophotometer, model DU, at approximately 25°. One-cm. cells were used. Extinction coefficient, 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 Experiments.—The extent of binding was measured by the differential dialysis technique described in detail previously.[12] All experiments were carried out in 0.15 *M* sodium nitrate solution. The protein concentration was 0.5%, unless otherwise indicated. The salt concentration chosen is adequate to decrease the Donnan effect to negligible proportions; the nitrate was used in preference to the chloride to minimize complex formation by the salt anion with the metal cation.

In experiments involving metal, dye and protein, three different stock solutions were used: (1) the metal salt in sodium nitrate solution, (2) dye dissolved in a portion of solution (1), and (3) protein dissolved in sodium nitrate solution. Each solution was brought to the desired *p*H by addition of small quantities of dilute sodium hydroxide or nitric acid. Dialysis tubes were then assembled with 10 ml. of solution inside the bag and 10 ml. outside. The outside solution was composed of a given quantity of solution (2) diluted with a known amount of (1). The inside solution contained protein in sodium nitrate, or in the control tubes sodium nitrate alone. Thus in each set of experiments the metal concentration in each tube was fixed, the dye concentration varied.

After equilibration for a period of approximately 18 hours, the external solutions were analyzed for total dye. In the absence of metal ion, the analysis was carried out in the usual manner[12] by measurement of the optical density at the wave length of maximum absorption of the dye (470 mμ for the azopyridines). In the presence of metal such a single measurement would be misleading, as well as of low precision, because the amount of free metal ion may differ between protein-containing and control tubes and hence the quantity of dye complexed to metal may differ between members in a pair of tubes. Optical density measurements were made, therefore, at two wave lengths for each external solution, and the total quantity of unbound dye, both in the free form and in the metal complex, was computed in each case. Computations of the quantity of protein-bound dye

(1) Presented at the 123rd Meeting of the American Chemical Society, Los Angeles, Calif., March 15–19, 1953.

(2) L. Hellerman, *Physiol. Rev.*, **17**, 454 (1937).

(3) E. L. Smith, *Proc. Natl. Acad. Sci.*, **35**, 80 (1949).

(4) For a recent review of this problem, see A. L. Lehninger, *Physiol. Rev.*, **30**, 393 (1950).

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

(6) O. N. Witt and F. Mayer, *Ber.*, **26**, 1072 (1893).

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

(8) H. A. Fiess and I. M. Klotz, *ibid.*, **74**, 887 (1952).

(9) J. H. Northrop, M. Kunitz and R. M. Herriott, "Crystalline Enzymes," 2nd ed., Columbia University Press, New York, N. Y., 1948, pp. 28–76.

(10) G. E. Perlmann, THIS JOURNAL, **74**, 6309 (1952).

(11) H. Fraenkel-Conrat, R. S. Bean and H. Lineweaver, *J. Biol. Chem.*, **177**, 385 (1945).

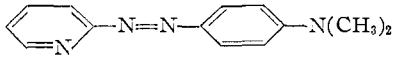
(12) I. M. Klotz, F. M. Walker and R. B. Pivan, THIS JOURNAL, **68**, 1486 (1946).

were then made in the standard fashion.[12] In some experiments an alternative procedure was followed in that an aliquot of solution to be analyzed was diluted first with 0.5 *M* citrate buffer, *p*H 6.3, or with 0.05 *M* tris-(hydroxymethyl)-aminomethane buffer, *p*H 7.9, to remove the metal from the dye complex.

In the dialysis experiments with zinc alone, analyses were carried out colorimetrically by the dithizone method.[13]

Results and Discussion

It has been shown recently[14] that uncharged organic molecules are bound only slightly (below *p*H 7) by serum albumin and not at all by any of the other proteins examined. This conclusion has since been further re-inforced by experiments with a number of additional neutral molecules, including pyridine-2-azo-*p*-dimethylaniline (I).



I

This substance is bound weakly by serum albumin but not to any significant extent by pepsin (Fig. 1).

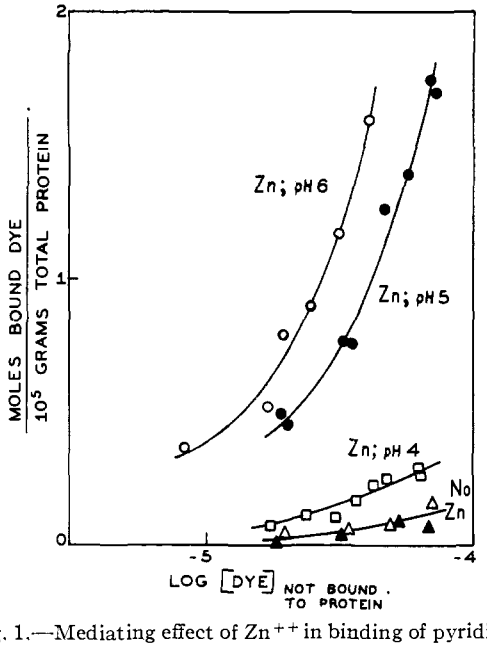


Fig. 1.—Mediating effect of Zn^{++} in binding of pyridine-2-azo-*p*-dimethylaniline by pepsin at 25° and at various *p*H's. In absence of Zn^{++}, Δ refers to *p*H 4.0, ▲ to *p*H 5.0. Concentration of Zn^{++}, when present, is 2.95×10^{-3} *M*; protein concentration, 0.5%.

In the presence of certain metal ions, however, substantial quantities of this dye are bound by each of several proteins. With pepsin (Fig. 1) in the presence of zinc, for example, the affinity for I becomes even greater than that of albumin for many anions or neutral molecules.

Since the complexes are made up of three components, protein, metal and small molecule, some of the characteristics of each participant have been investigated. It seems desirable, however, to present first the evidence that a ternary complex is actually formed.

(13) E. B. Sandell, "Colorimetric Determination of Traces of Metals," 2nd ed., Interscience Publishers, Inc., New York, N. Y., 1950, pp. 619–626.

(14) I. M. Klotz and J. Ayers, THIS JOURNAL, **74**, 6178 (1952).

Evidence for Ternary Complex.—The absorption spectrum of pyridine-2-azo-*p*-dimethylaniline is *p*H dependent,[7] as would be expected in view of the ability of two of the nitrogens to pick up a hydrogen ion. The divalent cation so formed, however, has acidity constants below *pK* 5 so that the spectrum of the dye becomes constant above *p*H 6. At this *p*H, nevertheless, the presence of Zn^{++} leads to a marked change in color (Fig. 2), due to complex formation between the dye and metal ion.

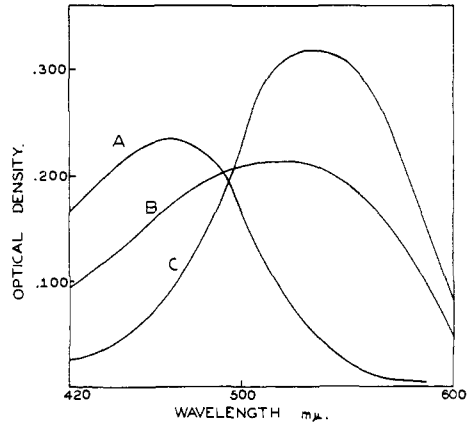
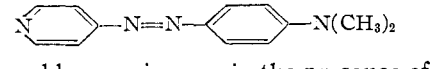


Fig. 2.—Absorption spectra of pyridine-2-azo-*p*-dimethylaniline at *p*H 6.1–6.6: A, dye alone; B, dye and 4.4×10^{-3} *M* $Zn(NO_3)_2$; C, dye and 4.4×10^{-3} *M* $Zn(NO_3)_2$ and 0.2% pepsin.

Addition of a protein such as pepsin to the zinc–dye solution produces a marked change in absorption (Fig. 2). Were the protein merely competing with the dye for the zinc ions, a drop in optical density at 538 mμ would be expected. The increase actually observed points to a short-range interaction between the protein and the zinc–dye complex.

The optical indications have been confirmed completely by dialysis experiments. Figure 1, for example, shows the effect of zinc ions on the binding of pyridine-2-azo-*p*-dimethylaniline by pepsin. Instead of the insignificant uptake observed when dye alone is present, the presence of metal results in the binding of a major fraction of the dye by the protein (except under unfavorable *p*H conditions).

Since metal ions such as zinc are bound extensively by pepsin, the effect of Zn^{++} might be ascribed to a change produced in the configurational pattern of the protein. Electrostatic factors have been shown to cause such changes in serum albumin,[15] with accompanying development of new binding sites. Likewise in the present situation, the effect of the metal ion might be merely to cause a change in the shape of pepsin, accompanied by the release of side chains capable of binding uncharged molecules. This explanation may be discounted, however, from the following observations. Pyridine-4-azo-*p*-dimethylaniline (II)



II

is not bound by pepsin even in the presence of Zn^{++} (Table I). Simultaneously the 4-azopyridine does

(15) I. M. Klotz, R. K. Burkhard and J. M. Urquhart, *ibid.*, **74**, 202 (1952).

not form a complex with zinc ion. Evidently when the metal has an effect, it acts truly as a mediator, by forming a mixed complex, the 2-azo-pyridine occupying two of the coördination positions and the protein one or both of the remaining coördination valences of Zn^{++}.

TABLE I

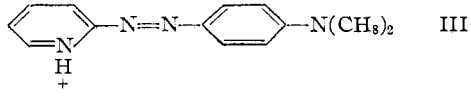
LACK OF BINDING OF PYRIDINE-4-AZO-*p*-DIMETHYLANILINE BY PEPSIN (*p*H 5, 0.15 *M* $NaNO_3$, 25°)

Concn. of unbound dye, moles/l.	Concn. of Zn^{++}, moles/l.	Moles bound dye per mole total protein
0.170×10^{-5}	0	0.000
.450	0	.002
.622	0	.002
.892	0	.005
0.59×10^{-5}	2.95×10^{-3}	0.000
1.52	2.95	.001
2.14	2.95	.008
3.05	2.95	.004

Identical conclusions were reached from optical experiments with Cu^{++} in place of Zn^{++}. The same absorption spectrum was observed for each of the following three solutions (all at *p*H 7): (a) pure pyridine-4-azo-*p*-dimethylaniline; (b) dye and 2.5×10^{-4} *M* $Cu(NO_3)_2$; (c) dye and 2.5×10^{-4} *M* $Cu(NO_3)_2$ and 0.5% pepsin. Evidently cupric ions do not form a complex with the 4-azo dye. Furthermore they do not mediate the binding of dye to pepsin despite the fact that large amounts of Cu^{++} are bound to this protein.[8]

With the ternary nature of the complex established, it is appropriate to consider some of the features of each participant which facilitate formation of the complex. A preliminary description of the effect of *p*H will be given first, since it reflects characteristics of each of the components.

Effect of *p*H.—The interaction of zinc and pepsin with the dye has been examined over the *p*H range of 4–6. Binding decreased progressively as the *p*H is lowered (Fig. 1). This trend is undoubtedly a reflection of three mutually-supporting effects. Increased hydrogen ion concentration decreases the concentration of free I by leading to the formation of the cation III



III

i.e., by competing with Zn^{++} for the pyridine nitrogen. Competition between H^+ and Zn^{++} for sites on the protein also occurs. Lowered *p*H furthermore decreases the net negative charge of the protein. The last two factors coöperate in decreasing the quantity of zinc bound by the protein and the first produces a decrease in available neutral dye molecules.

When mercuric or cupric ions are used in place of zinc, the effect of *p*H is more complicated since hydrolytic equilibria of the metal seem to be involved. Explanation of these cases may best be considered in connection with the comparison of metals discussed in a later section.

Comparison of Proteins.—The effect of a number of proteins was determined, first qualitatively by examination of their influence on the spectrum of the zinc–dye complex. In a solution at *p*H 6 (containing 4.42×10^{-3} *M* Zn^{++}, 1.1×10^{-5} *M* compound I and 0.15 *M* $NaNO_3$) ovalbumin (0.22%) produced no significant optical change, and β-lactoglobulin (0.1%), bovine serum albumin (0.2%) and gelatin (0.25%), respectively, produced only minor modifications. With slightly different conditions bovine γ-globulin (0.1%) and trypsin (0.1%) were also ineffective. With α-casein, however, marked optical effects were obtained even when the Zn^{++} concentration was reduced almost tenfold. Likewise, acetylated bovine albumin was found to bind a large fraction of the metal–dye complex, as judged from shifts in spectra. Definitive results could not be obtained with β-casein because of difficulties with precipitation.

Quantitative measurements, by the equilibrium dialysis technique, of the uptake of pyridine-2-azo-*p*-dimethylaniline by bovine serum albumin are summarized in Fig. 3. In this case, some dye is bound even in the absence of any zinc ions. Nevertheless addition of metal produces a significant increase in the uptake of azopyridine by the protein. However, the effect of the metal ion with serum albumin is substantially less than that with pepsin.

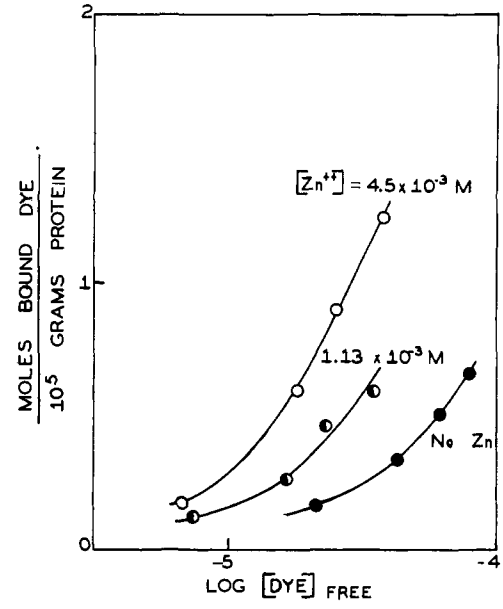


Fig. 3.—Mediating effect of Zn^{++} in binding of pyridine-2-azo-*p*-dimethylaniline by bovine serum albumin at 25° and *p*H 6; protein concentration, 0.5%. $[Dye]_{free}$ includes all the dye not bound to the protein.

The difference in behavior of these two proteins might be attributed at first glance to the difference in negative charge at *p*H's 5–6. Since pepsin, with its much larger negative charge, shows the greater effect on metal–dye binding, serum albumin was acetylated to block ε-ammonium side-chains and thereby to increase the net negative charge of the protein. As the results in Fig. 4 demonstrate, the effect of 1.13×10^{-3} *M* Zn^{++} with acetylated albumin is greater than that of 4.5×10^{-3} *M* Zn^{++} with the parent protein.

Analogous results were obtained with α-casein

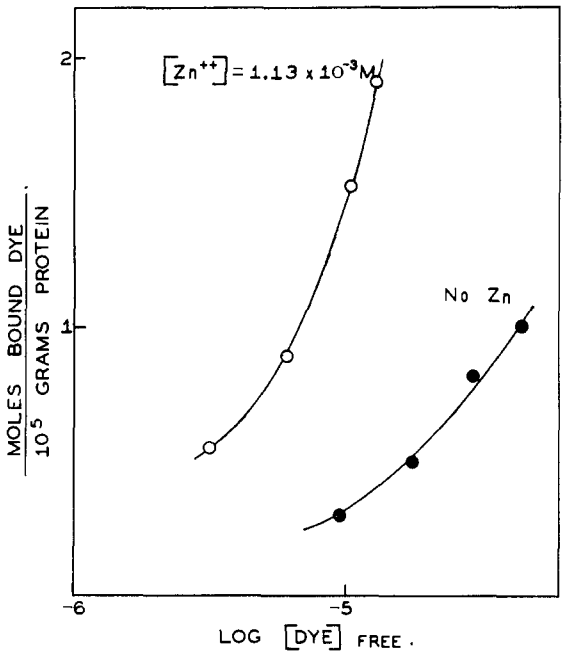


Fig. 4.—Mediating effect of Zn^{++} in binding of pyridine-2-azo-*p*-dimethylaniline by acetylated bovine serum albumin at 25° and *p*H 6.1; protein concentration 0.5%.

(Fig. 5), even 4.5×10^{-4} *M* Zn^{++} showing a pronounced effect in mediating the binding of the azopyridine molecule. Titration curves of this protein[16] show that it possesses a pronounced negative charge at *p*H 6.

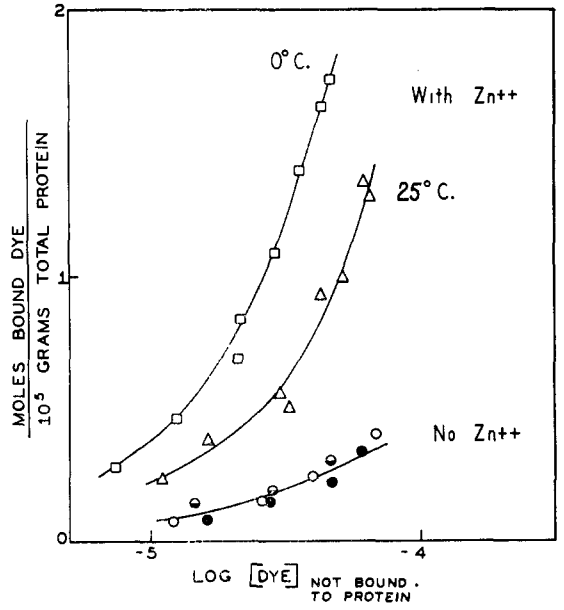


Fig. 5.—Mediating effect of Zn^{++} in binding of pyridine-2-azo-*p*-dimethylaniline by α-casein. In absence of Zn^{++}, O refers to *p*H 7, 0°; ●, to *p*H 6.6, 0°; ◒, to *p*H 6.6, 25°. Concentration of Zn^{++}, when present, is 4.5×10^{-4} *M*; protein concentration, 0.5%.

One of the expected results of a larger net negative charge on a protein is an increased affinity for metal cations. Limited solubility produced difficulties in attempts to measure the uptake of Zn^{++} by α-casein, but measurements were successfully made of the binding of Zn^{++} by acetylated serum albumin as well as by the parent protein (Fig. 6). (The results for bovine serum albumin agree reasonably well with data interpolated from the published values of Gurd and Goodman.[17]) As expected, the binding of zinc by the acetylated albumin is greater.

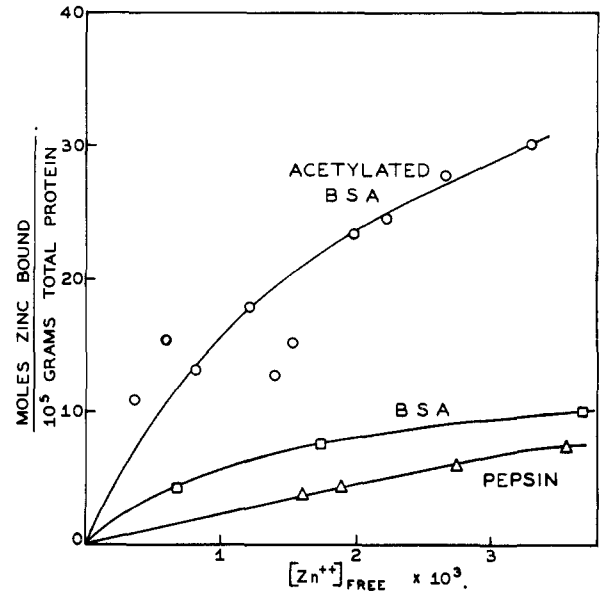
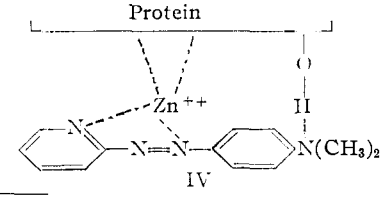


Fig. 6.—Binding of Zn^{++} by (1.0%) pepsin (*p*H 5), (0.5%) bovine serum albumin (*p*H 6) and (0.5%) acetylated bovine serum albumin (*p*H 6) at 25°; all experiments in 0.15 *M* $NaNO_3$.

Further examination of the data reveals, however, that the total uptake of azopyridine compound is not simply a function of the quantity of Zn^{++} bound by the macromolecule. Measurements of zinc binding by pepsin (at *p*H 5), although subject to much uncertainty because of difficulties described in the experimental section, lead to a curve (Fig. 6) even lower than that for (an equal weight of) serum albumin at *p*H 6. Nevertheless, pepsin shows a much greater affinity for the azopyridine dye in the presence of Zn^{++} than does serum albumin. Evidently some feature of pepsin adds to the affinity for the dye. Tentatively, it seems possible that the high content of hydroxy amino acids of pepsin may be the significant difference. These are probably internally hydrogen-bonded in the protein molecule under normal circumstances. When metal ions are attached to the protein, they may displace the hydrogen-bonded side-chains and thus make possible a bond such as



IV

(16) N. J. Hipp, M. L. Groves and T. L. McMeekin, THIS JOURNAL, **74**, 4822 (1952).

(17) F. R. N. Gurd and D. S. Goodman, *ibid.*, **74**, 670 (1952).

TABLE II

BINDING CONSTANTS[a] FOR PYRIDINE-2-AZO-*p*-DIMETHYLANILINE WITH ZINC ON PROTEIN

Protein	*p*H	Temp., °C.	Total Zn^{++}, moles/l.	$[r_D/(D)]_{(D)\to 0}$ Zn^{++} present	Zn^{++} absent	$(r_M)_{(D)=0}$	$k_{PM\text{-}D}$
Pepsin	4	25	2.95×10^{-3}	0.23×10^4	~0	...	.....
Pepsin	5	25	2.95×10^{-3}	2.44×10^4	~0	6.0	4.0×10^3
Pepsin	6	25	2.95×10^{-3}	3.74×10^4	~0	..	.....
Bovine albumin	6	25	1.13×10^{-3}	1.41×10^4	0.82×10^4	5.7	1.1×10^3
Bovine albumin	6	25	4.5×10^{-3}	1.66×10^4	0.82×10^4	10.5	0.80×10^3
Acetylated albumin	6.1	25	1.13×10^{-3}	12.4×10^4	(3.5×10^4)[b]	14	6.6×10^3
Acetylated albumin	6.6	25	1.13×10^{-3}	18.6×10^4	3.5×10^4	...	.....
α-Casein	6.8	25	0.45×10^{-3}	2.02×10^4	0.74×10^4	(20)[c]	0.64×10^3
α-Casein	6.8	0	0.45×10^{-3}	3.70×10^4	0.57×10^4	(20)[c]	1.6×10^3
None[7]	6–6.5	25	.....	.....	.....	...	0.229×10^3

[a] All measurements made in 0.15 *M* $NaNO_3$ solution. For purposes of comparison, *r*'s have been computed for a unit weight of 100,000 for each protein. [b] Binding of the uncharged, uncomplexed azopyridine at *p*H 6.1 has been assumed to be the same as at *p*H 6.6, where measurements were made. Such an assumption seems very reasonable from experience with other neutral molecules.[14] [c] This value was estimated very roughly from the low optical density at 538 mμ of the solution outside the bag in the dialysis experiments.

While the involvement of the dimethylamino nitrogen is primarily a guess, it is apparent, as will be shown shortly, that substituents on the benzene ring that are not involved in the metal–chelate formation do have an influence on the formation of these ternary complexes. Furthermore, it has been shown previously[14] that the binding of aminoazobenzene to serum albumin, in the absence of metal ions, probably involves the amino nitrogen atom.

Certain quantitative aspects of the effect of protein on the zinc–small molecule complex are also of interest. The metal, M, dye, D, and protein, P, may form a variety of ternary complexes which can be visualized concisely as

$$\begin{array}{ll} (D)_l\text{—P—}(M)_{i-j} & 0 \leqslant l \leqslant m \\ \quad\quad | & 0 \leqslant i \leqslant n \\ \quad (MD)_j & 0 \leqslant j \leqslant i \end{array} \qquad \text{V}$$

Diagram (V) in essence states that the protein has certain sites (*m* in number) which may form complexes with dye directly and other sites (*n* in number) which may form complexes with the metal, and that additional dye molecules may be bound to the protein through the metal bridge. Clearly, the number of dyes bound through metal cannot exceed the number of bound metal ions, *i.e.*, *j* cannot exceed *i*, even though the total bound dye may exceed the total bound metal, *i.e.*, $j + l$ may exceed *i*.

If we assume that the dye and metal bound directly to the protein are attached to mutually exclusive types of site, then it can be shown[18] that

$$\lim_{(D)\to 0}\left[\frac{r_D}{(D)}\right] = k_{PMD}\,(r_M)_{D=0} + k_{PD}m \quad (1)$$

where r_D represents the moles of total bound dye (*i.e.*, P–D and P–M–D) per mole of total protein, r_M the moles of bound metal (P–M and P–M–D), k_{PMD} the intrinsic constant for the binding of D to the metal on the protein, and k_{PD} the intrinsic constant for the binding of D directly to the protein. The primary objective at present is to evaluate k_{PMD}, *i.e.*, the *intrinsic* constant for the equilibrium

$$P(D)_l(M)_{i-j}(MD)_j + D = P(D)_l(M)_{i-(j+1)}(MD)_{j+1} \quad (2)$$

It is evident from equation 1 that we need first data for r_D as a function of (D) in the presence of metal as well as dye. Extrapolation of $r_D/(D)$ to zero dye concentration permits the evaluation of the sum of the two terms on the right-hand side of equation 1. If the protein binds an appreciable quantity of dye even in the absence of metal, $k_{PD}m$ can be evaluated from quantitative measurements of this equilibrium by procedures described previously.[8,12,14] Finally, it is necessary to determine $(r_M)_{D=0}$, *i.e.*, the quantity of metal ion bound by the protein in the complete absence of dye.

The measurements required have been made for zinc and the 2-azopyridine dye I with each of three proteins: pepsin, bovine serum albumin and acetylated bovine serum albumin. The data essential for the computations have been summarized already in Figs. 1, 3, 4 and 6, and the steps in the calculation of k_{PMD} are outlined in Table II.

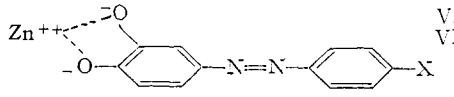
It is of particular interest to note in Table II that *k* for the binding of azopyridine by zinc is always larger when the zinc is on the protein than when it is in aqueous solution. The higher values of $k_{PM\text{-}D}$ correspond to increases in free energy of metal–dye chelation of from 600 to 2000 cal. per mole. These increases in stabilization of the metal–dye chelate may be due to the replacement of the two aquo ligands by the coördinating groups of the protein, this replacement in some way affecting the zinc bonds to the azopyridine nitrogens. It seems more likely, however, that the interaction of the organic ligand with the protein contributes primarily to this additional stabilization. For it is known that uncharged organic molecules can form complexes with serum albumin; complexes with other proteins have not proved measurable but it would be difficult to detect protein–small molecule complexes with total binding (free) energies of only 600–2000 cal. In addition the temperature coefficient of $k_{PM\text{-}D}$, as measured roughly with α-casein, corresponds in magnitude to that observed for the interaction between protein and uncharged organic molecule.[14] On the other hand, the temperature coefficient of *k* for the zinc dye chelate,[7] as well as for the binding of metal ions by proteins,[8,19] is small.

Effect of Dye Structure.—It has been pointed out already that metal mediation of binding does not occur with a molecule such as II, with which the

(18) I. M. Klotz and T. R. Hughes, unpublished work.

(19) S. Katz and I. M. Klotz, *Arch. Biochem. Biophys.*, **44**, 351 (1953).

metal does not form a complex. Even when a metal–dye complex is formed, furthermore, binding to the protein may not occur. Thus catecholazobenzene and catecholazobenzenesulfonate ion form complexes with Zn^{++} (VIa, b). Nevertheless, addition of pepsin to either



VIa, X = H
VIb, X = SO_3^-

VIa or VIb changes the spectrum in a manner indicating removal of Zn^{++} from the complex, rather than metal mediation of binding. With VIb and pepsin, this behavior might be attributed to the anionic character of both constituents. However, the same result is obtained with serum albumin which shows a general affinity for organic anions. Equilibrium dialysis experiments[20] confirm these interpretations of the spectroscopic observations. It seems apparent, therefore, that the $-SO_3^-$ substituent is of no assistance in facilitating the formation of the protein–metal–dye complex. Whether this behavior is due to the nature of the sulfonate group or to its position with respect to the other substituents can be determined only after further study with related molecules.

Comparison of Metals.—A variety of metal ions has been examined as possible mediators. It should be mentioned first that if the metal ion is incapable of forming a complex with I, it also fails to act as a mediator. Thus Ca^{++} and Mg^{++}, whose affinity for I is essentially zero,[7] do not change the spectrum of the dye when pepsin is added. On the other hand, Hg^{++}, Cu^{++}, Ag^+, Ni^{++}, Zn^{++}, Co^{++} and Mn^{++} do cause changes. Furthermore, equilibrium dialysis measurements confirm the conclusion that this latter group of cations can act as mediators. The extents of binding of the azopyridine dye I by pepsin, through the mediation of each of several of these metals, are illustrated in Fig. 7.

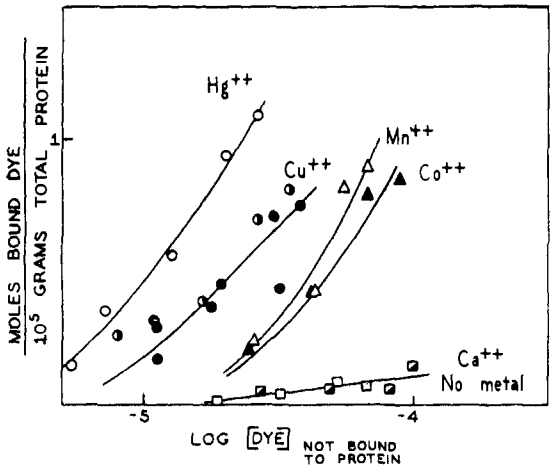


Fig. 7.—Comparison of metals as mediators in binding of pyridine-2-azo-*p*-dimethylaniline by pepsin at 25°: O, Hg^{++}, 1×10^{-4} *M*, *p*H 5; ●, Cu^{++}, 1×10^{-4} *M*, *p*H 5; ◑, Cu^{++}, 1×10^{-4} *M*, *p*H 6; △, Mn^{++}, 5×10^{-2} *M*, *p*H 5; ▲, Co^{++}, 5×10^{-4} *M*, *p*H 5; ◪, Ca^{++}, 5×10^{-2} *M*, *p*H 5; □, no divalent metal ion.

(20) M. Mellody, unpublished experiments.

It is of interest to note first that this group of mediating metals includes some (*e.g.*, Cu^{++} and Ni^{++}) which tend to form square-planar complexes, others (*e.g.*, Hg^{++}, Zn^{++}) which prefer tetrahedral coördination and still others (*e.g.*, Co^{++} and Mn^{++}) which are usually in an octahedral configuration. It is thus clear that the type of coördination arrangement preferred by the metal cation is not a primary factor in determining its ability to act as a mediator.

Nevertheless, there are striking differences in the mediating effectiveness of these coördinating metals. In the absence of complete information on the binding of each of these metals by pepsin (in the absence of dye), their relative efficiencies in acting as mediators in metal–dye–protein complexes may be evaluated in terms of equation 3, which is obtained directly from 1

$$\frac{1}{M_T}\left\{\lim_{(D)\to 0}\left[\frac{r_D}{(D)}\right]\right\} = k_{PMD}\frac{(r_M)_{(D)=0}}{M_T} = k_{PMD}f_M \quad (3)$$

Here M_T equals the total (initial) concentration of metal ion and f_M represents the product of a proportionality constant[20a] times the fraction of metal bound by the protein in the absence of dye. (The second term on the right-hand side of equation 1 is absent since k_{PD} is effectively zero for pepsin.) Values of $k_{PMD}f_M$ are listed in Table III.

TABLE III

RELATIVE EFFECTIVENESS OF VARIOUS METALS AS MEDIATORS IN AZOPYRIDINE BINDING BY PEPSIN[a]

Metal ion	*p*H	Total metal, moles/l.	$\lim_{(D)\to 0}\left[\frac{r_D}{(D)}\right]$ × 10^{-4}	$k_{PMD}f_M$ × 10^{-8}
Hg^{++}	5	1.0×10^{-4}	3.95	3.95
Cu^{++}	5	1.0×10^{-4}	2.04	2.04
Cu^{++}	6	1.0×10^{-4}	2.63	2.63
Co^{++}	5	5.0×10^{-4}	1.00	0.20
Zn^{++}	5	2.95×10^{-3}	2.44	.083
Zn^{++}	6	2.95×10^{-3}	3.74	.127
Mn^{++}	5	5.1×10^{-2}	1.04	.0020
Ca^{++}	5	5.1×10^{-2}	~0	~0

[a] All measurements made in 0.15 *M* $NaNO_3$ at 25°. To maintain uniformity with other computations, all *r* values have been expressed for 100,000 grams of protein.

It is of interest that the order observed, namely, $Hg^{++} > Cu^{++} > Co^{++} > Zn^{++} > Mn^{++} > Ca^{++}$ is the same as that of the chelate stability constants.[7] It should also be mentioned that the affinity of pepsin for the metals listed probably also falls in approximately the same order, if one may judge from investigations with other proteins.[21]

Despite the relative simplicity of the relationships described so far, there are a number of observations which point to influences which have not been fully appreciated in the past. Although the binding of metals alone to pepsin was not measured (except for zinc) because of difficulties in dialysis experiments with this protein, one can estimate from experiments with other proteins[8] that the greater fraction of the copper present must be on the pepsin. With this assumption, one may calcu-

(20a) The proportionality constant equals (total volume dialysis system)/(total moles protein) and is the same for all experiments listed in Table III.

(21) C. Tanford, THIS JOURNAL, **74**, 211 (1952).

late from the data at pH 6 that k_{PMD} for copper is 6×10^3. It is highly unlikely that this assumption is in error by more than a factor of two. The constant k_{PMD}, however, is a factor of almost twenty smaller than the chelate stability constant (1.3×10^5) for the copper–azopyridine complex[7] in the absence of protein. Similar computations with Hg^{++} lead to a value of approximately 1×10^4 for k_{PMD}, whereas the chelate stability constant, although highly sensitive to pH, is in the neighborhood of 2×10^5 (or probably even greater at pH 5). Thus Cu^{++} and Hg^{++} stand in marked contrast to Zn^{++} in that with the latter the protein markedly stabilizes the metal chelate, *i.e.*, k_{PMD} is 4.0×10^3 compared to 0.229×10^3 for the zinc chelate alone.

Mercury and copper are also distinguished from zinc and the other metals studied in another aspect of these mediating interactions. With all metals except the first two, the absorption spectrum on addition of pepsin to the metal chelate changes immediately to its final form. With Hg^{++} or Cu^{++} (Fig. 8), however, the spectrum changes slowly over a period of a day. Pepsin is not exceptional in this behavior. Analogous results were obtained with acetylated bovine albumin, the zinc dye interaction being very rapid, the copper dye interaction extending over 16 hours or more.

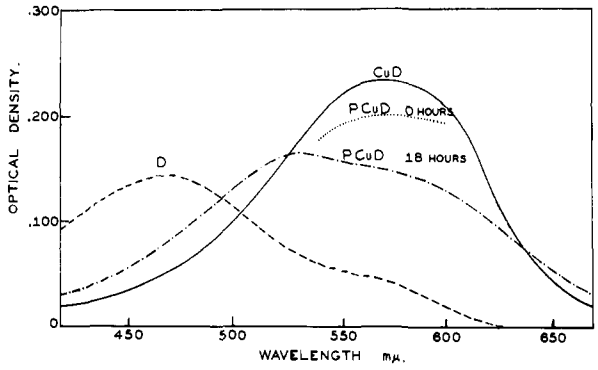


Fig. 8.—Absorption spectra of pyridine-2-azo-p-dimethylaniline at pH 5 in 0.15 M $NaNO_3$, by itself, in presence of (1×10^{-4} M) Cu^{++}, and in presence of (1×10^{-4} M) Cu^{++} and 0.5% pepsin.

The exceptional behavior of Hg^{++} and Cu^{++} parallels one of the major properties in which these two metal ions differ from the others studied—their hydrolytic equilibria. Copper and mercury have a much greater affinity for hydroxyl ion.[22-24] As a result, at pH's between 5 to 7, the general range of the current studies, the equilibrium

$$DMe(H_2O)_p + qOH^- \rightleftharpoons DMe(H_2O)_{p-q}(OH^-)_q + qH_2O \quad (4)$$

is largely to the right for Hg^{++} and Cu^{++}, but overwhelmingly to the left for the other metals examined. The presence of these hydroxo complexes is very clearly evident from the marked changes in spectra of the copper–azopyridine complex in the pH range of 6–7, in contrast to the constancy of optical density of the zinc chelate over the same range.

Likewise when the Hg^{++} or Cu^{++} ion is on the protein, aquo ligands may also be displaced to some extent by hydroxyl ions. For the azopyridine dye to be bound to the metal on the protein, OH^- ions must be displaced in turn, and since their affinity is greater than that of H_2O molecules for the metal, k_{PMD} may be substantially below that of k for the metal-dye chelate.

The slowness of the formation of the ternary complex when mercury or copper is the mediating metal is probably also a reflection of the hydrolytic equilibria. The replacement of H_2O on the metal by OH^- is likely to be a rapid exchange, since it can occur by a proton transfer from the water molecule. However, if we may judge from corresponding phenomena in inorganic complex-ion systems,[25] it is quite likely that the displacement of one or more OH^- ligands by the combining side chains of the protein is a slow process. Similarly other substitutions of ligands on metal complex ions may require substantial periods of time.[25] This behavior is of particular interest because of its possible relevance to the extended activation periods often observed in studies of metal-catalyzed enzymatic reactions.[3]

Hydrolytic equilibria of zinc ions become significant[22,23] at pH's near 7. One might expect, therefore, that at higher pH's interaction of the zinc dye with protein should reflect the competition of OH^- ions for this metal also. Although appreciable time effects could not be detected in this case, perhaps because the pH used is only at the verge of onset of OH^- complexes, the absorption spectrum (Fig. 9) of the zinc–dye–acetylated albumin mixture is substantially lower at pH 8 than at pH 7. Since increase in pH from 7 to 8 has no effect on the state of the azopyridine molecule, and since the rise in pH should increase the binding of metal by protein, the drop in extent of interaction as indicated by the spectrum must be a reflection of the competitive influence of OH^- ions for coördination positions of the cation.

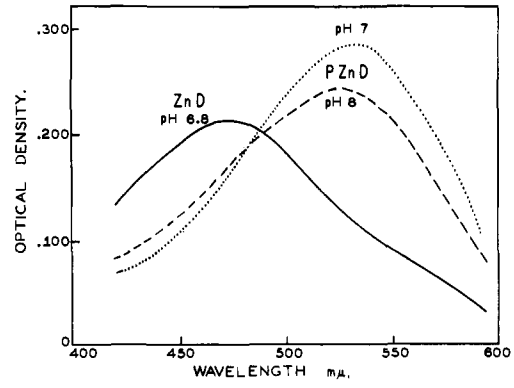


Fig. 9.—Absorption spectra of pyridine-2-azo-p-dimethylaniline in presence of 1.15×10^{-3} M $Zn(NO_3)_2$ with, and without, 0.25% acetylated bovine serum albumin.

Some Implications with Respect to Metal-catalyzed Enzymatic Reactions.—It is apparent from

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

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

(24) S. Hietanen and L. G. Sillen, *Acta Chem. Scand.*, **74**, 5057 (1952).

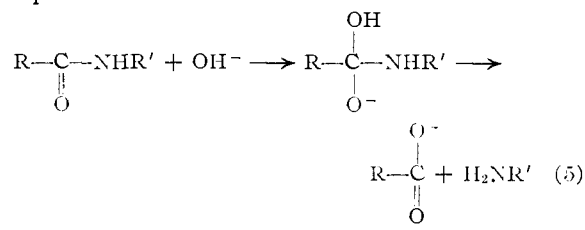
(25) F. Basolo, B. D. Stone and R. G. Pearson, THIS JOURNAL, **75**, 819 (1953).

the investigations described above that metals may actually serve as bridges in the formation of a complex between a protein and a small molecule. Such a mechanism has been postulated for some time for many metal-activated enzyme reactions, in particular decarboxylations[26-28] and hydrolyses.[2,3] Details of the mechanism of decarboxylations have been outlined convincingly by Steinberger and Westheimer.[28] Among hydrolytic reactions specific sites for the metal bridging have been described by Hellerman[2] and by Smith[3] for arginine in arginase activity and for various peptides in peptidase systems, respectively. In the latter systems particular emphasis has been placed on the formation of a chelate between the metal ion and the substrate as a requirement for enzymatic activity.

A number of questions have been raised in the past[4,29] in regard to the details of the chelate-formation picture of metal-activated enzymatic hydrolyses. It has been our feeling that the following questions are actually the most troublesome in connection with this picture. (a) The most commonly occurring activators are Mn^{++} and Mg^{++}, yet these are at the bottom of the list in chelating ability. In particular with amino acids and peptides the observed order is $Cu^{++} > Ni^{++} > Zn^{++} > Pb^{++} > Co^{++} > Mn^{++} > Mg^{++}$, with the first metal showing an avidity more than 10^5 times greater than the last two.[30,31] (b) The products of peptide hydrolyses are usually stronger chelating agents than the initial reactants. It has been shown by Kroll,[32] for example, that Mn^{++} forms complexes with leucine, but that no complexes can be detected with leucineamide. It is difficult to see, therefore, why leucineaminopeptidase, which is activated by Mn^{++}, should dissociate leucine, the product of the hydrolytic cleavage of leucineamide. Similar difficulties are encountered upon comparison of the stability constants of Mn^{++} (or Mg^{++}) with glycine and with glycylglycine.[31] (c) The chelation of metal to substrate is considered to produce a strain in the bond sensitive to hydrolysis. It is difficult to see, then, why the metal ion should not catalyze peptide hydrolysis about as well in the absence of specific protein as in its presence.

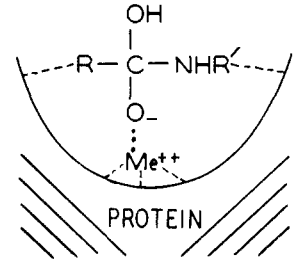
In our experience with metal bridging of dye–protein complexes, one of the more striking observations was that the metals with greatest chelating ability did not necessarily exhibit fullest efficiency as bridges to the protein because their strong complexing ability favored competing reactions. Thus although the k_{MD} of Cu^{++} is over 500 times greater than that for Zn^{++} (1.3×10^5 compared to 2.29×10^2), the values of k_{PMD} are practically equal (6×10^3 compared to 4×10^3, respectively). These results coupled with the fact that the poorest complexing metals (Mn^{++} and Mg^{++}) are most often encountered as activators of hydrolyzing enzymes, have led to the examination of a modified viewpoint of the role of metals in these enzymatic processes. The scheme to be described discounts the chelate-forming ability of the cation. By drawing attention instead to the activated state in the hydrolysis process, it suggests an alternative role for the metal ion which permits a broader unification of the behavior of a variety of systems.

If the mechanism of basic hydrolysis of simple esters[33-35] may be transposed to amides and peptides, the reaction course may be represented by equation 5



(5)

The most significant feature from our viewpoint is the nature of the intermediate complex in 5. A cationic metal on a suitable protein could favor the formation of this activated form in two ways. First, because of its positive charge it would increase the local concentration of OH^-; secondly, it would stabilize the transition state by the formation of a complex such as VII[36]



VII

Fig. 10.—Schematic representation of metal-stabilized activated state in enzymatic hydrolysis of peptides.

Both of these factors should speed up the hydrolysis reaction.

In this picture it is assumed that R and R′, the substituents that determine the specificity of the hydrolyzing enzyme are bound directly to the enzyme, rather than by a chelate linkage to the metal. Assumption of chelation of the substrate to the metal has been omitted on several grounds. The primary function of such an assumption has been to provide a basis for some of the specificities observed among the peptidases. Invariably, though, certain aspects of specificity behavior, particularly

(26) H. A. Krebs, *Biochem. J.*, **36**, 303 (1942).

(27) A. Kornberg, S. Ochoa and A. Mehler, *J. Biol. Chem.*, **174**, 159 (1948).

(28) R. Steinberger and F. H. Westheimer, THIS JOURNAL, **73**, 429 (1951).

(29) A. E. Martell and M. Calvin, "Chemistry of the Metal Chelate Compounds," Prentice-Hall, Inc., New York, N. Y., 1952, Chapter 8.

(30) A. Albert, *Biochem. J.*, **47**, 531 (1950); **50**, 690 (1952).

(31) C. B. Monk, *Trans. Faraday Soc.*, **47**, 297 (1951).

(32) H. Kroll, THIS JOURNAL, **74**, 2034 (1952).

(33) L. P. Hammett, "Physical Organic Chemistry," McGraw-Hill Book Co., New York, N. Y., 1940.

(34) A. A. Frost and R. G. Pearson, "Kinetics and Mechanism," John Wiley and Sons, Inc., New York, N. Y., 1953, pp. 5-7, 265-267.

(35) H. Kroll, THIS JOURNAL, **74**, 2036 (1952).

(36) In a discussion at the Symposium on the Mechanism of Enzyme Action of the McCollum-Pratt Institute at Johns Hopkins University, Professor L. P. Hammett suggested that the transition state might be better visualized with the metal ion more nearly in the vicinity of the amide nitrogen. In the latter position the electrostatic effect of the cation would be to draw electrons away from the C–N bond and hence promote its hydrolysis.

differences between stereoisomers, require one to fall back onto the configuration of the protein. It seems just as well, then, to place responsibility for specificity in general on the interaction of R and R′ groups with the apoenzyme directly. From this viewpoint it is also much easier to reconcile with enzymatic behavior our observations on protein–metal–dye complexes and to understand the outstanding positions of Mn^{++} and Mg^{++} as activators of the peptidases. Metals which form complexes with great affinity are not likely to have any open coördination positions, particularly at *p*H's as high as 8–9, at which these enzymatic reactions have been studied. At *p*H's above 7, most of the divalent cations having open coördination positions will bind OH^- ions strongly. If R includes an $-NH_2$ group, conceivably this group might replace an OH^- from the metal coördination sphere; it is very difficult to believe, however, that the very weakly-complexing >C=O or –NH– groups of the amide could displace a second strongly bound OH^- to form a chelate. On the other hand, with the assumption that they cannot do so, it becomes quite clear why metals such as Mn^{++} and Mg^{++} are the common activators. These metals being among the weakest coördinators, still have open coördination bonds. Even at *p*H's 8–9, the binding of OH^- ions is very small. As a result they are in a state in which they can stabilize the activated complex by formation of an additional linkage.

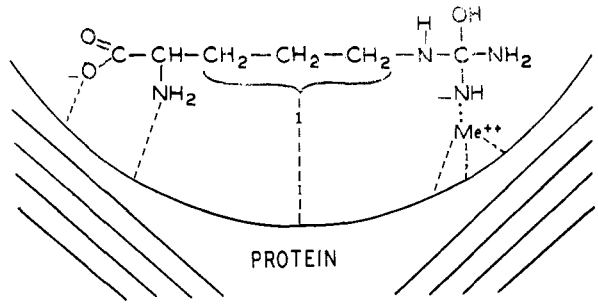
On the basis of the picture in VII it is also easier to understand the effect of hydrolysis products on the activity of the peptidases. The splitting of the amide bond produces a carboxyl and an amine group, which coördinate only weakly with Mn^{++} or Mg^{++}. Such weak bonds which might be formed with the metal are counterbalanced by the loss of a point of anchorage of the amino acid, as compared to the parent peptide, to the apoenzyme directly. Thus the products might be bound slightly to the enzyme but are hardly likely to have a high affinity for the active site.

Likewise, the proposed picture provides a simple explanation for the joint requirement of metal and specific protein. In the absence of protein, the juxtaposition of metal and substrate will be a relatively uncommon occurrence.

With a picture of metal stabilization of an intermediate complex in an hydrolysis reaction, it is a simple matter to extend the interpretation to the observed behavior of arginase.[2] The sensitive bond in the arginase system is of the same general type as in the amides and esters. In this case, as has been suggested by Hellerman and Perkins,[37] the metal ion may first destroy the resonance stabilization of the guanidinium group by displacing a hydrogen ion. From our viewpoint, it is in the next step, the stabilization of the intermediate complex VIII, that the metal plays its critical role for it facilitates the opening of the C=NH bond and the simultaneous introduction of the OH^- ion.

From this viewpoint some of the differences between arginase and the peptidases also seem reasonable. With the former enzyme, a hydrogen ion

(37) L. Hellerman and M. E. Perkins, *J. Biol. Chem.*, **112**, 175 (1935).



VIII

Fig. 11.—Schematic representation of metal-stabilized activated state in enzymatic hydrolysis of arginine.

is displaced from the amidinium group before the activated complex VIII is formed. This displacement can be brought about by a metal having some tendency to complex with nitrogen groups. On the other hand, too great an affinity for ligands would keep the metal complexed with OH^- ions so strongly that displacement by =NH groups would not be significant. On this basis, it seems reasonable that Co^{++}, Fe^{++} and Mn^{++} should be activators for arginase; Mg^{++} has a much weaker affinity for nitrogens; Cu^{++}, Fe^{+++} and Hg^{++} have too strong an affinity for hydroxyl ions. It is also of interest in connection with this interpretation that the activating effects of metals on arginase are more pronounced at *p*H 7.5 than at 9.5,[37] *i.e.*, at *p*H's where metal complexing with OH^- is decreased by the reduced hydroxyl concentration.

The appearance of Mn^{++} and Mg^{++} as primary activators in phosphatase action suggests that the role of metals in these hydrolyses may be similar to those described above. One may readily visualize an activated state, similar to those pictured in VII and VIII, stabilized by metal coördination. Likewise among the phosphate-transferring enzymes, the occurrence of Mn^{++} and Mg^{++} as activators suggests the desirability of writing a mechanism which emphasizes stabilization of a charge-separated type of activated state. In some of these systems the metal may function simultaneously as an agent for the increased binding of the substrate to the enzyme. Where such a role is a significant one, it seems likely that metals which form stronger complexes than Mn^{++} or Mg^{++} should also appear as activators. It is also of interest that beryllium is an outstanding inhibitor of alkaline phosphatase, for this metal ion shows an extremely strong affinity for OH^- ions.

The views represented by schemes such as VII are also suggestive on a wider scale. Many of the hydrolyzing enzymes do not require metal activators. Nevertheless, the type of intermediate shown in VII serves as an excellent basis of description of the activated state with the simple modification of merely replacing the metal ion by cationic $-NH-\overset{|}{\underset{NH_2}{C}}=\overset{+}{N}H_2$ or $-NH_3^+$ groups from arginine or lysine side chains. These positively charged loci, with their ability to form —NH · · · O— hydrogen bonds, could function in the same way as Me^{++} in favoring formation of the intermediate complex VII.

From this standpoint one might reasonably expect to find non-specific anionic inhibitors for these enzymes even though they are not metal-activated.

One is inclined also to wonder whether cationic detergent molecules would not catalyze reactions such as peptide hydrolyses in basic solution by a mechanism similar to those outlined above. It may be necessary to use a detergent of the type $R—NH_3^+$ rather than $R—N(R')_3^+$ in order to take advantage of hydrogen-bond formation in the stabilization of the intermediate complex. Substrates with a net negative charge and detergent concentrations above the critical micelle value should be particularly favorable.

It seems, therefore, that the behavior of metals as activators of hydrolyzing enzymes can be understood within the framework of the mechanisms established for simpler systems.[33,34] Little is gained and much that is perplexing is introduced by added assumptions of chelate formation. It does not follow, of course, that chelate formation is not an essential prerequisite in other metal-activated enzyme systems. A convincing case can only be established, however, if the postulated properties do not conflict with the known behavior of metals as chelating species and as mediators in the binding of small molecules to proteins.

Acknowledgments.—The authors are grateful to Professor Ralph G. Pearson for several discussions on the mechanisms of hydrolytic reactions. This investigation was supported by grants from the Rockefeller Foundation and the Office of Naval Research (Project No. NR124-054).

EVANSTON, ILLINOIS

[CONTRIBUTION NO. 1784 FROM THE GATES AND CRELLIN CHEMICAL LABORATORIES OF THE CALIFORNIA INSTITUTE OF TECHNOLOGY AND THE COLLEGE OF PHARMACY OF THE UNIVERSITY OF CALIFORNIA]

The Infrared Spectra of Nitroguanidine and Related Compounds

BY W. D. KUMLER

RECEIVED MARCH 6, 1953

The infrared spectra of nitroguanidine and six related compounds were obtained on mulls in perfluorokerosene. Six of the compounds have two or more bands in the 3 μ region giving evidence of considerable hydrogen bonding. 1-Nitro-2-nitrimino. imidazolidine has one narrow band at 3.02 μ which according to the current practice could be ascribed to a free N–H vibration. However, it appears probable that it may also arise from a hydrogen bonded N–H. The presence of a broad band in the 3 μ region for azo-bis-(chloroformamidine) indicates N–H $\cdots$ N bonds of appreciable strength. All the compounds have bands at 5.92–6.18 μ attributed to C=N and those containing nitro groups have bands at 6.11–6.38 μ attributed to NO_2. The frequency of the nitro bands changes in the same way as the C=N bands giving evidence that the two are conjugated. The shift in frequency is correlated with the contribution of resonating forms with a separation of charge. This behavior is further evidence that these compounds are nitrimines.

The infrared spectra of nitroguanidines and related compounds are of interest because they contain hydrogen bonds of the type N–H···O and N–H ···N and because recent work[1–5] based on chemical dipole moment and dissociation constant data has shown that a number of guanidine derivatives have structures different from those commonly assigned to them. Nitroguanidine, for example, is a nitroimine (nitrimine) $H_2N—\underset{HN}{\overset{NH_2}{C}}=N—NO_2$ and not a nitroamine (nitramine) $H_2N—\underset{\|}{C}—\underset{H}{N}—NO_2$, as it is usually written. The infrared spectra are in agreement with the recent extensive evidence that these compounds are nitrimines.

The only prior measurements of the infrared spectra of compounds of this type were carried out by Lieber, Levering and Patterson.[6] Their compounds were measured as mulls in white mineral oil and unfortunately this solvent itself has high absorption between 3 and 4 μ and 6 and 7 μ. This interferes with the measurement of the true absorption characteristics of the compounds themselves around 3 and 6 μ where the N–H stretching and double bond stretching frequencies occur. In interpreting their results they have assumed that the >C=NH group is present in all guanidine derivatives which recent evidence has shown is not the case.

The compounds in this work were all measured from 1 to 15 μ as mulls in perfluorokerosene, a solvent that is relatively free of absorption in the most interesting regions around 3 and 6 μ. A Perkin–Elmer Model 21 Infrared Spectrograph with a sodium chloride prism was used. The nitroguanidine and nitroaminoguanidine were those used in previous measurements.[3] The others were supplied to us by Professor George F Wright. These compounds gave no evidence of decomposition. The melting points of those compounds in which this property is an indication of purity were unchanged at the time of measurement from those originally reported.

All the compounds measured except 1-nitro-2-nitriminoimidazolidine have two or more bands in the 3 μ region, giving considerable absorption over a range of 300 or more cm.$^{-1}$. Four of the compounds, 2-nitroiminoimidazolidine, nitrosoguanidine, azo-bis-(chloroformamidine) and azo-bis-(nitroformamidine) have two bands around 3 μ, a narrow band in the 2.97–3.03 μ region and a broader band in the 3.21–3.30 μ region. In the

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