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Models for Metal Binding Sites in Zinc Enzymes. Syntheses of Tris[4(5)-imidazolyl]carbinol (4-TIC), Tris(2-imidazolyl)carbinol (2-TIC), and Related Ligands, and Studies on Metal Complex Binding Constants and Spectra

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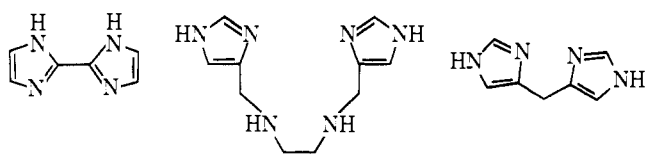
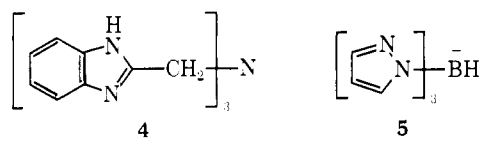
Abstract: Tris[4(5)imidazolyl]carbinol (4-TIC) and tris(2-imidazolyl)carbinol (2-TIC) have been synthesized as models for the zinc binding site of carbonic anhydrase and of alkaline phosphatase. Bis[4(5)imidazolyl]glycolic acid (4-BIG) has been synthesized to mimic the zinc binding site of carboxypeptidases and of thermolysin; bis[4(5)imidazolyl]carbinol (4-BIC) has also been synthesized. Basicities and metal binding constants have been determined for 4-TIC, 2-TIC, 4-BIG, and 4-BIC, and as well for the known bis(2-imidazolyl)methane (2-BIM) and 3-[bis(2-imidazolyl)]propionic acid (2-BIP). The data are compared with those reported for bis[4(5)imidazolyl]methane (4-BIM) and for human carbonic anhydrase B and carboxypeptidase A. 4-TIC and 2-TIC are tridentate ligands using three imidazole groups, but 4-TIC is more basic and a stronger metal complexing agent. The binding constants of 4-TIC are comparable to those of the enzymes for cobalt, nickel, and copper dications but not for zinc dication. Spectral and binding studies suggest that the geometry of 4-TIC is not quite right for a good mimic of carbonic anhydrase.

Extensive studies of various carbonic anhydrases[1] and alkaline phosphatases[2] indicate the presence of a catalytic Zn^{2+} bound to three imidazole residues of enzyme histidines. In the carboxypeptidases[3] and in thermolysin[4], the critical Zn^{2+} is bound to two imidazoles and a carboxylate group of the enzyme. In spite of the obvious interest such systems would have, few chelating ligands using imidazole rings have been made so far, and none which combine three simple imidazole rings as models for the metal binding sites of carbonic anhydrase.

Holmes et al.[5] have investigated metal binding by 2,2′-bis(imidazole) (**1**) while Gruenwedel[6] has studied Zn^{2+} and Co^{2+} binding by the tetradentate ligand **2.** An important study by Fruton[7] led to the synthesis and metal-binding constants for bis[4(5)-imidazolyl]methane (**3**), which we call 4-BIM. Fruton's synthesis, from histidine, is not adaptable for the preparation of related tris(imidazoles). Very recently,



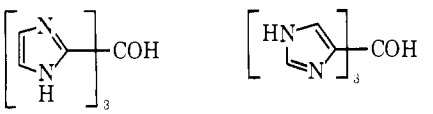
1 2 4-BIM (3)



4 5

Thompson et al.[8] have described some metal binding properties of a tris(benzimidazole) ligand system (**4**). Finally, the tris(pyrazolyl)borohydride ligand **5,** first reported by Trofimenko[9] but recently studied by Marks and Ibers,[10] is relevent to our studies.

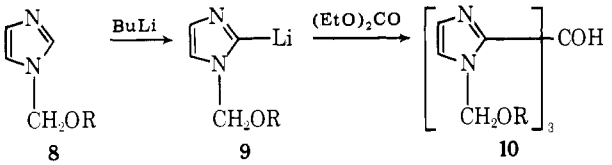
The x-ray studies[11] on carbonic anhydrase show that the three imidazole ligands have distorted tetrahedral coordination to the Zn^{2+}. Molecular models suggested that a similar geometry could be attained with a tris(imidazolyl)methane derivative. We now wish to report the synthesis of two isomeric chelating ligands, tris(2-imidazolyl)carbinol (2-TIC) (**6**) and



2-TIC (6) 4-TIC (7)

tris[4(5)-imidazolyl]carbinol (4-TIC) (**7**), and their metal binding properties.

Syntheses. Roe[12] and Shirley and Alley[13] have reported the metallation of *N*-benzylimidazole at C-2 with *n*-butyllithium, but in our hands significant benzylic lithiation also occurred. However, *N*-methoxymethylimidazole[14] (**8a**) and *N*-ethoxymethylimidazole (**8b**) were smoothly metallated at C-2; re-



8 9 10

HCl → 6

a, R = CH_3
b, R = C_2H_5

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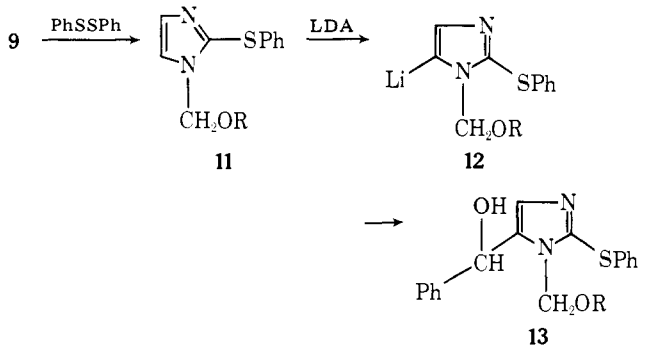
action of the lithio derivative **9b** with diethyl carbonate afforded the tris adduct **10b,** which with acid was deprotected to yield 2-TIC (**6**).

Basicity and metal-binding studies described below suggested that 4-TIC (**7**) would be a more interesting ligand. Its synthesis was more challenging. All attempts to make organometallic reagents from N-protected 4(5)-bromoimidazole[15] failed, leading either to reduction or to C-2 metallated derivatives. Thus we decided to block C-2. Reaction of **9a** with dimethyl disulfide afforded the 2-thiomethyl derivative, but with butyllithium this metallated at the methyl group of the thioether. Reaction of **9a** with diphenyl disulfide afforded the thiophenyl derivative **11a,** but this underwent sulfur–phenyl cleavage and sulfur–imidazole cleavage[16] with *n*-butyllithium or *tert*-butyllithium. However, lithium diisopropylamide proved strong enough[17] to metallate **11a** (and also **11b**) at C-5 without carbon–sulfur cleavage. The resulting lithio derivatives **12a** and **12b** could be added to various carbonyl compounds.

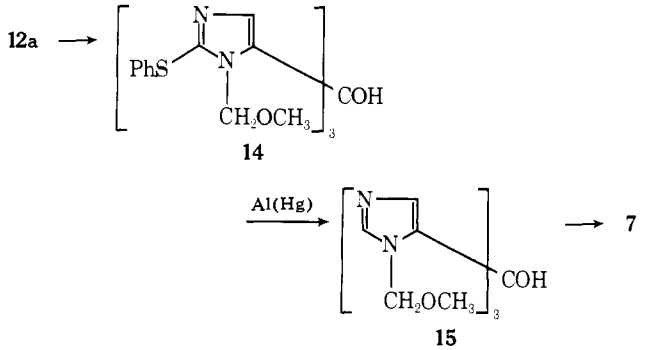


11 12 13

a, R = CH_3
b, R = C_2H_5

Reaction of **12a** with benzaldehyde afforded the adduct **13a** whose NMR spectrum was particularly revealing. In **13a** the CH_2 hydrogens on N appeared as an AB quartet, as expected since they are diastereotopic and close to the asymmetric phenylcarbinol group. If attachment had been at C-4 no significant splitting should have been seen, although the CH_2 hydrogens would of course still be diastereotopic. The metallation is apparently guided to C-5 by lithium coordination with the ether group.[18]

Reaction of **12** with diethyl carbonate yielded the tris adduct **14** in as much as 70% yield. Removal of the C-2 blocking group failed with Raney nickel, with $LiAlH_4$, with *n*-butyllithium, or with lithium in NH_3 or in $MeNH_2$. However, treatment of



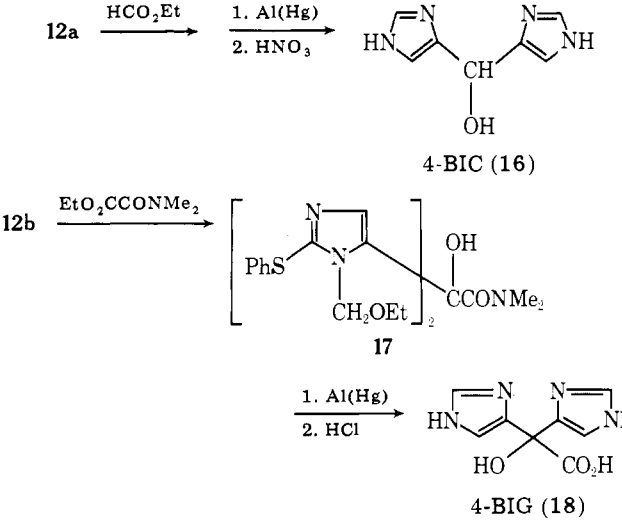
14 with aluminum amalgam[19] in aqueous ethanol gave good yields of **15.** This with HCl produced 4-TIC (**7**), which is of course a mixture of tautomers.

The lithio derivatives **9** and **12** are versatile synthetic intermediates. In particular, reaction of **12a** with ethyl formate (producing intermediates with diastereotopic CH_2's; cf. Experimental Section), then deblocking, produced bis[4(5)-imidazolyl]carbinol (4-BIC, **16**). This is a hydroxylated derivative of **3,** Fruton's 4-BIM. Reaction of **12b** with ethyloxalyl-*N*,*N*-dimethylamide afforded the bisadduct **17** which could be deblocked to produce bis[4(5)-imidazolyl]glycolic acid (4-BIG, **18**). This is of interest with respect to models for

Table I. pK_as of Imidazole Derivatives[a]

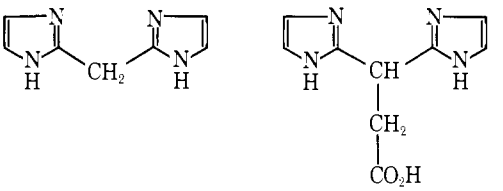
Compound	Structure	pK_1	pK_2	pK_3
Imidazole[b]		6.95 ± 0.02		
4-BIM[b]	**3**	7.39 ± 0.04	5.61 ± 0.02	
2-BIM	**19**	7.01 ± 0.03	4.83 ± 0.04	
4-BIC	**16**	6.89 ± 0.06	4.99 ± 0.03	
4-TIC	**7**	6.95 ± 0.01	5.23 ± 0.04	3.37 ± 0.02
2-TIC	**6**	6.12 ± 0.01	3.59 ± 0.01	<1.5
4-BIG	**18**	6.08 ± 0.04	3.92 ± 0.06	2.11 ± 0.13
2-BIP	**20**	7.11 ± 0.02	4.41 ± 0.05	1.90 ± 0.10

[a] At 25.0 °C and μ = 0.16. [b] Reference 7.



the Zn^{2+} binding site of the carboxypeptidases and thermolysin in which two imidazoles and a carboxylate are used.

Very recently chemists at ICI[20] have reported remarkable syntheses of bis(2-imidazolyl)methane (2-BIM, **19**) and of 3-[bis(2-imidazolyl)]propionic acid (2-BIP, **20**). We have

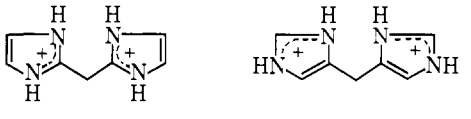


2-BIM (**19**) 2-BIP (**20**)

prepared these compounds by the reported procedures and incorporated them in our studies.

Basicities. Careful potentiometric titration and data analysis by a computer version[21] of the method of Simms[22] gave the pK_as listed in Table I. Several trends are apparent.

First of all, a comparison of 4-BIM with 2-BIM shows that an imidazole ring attached at C-4 is more basic, particularly



$[2\text{-BIM}\cdot H_2]^{2+}$ $[4\text{-BIM}\cdot H_2]^{2+}$

at pK_2. This can be understood in terms of charge separation. In the 2-isomer, all four nitrogens are close to each other. Diprotonation leads to strong electrostatic repulsion, and even in the monoprotonated 2-BIM the electronegative nitrogens of the other ring are destabilizing. By contrast, in protonated

Table II. pKs for Metal Binding[a]

Ligand	Structure	Zn^{2+}	Co^{2+}	Ni^{2+}	Cu^{2+}	
4-BIM	**3**	5.62 ± 0.02	5.72 ± 0.04	7.33 ± 0.02	10.41 ± 0.04	pK_1
		4.86 ± 0.03	4.81 ± 0.03	6.30 ± 0.03	8.18 ± 0.03	pK_2
2-BIM	**19**	5.73 ± 0.04	6.02 ± 0.05			pK_1
		4.98 ± 0.06	4.90 ± 0.07			pK_2
4-TIC	**7**	8.47 ± 0.14	9.06 ± 0.05	10.85 ± 0.13	11.51 ± 0.02	pK_1
		6.58 ± 0.15	7.78 ± 0.05	9.98 ± 0.14	7.93 ± 0.14	pK_2
2-TIC	**6**	6.77 ± 0.05	7.20 ± 0.02		7.52 ± 0.01	pK_1
		6.12 ± 0.09	5.92 ± 0.09		6.15 ± 0.02	pK_2
4-BIC	**16**	6.65 ± 0.10	6.51 ± 0.01			pK_1
		4.70 ± 0.04	4.07 ± 0.08			pK_2
4-BIG	**18**	6.03 ± 0.11	6.10 ± 0.07	7.57 ± 0.09	8.46 ± 0.09	pK_1
		4.01 ± 0.02	3.70 ± 0.03	5.24 ± 0.08	6.61 ± 0.05	pK_2
2-BIP	**20**	6.01 ± 0.03	6.20 ± 0.07			pK_1
		4.74 ± 0.05	4.13 ± 0.06			pK_2
Human carbonic anhydrase B,[c] pH 5.5		10.5	7.20	9.5	11.6	pK_1
Apocarboxypeptidase A,[d] pH 8.0		10.5	7.0	8.2	10.6	pK_1

[a] Negative logarithms of dissociation constants for the 1:1 and 2:1 ligand–metal complexes at 25.0 °C and μ = 0.16 (KNO_3). [b] Data from ref 7. [c] Data from ref 27. [d] Data from ref 28.

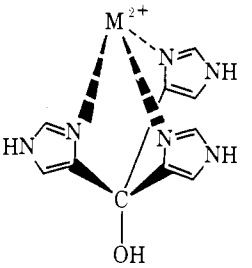
4-BIM the positive charge is shared with a nitrogen which is further from the other ring. A similar trend is seen in the comparison of 4-TIC with 2-TIC. Indeed, with 2-TIC the third protonation was not detected. A related finding for pyridylimidazoles was reported by Holmes et al.[23]

Further, the hydroxyl group in 4-BIC is base weakening, and more or less by the same amount at pK_1 and pK_2. This will be of interest with respect to metal binding by 4-BIC. Finally, the carboxyl group of 4-BIG is base weakening (by comparison with 4-BIC), while the more distant carboxyl of 2-BIP has little effect (judged by comparison with 2-BIM). Both these carboxyls are themselves quite acidic (pK_3).

Metal Binding Constants. Titration of the ligands in the presence of metal salts is a general method[24] for determining metal binding constants. We used the conditions described by Fruton[7] so that our results could be compared with his. Titration with an excess of ligand permitted us to determine both K_1 (formation of 1:1 complex) and K_2 (formation of 2:1 ligand to metal complex), the values listed in Table II. Our data were analyzed by a computer program[21] written for the purpose which successfully reproduced the results reported by Drey and Fruton,[7] Albert,[25] and Albert and Serjeant[26] from their raw data. The titrations occurred at such low pH, because of the strong binding, that no other ionizations occurred (e.g., of metal-bound water). Our data for Cu^{2+} were with $CuSO_4$; although sulfate titration could interfere, the same values were found for **7** using $Cu(NO_3)_2$.

Comparison of the binding constants in Table II reveals a number of effects. First of all, 4-TIC is a better ligand than is 2-TIC for all metals, and at both K_1 and K_2. This must reflect the same geometric situation outlined above for basicities: coordination of metal to the imidazole rings induces positive charge on the coordinated and also on the uncoordinated nitrogens. It is interesting in this respect that the x-ray structures of carbonic anhydrase, of carboxypeptidase, and of thermolysin show that in the imidazoles coordinated to Zn^{2+} the uncoordinated nitrogens are far apart, as in 4-TIC.

Curiously, the same effect is not seen in comparing 4-BIM with 2-BIM. The greater basicity of 4-BIM (Table I) does not lead to stronger metal binding. Although the uncertainty in comparing data from different laboratories[29] cannot be discounted, there are possible geometric explanations for all this. The essential point is that protonated 4-BIM and 2-BIM are free to twist, while in the metal complexes the rings are locked coplanar (with the uncoordinated nitrogens as distant as possible). By contrast, in 4-TIC and 2-TIC steric crowding maintains a similar geometry for protonated or for complexed ligand.



Comparison of 4-TIC with 4-BIC or 4-BIM shows clearly that 4-TIC is a tridentate ligand using all three imidazole rings for coordination to Zn^{2+}, Co^{2+}, or Ni^{2+}. All three metals also bind a second ligand well, going to octahedral coordination. With Cu^{2+} the evidence is less clear. Comparison of 4-TIC with 4-BIM suggests that the third imidazole in 4-TIC contributes only a little extra; Cu^{2+} prefers square planar coordination with only weak additional binding by axial ligands,[30] and 4-TIC can occupy only two planar and one axial positions. Thus, the 2:1 complex of Cu^{2+} with 4-TIC is only a little more stable ($pK_1 + pK_2$) than is that for 4-BIM. With Zn^{2+}, Co^{2+}, or Ni^{2+} the 2:1 complex with 4-TIC is strongly stabilized. Similar conclusions can be drawn for 2-TIC, although it is a weaker ligand.

The hydroxyl group of 4-BIC is probably a third ligand. Thus, K_1 for Zn^{2+} and Co^{2+} shows stronger binding by 4-BIC than by 4-BIM, despite lesser basicity (Table I). Glycolic acid is known to bind metals weakly with its hydroxyl group[31] in addition to the carboxyl group.

The binding constants with 4-BIG and 2-BIP show little evidence for carboxylate coordination. 4-BIG is not as good as 4-BIC, suggesting that in 4-BIG the third ligand could be the hydroxyl group. In 2-BIP, compared with 2-BIM, only minimal effects of the carboxyl may be discernible. Of course, the pK_as (Table I) show that these carboxylate ions are *very* weak bases.

Comparison with the two enzymes is instructive. 4-TIC is certainly comparable with carbonic anhydrase in its affinity for Co^{2+}, Ni^{2+}, and Cu^{2+}, but not for Zn^{2+}. The preference of the two enzymes for Zn^{2+} over Co^{2+} or Ni^{2+} is actually not mirrored in any of our ligands. Although various factors can be invoked, we believe that the imidazole rings in 4-TIC may be a little too close, favoring octahedral (50% coverage of the metal sphere by a single molecule of 4-TIC) rather than tetrahedral (75% coverage) coordination. This is also revealed in the high second binding constants for our ligands. The other comparison, with carboxypeptidase, shows that the enzyme

gets more from the carboxylate ligand than we do in either 4-BIG or 4-BIP.

Finally, it should be noted that the metal binding constants for 4-TIC in Table II are higher than those of common chelating ligands (except polydentate EDTA). Thus, these new ligands may also be of interest in areas other than biomimetic chemistry.

Spectra. Extensive studies have been made of the electronic spectra of Co^{2+}-substituted zinc enzymes, in both the visible and near-infrared region.[32] The general situation is that octahedral Co^{2+} is pink, and tetrahedral or pentacoordinate Co^{2+} is blue or violet. While detailed studies are not yet complete for our compounds, some rough qualitative observations can be reported. In water at pH 5.0 the pink hexaaquocobaltous ion forms a pink 1:1 (and 2:1) complex with 4-BIG (**18**) and with 4-TIC. Apparently these ligands displace three waters, but the Co^{2+} stays hexacoordinate. When the pH is raised to 7.5–8.0 the pink 1:1 Co^{2+} complex with 4-BIG changes to violet, with peaks at 525, 550, and 590 nm. This suggests that titration of a water produces a ligand so basic that the other waters are released, so the Co^{2+} complex carries only 4-BIG and a hydroxide and is tetracoordinate.

In nonaqueous solution the situation is different. The Co^{2+} solvate itself is blue in hexamethylphosphoramide, dimethylacetamide, or isopropyl alcohol, indicating that six bulky solvents cannot fit around Co^{2+}. The 1:1 complex with 4-BIG, with 2-BIP (**20**), or with 2-BIM (**19**) is also blue, but the 2:1 complexes with 2-BIP or 2-BIM are pink in hexamethylphosphoramide. As expected the 2:1 complex with 2-BIP can be octahedral, since the solvent is not involved. Apparently with 2-BIM, a bidentate ligand, the two molecules of ligand occupy positions (equatorial?) which permit coordination of two additional solvents.

More detailed studies will be needed to clarify all this. In any event, it is already clear that the Co^{2+} complex with 4-TIC or with 4-BIG in nonbasic water solution does not have the blue color characteristic of tetracoordinate Co^{2+}, as in carbonic anhydrase or carboxypeptidase complexes of Co^{2+}. Although the nonaqueous solvents may be better mirrors of the enzyme interior, we believe that the differences in spectra of Co^{2+} complexes with our ligands and with the enzymes reflect a deficiency in our ligands. They are a bit too small, so that octahedral complexing is facile. A somewhat larger ligand related to 4-TIC might well mimic better both the spectroscopic behavior of carbonic anhyrase and also the extraordinary Zn^{2+} affinity of the enzyme.

Experimental Section[33]

Tris[2-(*N*-methylimidazolyl)]carbinol. A solution of 2.2 g of *N*-methylimidazole in 40 mL of ether under N_2 was cooled to −60 °C (suspension) and treated with 11 mL of 2.4 M *n*-butyllithium in hexane for 1 h. Then 0.925 g of diethyl carbonate was added, the mixture was warmed to 10 °C over 2.5 h, and the reaction mixture was quenched with water. Isolation by continuous extraction with ethyl acetate and two crystallizations from benzene afforded 644 mg (30%) of the product as white plates, mp 177.5–179.5 °C, characterized by spectra and analysis. Anal. ($C_{13}H_{16}N_6O$) C, H, N.

Bis[2-(*N*-methylimidazolyl)]carbinol. This was prepared in a similar fashion, but using ethyl formate instead of diethyl carbonate. The carbinol was isolated as white needles, mp 188–189.5 °C, in 44% yields, and characterized by spectra and analysis. Anal. ($C_9H_{12}N_4O$) C, H, N.

***N*-Ethoxymethylimidazole (8b).** A stirred suspension of imidazole (120 g, 1.55 mol) in 400 mL of benzene was treated with chloromethyl ethyl ether (80.5 g, 0.81 mol) in 200 mL of benzene for 2 h (room temperature). Distillation afforded a fraction of bp 60–100 °C (0.2 Torr) which was redistilled to yield 87 g (85%) of **8b**, bp 75–77 °C (1 Torr).

Tris(2-imidazolyl)carbinol, 2-TIC (6). Treatment of 2.54 g (20 mmol) of **8b** in 70 mL of dry THF at −60 °C with 10 mL (20 mmol) of 2.0 M *n*-butyllithium in hexane for 45 min to form **9b**, followed by addition of 0.81 mL (7 mmol) of diethyl carbonate and warming to room temperature, was followed by quenching and several ethyl acetate extractions. Crystallization from ether–hexane gave 50–70% yields of **10b**, mp 101–103 °C, characterized by spectra and analysis. Anal. ($C_{19}H_{28}N_6O_4$) C, H, N.

A solution of 942 mg of **10b** in 100 mL of 50% aqueous ethanol with 40 mL of concentrated HCl was heated at reflux for several hours. Solvent removal in vacuo and trituration with methanol–chloroform afforded 2-TIC (**6**) trihydrochloride, mp 172 °C dec; ^1H NMR δ 7.67 (s) (CH_3CN–D_2O).

***N*-Methoxymethyl-2-phenylmercaptoimidazole (11a) and *N*-Ethoxymethyl-2-phenylmercaptoimidazole (11b).** The solution of **9b** prepared as above was transferred into 1 equiv of diphenyl disulfide as a 10% solution in THF at −50 °C. After 2 h at −70 °C, then warming to room temperature and quenching, the product was isolated by ether extraction and distilled to afford **11b** as a colorless oil, bp 128–130 °C (0.2 Torr). In a similar fashion the *N*-methoxymethyl analogue **11a** was prepared from known[12] *N*-methoxymethylimidazole (**8a**).

5-[*N*-Methoxymethyl-2-phenylmercapto]imidazolylphenylcarbinol (13a). A solution of lithium diisopropylamide was prepared from 3 mL of THF, 0.15 ml of diisopropylamine, and 0.5 mL of 2.10 M *n*-butyllithium in hexane, and added dropwise to 220 mg of **11a** in 8 mL of THF at −60 °C under N_2 to produce **12a.** After 25 min benzaldehyde (0.11 mL) was added and the mixture was allowed to warm to room temperature. Extraction and chromatography afforded **13a** as white cubes from ethyl acetate–hexane, mp 103–105 °C, in 38% yield. The ^1H NMR spectrum showed the expected signals, including an AB quartet for the CH_2 at δ 5.25 and 5.33.

Tris[4(5)imidazolyl]carbinol, 4-TIC (7). A solution of **12a** prepared as above from 25 mmol of **11a** and 24.55 mmol of LDA was treated with 7.58 mmol of diethyl carbonate. Ether extraction and silica chromatography afforded 709 mg of recovered **11a** and 3.77 g (70%) of **14**, mp 139.5–142 °C. Anal. ($C_{34}H_{34}N_6O_4S_3$) C, H, N.

A solution of 2.323 g of **14** in 250 mL of 15% aqueous ethanol was stirred while 10.4 g of aluminum amalgam, prepared by either of two literature methods,[34,35] was added in 1.2–1.3-g portions over 13 h. The product was filtered (Celite, ethanol washing) and isolated by silica chromatography yielding 61–92% of **15** as a white gum.

Heating 900 mg of **15** in 90 mL of 1:1 ethanol–water with 15 mL of concentrated HCl for 8.5 h, evaporation, and crystallization from methanol–chloroform afforded 355 mg (42%) of **7** as the trihydrochloride, mp 165 °C dec. Anal. ($C_{10}H_{13}N_6OCl_3$) C, H, N.

Bis[4(5)-imidazolyl]carbinol, 4-BIC (16). In a similar fashion to that above 12 mmol of **11a** was converted to **12a** and treated with 6 mmol of ethyl formate. The adduct had mp 99–101 °C, and diastereotopic CH_2 ^1H NMR signals at δ 5.28 and 5.48. The product from aluminum amalgam treatment also had diastereotopic CH_2 signals at δ 5.13 and 5.33. 4-BIC (**16**) was prepared as the dinitrate, mp 148–150 °C dec.

Bis[4(5)imidazolyl]glycolic Acid, 4-BIG (18). A solution of **12b** from 23 mmol of **11b** was treated with 11.5 mmol of ethyl *N,N*-dimethyl oxamate. The product **17** was isolated by chromatography, and desulfurized with aluminum amalgam to afford the intermediate, mp 147–148 °C, in 40% yield. [Anal. ($C_{16}H_{25}N_5O_4$) C, H, N.] This was cleaved by heating 702 mg in 20 mL of 70% aqueous ethanol with 1 mL of concentrated HCl for 6 h. Evaporation and crystallization from methanol–chloroform afforded **18** as the dihydrochloride, mp 170–172 °C dec.

pK_a Determinations. Triplicate titrations were performed in a thermostated cell (25.0 ± 0.1 °C) under argon. A Radiometer TTT 60 pH meter, Radiometer G222C glass electrode, K 4112 calomel electrode, and digital calibrated buret were used. Linearity of the electrodes was checked before each run with standard buffers, and freshly standardized 0.1 N NaOH was employed. The initial volume was 4.50 mL, and was 0.15 M in KNO_3 and ca. 5 mM in substrate. All data were read directly from the digital pH meter and buret.

A computer version[21] of Simm's method[22] was used for data analysis. Similar titration of histidine hydrochloride gave pK_as of 6.22 and 9.11 (reported[36] 6.05 and 9.12). The data are listed in Table I.

Metal Binding Constants. Stock solutions of $Co(NO_3)_2$, $Ni(NO_3)_2$, $CuSO_4$, $Cu(NO_3)_2$, and $Zn(NO_3)_2$ were prepared from reagent grade hydrate salts and assayed by atomic absorption spectroscopy. A three- to fourfold excess of ligand to metal was used and the solution titrated as above. When needed, standard HNO_3 was added before titration to reduce $\bar{n}$. Triplicate titrations were averaged, and the data were

analyzed by a computer program[21] using equations we have derived for complexation of tribasic species which are identical with the equations of Albert.[25] Our program was able to reproduce literature results from published data.[7,25,26] Stability constant calculation involves a determination of $\bar{n}$, the average number of ligands bound per metal. We used from 7 to 11 values of $\bar{n}$ between 0.3 and 0.7 to determine K_1, and an equivalent number between 1.3 and 1.7 to determine K_2.[33] The results are listed in Table II.

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Interactions of Melanin with Metal Ions. Electron Spin Resonance Evidence for Chelate Complexes of Metal Ions with Free Radicals

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Abstract: ESR experiments reveal, for the first time, that di- and tripositive metal ions bind to *o*-semiquinone radical centers within melanin polymers to yield chelate complexes. The binding is often accompanied by large increases in total radical concentration, which are considered to arise via metal ion induced shifts in a comproportionation equilibrium. The effects observed are likely to be general for radicals with chelating ability, and may find use in the separation and identification of composite ESR signals in other biological systems.

There have been several reports that naturally occurring melanin from various sources will incorporate a variety of metal ions, and that synthetic melanin has a similar affinity for these same ions.[2,3] This similarity has led to the suggestion[2] that the protein associated with natural melanins is not of major importance in metal ion binding.

The melanin polymer is heterogeneous[4] and contains carboxyl, quinol, and amine groups which have been suggested to be the binding sites. From similarities in metal ion affinity between melanins and carboxylic ion exchange resins it has been proposed[2,3] that at both pH 5.0[3] and 7.6[2] melanin metal binding is a function of free carboxyl groups within the polymer. However, it seems likely that other groups also can participate in binding. Thus, dependent upon pH, L-Dopa, a melanin precursor with many of the structural features attributed to the polymer, appears[5,6] to form at least three kinds

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