[CONTRIBUTION NO. 853 FROM THE CENTRAL RESEARCH DEPARTMENT, EXPERIMENTAL STATION, E. I. DU PONT DE NEMOURS AND CO., INC., WILMINGTON 98, DEL.]

A Nuclear Magnetic Resonance Study of Structures of Cobalt(II)–Histidine Complexes

BY C. C. McDONALD AND W. D. PHILLIPS

RECEIVED JULY 26, 1963

Complexes of histidine with Co(II) in aqueous solution have been examined by proton magnetic resonance spectroscopy. Coordinate interaction of histidine with paramagnetic Co(II) produces large contact interaction shifts in the histidine proton resonance spectra. Four histidine–Co(II) complexes have been distinguished: a weak 1:1 histidine–Co(II) complex at low pH (<4) in which only the histidine carboxyl group is bonded to octahedral Co(II); strong 1:1 and 2:1 histidine–Co(II) complexes at intermediate pH values (4–10) in which histidine behaves as a tridentate ligand; and a tetrahedral 2:1 histidine–Co(II) complex at high pH (>11) in which histidine is bonded to cobalt *via* the NH_2 group and an imidazole nitrogen atom. It was found that ΔF° for the 2:1 octahedral histidine-Co(II) complex composed of one D-histidine and one L-histidine is about 0.7 kcal./mole less than for the complex containing two histidine ligands of the same configuration (DD or LL). Proton magnetic resonance spectra of complexes of several histidine derivatives with Co(II) confirm the structures assigned to the various histidine–Co(II) complexes.

Introduction

One of the more important transition metals from the standpoint of biological significance is cobalt. Cobalt ions act as activators for cholinesterase, carboxylase, and phosphoglucomutase and afford protection of brain respiration against high oxygen pressure. In addition, cobalt ions act as bacteriostatic agents at concentrations which make them competitive with antibiotics in effectiveness.[1]

Histidine as a component of cytochrome-c and hemoglobin has been implicated in the respective electron transport and oxygen transport functions of these proteins. It is likely by virtue of the basic and chelating characteristics of histidine that the histidyl group is present at the active or allosteric sites of certain enzymes. A particularly intriguing aspect of the histidine–Co(II) complex is the ability of the complex to absorb oxygen reversibly. A number of the effects of cobalt in biological systems may be due to this property of the histidine–Co(II) complex.

It is apparent that elucidation of the structure of the histidine–Co(II) complex in solution would be of importance as a first step to the understanding of the mode of action of cobalt in biological systems. Spectral, magnetic susceptibility, and potentiometric studies of the histidine–Co(II) complex have been performed by a number of investigators,[1-3] none of which have led to the unequivocal establishment of the structure of the complex. Recently, Milner and Pratt[4] published some results of a nuclear magnetic resonance (n.m.r.) study of interactions between transition metals and amino acids. As a part of a general investigation of interactions between transition metal ions and molecules of biological significance, we would like to present the results of some studies on the solution structures of four histidine–Co(II) complexes employing proton contact interaction shifts.

Contact Interaction Shifts in N.m.r. Spectra of Coordination Compounds of Paramagnetic Transition Metal Ions.—The presence of a transition metal ion in a coordination compound can give rise to a number of effects on the observables of the n.m.r. spectrum of the coordinating group. One such effect is the marked shortening of T_{2N}, the transverse nuclear relaxation time or line width parameter.[5] For this reason, n.m.r. line widths of paramagnetic species often are broadened to the point that chemical shifts and nuclear spin–spin splittings can no longer be resolved.

The effectiveness of an electronic magnetic dipole in relaxing nuclear magnetic dipoles is, however, a sensitive function of the electronic relaxation time T_{1e}. As T_{1e} decreases beyond a certain value, electronic dipoles become increasingly ineffective in relaxing nuclear dipoles[5] and hence less effective in broadening n.m.r. spectra. It adds little to the present discussion to couch the above description of the relationship between n.m.r. line widths and electronic relaxation times in paramagnetic systems in more quantitative terms.[6] However, as T_{1e} of a paramagnetic system decreases, important benefits accrue in the form of large shifts in the n.m.r. spectra that result from nucleus–electron isotropic hyperfine contact interactions[7] and pseudocontact interactions.[8]

Contact shifts have been observed primarily in ligands of transition metal chelates in which the contact shifts are produced by effects of the unpaired electrons of the metal ion on the local magnetic environments of the ligand's component nuclei. Unpaired electrons can be transferred to π-orbitals of conjugated ligands as the result of $p\pi$–$d\pi$ bonding or spin polarization effects. Spin density can be introduced into the σ-orbitals of ligands by similar mechanisms. Alternatively, nuclear resonance frequencies of the atoms of ligands of paramagnetic chelates can be influenced by field effects which originate at the paramagnetic ion and depend on the existence of an anisotropy in the ion's magnetic properties.

All of these topics have been discussed elsewhere in connection with studies that have provided new information concerning metal–ligand bonding and ligand spin density distributions in chelate systems.[4,9-11] In the present study we wish to use the contact shift effect only to explore the nature of the coordinate interaction between Co(II) and histidine. For this purpose, we need bear in mind that magnitudes of contact shifts reflect sites and strengths of coordinate interaction between paramagnetic ions and ligands. Attempts to translate observed contact shifts into ligand spin densities and details of metal–ligand binding in these Co(II)–histidine complexes will be left to a future, more general study of interactions between metal ions and biological molecules.

(1) For a review of the role of cobalt in biological processes, see J. Z. Hearon, D. Burk, and A. L. Schade, *J. Natl. Cancer Inst.*, **9**, 337 (1949).

(2) J. M. White, T. J. Weissmann, and N. C. Li, *J. Phys. Chem.*, **61**, 126 (1957).

(3) A. Earnshaw and L. F. Larkworthy, *Nature*, **192**, 1068 (1961).

(4) R. S. Milner and L. Pratt, *Discussions Faraday Soc.*, **34**, 88 (1962).

(5) N. Bloembergen, E. M. Purcell, and R. V. Pound, *Phys. Rev.*, **73**, 679 (1948).

(6) See, however, N. Bloembergen and L. O. Morgan, *J. Chem. Phys.*, **34**, 842 (1961), for a thorough discussion of this topic.

(7) H. M. McConnell and C. H. Holm, *ibid.*, **27**, 314 (1957).

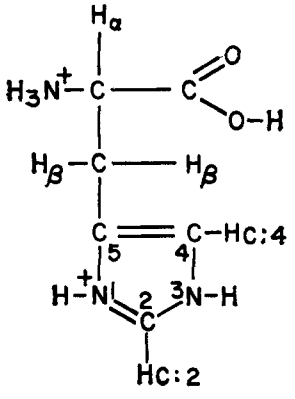
(8) H. M. McConnell and R. E. Robertson, *ibid.*, **29**, 1361 (1958).

(9) H. M. McConnell and C. H. Holm, *ibid.*, **28**, 750 (1958).

(10) D. A. Levy and L. E. Orgel, *Mol. Phys.*, **3**, 583 (1960).

(11) (a) D. R. Eaton, A. D. Josey, W. D. Phillips, and R. E. Benson; *J. Chem. Phys.*; **37**, 347 (1962); (b) D. R. Eaton, W. D. Phillips, and D. J. Caldwell; *J. Am. Chem. Soc.*, **85**, 397 (1963).

Proton Spectra of Histidine.—The proton magnetic resonance (p.m.r.) spectrum of histidine in aqueous solution at a pH of 0.9 is presented in Fig. 1. As in most of the experiments reported below, D_2O was used as solvent to reduce the strong and obscuring proton resonance of H_2O. However, exchange of labile protons of histidine with D_2O introduced sufficient protons to give a strong HDO proton resonance. The solution also contained tetramethylammonium chloride to serve as an internal proton reference for spectral calibration. In all experiments, positions of the various proton resonance lines (at 60 Mc./sec.) were measured with respect to their displacements to lower (−) or higher (+) fields (or equivalent frequency displacements) relative to the position of the single proton resonance of the tetramethylammonium ion. Histidine proton resonances were observed only for the hydrogen atoms bonded to carbon atoms designated α, β, C:2, and C:4 in Fig. 1. The other protons of histidine exchange rapidly with water. Assignments of the resonances to the nonequivalent histidine C–H protons from which they arise are relatively straightforward and are based on nuclear spin–spin splitting, expected chemical shifts, and pH dependences.



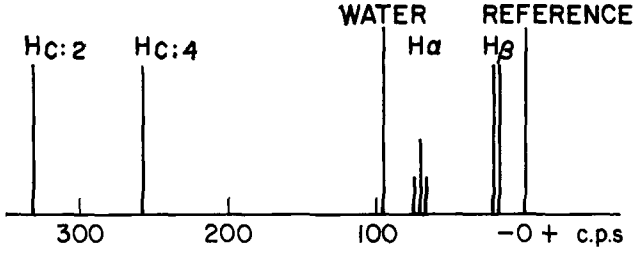


Fig. 1.—P.m.r. spectrum at 60 Mc./sec. of aqueous histidine at pH 0.9.

The pH dependences of the chemical shifts of the four types of nonexchanging hydrogen atoms of histidine in aqueous solution are shown in Fig. 2. The zero for each of the four chemical shifts is taken as the position of the resonance at pH 0.9. Large pH dependences for the chemical shift of H_α are observed only at pH 1–3 and pH 7–10 and result from the ionization of the $-COOH$ (pK 1.8) and $-\overset{+}{N}H_3$ (pK 9.2) groups, respectively. On the other hand, the chemical shift for $H_{C:2}$ is pH dependent only in the range 5–7 where the proton bonded to the 1-nitrogen of the imidazole ring is released (pK 6.0). The chemical shifts of H_β and $H_{C:4}$ are seen to respond to a smaller degree to all of the proton ionizations. The position of the proton resonance of water is not significantly affected by pH changes over the range 1–11. It is thus clear that precise, specific, and unambiguous information concerning ionization phenomena in amino acid systems can be obtained from n.m.r. studies.

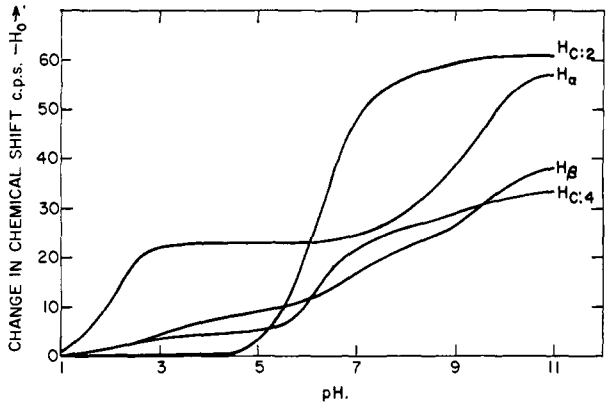


Fig. 2.—Effect of pH on chemical shifts at 60 Mc./sec. of histidine p.m.r. spectrum.

Proton N.m.r. Spectrum of Water Containing Co(II).—Cobaltous ion dissolves readily in water in the pH range 1–7 to form the pink, octahedral, hexahydrate complex. If the pH is raised above about 7, Co(II) precipitates as the hydroxide. As Co(II) in D_2O is increased in concentration from 0 to 2 M, the proton resonance of residual HDO in the solvent is observed to shift to low field in linear fashion at a rate of about 550 c.p.s./mole of Co(II). This shift is attributed to a contact shift of about −5100 c.p.s. (at 60 Mc./sec.) for the protons of water in the first coordination sphere of Co(II) and results from spin polarization of the ligand water molecules by Co(II). The full contact shift is not observed because proton exchange between bound water and free water is fast relative to the 5100 c.p.s. shift between the proton resonances of free and cobalt-bound water. Thus, the proton resonances of coordinated and free water are observed as a single, composite line. The shift of this line (the observed contact shift) away from the position of the Co(II)-free water line is proportional to the ratio of complexed water to free water and hence to the Co(II) concentration.[12] For a fixed Co(II) concentration, displacement of water from the first coordination sphere of Co(II) by another ligand is reflected in a shift of the water proton resonance toward the cobalt-free position. As will be shown, observation of such a reverse shift of the water resonance is useful in estimating the number of coordination sites on the metal ion that are utilized by a ligand and that are not accessible to water. This technique is useful, of course, only in the pH range in which all Co(II) remains in solution. Previous in-

(12) This is the first of several examples in this investigation where the observables of nuclear magnetic resonance are affected by intramolecular and intermolecular rate processes (such as proton exchange, ligand exchange, or exchange between chelated and nonchelated ligand) which exchange nuclei between nonequivalent environments. Resonances of two protons in nonequivalent environments appear as two discrete spectral lines if environmental exchange processes occur at rates that are small compared to $2\pi\delta$, where δ (expressed in c.p.s.) is the chemical or contact shift difference between the two lines. If an exchange rate approaches $2\pi\delta$, the lines broaden and move together. When the environmental exchange process proceeds at a rate exceeding $2\pi\delta$, the individual spectral lines collapse to a single line at a position between the original lines that depends on the relative concentrations of the two types of protons. A discussion of effects of nuclear exchange phenomena on the observables of n.m.r. spectroscopy may be found in "High-Resolution Nuclear Magnetic Resonance," J. A. Pople, W. G. Schneider, and H. J. Bernstein, McGraw-Hill Book Co., Inc., New York, N. Y., 1959, p. 218.

vestigators[13-16] have measured proton relaxation times of water to investigate accessibility of coordination sites on paramagnetic ions in proteins or nucleic acids to water. It would appear that proton shifts of water may be useful in a similar vein.

Complexes of Co(II) with Histidine in D_2O Solutions.—Proton magnetic resonance spectra were obtained over the pH range 0.5–12 for D_2O solutions which were 0.4 *M* in histidine and contained various concentrations of Co(II). Coordinate interactions of Co(II) with histidine resulted in large contact shifts and these spectra were used to deduce the characteristics of the several types of histidine–Co(II) complex that were formed.

Complex I: pH 1.0–3.5.—If Co(II) is added to an aqueous solution of histidine at pH < 1 wherein histidine is in the completely protonated form illustrated in Fig. 1, the histidine p.m.r. spectrum is not changed. The proton resonance of water shifts to low field in proportion to the Co(II) added as described previously. Evidently in this environment there is no interaction of Co(II) hexahydrate with histidine.

However, as the pH of a solution 0.4 *M* in histidine and 0.2 *M* in Co(II) is increased through the range from 1.0 to 3.5, small contact shifts of H_α and H_β to lower fields are observed as shown in Fig. 3. There is

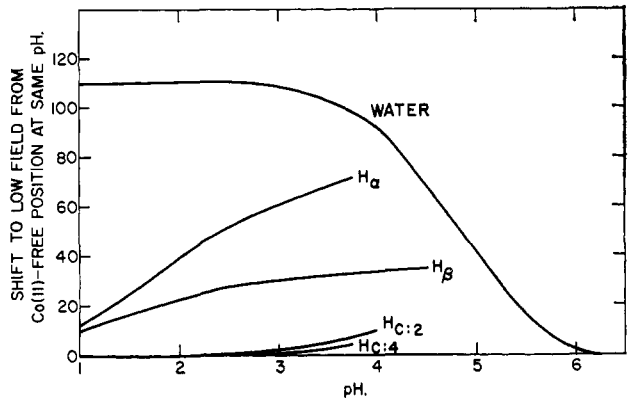


Fig. 3.—Effect of pH on contact shifts of p.m.r. spectrum of complex I: D-histidine 0.4 *M*, Co(II) 0.2 *M*.

little, if any, shift of $H_{C:2}$ or $H_{C:4}$ under these conditions. In this pH range the histidine carboxyl group ionizes. The carboxyl anion apparently competes with water for a binding site in the coordination sphere of Co(II). Thus, a Co(II)–histidine complex is formed which we designate complex I. The structure of complex I is suggested in Fig. 4. The "true" contact shifts of complex I are not observed because the rate of exchange of free histidine ligand with complexed histidine apparently is fast compared to the largest contact shift of the complexed form. Thus, only one histidine p.m..r. spectrum appears (instead of separate spectra for complexed and noncomplexed histidine), and the observed contact shifts depend on the ratio of complexed histidine to total histidine. It is believed that this ratio is small (*i.e.*, the histidine ion competes unfavorably with water for a site on Co(II) over this pH range) and consequently that the actual contact shifts of protons of complex I are much greater than the observed shifts. The above reasoning is supported by the very small reverse shift of the water resonance which indicates that there has been little displacement of water

(13) J. Eisinger, R. G. Shulman, and B. M. Szymanski, *J. Chem. Phys* **36**, 1721 (1962).
(14) M. Cohn and J. S. Leigh, *Nature*, **193**, 1037 (1962).
(15) N. Davidson and R. Gold, *Biochim. Biophys. Acta*, **26**, 370 (1957).
(16) R. Lumry, H. Matsumiya, F. A. Bovey, and A. Kowalsky, *J. Phys Chem.*, **65**, 837 (1961).

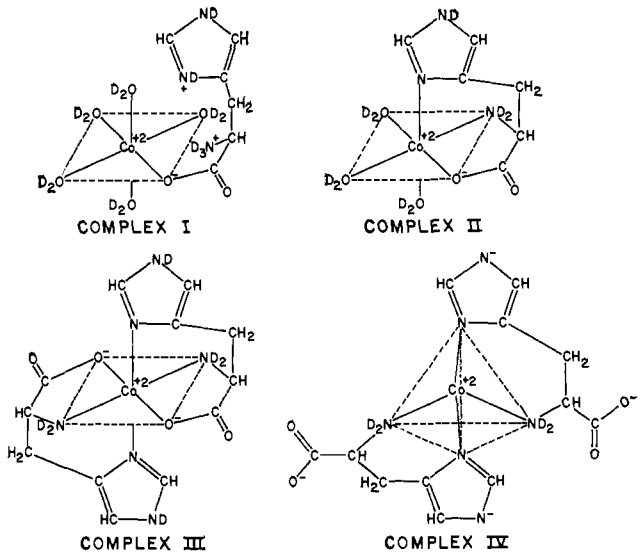


Fig. 4.—Schematic structures of histidine–Co(II) complexes.

from the first coordination sphere over this pH range. Moreover, the observed contact shifts can be increased (as shown in Fig. 5) by increasing the ratio of complexed histidine to free histidine by the addition of more Co(II) to the solution. In these experiments, the Co(II)

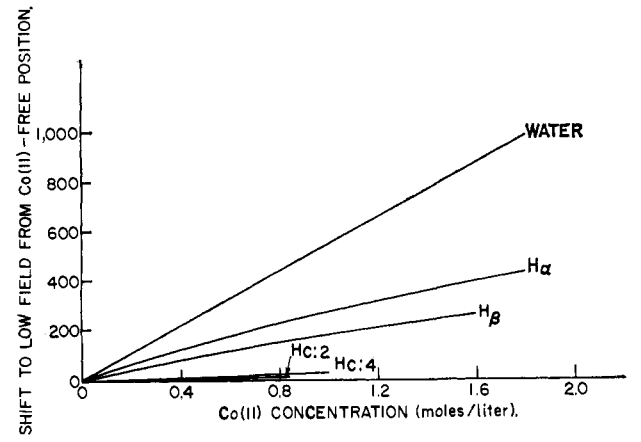


Fig. 5.—Effect of Co(II) concentration on contact shifts of p.m.r. spectrum of complex I: D-histidine 0.4 *M*, pH 3.0, 60 Mc./sec.

concentration was increased until the histidine spectrum became too broad to measure, but even when the Co(II) concentration was made 1.8 *M* the observed shifts were still increasing. It is clear, then, that the rate of ligand exchange must be greater than about 10^3 sec.$^{-1}$. When interactions of Co(II) with histamine or histidine methyl ester at pH 1 to 3.5 were examined, no proton contact shifts were observed. These observations provide further verification that only the carboxyl group of histidine is involved in the binding of this ligand to Co(II) in complex I. Since a solution containing complex I remains pink and has the same magnetic susceptibility as a solution containing only Co(II) hexahydrate (Table I), it is concluded that Co(II) in complex I remains octahedrally coordinated.

Complexes II and III: pH 4.5–10.5.—The proton magnetic resonance spectrum of histidine broadens, decreases in peak height intensity, and finally disappears as the pH of a solution 0.4 *M* in D- or L-histidine and 0.2 *M* in Co(II) is increased through the range from 3 to 7. In this same pH region the HDO proton resonance shifts to that of water free of Co(II) (Fig. 3) which indicates complete displacement of water from

the first coordination sphere of Co(II) by histidine. At a pH of about 4.5 a p.m.r. spectrum is observed which exhibits large contact shifts and arises from a new species designated complex II. An additional, separate spectrum appears at a pH of about 5 and is attributed to another new species designated complex III. The spectra of complexes II and III are presented in Fig. 6 and are seen to be qualitatively similar except

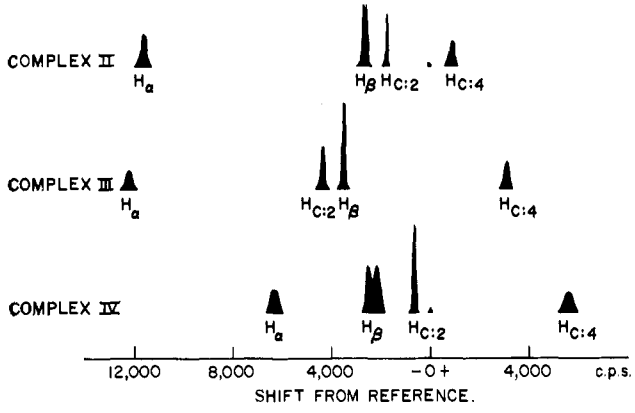


Fig. 6.—P.m.r. spectra at 60 Mc./sec. of histidine–Co(II) complexes.

that the contact shifts for complex III are somewhat larger than for complex II. The intensity of the spectrum of complex II increases as the solution is made more alkaline up to a pH of about 5.8; the intensity then decreases as pH is increased further and the spectrum of this species disappears at a pH of about 7. The intensity of the spectrum of complex III increases with pH until complex II disappears and then remains constant to a pH of about 10.5. If the histidine–Co(II) ratio is increased above 2, the ratio of complex III to complex II is increased and a severely broadened spectrum of uncomplexed histidine persists at pH values above 7. On the other hand, if the histidine–Co(II) ratio is reduced to one, complex II is favored and is not completely converted to complex III until a pH of about 8. In the latter case, the spectrum of uncomplexed histidine is not observed at pH > 4.5 and some Co(II) hydroxide precipitates at pH > 7. No precipitate is observed when the histidine–Co(II) ratio is two. Since in the pH range 4–10 oxygen-free, histidine–Co(II) solutions remain pink and exhibit magnetic susceptibilities of about 5.1 B.M. (Table I), it is concluded that Co(II) is octahedrally coordinated in complexes II and III.

TABLE I

pH DEPENDENCE OF MAGNETIC PROPERTIES OF HISTIDINE–Co(II) COMPLEXES

Soln. pH	Predominant Co(II) species in soln.	Co(II) magnetic moment μ_{eff} (Bohr magnetons)
0.95	Co(II) hexahydrate	5.08
4.1	Co(II) hexahydrate	5.12
	Complex I	
5.7	Complex II	5.02
	Complex III	
9.3	Complex III	5.16
11.45	Complex IV	4.48

The pH region in which complexes II and III form is the region in which Co(II) forms complexes with imidazole itself. It is concluded that Co(II) replaces the proton at the 1-nitrogen and concurrently, insofar as our measurements can detect, the amine and carboxyl group also bind to Co(II) with expulsion of a proton from the protonated amine group. Therefore, complex II is considered to be a 1:1 histidine–Co(II) complex that contains three water molecules and in which histidine is tridentate. Complex III appears to be a 2:1 histidine–Co(II) complex in which tridentate histidine has replaced all the water molecules in the first coordination sphere of Co(II). These complexes are illustrated schematically in Fig. 4.

The assignment of the structures of complexes II and III is clear from the dependence of the relative intensities of the spectra of these species on the histidine–Co(II) concentration ratios in the solutions investigated. Additional confirmation of these structures is provided by the monotonic, reverse shift of the water resonance as water is excluded from coordination with Co(II) over the pH range in which complexes II and III form. When the solution composition is reached in which Co(II) is all bound in complex III, the water resonance line returns to the unbound water position, demonstrating apparently complete exclusion of water from coordination with Co(II).

Further evidence for the identity of the functional groups of histidine that bind to Co(II) in complexes II and III was provided by examination of p.m.r. spectra of solutions containing derivatives of histidine and Co(II) in 2:1 ratio in the pH range from 6 to 9. Representations of these spectra are presented in Fig. 7.

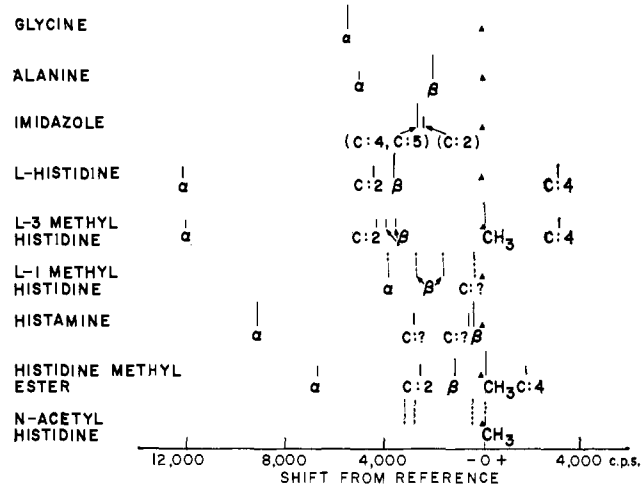


Fig. 7.—P.m.r. spectra of Co(II) complexes. Dotted lines are used where intensity relationships are uncertain.

When the carboxyl, amine, or imidazole 1-nitrogen position is blocked or removed as in histidine methyl ester, N-acetylhistidine, 1-methylhistidine, or histamine, complexes with Co(II) still form and the p.m.r. spectra of these complexes exhibit large contact shifts, but the spectra are quite different from those of complexes II or III of histidine. Complexes of Co(II) with these histidine derivatives are less stable than histidine complexes and precipitation of Co(II) occurs in these systems at pH > 7 unless a considerable excess of ligand is present. It is apparent that the 3-nitrogen position of histidine does not bind to Co(II) since the complex of 3-methylhistidine with Co(II) in which this position is blocked exhibits a p.m.r. spectrum very similar to that of complex III of histidine.

Certain conclusions concerning ligand exchange reactions of complexes II and III can be drawn from their proton magnetic resonance spectra. If the complexes are prepared in H_2O, resonance lines are observed for

each of the amine hydrogens in each complex at positions shown in Fig. 8. Therefore, the exchange rate of these protons in complexes II and III with the solvent is less than 6×10^4 sec.$^{-1}$. The proton at the 3-nitrogen position is believed to be more labile and a resonance line for this proton has not been observed. Since discrete n.m.r. spectra can be observed for complexes II and III in solutions containing an excess of histidine, the rates of exchange of bound ligand and free ligand for complexes II and III must be less than 6×10^3 sec.$^{-1}$ and 2×10^4 sec.$^{-1}$, respectively. These exchange rates are not negligible, however, as there is evidence of broadening of the resonances of the free ligand and complexes in these solutions due to ligand exchange. This broadening appears to increase as the solution pH is increased through the range from 7 to 10. Shifts (and severe broadening) induced by nuclear exchange phenomena also are observed in the spectra for the ligand and complex in the pH region from 4.5 to 6 where the complexes are forming, as may be seen in Fig. 8. These exchange effects, however, are believed to result from exchange of free ligand and of the bound ligand of complexes II and III with some intermediate species, perhaps a complex in which only the imidazole group of histidine binds to Co(II). A p.m.r. spectrum of this intermediate is not observed because of its low concentration and fast exchange with other species. Some evidence for such an intermediate was obtained in the course of examination of spectra of complexes of histamine with Co(II). In the pH region where the complex was beginning to form, a proton spectrum similar to that of the complex of imidazole with Co(II) was obtained rather than the spectrum presented in Fig. 7 for the stable histamine–Co(II) complex that appeared in a higher pH range.

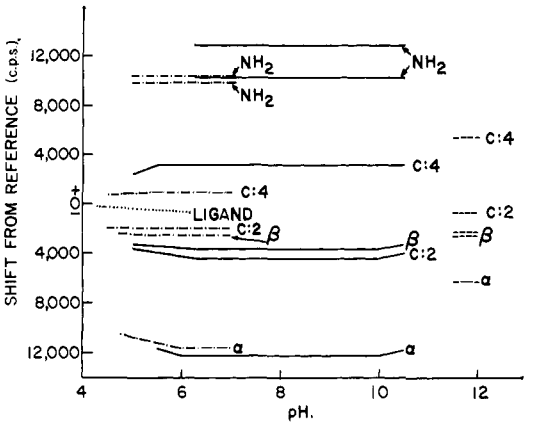


Fig. 8.—Effect of pH on p.m.r. spectra of histidine–Co(II) complexes; L-histidine 0.4 *M*, Co(II) 0.2 *M*; complex II, – · – · –; complex III, ——; complex IV, – – – –.

Complex IV: pH > 11.5.—If the pH of a solution 0.4 *M* in histidine and 0.2 *M* in Co(II) is increased through the range from 10.5 to 12, further changes are observed in the proton spectrum of the solution (Fig. 8). First, the spectrum of complex III broadens and shifts to higher field positions. At a pH of about 11, this spectrum becomes too broad to be readily observed. As the pH is increased further, a new spectrum appears, broad at first, and then well defined in the pH region from 11.5 to 12. The spectrum of this new histidine–Co(II) species, designated complex IV, is shown in Fig. 6. Concomitant with these changes in the proton spectrum, the solution changes from pink to blue and the magnetic susceptibility decreases from 5.1 to 4.5 B.M. The blue color and latter magnetic susceptibility are characteristic of tetrahedral Co(II) complexes.[17]

Complex IV is almost certainly the tetrahedral 2:1 histidine–Co(II) complex in which the 1-nitrogen position and either the amine nitrogen or the carboxyl group of histidine bind to Co(II). The amine nitrogen is the most probable second binding group as it is more basic than the carboxyl group. Furthermore, the recent X-ray study of a solid zinc–histidine chelate by Kretsinger, Bryan, and Cotton[18] has shown the zinc to be tetrahedral and that coordinate binding between zinc and the carboxyl group is absent. A representation of complex IV is presented in Fig. 4.

The octahedral to tetrahedral conversion of complex III to complex IV is thought to result from an increase in the binding energy at the 1-nitrogen position of the imidazole ring that is a consequence of ionization of the proton at the 3-nitrogen position. When either the 1-nitrogen or the 3-nitrogen position of histidine was blocked as in 1-methylhistidine or 3-methylhistidine, the tetrahedral complex was not observed. One might expect to get complexes similar to complex IV in solutions wherein histidine is replaced by histidine methyl ester or histamine but not when N-acetylhistidine is used. However, in the pH region where complex IV was formed histidine methyl ester hydrolyzed, histamine formed a deep blue precipitate, and N-acetylhistidine produced a blue solution which did not exhibit detectable contact shifts. Thus, the study of p.m.r. spectra of histidine derivatives was not fruitful in confirming the assigned structure for complex IV. The severe broadening of the proton spectra of complexes III and IV in the pH range from 10.5 to 11.5 is attributed to exchange between these two chelate structures.

The described interconversion of histidine among the various complexes as the pH or histidine–Co(II) ratio is varied is rapidly reversible.

Mixed Chelates of Co(II) with D- and L-Histidine.—The p.m.r. spectrum of complex III shown in Fig. 6 is observed when the complex is prepared from D-histidine or from L-histidine. If the histidine employed contains both the D- and L-optical isomers, one obtains for complex III the same spectrum described above plus a new spectrum with larger contact shifts shown schematically in Fig. 9. It seems clear that the new spectrum must be ascribed to a form of complex III wherein one of the two histidine molecules which are acting as tridentate ligands is the D-isomer and the other is the L-isomer. The identical contact shifts for the DD- and LL-complexes show, as expected, that the

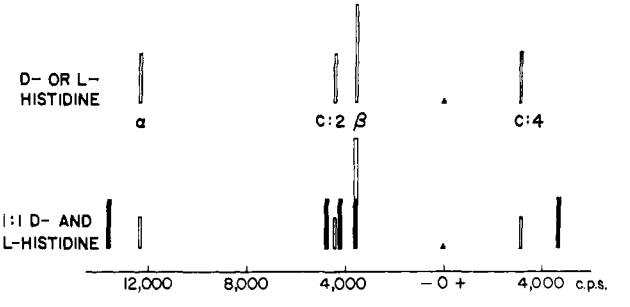


Fig. 9.—P.m.r. spectra of DD-, LL-, and DL-forms of complex III.

(17) F. A. Cotton and R. H. Holm, *J. Am. Chem. Soc.*, **82**, 2979, 2983 (1960).

(18) R. H. Kretsinger, R. F. Bryan, and F. A. Cotton, *Proc. Chem. Soc.*, 177 (May, 1962).

strengths of metal–histidine binding in these two forms are the same. The larger contact shifts for the DL-form suggest that the strength of binding in this form is greater than in the DD- or LL-forms. As we shall see, equilibrium studies do indeed show the DL-complex to be thermodynamically more stable than the DD- and LL-complexes.

The stability of the DL- form of complex III relative to the DD- or LL-forms was further investigated by examination of the intensity ratios of analogous resonances of the DL and (DD + LL) forms as the proportion of D-histidine in the histidine charge was varied from 20 to 80%. Concentration ratios, DL/(DD + LL), obtained from spectral intensity ratios are compared in Table II with the statistical ratios expected for random distributions among DD-, LL-, and DL-species. It is evident that the concentration of the DL-form is greater in all cases than the concentration predicted for a random distribution. The equilibrium constant, K, for the reaction

$$\text{DD} + \text{LL} \rightleftarrows 2\text{DL}$$

is given by

$$K = 4\alpha^2/(\alpha^2 - \alpha + x - x^2) \qquad (1)$$

where

$$\alpha = 1/2(R + 1),\ R = (\text{DD} + \text{LL})/\text{DL}$$

and x is the mole fraction of D-histidine in the total histidine charge. Values of K calculated from eq. 1 for each experimental value of x are presented in Table II. The average value of K is 11.5 and indicates that the *additional* stability of the DL-form over that of the DD- or LL-forms is about 0.7 kcal./mole. Ligand exchange rates among the three forms of complex III must be less than 10^3 sec.$^{-1}$ since separate and not averaged proton resonances are observed for the DL- and DD,LL-forms.

TABLE II

EQUILIBRIA OF MIXED CHELATES OF D- AND L-HISTIDINE WITH Co(II)

Mole fraction D-histidine	DL/(DD + LL) random distribution	DL/(DD + LL) observed intensity ratio	K
0.2	0.469	0.598	17.5
.3	.725	0.877	6.9
.4	.927	1.30	8.7
.5	1.00	1.59	10.1
.6	0.927	1.51	12.2
.7	.725	1.05	13.9
.8	.469	0.581	11.5

In contrast with complex III, the characterisics of the proton spectra of complexes I, II, and IV did not depend on the optical isomeric composition of the histidine charge that was used to prepare these complexes.

Complexes of Co(II) with Other Compounds.—Representations of p.m.r. spectra of aqueous complexes of Co(II) with various ligands having some similarity to histidine are presented in Fig. 7. Some of these spectra have been used to confirm structures assigned to Co(II)–histidine complexes; others are used below in discussion of the assignment of the spectral lines of the p.m.r. spectra of Co(II)–histidine complexes.

The spectra of Fig. 7 were obtained from aqueous solutions of the complexes at pH 6–9 and for ligand–Co(II) ratios > 2. In general, these complexes are weaker than complexes II and III of histidine, and Co(II) hydroxide is precipitated as the solutions become alkaline. The decrease in stability of the complexes is accompanied by a decrease in the magnitude of the contact shifts of the p.m.r. spectra. In several of the less stable complexes, resonances are very broad and poorly resolved. In many cases exchange effects produce a greater degree of broadening than was observed in the histidine spectra. It is not certain, of course, that all of the spectra of Fig. 7 represent octahedral, 2:1 ligand–Co(II) complexes since other configurations may be more stable for ligands such as glycine or imidazole. With these reservations in mind, it is quite apparent that modification of the histidine structure results in dramatic changes in the p.m.r. spectrum of the complex of the modified ligand with Co(II).

Assignment of P.m.r. Spectra of Histidine–Co(II) Complexes.—Detailed assignment of the individual proton magnetic resonance lines of the spectra of complexes II, III, and IV of histidine and Co(II) is not essential for the assignment of the structures of these complexes but is perhaps of some spectroscopic interest. Identification of the separate resonances with the various protons of complexes II, III, and IV is somewhat complicated by the fact that the lines are too broad to permit resolution of spin–spin structure. Contact shifts in these spectra may arise from both isotropic hyperfine contact interactions[7] and pseudocontact interactions.[8] The contributions of isotropic contact interactions to resonance shifts are complicated by the fact that spin density may be introduced onto the ligands from the Co(II) ion at the two or three binding sites of each ligand and by way of both the π- and σ-bond systems. Neither theoretical nor empirical methods have yet been developed sufficiently to permit confident predictions to be made of directions and magnitudes of contact shifts in p.m.r. spectra of complexes of this type. Thus, the spectral assignments shown in Fig. 6 are based primarily on intensity relationships and on the nature of the spectra shown in Fig. 7 for similar species.

The p.m.r. spectra of complexes II and III are very similar and are considered together. The two spectral lines of each complex that are observed when H_2O rather than D_2O is used as solvent have about the same intensities and appear in the same field region. These lines must be associated with protons that are nearly equivalent and that exchange rapidly with D_2O before the complex is formed. These two lines are assigned, therefore, to the hydrogen atoms bonded to the amine nitrogen. In complex I the contact shifts of H_α and H_β are to low field and the ratio of the magnitudes of the shifts of H_α/H_β is about 2. Furthermore, in the p.m.r. spectra of the complexes of glycine or alanine with Co(II), H_α exhibits a large contact shift to low field. H_β of alanine (H_α and H_β are distinguished by their 1:3 intensity relationship) is also shifted to low field and the ratio of the magnitudes of the shifts of H_α/H_β is 2.5. Therefore, in the p.m.r. spectra of complexes II and III we have assigned the low field line that is twice as intense as the other lines to H_β and the line that exhibits a low field shift about 3.5 times greater than H_β to H_α. Two resonances then remain in the spectra of complexes II and III to assign to the C–H protons of the imidazole ring, $H_{C:2}$ and $H_{C:4}$. These lines have about the same intensity as H_α and thus represent one proton each. The assignment of the low field line to $H_{C:2}$ and the high field to $H_{C:4}$ is tentative as it is based on concepts of electron spin density introduction onto ligands that will be developed in a future communication. It was noted earlier in this paper that the p.m.r. spectra of complexes II and III are similar except that the contact shifts are somewhat greater for

complex III and this is taken to indicate a stronger bonding of histidine to Co(II) in complex III. Further comparison of the contact shifts suggests that the major enhancement of binding strength in complex III comes from an increase in the strength of the Co(II)–imidazole nitrogen coordinate link.

Turning now to the assignment of the p.m.r. lines of the spectrum of complex IV, we note that the contact shifts are quite different from those of complexes II and III. Such differences are to be expected since the change from octahedral to tetrahedral symmetry may affect both the binding energy of the ligand atoms to Co(II) and the geometry of interaction of electron orbitals of the ligand with Co(II) orbitals, and does indeed, as discussed earlier, affect the magnetic susceptibility of the complex. In the spectrum of complex IV, H_β appears as a doublet (because of nonequivalence of the protons of the CH_2 group) and is therefore easily assigned; H_α, $H_{C:2}$, and $H_{C:4}$ are assigned on the same grounds that were employed for complexes II and III.

Effects of O_2 on Histidine–Co(II) Complexes.— Other investigators[1] have made extensive studies of interactions of molecular oxygen with complexes of amino acids and Co(II). They have found that the aqueous histidine–Co(II) complex reacts reversibly with O_2 to form a diamagnetic complex that is amber in color. While we have not made detailed studies of effects of O_2 on histidine–Co(II) solutions, we have observed that solutions of complexes II, III, or IV all turn amber rapidly in air. As the oxygen complex forms, both the paramagnetic susceptibility of the solution and the intensity of the p.m.r. spectrum of the original histidine–Co(II) complex decreases. A rather poorly resolved p.m.r. spectrum appears in the resonance field region where diamagnetic species are observed. Thus, it appears that all of the strong histidine–Co(II) complexes react with O_2 to give diamagnetic complexes which may or may not be identical. Furthermore, the behavior in p.m.r. of histidine–Co(II) complexes in the presence of O_2 is consistent with previous studies of magnetic behavior of the complexes.[1,19]

Experimental

Complexes of Co(II) with histidine were prepared in D_2O with rigorous exclusion of oxygen. A typical experiment was performed in the following manner. A solution of tetramethylammonium chloride (0.23 *M*) was prepared in 10 ml. of D_2O (99.5 + % D_2O). The solution was stirred under a stream of nitrogen for 10 min. and kept under nitrogen during the remainder of the experiment. Weighed amounts of histidine and $CoCl_2 \cdot 6D_2O$ were added to bring the solution to the desired molarity in these reagents. The pH of the solution was adjusted to a value less than 3, and the solution was stirred under nitrogen for an additional 10 min. No reaction of oxygen with histidine or Co(II) occurs under these conditions. The solution was then adjusted to the required pH by stirring in small amounts of concentrated solutions of HCl or NaOD. A 0.2-ml. aliquot of the solution was transferred under nitrogen to an n.m.r. sample tube and sealed therein. The proton magnetic resonance spectrum of the solution was examined at room temperature with a Varian HR-60 n.m.r. spectrometer. Additional samples were taken in a similar manner after appropriate variation of the pH of the solution. Equilibrium was reached in all of the experimental solutions within a few minutes as judged by the constancy and reproducibility of the p.m.r. spectra of the samples. Solutions contaminated with only a small amount of oxygen turned from pink or blue to brown and were discarded. Solutions of complexes of other ligands with Co(II) were prepared in a similar manner.

Reagent grade chemicals or, in the case of the amino acids, the purest compounds commercially available from Calbiochem Inc. or Nutritional Biochemicals Corp. were used. $CoCl_2 \cdot 6D_2O$ was prepared by exchanging $CoCl_2 \cdot 6H_2O$ with D_2O. NaOD was made by addition of sodium metal to D_2O. Measurements of pH were taken as read from a commercial potentiometric pH meter employing a glass electrode without correction to pD values.

Frequency displacements of all p.m.r. spectral lines with respect to the position of the resonance of tetramethylammonium ion employed as internal standard were measured by the side band modulation technique. Paramagnetic susceptibilities of the histidine–Co(II) solutions were measured by an n.m.r. technique.[20]

(19) L. Michaelis, *Arch. Biochem.*, **14**, 17 (1947).

(20) D. F. Evans, *J. Chem. Soc.*, 2003 (1959).