Metal Chelates of a Bis-imidazole*

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ABSTRACT: The stability constants of metal chelates of 4,4′-(5,5′)-bis-imidazolylmethane (BIM) with Mn(II), Fe(II), Co(II), Ni(II), Cu(II), Zn(II), and Cd(II) have been determined at $\gamma/2 = 0.16$ and 30° by potentiometric titration, using the glass electrode. The data indicate an appreciable chelate effect when stability constants of comparable BIM–metal ion and imidazole-metal ion systems are compared. The Co(II) and Fe(II) complexes of BIM were found to be autoxidizable. The possible relation of the data on BIM–metal ion systems to the study of protein–metal ion interaction is discussed.

In an earlier communication from this laboratory (Drey and Fruton, 1965), the synthesis of 4,4′(5,5′)bis-imidazolylmethane (BIM)[1] was reported, and several of its properties were described. Our interest in compounds of this type stems from the possibility that, in some proteins and especially in the active centers of some enzymes, two imidazolyl groups may be held near each other by the three-dimensional structure of the peptide chains (Crestfield *et al.*, 1963; Walsh *et al.*, 1964). In this connection, it seemed desirable to examine the properties of a series of simple model compounds in which two imidazolyl groups were linked to each other through an aliphatic bridge between their 4(5) positions. In addition to the synthesis of BIM, we had initiated synthetic work leading to the higher homologs of such a series; since the submission of our previous paper we have seen the report of Schubert *et al.* (1964), who have described the synthesis of bis-imidazoles linked at their 4(5) positions by $(CH_2)_n$ bridges, where $n = 5, 6, 7$, and 8. These investigators prepared the bis-imidazoles by desulfurization of the corresponding bis-(2-mercaptoimidazolyl) compounds, which were made by treatment of the appropriate ω,ω'-diaminoalkanediones with NH_4SCN. Our synthetic route to the homologous bis-(2-mercaptoimidazolyl) compounds has involved reduction of the α,α'-diamino derivatives of dimethyl adipate, dimethyl pimelate, and dimethyl suberate with NaHg in the presence of KSCN; desulfurization then yields the bis-imidazoles with two, three, and four CH_2 groups between the two imidazole rings.

In the present communication, we report the stability constants of metal complexes of BIM with several divalent metal ions, and present evidence for the efficient chelation of these metals by the bis-imidazole. Consideration has long been given to the possibility that the binding of metal ions by some proteins may involve chelates in which two imidazolyl groups are bound to a single metal ion, and the data reported in what follows are intended as a contribution to the study of metal-protein interaction. A valuable review of this field has been prepared by Gurd and Wilcox (1956); for comprehensive reviews on metalloenzymes, see Malmström and Rosenberg (1959) and Vallee and Coleman (1964).

Experimental

Reagents. All the aqueous solutions were prepared from freshly distilled and reboiled water. BIM was an analytically pure sample of the free base, mp 229-231° (Drey and Fruton, 1965); the stock solution was 0.0265 M. Imidazole was a recrystallized sample (mp 89.5°) of the free base; the stock solution was 0.0250 M. The metal-ion stock solutions were prepared from the following reagent grade salts: $Co(NO_3)_2 \cdot 6\,H_2O$ (0.0101 M), $Ni(NO_3)_2 \cdot 6\,H_2O$ (0.00981 M), $Cu(NO_3)_2 \cdot 3\,H_2O$ (0.01000 M), $Cd(NO_3)_2 \cdot 4\,H_2O$ (0.00910 M), $Zn(NO_3)_2 \cdot 6\,H_2O$ (0.00989 M), $ZnSO_4 \cdot 7\,H_2O$ (0.0106 M), $FeSO_4 \cdot 7\,H_2O$ (0.00925 M), and the Mn(II) solution was prepared from a commercial reagent-grade 50% aqueous solution of $Mn(NO_3)_2$ to give a stock solution of 0.0154 M. The metal content of the stock solutions was determined by the pyridine thiocyanate method (Vogel, 1953) for Co(II), Ni(II), Cu(II), and Cd(II); by the 8-hydroxyquinoline method (Vogel, 1953) for Zn(II) and Fe(III); by EDTA titration (Flaschka, 1964) for Zn(II) and Mn(II). Ferrous sulfate was used because the desired ferrous nitrate was not readily available; for analysis, the $FeSO_4$ solution was oxidized to the ferric form.

pH Measurements. A double-walled glass cell (15 ml) was fitted with a rubber stopper with inlets for titrant (0.0993 M KOH), nitrogen, a Radiometer glass electrode G202B, and a Radiometer calomel electrode K130. The cell was maintained at 30 ± 0.1° throughout the course of the measurements. The *p*H was recorded by means of a Radiometer TTTla meter in conjunction with a Radiometer scale expander PHA630Ta. Linearity of the electrodes was checked before and after each complete titration using Beckman buffer solutions 3501 (*p*H 7.0) and 3506 (*p*H 4.0); the maximum allowable

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[1] Abbreviations used in this paper: BIM, 4,4′(5,5′)bis-imidazolylmethane; IM, imidazole.

deviation was ±0.05 *p*H unit. The ionic strength was 0.16 M in KNO_3. The molar ratio of ligand to metal ion was 10:1 for imidazole and 5:1 for BIM.

Air was excluded from the cell by passing a gentle stream of nitrogen through the solution. In all titrations, except those involving Co(II) and Fe(II), the nitrogen was passed successively through a solution of $Ba(OH)_2$ and of water before entering the cell. With Co(II) and Fe(II), the gas was passed successively through a 15% solution of pyrogallol in 50% KOH, water, barium hydroxide solution, and water. During the titration, increments of 0.0993 N KOH were added from a 0.5-ml Agla micrometer syringe. Each titration was performed in duplicate. At no stage in the titrations was there evidence of precipitation of metal hydroxide.

The potentiometric titration curves for BIM and the BIM-metal ion systems are shown in Figure 1. The curve for BIM was used for the determination of its acid-dissociation constants ($pK_1' = 5.61 \pm 0.02$; $pK_2' = 7.39 \pm 0.03$) at $\gamma/2 = 0.16$ and 30°, according to the semimicro method of Albert and Serjeant (1962). Under these conditions, imidazole gave a pK' of 6.95 ± 0.02; this value is in satisfactory agreement with those previously reported for the ionic strength used in the present study (Edsall *et al.*, 1954; Tanford and Wagner, 1953). The above pK' values for BIM and imidazole are somewhat lower than those previously found in the absence of added KNO_3 (Drey and Fruton, 1965).

The stability constants were calculated according to the procedure developed by Bjerrum (1941) in studying the metal complexes of ethylenediamine. In the case of BIM, we define the following terms:

$C_M \equiv$ total concentration of metal ion
$C_L \equiv$ total concentration of BIM
$[B] \equiv$ concentration of unprotonated BIM
$pB \equiv -\log [B]$
$[BH^+] \equiv$ concentration of monoprotonated BIM
$[BH_2^{2+}] \equiv$ concentration of diprotonated BIM
$k_1 \equiv$ acid dissociation constant of BH^+
$k_2 \equiv$ acid dissociation constant of BH_2^{2+}
$C_B \equiv [B] + [BH^+] + [BH_2^{2+}]$
$C_S \equiv [BH^+] + 2[BH_2^{2+}]$
$\alpha \equiv [B]/C_B$

The average number of hydrogen ions bound to BIM is given by the expression

$$\bar{n}_L = C_S/C_B = \frac{k_2[H^+] + 2[H^+]^2}{k_1k_2 + k_2[H^+] + [H^+]^2}$$

Since $C_L = [B] + [BH^+] + [BH_2^{2+}] + \bar{n}C_M = C_B + \bar{n}C_M$, the term $\bar{n}$ (the average number of BIM molecules bound per metal atom) is given by the expression

$$\bar{n} = \frac{C_L - \dfrac{C_S}{\bar{n}_L}}{C_M}$$

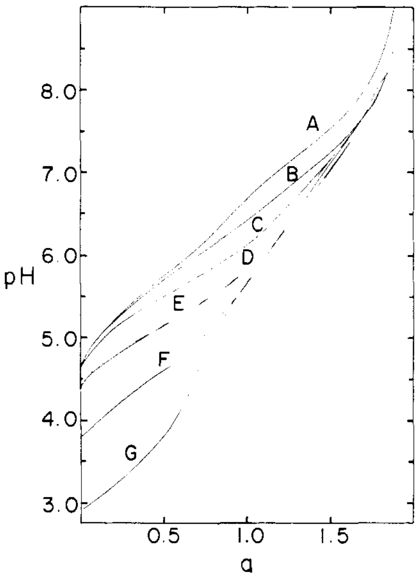

FIGURE 1: Potentiometric titration curves of BIM–metal ion systems. Curve A, BIM (no added metal ion); curve B, BIM-Mn(II); curve C, BIM-Fe(II); curve D, BIM-Zn(II); curve E, BIM-Co(II); curve F, BIM-Ni(II); curve G, BIM-Cu(II). The curve for the BIM-Cd(II) system is omitted because it falls on curves D and E. *a* = moles of base added per mole of ligand present.

The concentration of unprotonated, noncomplexed BIM is given by the expressions

$$[B] = \alpha C_S/\bar{n}_L$$

$$pB = \log \frac{\bar{n}_L}{\alpha} - \log C_S$$

The value of C_S was calculated from the data obtained during the titration, by subtracting the equivalents of OH^- added as KOH from the equivalents of H^+ added as HNO_3 (corrected for the ionization of water; this correction becomes negligible at *p*H values of 5 and above). Upon plotting $\bar{n}$ against *p*B, formation curves were obtained, and the values of the stability constants $\log K_1$ and $\log K_2$ were computed both by the "correction-term" method and least-squares treatment of Irving and Rossotti (1953). The terms $\log K_1$, $\log K_2$, and $\log \beta_2$ are used in accordance with the definitions given in Bjerrum *et al.* (1957) and Sillén and Martell (1964). A typical set of data is presented in Table I, for a titration of the BIM-Ni(II) system.

The data for the other metal ions were processed in a similar manner, and from the results the formation curves shown in Figures 2 and 3 were constructed. Because of the unavailability of ferrous nitrate, ferrous sulfate was used as a source of Fe(II). To gain some measure of the sulfate effect in the titration of a BIM–metal ion system, a comparison was made between zinc

TABLE I: Titration of Bis-imidazole in Presence of Ni(II).[a]

Volume (ml)	pH	C_S (mmoles)	$\bar{n}_L$	$\bar{n}$	α	pB
4.75	3.79	0.04932	1.985	0.33	3.66×10^{-6}	7.72
4.79	4.05	0.04584	1.973	0.66	1.23×10^{-5}	7.23
4.82	4.25	0.04276	1.958	0.94	2.96×10^{-5}	6.87
4.85	4.40	0.04052	1.939	1.13	5.94×10^{-5}	6.59
4.89	4.65	0.03591	1.900	1.54	1.80×10^{-4}	6.16
4.92	4.85	0.03293	1.850	1.76	4.26×10^{-4}	5.81
4.95	5.04	0.03048	1.786	1.91	9.46×10^{-4}	5.48
4.97	5.25	0.02811	1.692	2.00	2.20×10^{-3}	5.13
5.02	5.65	0.02365	1.452	2.07	9.21×10^{-3}	4.53
5.06	6.04	0.01962	1.230	2.14	3.16×10^{-2}	4.00
5.10	6.45	0.01533	1.023	2.34	9.11×10^{-2}	3.57
5.13	6.85	0.01203	0.828	2.43	2.14×10^{-1}	3.22
5.17	7.24	0.00836	0.605	2.58	4.09×10^{-1}	2.96
5.19	7.45	0.00643	0.476	2.63	5.31×10^{-1}	2.86

[a] The initial solution (4.75 ml) contained 1.0 ml 0.0265 M BIM, 0.5 ml 0.00981 M nickel nitrate, and 0.5 ml 0.100 M HNO_3. The increments in volume indicate the amount of 0.0993 N KOH added during the titration. The ionic strength was 0.16 in KNO_3. The terms at the head of the last five columns of the table are defined in the text.

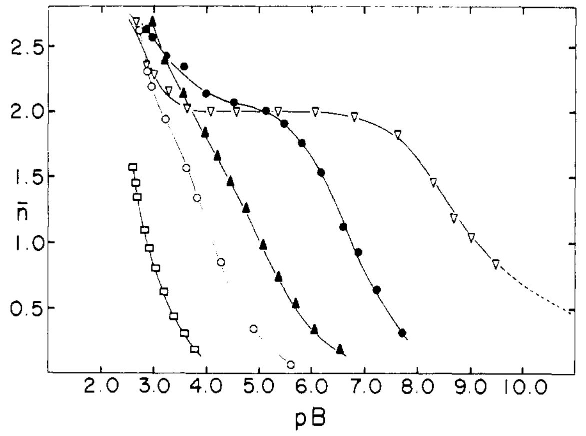

FIGURE 2: Formation curves of BIM-metal ion complexes. □, Mn(II); ○, Fe(II); ▲, Cd(II); ●, Ni(II); ▽, Cu(II).

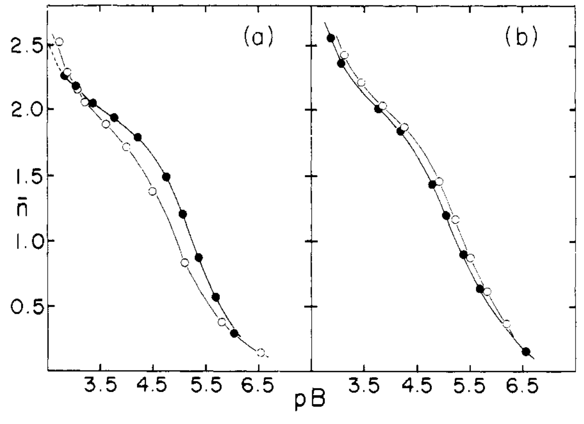

FIGURE 3: Formation curves of BIM-metal ion complexes. (a) ●, $Zn(NO_3)_2$; ○, $ZnSO_4$; (b) ●, Co(II), nitrogen; ○, Co(II), air.

sulfate and zinc nitrate. As can be seen from the formation curves in Figure 3a, the replacement of nitrate by sulfate caused a small effect in the direction of lower stability constants ($\Delta \log \beta_2 = 0.59$). This effect was judged to be insufficient to invalidate the results obtained with ferrous sulfate.

Results

The data for all the stability constants determined in the present work are collected in Table II. When the comparable stability constants for BIM-metal ion systems are plotted as a function of the atomic number of the metal (Irving and Williams, 1948), the expected order Mn < Fe < Co < Ni < Cu > Zn is obtained (Figure 4). For the determination of the magnitude of the chelation effect, the stability constants of the imidazole-Cu(II) system were determined under the same conditions as those employed for the BIM-Cu(II) system. The values obtained were $\log K_1 = 4.38$, $\log K_2 = 3.44$, $\log K_3 = 2.75$, $\log K_4 = 1.82$. Nozaki *et al.* (1957) reported values of 4.33, 3.54, 2.82, and 2.03 for the imidazole-Cu(II) system at $\gamma/2 = 0.15$–0.16 and 25°; the corresponding log K values for the 4(5)-methylimidazole-Cu(II) system under these conditions were reported to be 4.13, 3.49, 2.87, and 1.96, respectively.

The magnitude of the chelation effect in BIM-metal

TABLE II: Stability Constants of BIM–Metal Ion Complexes.

Metal Ion	log K_1	log K_2	log β_2
Mn(II)	2.96 ± 0.03	2.84 ± 0.05	5.80
Fe(II)[a]	4.47 ± 0.05	3.90 ± 0.04	8.37
Co(II)	5.72 ± 0.04	4.81 ± 0.03	10.53
Co(II)[b]	5.87 ± 0.03	4.90 ± 0.05	10.77
Ni(II)	7.33 ± 0.02	6.30 ± 0.03	13.63
Cu(II)	10.41 ± 0.04	8.18 ± 0.03	18.59
Zn(II)	5.62 ± 0.02	4.86 ± 0.03	10.48
Zn(II)[a]	5.45 ± 0.03	4.44 ± 0.02	9.89
Cd(II)	5.50 ± 0.05	4.55 ± 0.04	10.05

[a] The sulfate was used in the titration. [b] The titration was conducted in the presence of air.

ion systems is most clearly evident in the data for Cu(II). Comparison of the stability constant for $[CuBIM]^{2+}$ (log K_1 = 10.4) and for $[Cu(IM)_2]^{2+}$ (log β_2 = 7.8) indicates that the comparable complexes differ in stability constants by a factor of about $10^{2.6}$. The comparable data for the pair $[Cu(BIM)_2]^{2+}$ (log β_2 = 18.6) and $[Cu(IM)_4]^{2+}$ (log β_4 = 12.4) indicate an enhancement in stability for the 2:1 BIM-Cu(II) chelate of approximately $10^{6.2}$. The striking stability of BIM-Cu (II) complexes is further emphasized by the fact that the $[CuBIM]^{2+}$ complex is completely formed at *p*H 3.2, whereas formation of the $[Cu(IM)_2]^{2+}$ complex is not complete until *p*H 6.0. The formation of $[Cu(BIM)_2]^{2+}$ is complete at *p*H 4.7; the corresponding *p*H values for $[NiBIM]^{2+}$ and $[Ni(BIM)_2]^{2+}$ are 4.4 and 5.2, respectively.

It will be noted from Figures 2 and 3 that the potentiometric titrations indicate the release of protons at values of $\bar{n}$ greater than 2. Such behavior at alkaline *p*H values cannot in itself be interpreted as evidence of the formation of presumed Tris complexes. In the special case of Cu(II), where square planar complexes are to be expected, it has been reported that Tris complexes have been obtained with ethylenediamine (Gordon and Birdwhistell, 1959), as well as with 2,2′-dipyridyl and with 1,10-phenanthroline (Harris and Livingstone, 1964). Although the possibility exists, therefore, that Tris complexes of BIM are in fact formed with metal ions such as Cu(II), Ni(II), and Cd(II), as well as with Fe(II) and Co(II), where octahedral complexes are favored, equal consideration must be given to the occurrence of "olation" reactions (Rollinson, 1956; Gustafson and Martell, 1959). In the latter case, the addition of OH^- would cause the ionization of coordinated water molecules (sometimes termed "hydrolysis") in the bis-BIM complexes, with subsequent polymerization.

It should be added that, after the titration of the BIM-Co(II) system had been completed, exposure of the solution to air resulted in the appearance of a yellow-orange color, with an absorption maximum at 385 mμ (ϵ = 1700). Hearon *et al.* (1949) have shown that the complex formed anaerobically between Co(II) and two histidines combines reversibly with O_2 to form an oxygenated derivative with an absorption maximum at 385 mμ (ϵ = 1620); on prolonged oxygenation, the process gradually becomes irreversible, with probable oxidative decomposition of the organic ligand. Hearon *et al.* (1949) suggested that histidine might be decarboxylated to yield histamine; the oxidation of the imidazole ring is also a possibility, and merits additional study. The oxygenation of the Co(II) complex of 1,2-bis(salicylideneamino)ethane has also been described (Tsumaki, 1938). In the case of BIM, the oxygenation of the Co(II) complex was not found to be reversed upon bubbling N_2 through the solution. In Figure 3b are shown the formation curves for the BIM-Co(II) system from titrations performed in the presence and absence of air. The oxygenated complex has slightly higher stability constants, although the effect of oxygenation does not appear to be so pronounced as in the case of the histidine-Co(II) system studied by Hearon *et al.* (1949). It may be added that the apparent occurrence of an autoxidation reaction was also observed upon exposure of the BIM-Fe(II) system to air after the titration had been completed; a yellow precipitate formed rapidly, and the solution contained Fe(III) as judged by a test with potassium thiocyanate.

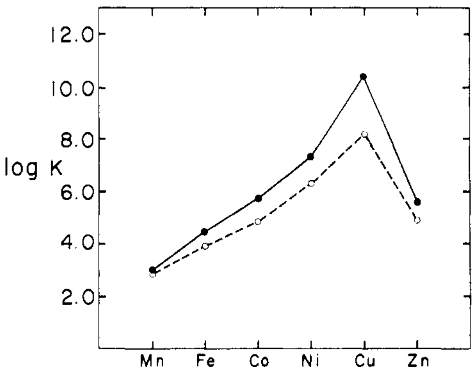

FIGURE 4: Irving-Williams plot of stability constants of BIM–metal ion systems. ———, log K_1; - - -, log K_2.

Discussion

The marked chelating ability of BIM is further indicated by comparison of its log β_2 values with those recorded in the literature for several other nitrogenous ligands (Table III). In particular, it will be noted that propane-1,3-diamine (pK_1' = 8.98, pK_2' = 10.72) which, like BIM, forms a six-membered chelate ring (Irving *et al.*, 1954), appears to form less stable complexes with Ni(II) and Cu(II) than does BIM. It may be concluded that the stereochemistry of the BIM–metal ion chelates and the π-bonding capacity of the imidazole rings strongly promote the stability of the chelates. In view of the markedly lower pK' values of BIM its metal chelates may be expected to be much more stable

TABLE III: Stability Constants of Metal Chelates.[a]

Ligand	log β_2 Ni(II)	Cu(II)	Zn(II)
Ethylenediamine	14.1	19.6	11.1
Propane-1,3-diamine	10.4	16.9	
2,2′-Bipyridyl	14.0	16.3	9.8
1,10-Phenanthroline	16.7	17.0	12.2
L-Histidine	15.8	18.6	12.9
Histidine	11.8	16.0	10.5
Carnosine[b]	5.4	9.7	
L-Histidyl-L-histidine[c]	13.3	12.0	8.2
BIM	13.6	18.6	10.5

[a] As the data listed in this table were obtained under different conditions of ionic strength (0.1–1.0) and temperature (20–30°), only approximate comparisons are valid. Except for BIM, and as noted for other ligands, the data were taken from Sillén and Martell (1964). [b] Data taken from Lenz and Martell (1964) for 1:1 complex ML^+. [c] Data for Cu(II) taken from Doran *et al.* (1964) for 1:1 complex CuL^+; data for 1:1 complexes of Ni(II) and Zn(II) taken from Sillén and Martell (1964).

at *p*H values below 7 than are the chelates involving the more basic ligand groups of propane-1,3-diamine.

Several groups of investigators have determined the stability constants of the metal ion complexes of L-histidine (Li *et al.*, 1957; Leberman and Rabin, 1959), histamine (Mickel and Andrews, 1955; Doran *et al.*, 1964), β-alanyl-L-histidine (Lenz and Martell, 1964), and L-histidyl-L-histidine (Martin and Edsall, 1960; Doran *et al.*, 1964) (Table III). Of particular interest is a comparison of BIM with L-histidyl-L-histidine. The data of Doran *et al.* (1964) for the 1:1 complex of Cu(II) with the dipeptide indicate a stability constant log K = 12.0 for the tridentate chelate considered to involve both imidazolyl groups and probably the α-amino group as ligands. The carboxylate groups and (at alkaline *p*H values) the peptide bond of the dipeptide provide additional ligand groups for chelation.

Studies are in progress on the isolation and characterization of the BIM–metal ion chelates, and the details will be reported in a subsequent communication. It may be noted here that the absorption spectrum of $[Cu(BIM)_2]^{2+}$ exhibits maxima at 568 and 752 mμ. The position of the major band at 568 mμ may be compared with the value of 600 mμ reported for the d-d band of $[Cu(IM)_4]^{2+}$ (Koltun *et al.*, 1959). The two-banded spectrum of $[Cu(BIM)_2]^{2+}$ in the region 500–800 mμ is of special interest in relation to the studies of Jørgensen (1955) on the absorption spectra of the Cu(II) complexes of 1,10-phenanthroline and of 2,2′-bipyridyl.

As indicated in the introduction, the primary aim of the present investigation has been to contribute to the study of the chelation of metal ions by imidazolyl groups suitably located in proteins. Several proteins have been found to bind metal ions more firmly than can be explained solely in terms of independent (1:1) interaction with potential ligand groups. In particular, the possibility of chelation involving imidazolyl groups in the binding of metal ions by proteins has been raised in the past for the binding of Zn(II) by insulin (Tanford and Epstein, 1954; see also Marcker, 1960) and enolase (Malmström and Westlund, 1956), of Mn(II) by bovine serum albumin (Mildvan and Cohn, 1963), and of Cu(II) by bovine serum albumin (Rao and Lal, 1958), ascorbic acid oxidase (Dawson, 1960), and myoglobin (Breslow and Gurd, 1963; Breslow, 1964). On the other hand, Gurd and Goodman (1952) concluded that the data on the binding of Zn(II) by human serum albumin could be satisfactorily interpreted in terms of 1:1 interaction of the metal ion with imidazolyl residues. The difficulties and uncertainties in the assignment of ligand groups in the interpretation of metal ion binding by proteins have been discussed by many authors, notably by Gurd and Wilcox (1956) and Malmström and Rosenberg (1959), and more recently by Breslow and Gurd (1963) and Breslow (1964). In particular, combination with metal ions may lead to conformation changes in the protein (Breslow and Gurd, 1963).

Of special interest in this connection is ribonuclease, where the imidazolyl groups of histidines 12 and 119 are held near each other (Crestfield *et al.*, 1963). The *p*H dependence of the K_m values of ribonuclease has suggested the involvement, in the active center of the enzyme, of two groups having *pK′* values of 5.2 and 6.8 (Herries *et al.*, 1962). These values are close to those (5.3 and 7.0) obtained for BIM in the absence of added salt (Drey and Fruton, 1965), and further support the suggestion that two imidazolyl groups of ribonuclease interact in the native protein. In the light of the results reported in the present communication, it clearly would be useful to examine the binding of metal ions by ribonuclease. To our knowledge, no equilibrium measurements of this kind have been recorded in the literature. Dr. E. Breslow (personal communication) has informed us, however, that studies on the binding of metal ions by ribonuclease are under way in her laboratory. The inhibition of ribonuclease by Zn(II) and Cu(II) has been studied by Davis and Allen (1955) and Ross *et al.* (1962). It will be of interest to learn whether some metal ions are bound to ribonuclease more tightly than can be accounted for solely by independent interaction with single imidazolyl groups.

Several chelating agents (1,10-phenanthroline, 2,2′-bipyridyl, EDTA, 8-hydroxyquinoline, and the like) have been examined for their ability to remove, or to combine with the metal component of various metalloproteins (Westerfeld, 1961). In view of the excellent chelating capacity of BIM, this bis-imidazole may be a useful addition to this group of reagents.

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