The Structure of the Copper(II)–L-Histidine 1:2 Complex in Solution[1]

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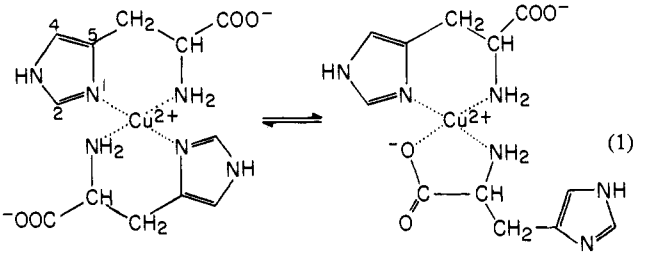
Abstract: The tridentate ligand, histidine, can coordinate to Cu^{2+} ion in a histamine- or glycine-like way. By means of nmr spectroscopy and the line-broadening effect of a paramagnetic metal ion, the structure of the Cu^{2+}–L-histidine 1:2 complex in aqueous solution (D_2O) at pD 8.1 was investigated. The experimental data suggest that an equilibrium exists between a complex species where both histidines are bound histamine-like and another predominant species where one histidine is bound histamine-like and the other glycine-like. These results were obtained by measuring the effects of Cu^{2+} in a histidine system in comparison with systems containing the partially blocked ligands, L-histidinol, histamine, and the L-1- and L-3-methylhistidines. The line broadening in systems containing histamine or L-histidinol is greater and with L-1-methylhistidine smaller than that observed in the histidine system. The results obtained in solutions containing the mixed systems, L-histidine–L-histidinol = 1:1, and L-histidine–L-1-methylhistidine = 1:1, are in agreement with the suggested structure of the Cu^{2+}–histidine 1:2 complex. L-Histidine acts in the first case as a glycine-like and in the second as a histamine-like ligand. In both cases, the same line broadening is observed as in the L-histidine system alone. The evidence given for the "mixed" structure of the Cu^{2+}–L-histidine 1:2 complex in solution is in agreement and, in part, even further supported by results described in the literature.

Histidine is a tridentate ligand that has an amino, imidazole, and carboxylate group as metal ion binding sites, but only a metal ion with an octahedral coordination sphere can form a tridentate chelate. With metal ions having a square-planar coordination sphere, for steric reasons, only two of the three binding sites can coordinate, *i.e.*, either histamine-like or glycine-like complexes have to be formed.

These facts led to contrary suggestions for the structure of Cu^{2+}–histidine complexes, because Cu^{2+} in aqueous solution prefers a square-planar (or grossly distorted octahedral) coordination sphere, as can be judged from the stabilities[2,3] of its complexes and their catalytic behavior.[4] Recently, by using the peroxidase-like activity of Cu^{2+} as a probe,[4] it was shown that the imidazole group of histidine participates in formation of the 1:1 complex with Cu^{2+}. This suggests a histamine-like coordination.[5] In addition, data from nmr[5] and ORD[6] measurements revealed that the imidazole group is also involved in formation of the Cu^{2+}–histidine 1:2 complex. These results are in contrast to an earlier paper[7] that suggested the formation of purely glycine-like complexes, but are in agreement with a recent X-ray crystallographic analysis[8] of the mixed L-histidinato–Cu^{2+}–L-threoninato–aquo complex. The latter showed that histidine binds to the coordination square of Cu^{2+} in a histamine-like manner.

In a very recent paper,[9] it was suggested that in the Cu^{2+}–histidine 1:2 complex, one histidine may coordinate histamine-like and the other glycine-like. As histidine complexes are important in biological systems, it is desirable to know their structure in solution. From the earlier investigations,[5,6] it is quite clear that an imidazole group is involved in the 1:2 complex; therefore, structures of the two complexes given in equilibrium 1 had to be considered in the present study. The concentration of a complex with a structure where both histidines are coordinated in a glycine-like manner can be considered as low even from the beginning.[9]



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For the investigation of complex structures in solution, nmr spectroscopy is a useful tool. Because of proton relaxation effects, complexation with a paramagnetic metal ion, *e.g.*, Cu^{2+}, results in a selective broadening of the signals from protons sufficiently close to the binding sites.[10] Due to rapid exchange be-

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(2) L. G. Sillén and A. E. Martell, *Chem. Soc. Spec. Publ.*, **No. 17** (1964).

(3) D. D. Perrin, I. G. Sayce, and V. S. Sharma, *J. Chem. Soc. A*, 1755 (1967).

(4) H. Sigel, *Angew. Chem.*, **81**, 161 (1969); *Agnew. Chem., Int. Ed. Engl.*, **8**, 167 (1969).

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(10) M. Cohn and T. R. Hughes, Jr., *J. Biol. Chem.*, **237**, 176 (1962); T. J. Swift and R. E. Connick, *J. Chem. Phys.*, **37**, 307 (1962); R. Mathur and N. C. Li, *J. Amer. Chem. Soc.*, **86**, 1289 (1964).

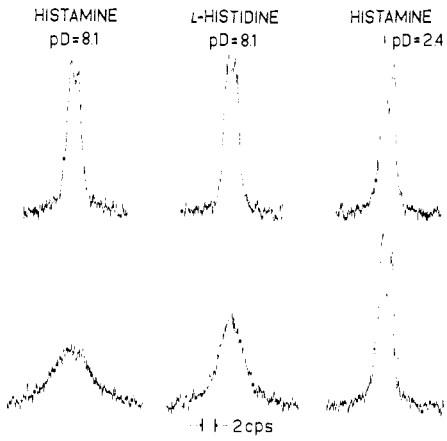

Figure 1. Proton magnetic resonance signals of the proton at C-2 of the imidazole ring in histamine and L-histidine taken in 0.2 *M* solutions of the ligands alone (upper part) and in the presence of 10^{-4} *M* $Cu(ClO_4)_2$ (lower part) in D_2O at 37°.

tween the complexed and free ligand, a trace of the paramagnetic ion is sufficient to broaden the signals from protons adjacent to the binding sites. Therefore, Cu^{2+}-elicited line broadening of the signal due to the hydrogen bound at the carbon in position 2 was measured (for numbering see equilibrium 1). To be able to judge the effect of Cu^{2+} on the hydrogen atoms of bonded and free ligand (or ligand binding sites), and thereby draw conclusions with regard to equilibrium 1, not only L-histidine was investigated but also the following derivatives wherein a part of the binding sites is selectively blocked: L-histidinol, histamine, L-1-methylhistidine, and L-3-methylhistidine. Hence, the present study encompasses the system where a ligand (histidine) may assume more than one conformation, as well as systems (histidine plus derivatives) that involve two different, though similar, ligands.

Experimental Section

Materials. L-Histidine monohydrochloride was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. L-Histidinol dihydrochloride, L-1-methylhistidine monohydrate, and L-3-methylhistidine were from Calbiochem, Los Angeles, Calif. Histamine dihydrochloride, $Cu(ClO_4)_2 \cdot 6H_2O$, and D_2O (99.75%) were from Fluka AG, Buchs, Switzerland. Sodium 3-(trimethylsilyl)propanesulfonate was from Merck AG, Darmstadt, Germany.

Solutions of NaOD and D_2SO_4 were prepared by dissolving NaOH and H_2SO_4, respectively, in D_2O.

Nmr Experiments. The nuclear magnetic resonance spectra were taken in 0.2 *M* solutions of the ligands in D_2O, usually at pD ~ 8.1 (adjusted with NaOD and D_2SO_4 and a Metrohm potentiometer E 353), with a Varian NMR spectrometer A-60 at 37°. The assignments of the signals were done as earlier;[5] sodium 3-(trimethylsilyl)propanesulfonate was used as an internal standard (0.02 *M*).

In all cases, the readings of the pH meter plus 0.4 were used to calculate pD according to the relationship: pD = pH + 0.4.[11]

Results

All the ligands investigated here bind Cu^{2+} either in a histamine-like or a glycine-like way. A comparison of the stability constants of the Cu^{2+} complexes of glycine or histamine shows that the glycine complex is a little less stable.[2] To be on the safe side, it is reasonable to use the constants of a substituted amino acid for the calculation. In an aqueous solution which is 0.2 *M* in *N*-ε-acetyl-L-lysine[12] and 10^{-4} *M* in Cu^{2+} and has a pH of 8.0, Cu^{2+} exists completely as the Cu^{2+}–amino acid 1:2 complex (CuL_2). As this calculation was done for the extreme limits, this means that under our experimental conditions with all the ligands used, the added Cu^{2+} is in the form CuL_2 (*cf.* also ref 5). This equality in degree of complex formation is a preferred condition for a meaningful comparison of the effect of Cu^{2+} on the line width in the nmr spectra of the several ligands.

In addition, the exchange rate of the metal ion according to equilibrium 2 can be assumed to be the same for those complexes where the imidazole group is in-

$$LCuL + L^* \longrightarrow LCuL^* + L \qquad (2)$$

volved, as the ligands have the same substituent, $-CH_2-CH(NH_2)R$, at the imidazole ring.[13] Therefore, the observed differences in line broadening of the signals of H at position 2 (and 4) are not due to different exchange rates or different degrees of complex formation; they are due to different degrees of participation of the imidazole group in complex formation. This means the line broadening effect in the presence of Cu^{2+} is proportional to the concentration of those complex species where an imidazole ring is involved.

In Figure 1, as an example, the nmr signals due to H-2 of histamine and histidine are shown in the absence and presence of 10^{-4} *M* Cu^{2+}. If the metal ion is not bonded to the ligands, there is no evident effect on the shape of the signal, as the example with histamine at pD 2.4 shows. However, if the pD is raised to 8.1, the effect of the paramagnetic metal ion on the signal is quite remarkable in the case of histidine and dramatic with histamine.[14] Already this result evidently suggests a less pronounced participation of the imidazole ring in the complex formation of histidine with Cu^{2+}.

The line widths of the signals due to the hydrogens of the imidazole group of the ligands studied in the absence and presence of Cu^{2+} are given in Table I. As expected with increasing amounts of the paramagnetic metal ion, the signals due to the protons of the imidazole ring are increasingly broadened. A comparison of the data reveals that the involvement of the imidazole group in complex formation with Cu^{2+} decreases in the order: L-histidinol ~ histamine > L-histidine > L-1-methylhistidine.

The data in Table I show for all cases that the proton on C-4 is about as strongly influenced as is that on C-2. This is due to the known[15] formation of π bonds between the d orbitals of Cu^{2+} and the π orbitals of the aromatic imidazole group.[16] That the formation of such π bonds (back donation) is important also became obvious from studies of the stability of mixed-ligand Cu^{2+} complexes containing the imidazole group.[17]

(11) R. Lumry, E. L. Smith, and R. R. Glantz, *J. Amer. Chem. Soc.*, 73, 4330 (1951).

(12) The constants are: $pK_{H_2L}^{H} \approx 2.24$; $pK_{HL}^{H} = 9.63$; $\log k_{CuL}^{Cu} = 8.09$; and $\log K_{CuL_2}^{CuL} = 6.83$. These data are taken from R. Griesser, B. Prijs, H. Sigel, W. Föry, L. D. Wright, and D. B. McCormick, *Biochemistry*, 9, 3285 (1970).

(13) Except for L-1- and L-3-methylhistidine; the additional substituent at the imidazole ring may have an influence.

(14) It is of interest to note in this connection that the line broadening by Cu^{2+} of the signals due to $-CH_2(NH_2)$ of histamine is less than that due to $-CH(NH_2)COO^-$ of histidine.[5]

(15) W. J. Eilbeck, F. Holmes, G. G. Phillips, and A. E. Underhill, *J. Chem. Soc. A*, 1161 (1967).

(16) Another explanation could be the presence of complexes wherein the metal ion is bound to N-3 and the proton to N-1, but this is highly improbable, since coordination to N-3 would exclude formation of a chelate. Quite generally, the complexes formed with histidine or histamine are much more stable than the simple corresponding imidazole complexes.

(17) P. R. Huber, R. Griesser, and H. Sigel, *Inorg. Chem.*, in press.

Table I. Line Widths of Nmr Spectra[a] of L-Histidine and Histidine Derivatives with Increasing Amounts of Cu^{2+}

Ligand[b]	Line widths[c] in cps in the presence of $[Cu^{2+}]_{tot}$ = 0	5×10^{-5}	10^{-4}	2×10^{-4}
L-Histidinol	2.80 (2.84)	6.2 (6.0)	9.5 (10.4)	19 (19)
Histamine	2.39 (2.61)	4.1 (4.6)	7.1 (7.2)	15 (14)
L-Histidine	2.62 (2.42)	3.2 (3.4)	4.6 (4.5)	7.4 (7.4)
L-Histidine; L-1-methylhistidine[d,e]	5.10 (5.36)	6.2 (7.4)	8.8 (8.6)	14.8 (11.0)
L-Histidine; L-histidinol[d,f]	2.60 (3.10)	3.2 (3.9)	4.2 (4.7)	6.8 (7.0)
Histamine (pD = 2.4)	2.44 (2.70)	2.50 (2.84)	2.66 (3.10)	2.80 (3.22)
L-1-Methylhistidine	2.82 (2.75)	3.26 (3.02)	3.94 (4.00)	5.5 (5.5)
L-3-Methylhistidine	2.94 (3.02)	16 (16)	~27 (~27)	

[a] The numbers given are due to the signal of H-2 of the imidazole group; the numbers due to H-4 are given in parentheses. [b] If no other condition is mentioned, [ligand] = 0.2 *M*, pD = 8.1, and $t = 37°$. [c] Measured at the half-height of the peaks on an expanded scale (100 cps). [d] The ratio of 0.1 *M* ligands at pD 8.1 was 1:1. [e] The sum of the line width due to the signals of the two ligands is given. [f] The signals due to the H and the imidazole group are the same for both ligands, *i.e.* only one peak is observed.

To get a better view of the data in Table I, it is reasonable to define a "line broadening factor," which in this case means to divide the measured line width in the presence of Cu^{2+} by the line width in the absence of the metal ion. In Figure 2, such line broadening factors for H-2 are plotted against the total concentration of Cu^{2+} added.

It can easily be seen that the data separate into three main groups: (a) histamine at pD 2.4; in this case, the metal ion has almost no influence on the line width; (b) L-histidinol and histamine at pD 8.1; as the only possible chelate coordination for these ligands is through the amino group and N-1 of the imidazole ring, the line width due to H-2 is, as expected, strongly broadened by a factor of about 6; (c) the third group is of interest with L-histidine also at pD 8.1. In this case, the maximal line broadening reaches a factor of about 3, which is half of the influence observed under b. This strongly suggests that only half of the imidazole groups are coordinated. In other words, in the Cu^{2+} 1:2 complex, one histidine is bound glycine-like and the other histamine-like (structure II); thus, equilibrium 1 lies to a considerable extent to the right side.[18]

Since the Cu^{2+}–histidine 1:2 complex in solution evidently has mainly structure II, histidine is an ambidentate ligand that should be able, depending on the situation, to coordinate in one case histamine-like and in another glycine-like. That this is so was shown in the mixed-ligand experiments where one ligand group was prevented from coordination. Thus, L-histidinol can coordinate only histamine-like and L-1-methylhistidine only glycine-like; therefore, in a 1:1 mixture with the first ligand, histidine should be bound mainly glycine-like and with the second ligand mainly histamine-like. In other words, equilibrium 3 should be

$$CuA_2 + CuB_2 \rightleftharpoons 2CuAB \qquad (3)$$

considerably on the right side which is, in addition, statistically favored.[19] That these two mixed-ligand complexes reach about the same concentration as does the Cu^{2+}–histidine 1:2 complex with the mixed binding mode (structure II) is obvious from Figure 2 (and Table I); the line broadening factors are about the same for all three systems. In addition, the mixed-ligand experiments prove that the assumptions made at the beginning are reasonable, as the observed line broadening factor is not the average of those observed for the experiment with the pure ligands.

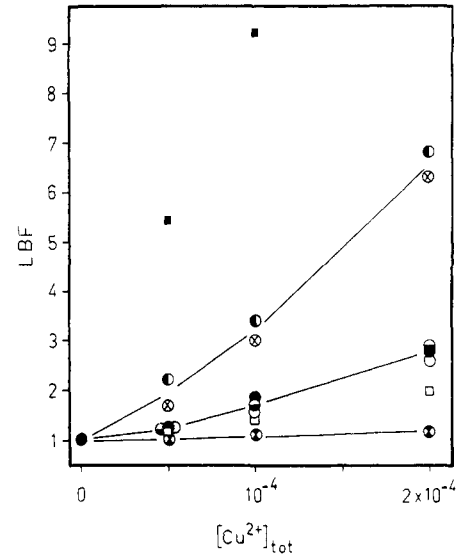

Figure 2. Relation between the line-broadening factor, LBF, for the proton at C-2 of the imidazole ring and the concentration of $Cu(ClO_4)_2$ in 0.2 *M* solutions of the ligands in D_2O at pD 8.1 (*cf.* Figure 1 and Table I) for: L-3-methylhistidine, ■; L-histidinol, ◐; histamine, ⊗; L-histidine, ●; L-1-methylhistidine, □; histamine at pD 2.4, ⊗; and 1:1 mixtures (each 0.1 *M*) of L-histidine–L-histidinol, ○; and L-histidine–L-1-methylhistidine, ◒.

As L-1-methylhistidine is forced to form glycine-like complexes by the prevention of N-1 from coordination, one expects to find almost no line broadening by the addition of Cu^{2+}. In fact, the observed line broadening is considerably smaller in the histidine system (Figure 2).[20] L-3-Methylhistidine has the same binding sites available as L-histidine. However, the line broadening factor is enormous, even larger than with histamine. The NCH_3 group influences[21] the π system of the imidazole

(18) This is further supported by earlier data[5] also showing that the broadening of the protons of C-2 and C-4 is about twice as strong in the case of histamine than it is with histidine. In addition, the proton neighbored to the NH_2 group is influenced nearly twice as much in the case of histidine than are the protons of histamine.

(19) R. DeWitt and J. I. Watters, *J. Amer. Chem. Soc.*, **76**, 3810 (1954); S. Kida, *Bull. Chem. Soc. Jap.*, **29**, 805 (1956).

(20) The weak broadening may be due to a random interaction between Cu^{2+} and N-3, becoming evident through the presence of the NCH_3 group; *cf.* the very strong line broadening observed with the L-3-methylhistidine in the presence of Cu^{2+}.

(21) This can also be seen from the chemical shifts toward higher field observed for the protons of the imidazole group by comparing L-1-methylhistidine with L-histidine: H-2, 0.17 ppm, and H-4, 0.07 ppm.

ring and, therefore, also the interaction between the metal ion and the imidazole ring. The very strong broadening of the line width suggests that both molecules coordinate in a histamine-like manner to Cu^{2+}, thus showing how easily the mixed binding mode of the Cu^{2+}–histidine 1:2 complex may be changed and equilibrium 1 shifted from one side to the other.

Discussion

The nmr data presented herein give direct evidence that in aqueous solution the Cu^{2+}–histidine 1:2 complex exists mainly in a mixed binding mode as described by structure II. These conclusions are further supported by a consideration of the stability differences of CuL and CuL_2 for the Cu^{2+} complexes of L-histidine, histamine, L-alanine, and *N*-ε-acetyl-L-lysine (Table II). The Δ log *K* value for histidine is considerably smaller than that for histamine. For the formation of a pure histamine-like complex (structure I) in the case of histidine, one would expect, however, a similar or even somewhat greater value for Δ log *K* due to repulsion of the negative charges of the carboxylic acid groups. In reality, though, the Δ log *K* value of histidine is between those of histamine and L-alanine or *N*-ε-acetyl-L-lysine, which is in agreement with the location of equilibrium 1 and the proposed structure II for CuL_2.

Table II. Comparison of the Stability Constants of Several CuL and CuL_2 Complexes and the Corresponding Values of Δ log *K*

Ligand	log K_{CuL}^{Cu} [a]	log $K_{CuL_2}^{CuL}$ [b]	Δ log *K* [c]
Histamine[d]	9.43	6.43	3.00
L-Histidine[d]	10.30	8.20	2.10
L-Alanine[e]	8.25	7.05	1.20
N-ε-Acetyl-L-lysine[e]	8.09	6.83	1.26

[a] $K_{CuL}^{Cu} = [CuL]/([Cu][L])$. [b] $K_{CuL_2}^{CuL} = [CuL_2]/([CuL][L])$. [c] Δ log K = log K_{CuL}^{Cu} − log $K_{CuL_2}^{CuL}$. [d] A. Chakravorty and F. A. Cotton, *J. Phys. Chem.*, **67**, 2878 (1963). [e] Reference 12.

In this connection, it is of interest to consider equilibrium 3 and the corresponding constant *X* (defined by equilibrium 4), for the ternary L-histidine–Cu^{2+}–L-histidine methyl ester system.[22] The calculated value

$$X = [CuAB]^2/([CuA_2][CuB_2]) \quad (4)$$

is log X = 1.17,[6] which indicates a greater stability of the ternary complex than expected on purely statistical grounds (log X = 0.6[19]). From X = 14.8, it follows that the mixed complex in equilibrium 3 is about 65% formed. Hence, there are only about 17% of each of the two binary complexes, Cu^{2+}–histidine 1:2 and Cu^{2+}–histidine methyl ester 1:2, left. In the L-histidine–Cu^{2+}–L-threonine system,[23] the ternary complex is even more favored. Using the data from ref 23, the value calculated (*cf.* ref 24) for log X is 2.31. From X = 204, it follows that the mixed complex in equilibrium 3 is formed for 88% and the two corresponding binary complexes occur only in the minor concentration of 6% each. Of similar order are the data for the related histamine–Cu^{2+}–L-serine system[25] with log X = 2.95.[26]

The stabilizing effect of the imidazole group[17,26,27] on the formation of ternary Cu^{2+} complexes, especially marked in those containing a binding group with O as donor atom, should also be mentioned here; the same is known about ternary 2,2′-bipyridyl–Cu^{2+}–O-ligand complexes.[24,25] Along the same line is the relatively short Cu–N (imidazole) bond length (1.95 Å) observed in the mixed imidazole–Cu^{2+}–glycine peptide complexes[28] and in the mixed L-histidinato–Cu^{2+}–threoninato–aquo complex,[8] compared with the values otherwise observed (2.00 Å).[29] Taking these things into account, one starts to understand why a mixed binding mode is preferred in the Cu^{2+}–histidine 1:2 complex.[30]

Structure II is also in agreement with the observation[7] that in D_2O in a 1:2 mixture of Cu^{2+}–histidine (pD 7.5), the antisymmetric COO^- stretching in the ir is shifted in a way typical for the metal-coordinated carboxylate group. Whether or not the carboxylate group of the histamine-like bound histidine (structure II) is bonded to a distorted axial position of Cu^{2+} in aqueous solution is hard to decide. Such an interaction is assumed[8] due to the mentioned ir investigation[7] and also to the red shift of the Cu^{2+}–histidine 1:2 complex compared to the Cu^{2+}–histamine 1:2 complex (638 nm *vs.* 600 nm).[9] In fact, in crystals of the mixed L-histidinato–Cu^{2+}–threoninato–aquo complex, L-histidine can act as a tridentate ligand.[8] But, the distance of the O(carboxyl) atom of L-histidine from Cu^{2+} is 2.58 Å, compared with only 1.97 Å for the O(carboxyl) atom of L-threonine; moreover, the O(carboxyl) atom of L-histidine is in an irregular axial position (angle N(amino)–Cu–O(carboxyl) = 68.3°). Thus, it seems that this latter bond is not very strong, and in aqueous solution the axial position (at least in equilibrium) may well be occupied by water. It also follows from such considerations that coordination of a single histidine in a tridental fashion is certainly not preferred.

Acknowledgments. The experiments were performed with the skillful technical assistance of Mr. K. Aegerter (Institute for Organic Chemistry, University of Basel) and Miss R. Baumbusch.

(22) R. W. Hay and P. J. Morris, *Chem. Commun.*, 18 (1969).
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(24) R. Griesser and H. Sigel, *Inorg. Chem.*, **9**, 1238 (1970).
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(30) Such an ambivalent coordination of the same ligand within a 1:2 complex was also suggested for Cu^{2+}–(L-*S*-methylcysteine)$_2$ in solution: D. B. McCormick, H. Sigel, and L. D. Wright, *Biochim. Biophys. Acta*, **184**, 318 (1969).