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Histidine Complexes of Molybdenum(V) and Molybdenum(VI)[1]

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It has been determined by optical rotation and p.m.r. measurements that L-histidine forms complexes with Mo(VI) in the pH range 4.8–8.0. The ratio of histidine to Mo(VI) was found to be 1:1 at pH 6.0, but the ratio was higher at lower pH values. Studies with L-histidine methyl ester, L-alanine, and N-acetyl-L-histidine indicated that the binding sites of the complex are the amino group and the 1-nitrogen of the imidazole ring. The apparent formation constant for the 1:1 Mo(VI) complex at pH 6.0 was estimated. Kinetic measurements of oxidation rates of Mo(V) showed that L-histidine forms a weak complex with Mo(V) at pH 4.6. E.p.r. measurements indicated that this complex is dimerized.

L-Histidine has been proposed to be present at the active site of a number of enzymes.[3–5] In addition, as part of cytochrome-*c*, it is apparently involved in the electron-transport system. Its ability to complex metal ions has been extensively studied and a number of formation constants have been determined.[6]

Molybdenum has been reported to be present in four enzymes[7] and has been implicated in a number of biological oxidation–reduction reactions. As part of a study of the biological function of the metal,[8] it seemed of considerable interest to investigate possible complexes of Mo(V) and Mo(VI) with L-histidine as potential models for molybdenum binding in the enzymes.

Experimental

Polarimetric data was obtained with a Rudolph high precision polarimeter and monochromator using a 100-watt zirconium-concentrated arc light source. The precision of the measurements was found to be ±0.003° of arc. All solutions were allowed to stand until the rotations were constant before measurements were made.

Spectrophotometric measurements were made with two instruments, a Cary Model 15 recording spectrophotometer and a Beckman Model DU spectrophotometer, using cells of the appropriate path length. A specially constructed vessel, joined to an absorption cell that could be evacuated,[8] was used for Mo(V) measurements. All solutions for the polarimetric and spectrophotometric measurements were kept in a thermostat at 25.0 ± 0.1° for at least 30 min. before use. The cell compartment of the DU spectrophotometer was water-jacketed to assure a constant temperature.

The p.m.r. spectra were obtained with a Varian A-60 high resolution spectrometer equipped with a 60-Mc. oscillator. A 6% solution of tetramethylsilane in chloroform was used as external standard. The precision in measuring the peak positions was estimated to be ±0.5 c.p.s. Relative peak heights were determined with the use of an integrator.

The oxidation of Mo(V) was investigated as follows: The deaerated solution containing Mo(V) was added to the evacuated absorption cell which was then placed in the cell compartment of the DU spectrophotometer. After completing the initial absorbance reading at 298 mμ, the cell was opened and shaken to allow oxygen to enter. In some cases, air or oxygen was bubbled through the solution for 5 min. It was then replaced in the instrument and the absorbance followed with time.

The attempted measurement of the e.p.r. spectrum of the Mo(V) complex was made with a Varian V-4502 spectrometer equipped with a 100-kc. field modulation unit, using a flat quartz cell.

Solutions of Mo(V) and Mo(VI) were prepared and standardized as previously reported.[8,9] L-Histidine, obtained from Nutritional Biochemicals Corp., was recrystallized twice from ethanol before use. L-Histidine methyl ester, N-acetyl-L-histidine, and L-alanine were purchased from Nutritional Biochemicals Corp. and were used without further purification. D_2O (99.5%) was obtained from Nichem, Inc., for use in the p.m.r. studies. All buffers were prepared from reagent grade chemicals.

Results

Polarimetric Studies with Mo(VI).—When Mo(VI) was added to a solution of L-histidine at a pH between 5 and 7 a large increase in the optical rotation (α) of the solution at wave lengths shorter than 450 mμ was observed, suggesting complex formation. The effect of pH on the optical rotation of such solutions at 350 mμ is graphed in Figure 1. In order to determine the composition of the complex, a continuous variations study, according to the method of Job, was made at pH 6.00. The results of this study for histidine and histidine methyl ester are found in Table I. As can be seen, a rather sharp maximum at a mole fraction of Mo(VI) of 0.5 was obtained, indicating a 1:1 complex. A similar result was also obtained at pH 7.0. At pH 5.00, the maximum shifted to a lower value, indicating that complexes having higher ratios of histidine to Mo(VI) were present. Because of the limitations of the method,[10] no definite conclusions concerning the structure of the complex at this lower pH can be drawn.

Experiments with compounds of similar structure were carried out in order to determine which functional groups of histidine are involved in complex formation. No evidence for complex formation was found with L-alanine or N-acetyl-L-histidine, but L-histidine methyl ester gave results very similar to those obtained with histidine (Figure 1 and Table I).

(1) Journal Paper No. 428, Utah State Agricultural Experiment Station.
(2) Abstracted from the thesis submitted by J. Y. L. for the M.S. degree, Utah State University, 1964.
(3) B. Margoliash, *Nature*, **175**, 293 (1955).
(4) H. G. Gundlach, W. H. Stein, and S. Moore, *J. Biol. Chem.*, **234**, 1754 (1959).
(5) W. H. Stein, S. Moore, and A. M. Crestfield, *ibid.*, **238**, 2421 (1963).
(6) J. Bjerrum, "Stability Constants. Part I: Organic Ligands," Special Publication No. 6, The Chemical Society, London, 1957.
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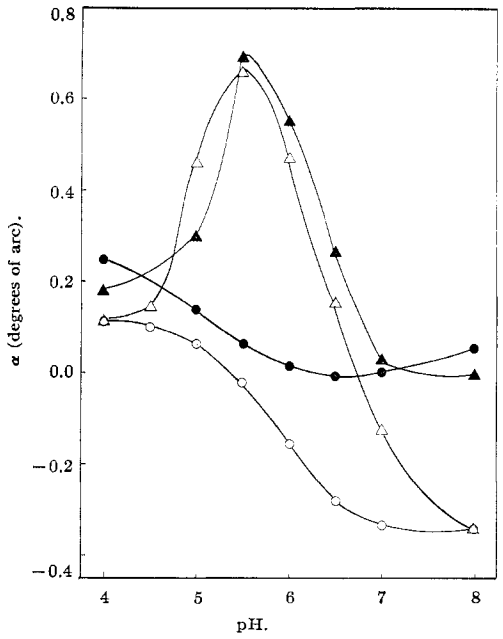
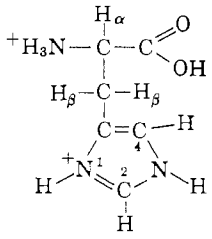


Figure 1.—Effect of pH on optical rotation (α) of Mo(VI) complexes with L-histidine and L-histidine methyl ester: O, L-histidine, 1.00×10^{-2} *M*; △, L-histidine, 1.00×10^{-2} *M*, + Mo(VI), 1.00×10^{-2} *M*; ●, L-histidine methyl ester, 1.00×10^{-2} *M*; ▲, L-histidine methyl ester, 1.00×10^{-2} *M*, + Mo(VI), 1.00×10^{-2} *M*; λ 350 mμ; tube length 20 cm.

P.m.r. Studies with Mo(VI).—The lines of the p.m.r. spectrum of L-histidine in D_2O have been assigned by McDonald and Phillips,[11] and our results agree with theirs. The assignments and the nomenclature of the various protons are as follows.



McDonald and Phillips have also reported the effect of pH on the position of the lines.[11] They state that as the pH is raised, between pH 1 and 3 and between pH 7 and 10 the position of H_α undergoes large changes that correspond to the ionization of the carboxyl and charged amino groups. The chemical shift for Hc:2 is reported to be pH-dependent only from 5 to 7, where the 1-nitrogen proton of the imidazole ring ionizes. McDonald and Phillips also found that H_β and Hc:4 showed much smaller changes over the entire pH range. Our work confirmed these results.

In the presence of Mo(VI), the p.m.r. spectrum of L-histidine showed considerable dfferences in the pH range 4.8–8.0. Below or above this range, the spectrum was essentially the same as that of histidine alone. The p.m.r. spectrum of a solution of Mo(VI) and L-histidine is illustrated as a function of pH in Figure 2. In contrast to the pH dependency of the resonance lines of the Hc:2 and Hc:4 protons in histidine, the position of these lines in the complex remained relatively constant in the pH region 5.0–7.5. The H_α proton was also pH-independent in this region, as it is in histidine itself, but its position was shifted in the complex. The position of the H_β proton was essentially unaffected by Mo(VI). In a 3:1 mixture of histidine and Mo(VI) two sets of resonance lines were observed for the H_α, Hc:2, and Hc:4 protons as the pH was raised above 4.8. The lines corresponded to those of free histidine and the complex. From pH 5.4 to 6.8 the ratio of the heights of the two sets of lines remained almost constant at approximately 2:1, indicating that a 1:1 complex is formed over this pH range at the concentrations used. Above pH 6.8, the ratio decreased again, and apparently little complex exists above pH 8.0 (or below pH 4.8). These results are in excellent agreement with those of our polarimetric studies.

(11) C. C. McDonald and W. D. Phillips, *J. Am. Chem. Soc.*, **85**, 3736 (1963).

TABLE I

CONTINUOUS VARIATION DATA; OPTICAL ROTATION (α) AS A FUNCTION OF COMPOSITION[a]

Mole fraction Mo (VI)	α-Complex	α-Ligand[b]	Δα
Mo(VI)–L-histidine complex			
[Mo(VI)] + [histidine] = 1.00×10^{-2} *M*			
0.0	−0.151	−0.151	0.000
0.1	−0.049	−0.136	0.087
0.2	0.037	−0.121	0.158
0.3	0.091	−0.106	0.197
0.4	0.137	−0.091	0.228
0.5	0.158	−0.076	0.234
0.6	0.162	−0.060	0.222
0.7	0.133	−0.045	0.178
0.8	0.088	−0.030	0.118
0.9	0.059	−0.015	0.074
1.0	0.000	0.000	0.000
[Mo(VI)] + [histidine] = 2.00×10^{-2} *M*			
0.0	−0.297	−0.297	0.000
0.1	−0.041	−0.267	0.226
0.2	0.168	−0.238	0.406
0.3	0.325	−0.208	0.533
0.4	0.417	−0.178	0.595
0.5	0.466	−0.149	0.615
0.6	0.461	−0.119	0.580
0.7	0.390	−0.089	0.479
0.8	0.289	−0.060	0.349
0.9	0.153	−0.030	0.183
1.0	0.000	0.000	0.000
Mo(VI)–L-histidine methyl ester complex			
[Mo(VI)] + [histidine methyl ester] = 1.00×10^{-2} *M*			
0.0	0.013	0.013	0.000
0.1	0.147	0.012	0.135
0.2	0.263	0.010	0.253
0.3	0.360	0.009	0.351
0.4	0.444	0.008	0.436
0.5	0.465	0.006	0.459
0.6	0.384	0.005	0.379
0.7	0.317	0.004	0.313
0.8	0.221	0.003	0.218
0.9	0.110	0.001	0.109
1.0	0.000	0.000	0.000

[a] All solutions at pH 6.00 in phosphate buffer 1.5×10^{-2} *M* in Na_2HPO_4 and 1.0×10^{-1} *M* in NaH_2PO_4; exact pH adjusted with small amounts of concentrated HCl or NaOH. [b] Concentration of ligand same as in mixture of ligand + Mo(VI).

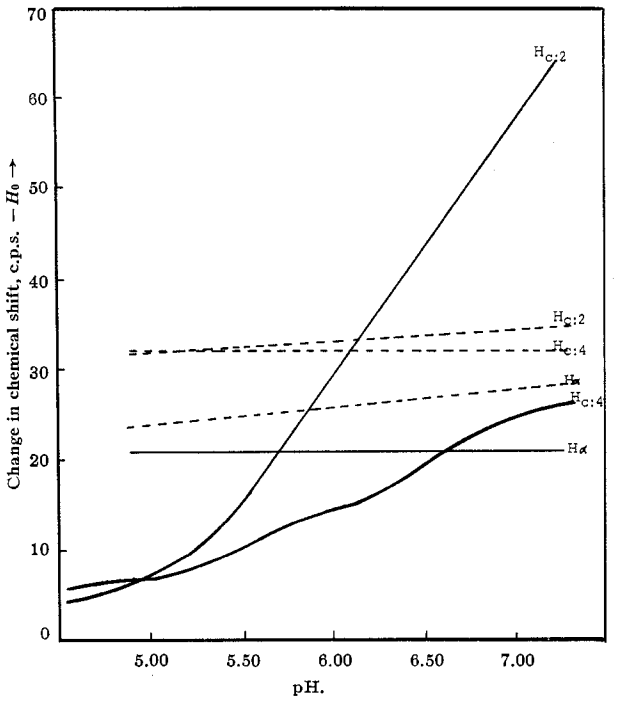


Figure 2.—Effect of pH on chemical shifts of p.m.r. spectrum of L-histidine (solid lines) and L-histidine–Mo(VI) complex (dashed lines) in D_2O. Zero taken as position of resonance at pH 0.93 of L-histidine. Both L-histidine and Mo(VI) 0.100 *M*.

Kinetic Studies with Mo(V).—Mo(V) in weakly acidic solutions is easily oxidized by atomospheric oxygen. In the presence of L-histidine, however, the rate of oxidation is considerably reduced, probably due to stabilization of Mo(V) by complex formation. Figure 3 shows the oxidation rates of Mo(V) at pH 4.60 in the presence of various concentrations of L-histidine. As the ratio of histidine to Mo(V) approached 1:2, little further decrease in the rate was observed, suggesting a 1:1 complex is formed at this pH. The oxidation rate appears to be independent of oxygen concentration since solutions through which oxygen or air had been bubbled showed no difference in rates. This has been confirmed by other work in this laboratory.

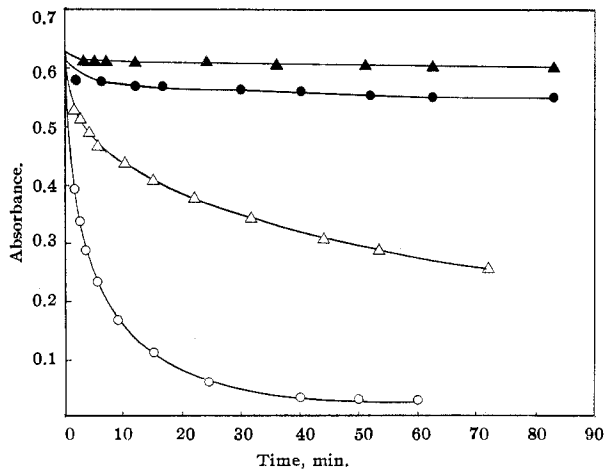


Figure 3.—Effect of L-histidine on oxidation rate of Mo(V) at pH 4.60, 20.0°; Mo(V), 2.31×10^{-4} *M*: ○, no histidine; △, 2.00×10^{-4} *M* histidine added; ●, 4.00×10^{-4} *M* histidine added; ▲, 6.00×10^{-4} *M* histidine added. Absorbance at 298 mμ is plotted *vs.* time. Acetate buffer, 1.0×10^{-2} *M* in sodium acetate and 1.0×10^{-2} *M* in acetic acid, exact pH adjusted with concentrated HCl or NaOH.

E.p.r. Studies with Mo(V).—No e.p.r. signal could be detected over a wide range of magnetic field strengths for solutions of Mo(V) and L-histidine at pH 4.60, indicating that no paramagnetic species were present.

Discussion

Results of the optical rotation studies showed that at pH 6.00 a 1:1 complex is formed between L-histidine and Mo(VI). Three histidine functional groups (the carboxyl group, the α-amino group, and the 1-nitrogen of the imidazole ring) may coordinate with metal ions. Therefore, it may act as a bi- or tridentate ligand. Since L-alanine and N-acetyl-L-histidine apparently do not complex with Mo(VI), while L-histidine methyl ester does, histidine probably is bidentate and uses the amino group and the 1-nitrogen of the imidazole ring for complex formation. These conclusions are further strengthened by the p.m.r. studies.

Histidine exists in solution in one or more of four forms, depending on the solution pH.[12]

$$LH_3^{2+} \underset{pk_1 = 1.82}{\rightleftharpoons} LH_2^{+} \underset{pk_2 = 6.08}{\rightleftharpoons} LH^{\pm} \underset{pk_3 = 9.20}{\rightleftharpoons} L^{-}$$

LH_3^{2+}, LH_2^{+}, $LH^{\pm}$, and L^{-} refer to the different forms of histidine existing at different pH values. The first dissociation (pK_1) is the carboxyl group, pK_2 is the 1-nitrogen of the ring, and pK_3 is the charged amino group. At pH 6.00, H_2L^{+} and $HL^{\pm}$ are the major species and are present in approximately equal amounts. Since the complex apparently involves the amino group and the 1-nitrogen of the ring, however, L^{-} must be the species which complexes.

The apparent formation constant for the 1:1 complex at pH 6.00 was estimated from a continuous variations plot of the data of Table I. The first equation is based on the fact that the total rotation (α_M) at the maximum (mole fraction Mo(VI) = 0.5) is due to the rotation of the complex and of the free histidine species.

$$\alpha_M = \alpha_0 + \alpha H_3L^{+2} + \alpha H_2L^{+} + \alpha HL^{\pm} + \alpha L^{-} \quad (1)$$

At pH 6.0, the concentrations of H_3L^{2+} and L^{-} are negligible and the rotation due to them may be neglected. Since the rotation of each species is proportional to the concentration of that species, eq. 1 may be written as

$$\alpha_M = K_C[C] + K_{H_2L^+}[H_2L^+] + K_{HL^\pm}[HL^\pm] \quad (2)$$

where the K's are proportionality constants. Two more equations were then written

$$C_L = [C] + [H_2L^+] + [HL^\pm] \quad (3)$$

$$\frac{[H^+][HL^\pm]}{[H_2L^+]} = K_2 \quad (4)$$

where C_L is the total ligand concentration. The values of $K_{H_2L^+}$ and $K_{HL^\pm}$ were obtained from the plot of α *vs.* pH for histidine at the respective pH values where these forms are maximized and the other species

(12) A. Albert, *Biochem. J.*, **50**, 690 (1952).

can be neglected (pH 3.92 and 7.61). The value of K_C was obtained from a measurement of α at pH 6.0 for a solution 1.90×10^{-2} M in Mo(VI) and 1.00×10^{-3} M in histidine in which the histidine is essentially all complexed. The values obtained were: $K_{H_2L^+} = 11.5$, $K_{HL^\pm} = -34.5$, and $K_C = 76.5$. Equations 2, 3, and 4 were then solved for [C] and [$HL^\pm$]. The concentration of L^- was calculated from

$$\frac{[H^+][L^-]}{[HL^\pm]} = K_3 \tag{5}$$

The free metal ion concentration, [M], was obtained from

$$C_M = [M] + [C] \tag{6}$$

The formation constant, K_f, was calculated with the equation

$$K_f = \frac{[C]}{[M][L^-]} \tag{7}$$

This gave the value of $\log K_f = 6.2 \pm 0.1$, for two calculations at total concentrations of 1.00×10^{-2} and 2.00×10^{-2} M. A similar method was used to estimate K_f for the histidine methyl ester complex, except that the complex concentration, [C], was obtained directly from the plot by the usual extrapolation method,[8] since the free ligand has essentially no rotation at pH 6.0 (see Figure 1). This gave a value for $\log K_f$ of 4.6. It should be emphasized that these estimates are valid only at the specified pH values, since the nature and concentration of the Mo(VI) species depends in an unknown way on pH. The fact that both these constants are of roughly the same order of magnitude further strengthens the postulate that the same sites, the amino group and the 1-nitrogen of the ring, are involved in both the histidine and histidine methyl ester complexes.

Proton magnetic resonance has been used recently to determine the structures of complexes of histidine with Co(II)[11] and of EDTA with Mo(VI).[13] When a ligand complexes with a metal ion, the environment of the protons in proximity to the metal ion is changed and this is reflected in the p.m.r. spectrum.

In the pH region 5.0–7.5, the positions of the resonance lines for the Hc:2 and Hc:4 protons of the complex are pH independent, indicating a constant environment, as contrasted with the pH dependency of these lines for histidine. Because of their position, these protons are affected primarily by changes at the 1-nitrogen of the ring. Once this group is bonded to the metal ion, the environment is essentially fixed for these protons and their resonance lines do not change with pH. In the complex, the position of the H_α proton is shifted somewhat from its position in histidine, no doubt because of the bonding of the amino group to the metal ion, and is also pH independent in this region. The p.m.r. data therefore are in agreement with the optical rotation results and substantiate the participation of the amino group and the 1-nitrogen of the ring in bonding to the metal ion. Furthermore, the absence of an H_α line for the complex below pH 4.8 provides further evidence that the carboxyl group is not involved in complex formation, since its pK (1.80) is low enough that it is ionized at pH below 4.8. If the carboxyl group was bonded to the metal ion, some differences in the H_α line would be anticipated because of the proximity of this proton to this group. Finally, the almost constant 2:1 ratio of the heights of the resonance lines of histidine to those of the complex indicates that the main species present at this concentration is a 1:1 complex.

Our kinetic studies with Mo(V) and histidine indicated that a complex, probably of a 1:1 ratio, is formed. This complex is apparently much weaker, however, than those of Mo(V) with EDTA[14] or with 8-hydroxyquinoline-5-sulfonic acid.[15] These complexes stabilize Mo(V) toward oxidation to a much greater degree, even at higher pH. Little more can be said of the rather weak Mo(V)–histidine complex, except that it must be dimerized or polymerized in some way that allows pairing of the spins of the Mo(V) ions, since no e.p.r. signal could be detected.

In summary it appears that, in the molybdenum-containing enzymes, Mo(VI) could be bound by the imidazole ring of a histidine residue, perhaps in connection with an adjacent amino group. The weak interaction of Mo(V) with L-histidine is very likely of little biological significance.

Acknowledgment.—Thanks are expressed to The Public Health Service (Grant GM 08347-03, Division of General Medical Sciences) for financial support.

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