Complexes formed between Zinc(II) and L-Histidine: a Carbon-13 Nuclear Magnetic Resonance Study†

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Carbon-13 NMR spectral data are reported as a function of pH for mixtures of L-histidine and zinc nitrate, and of L-histidine + zinc + 1,2-diaminoethane (en), 1,3-diaminopropane (pn), or glycine (gly), and also for L-histidine alone. The pH profiles have been analysed by computer and the chemical shifts of the carbon atoms determined for the following species: $HisH_{-1}^{2-}$, His^-, HHis, H_2His^+, H_3His^{2+}, $[Zn(His)]^+$, $[Zn(His)_2]$, $[Zn(His)(HisH_{-1})]^-$ and $[Zn(HisH_{-1})_2]^{2-}$. The ^{13}C chemical shifts have also been determined for en, pn and gly (methylene only) in the complexes $[Zn(His)_2X]$ (X = en, pn or gly), $[Zn(His)_2(HX)]^+$ (X = en or pn), $[Zn(His)_2(glyO)]^-$, $[Zn(His)(HisH_{-1})X]^-$ (X = en or pn) and $[Zn(His)(HisH_{-1})(glyO)]^{2-}$. Stability constants have been determined for the complexes. Potentiometric titrations with zinc and histidine solutions provide support for the interpretation of the chemical shift data. It is argued that $[Zn(His)_2]$ contains distorted octahedrally co-ordinated zinc, and that deprotonation occurs at the pyrrole-ring nitrogen rather than zinc-bound water.

Zinc is an essential metal in many enzyme systems yet little is known about its mechanistic role other than that it acts as a Lewis acid and that its co-ordinational structure frequently changes during turnover. The most widely studied zinc enzymes are carbonic anhydrase and carboxypeptidase, in both of which the active-site Zn^{2+} ion is bound through the imidazole side-chain of one or more histidine residues.[1] For this reason there have been more reports on the thermodynamics of zinc complex formation with histidine than with almost any other amino acid but there still remain ambiguities over the identities of some of the complexes formed. While the earlier results[2,3] were analysed in terms of only the mono and bis complexes of the histidine monoanion ([ML] and $[ML_2]$), three of the more recent studies[4-6] have included contributions from one or more of the protonated forms [M(HL)], [ML(HL)] and $[M(HL)_2]$, and one[5] has invoked the formation of a 'high-pH' form.

The underlying cause of these uncertainties is the d^{10} configuration of Zn^{2+} and the consequent lack of suitable spectroscopic properties for identifying individual complexes formed in solution or directly probing their co-ordination geometries. This has led to a reliance on indirect methods, principally pH titrations, and comparison with analogous systems involving Co^{2+}, Cu^{2+} and other more readily characterized metals. Carlson and Brown[7] used IR and proton NMR spectroscopy to investigate the structures of a range of metal–histidine systems in D_2O but they were prevented by precipitation between pH 5.5 and 11.5 from studying the zinc system in the biologically important range around neutrality.

We have recently shown[8-14] that the ^{13}C NMR chemical shifts of an organic ligand L can be used to characterize the Zn–L complexes formed in solution, even when (as is generally the case) exchange is rapid on the NMR time-scale. This is done by comparing computer-generated pH profiles of L (which are matched with the experimental data points) in the presence and absence of the metal. Subsequent addition of a potentially bidentate ligand A (such as en, pn or glyO) to the Zn + L system generates a new set of pH profiles from which the chemical shifts and stability constants for any Zn–L–A ternary complexes formed can be determined. For many of the systems we have studied,[9-14] in which L was an aliphatic polyamine, it proved possible to use this information to deduce the co-ordinational structures of the individual complexes in solution.

In the present paper we provide ^{13}C NMR data and supporting potentiometric evidence which confirm the formation of mono and bis complexes with the L-histidine monanion, [ML] and $[ML_2]$. We find no evidence for the formation of protonated complexes in acidic solution but in the alkaline region the bis complex loses two protons, with estimated pK_a values (pK_D^c) of 11.15 and 11.80. Comparison of the chemical shifts for the species $[ML_2H_{-1}]$ and $[ML_2H_{-2}]$ with those for the various forms of the free ligand suggests that the protons have been lost from the pyrrole nitrogens of the histidine ligands rather than metal-co-ordinated water molecules. Comparatively weak ternary complexes $[ML_2(A)]$ are formed with glyO, en and pn (A). We argue that the distorted-octahedral co-ordination geometry for $[ML_2]$ observed in the solid state is retained in solution, and our present results are compared with those for $[Zn(dien)_2]^{2+}$, which also retains a distorted-octahedral geometry in solution. Our findings on the nature of the deprotonated complexes are discussed briefly in the light of recent work on zinc enzymes.

Experimental

L-Histidine (BDH, >98%) was used without further purification. The other chemicals were purified and the solutions made up in D_2O as described previously;[15,16] pD = pH meter reading +0.40. The NMR spectra were recorded[15,16] at 21 ± 1 °C with a JEOL FT-100 or GX-271 instrument, using air-conditioning and compressed air to achieve thermostatting. Chemical shifts were measured relative to internal 1,4-dioxane (δ 67.71) and are quoted on the δ scale; they are estimated to be reliable to ±0.12 ppm or better. The potentiometric titrations were done in D_2O under a CO_2-free atmosphere using initial volumes of 5.70 cm^3 and [NaOD] = 0.98 mol dm^{-3}, and the analysis of these and the NMR titrations was performed as described previously.[11,15,16] Following recent practice, we have

† *Supplementary data available* (No. SUP 56859, 5 pp.): observed chemical shifts for solutions containing histidine.

See Instructions for Authors, *J. Chem. Soc., Dalton Trans.*, 1992, Issue 1, pp. xx–xxv. The notation used in this paper is as follows: gly = glycine, glyO = glycinate(1−), en = 1,2-diaminoethane, pn = 1,3-diaminopropane, dien = diethylenetriamine; ligand X = A (glyO, en or pn) or HA (gly, Hen^+ or Hpn^+); ligand L = His = L-histidinate(1−).

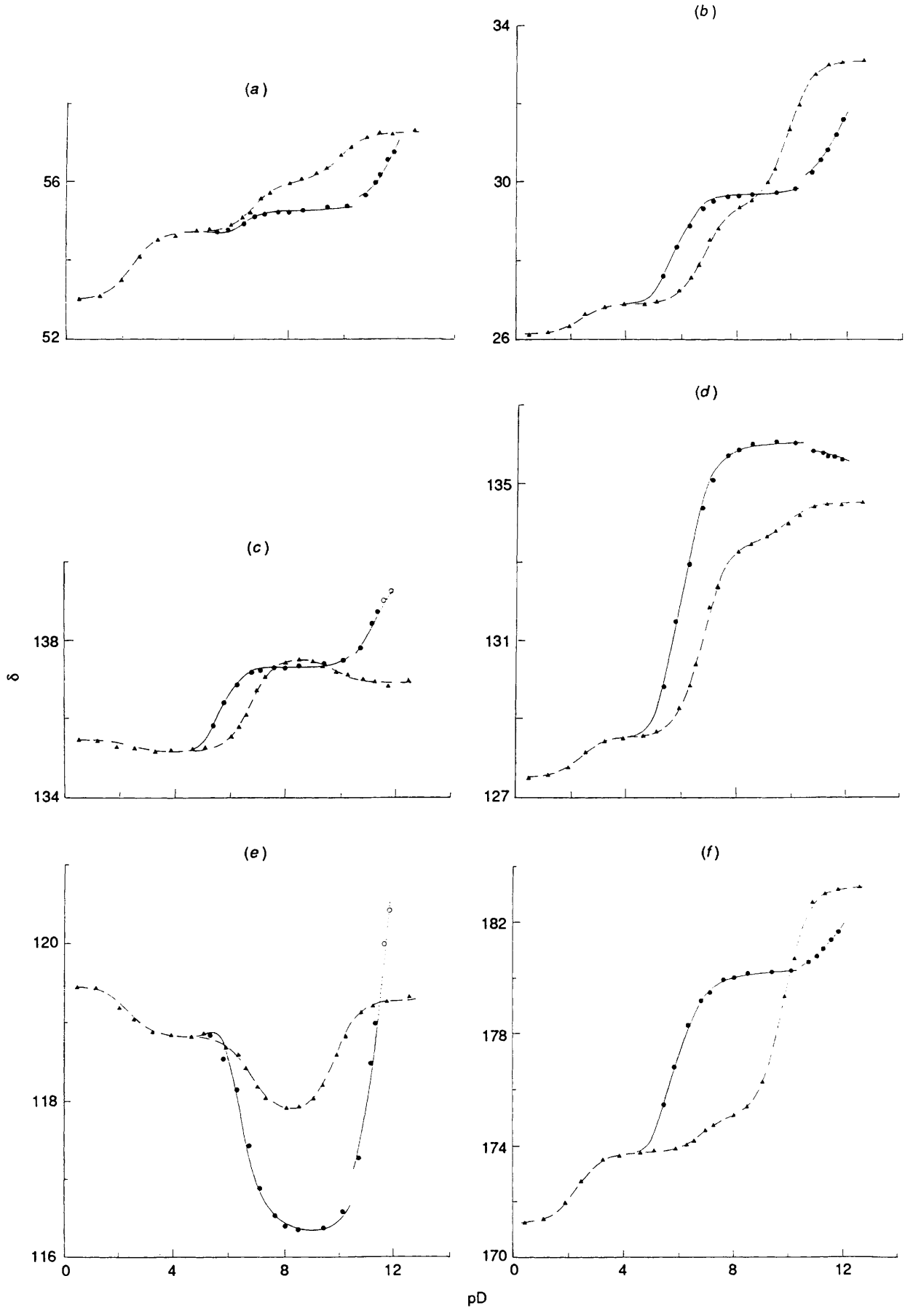
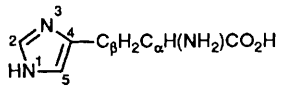


Fig. 1 Carbon-13 NMR chemical shifts as a function of pD for histidine (0.20 mol dm^{-3}) and zinc (0.045 mol dm^{-3}) + histidine (0.090 mol dm^{-3} at pD < 10.5 or 0.103 mol dm^{-3} at pD > 10.5). Atoms C_ε (*a*), C_β (*b*), C^2 (*c*), C^4 (*d*), C^5 (*e*) and C_α (*f*)

indicated the quality of fit of the pD profiles by the standard deviations calculated from the difference between the observed δ or (volume added) values and those computed on the basis of the parameters listed.

Results

Histidine.—The non-equivalent C atoms in histidine are identified according to IUPAC nomenclature as shown. (Under common biochemical usage, which was employed in some of the earlier[17,18] and even more recent[19] work on histidine, the numbering of the ring atoms starts with the



pyridine N rather than the pyrrole N such that N^1 and N^3 are interchanged, as are C^4 and C^5.) The measured chemical shifts δ for the six C atoms at different pD are represented by the triangles in Fig. 1. The spectral lines were sharp throughout, though their relative heights were somewhat variable, as has been noted previously.[17,20] The titration curves (dashed lines) were computed from the data assuming rapid exchange and using the pK_a values (pK_D^c) and chemical shifts listed in Table 1.

The assignment follows that of Horsley *et al.*[17] and Freedman *et al.*,[18] and also of Quirt *et al.*,[25] who provided a theoretical rationalization for it in the form of CNDO/2 calculations for their observed titration shifts. (It should be noted that in other places, *e.g.* refs. 26–28, the assignment of the C^2 and C^4 resonances is reversed. Our observation of line broadening at high pH in the resonances attributed to C^2 and C^5 for histidine solutions containing zinc, described below, provides further evidence for the correctness of the assignment of Horsley *et al.*) When allowance is made for differences in referencing, the agreement between our own chemical shifts and other published data[18,25,29] is good.

There is also good agreement between our derived pK_a values (pK_D^c = 9.82, 6.75 and 2.34) and those in the literature. Li *et al.*[21] reported values of 9.83, 6.65 and 2.14 under similar conditions (D_2O solvent, I = 0.11 mol dm^{-3}, 25 °C) to our own. In addition, there have been[2] several pK_a determinations in water, of which representative sets at similar ionic strength and temperature (refs. 22–24) give average values of 9.15, 6.09 and 1.86. These are pK_H^c values and imply deuterium isotope effect corrections ΔpK ($= pK_D^c - pK_H^c$) of 0.67, 0.66 and 0.48, respectively, for our pK_D^c values, which are well in line with other published results.[16,30]

The Zinc + Histidine System.—There is general agreement in the literature[2] that around neutrality histidine forms two principal zinc complexes, $[Zn(His)]^+$ and $[Zn(His)_2]$ ([ML] and [ML_2]), whose respective (logarithmic) stability constants are *ca.* 6.5 and 5.5. Preliminary computation indicated that it would not be possible to find L:M concentration ratios at which the ligand was present entirely in one or other of these forms, but that the best conditions for characterizing them

Table 1 'Best' ^{13}C NMR chemical shift[a] and pK_a values for His (L)

	$\delta(LH_{-1}^{2-})$	$\delta(L^-)$	$\delta(HL)$	$\delta(H_2L^+)$	$\delta(H_3L^{2+})$
C_α	57.55[b]	57.22	55.98	54.71	53.02
C_β	35.00[b]	33.16	29.42	26.90	26.12
C^2	146.20[b]	136.84	137.57	135.12	135.46
C^4	137.60[b]	134.52	133.49	128.52	127.48
C^5	124.90[b]	119.26	117.82	118.80	119.44
C_0	184.35[b]	183.27	175.04	173.72	171.22

Measured[c]	pK_D^c	—	9.82	6.75	2.34
Literature[d]	pK_D^c	(13.93[e])	9.83	6.65	2.14
Literature[f]	pK_H^c	—	9.15	6.09	1.86

[a] The estimated errors for all except $\delta(LH_{-1}^{2-})$ are ±0.1 ppm. The 'best' values were determined from 23 sets of data points which yielded the following standard deviations (in ppm): 0.047 (C_α), 0.067 (C_β), 0.078 (C^2), 0.086 (C^4), 0.058 (C^5) and 0.130 (C_0). [b] Determined in 10 mol dm^{-3} NaOD. The δ values quoted are lowest limits in each case but no estimates of the errors are possible. [c] At 21 ± 1 °C, various I. The estimated errors (see also footnote *a*) are ±0.1. [d] Data from ref. 21. [e] Value quoted in ref. 7. [f] The average of data from refs. 22 (20 °C, I = 1 mol dm^{-3}), 23 (25 °C, I = 0.2 mol dm^{-3}) and 24 (25 °C, I = 0.25 mol dm^{-3}) (see text).

Table 2 'Best'[a] ^{13}C NMR chemical shifts[b] and formation constants[c] for zinc(II)–histidine complexes

Complex		δ(histidine)[d]						δ(X)		log K
		C_α	C_β	C^2	C^4	C^5	C_0			
$[Zn(His)]^+$	[ML]	54.40	29.90	137.90	133.70	119.30	180.50			6.90[e]
$[Zn(His)_2]$	$[ML_2]$	55.26	29.67	137.25	136.12	116.23	180.20			5.30[f]
$[Zn(His)(HisH_{-1})]^-$	$[ML(LH_{-1})]$	55.90	30.60	139.95	136.00	119.50	180.60			11.15[g]
$[Zn(HisH_{-1})_2]^{2-}$	$[M(LH_{-1})_2]$	58.25	32.80	144	135.40	124	183.00			11.80[g]
$[Zn(His)_2(en)]$	$[ML_2(A)]$								40.80	1.85[h]
$[Zn(His)_2(pn)]$	$[ML_2(A)]$							C_a	41.20	1.78[h]
								C_b	30.90	
$[Zn(His)_2(glyO)]^-$	$[ML_2(A)]$							CH_2	43.30	1.30[h]
$[Zn(His)(HisH_{-1})(en)]^-$	$[ML(LH_{-1})A]$								42.60	1.48[i]
$[Zn(His)(HisH_{-1})(pn)]^-$	$[ML(LH_{-1})A]$							C_a	39.60	1.30[i]
								C_b	36.00	
$[Zn(His)(HisH_{-1})(glyO)]^{2-}$	$[ML(LH_{-1})A]$							CH_2	45.90	1.30[i]
$[Zn(His)_2(Hen)]^+$	$[ML_2(HA)]$								40.60	0.70[h]
$[Zn(His)_2(Hpn)]^+$	$[ML_2(HA)]$							C_a	39.20	0.78[h]
								C_b	26.40	
$[Zn(His)_2(gly)]$	$[ML_2(HA)]$							CH_2	43.10	1.00[h]

[a] The 'best' values for the binary system were determined from 27 sets of data points, with standard deviations of the experimental chemical shifts (shown by circles in Fig. 2) from the values calculated on the basis of the parameters listed of 0.081 (C_α), 0.068 (C_β), 0.113 (C^2), 0.130 (C^4), 0.210 (C^5) and 0.080 (C_0) ppm. For the ternary systems, the numbers of data points (in parentheses) and standard deviations for the ligand (ppm) were, respectively: en (10), 0.055; pn (C_a)(10), 0.096; pn (C_b)(10), 0.120; gly (CH_2)(9), 0.042. [b] The estimated errors (ppm) are as follows: ±0.1 for [ML_2], ±0.3 for [ML] and [$ML(LH_{-1})$], ±0.8 for C_α, C_β, C^4, C_0 in [$M(LH_{-1})_2$], ±2 for C^2, C^5 in [$M(LH_{-1})_2$] (less reliable values), and ±0.2 for the ligand X in the ternary complexes. [c] At 21 ± 1 °C; various I. The estimated errors are ±0.2 for [ML], [ML_2], and [$ML(LH_{-1})$], ±0.4 for [$M(LH_{-1})_2$] and ±0.3 for ternary complexes. [d] Where values are not given, reliable values were unobtainable. [e] K_1 = [Zn(His)]/[Zn][His]. [f] K_2 = $[Zn(His)_2]/[Zn(His)][His]$. [g] pK_a values pK_D^c for $[Zn(His)_2]$. [h] K_A = $[Zn(His)_2X]/[Zn(His)_2][X]$ with X = Hen^+, Hpn^+, en, pn, gly or glyO. [i] K_A = $[Zn(His)(HisH_{-1})X]/[Zn(His)(HisH_{-1})][X]$ with X = en, pn or glyO.

would be about 1.1:1 and 2:1, respectively (a ratio closer to 1:1 or an excess of metal being ruled out by the very low solubility of zinc hydroxide). We experienced no problems with the higher ratio but found that, at decimolar (or even lower) concentrations, solutions of the lower ratio tended to be unstable over the time-scale necessary for the accumulation of NMR data and the measurement of pH (owing to precipitation of a fine white solid which elemental analysis indicated to be the mono complex, see also ref. 7). We therefore used the ratio 1.5:1 to determine the parameters for [ML], having confirmed the $[ML_2]$ parameters through use of the L:M ratio of 3:1. (There is no evidence in the literature for the formation of a tris complex.) The chemical shifts for the individual solutions containing histidine + zinc at concentrations of 0.090 + 0.045, 0.103 + 0.045, 0.15 + 0.05 and 0.15 + 0.10 mol dm^{-3}, respectively, at different pD are given in SUP 56859, while the first two sets of data are shown by the circles in Fig. 1.

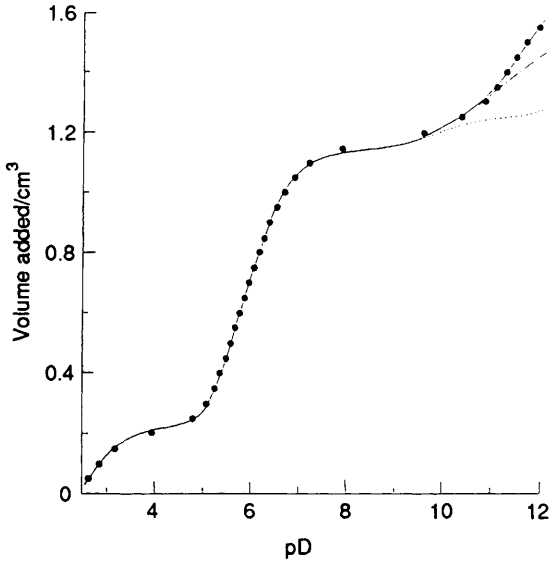


Fig. 2 Potentiometric titration of zinc + histidine (0.035 + 0.088 mol dm^{-3}) in D_2O; NaOD (0.98 mol dm^{-3}) was added to 5.70 cm^3 of the solution

The six spectral lines were consistently sharp (with the exception of those assigned to C^2 and C^5 at pD above about 11), indicating that the system is in the fast-exchange regime. Since the individual resonances remained discrete throughout, there is no ambiguity over assignment. Computer analysis of the chemical shifts on the basis of the formation of [ML] and $[ML_2]$ provides a good fit to the experimental data up to about pD 9.5 (Fig. 1, solid lines) but above this there is clearly at least one other species involved since the plateau regions which start at about pD 7 would otherwise extend indefinitely to high pD until the complexes break up under competition from hydroxide, and histidine is released. (The possibility that this break-up of the complexes itself is the cause of the divergence above pD 9.5 can be ruled out by considering the movement of the C^2 resonance: if histidine were being released the observed δ value would converge on the free-ligand line whereas it actually moves away from it.) Of the potentiometric investigations of the zinc–histidine system in the literature only one, that by Sovago *et al.*,[5] indicates the formation of a 'high-pH' complex but the authors do not provide any quantitative information about it. Further analysis of our own ^{13}C NMR data shows that it is possible to obtain a good match up to at least pD 11.5 if it is assumed that $[ML_2]$ loses two protons* with pK_a values (pK_D^c) of 11.15 and 11.80, respectively (Fig. 1 and Table 2).

In order to confirm this interpretation, we performed potentiometric titrations on 0.088 mol dm^{-3} solutions of histidine in D_2O containing 0.035 mol dm^{-3} zinc, as shown in

* For simplicity, we have followed the convention that mixtures of protonated and deuteriated forms are referred to as protonated and represented by [HL], *etc.*

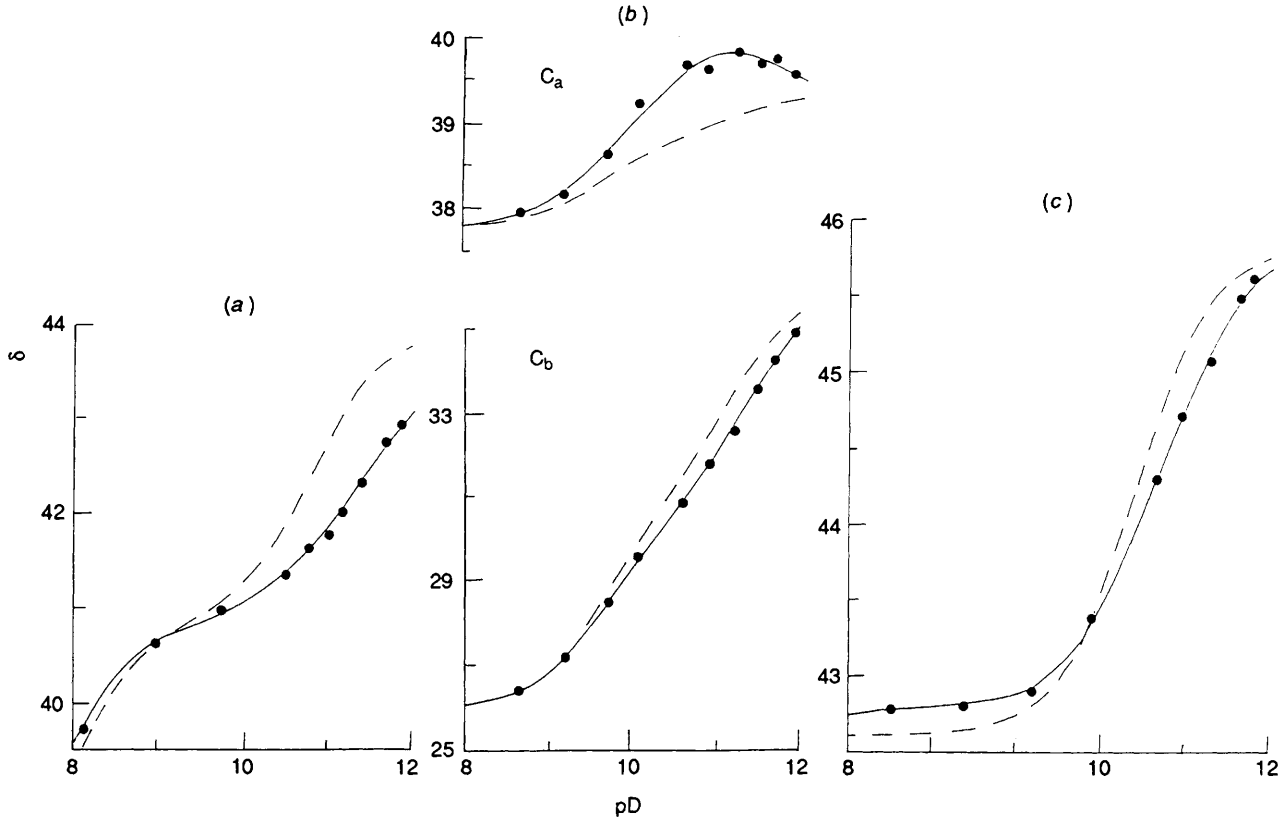


Fig. 3 Carbon-13 NMR chemical shifts as a function of pD for X in $Zn(NO_3)_2$ + histidine + X:X = en (*a*), pn (*b*) or gly (*c*). Concentrations were 0.10 + 0.20 + 0.10 mol dm^{-3}, respectively

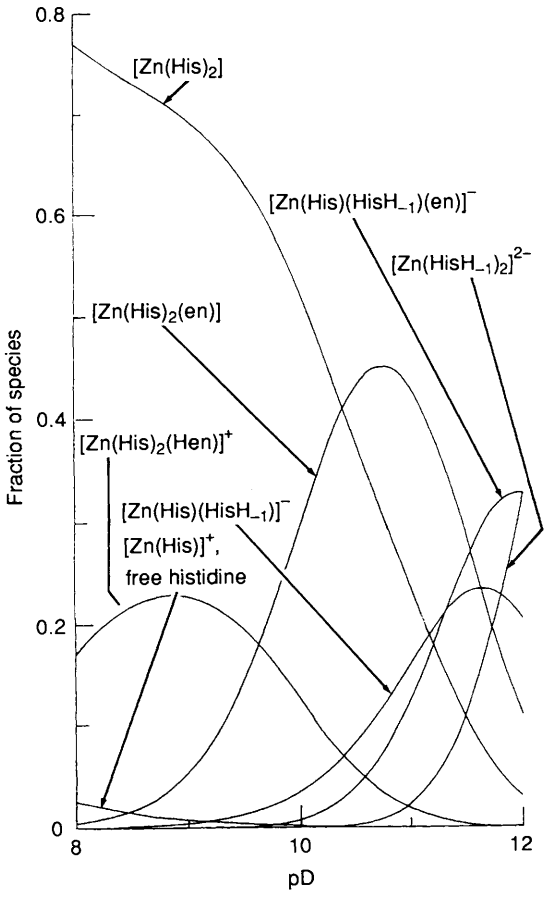


Fig. 4 Distribution diagram for the various complexes and uncomplexed histidine in Zn (0.10 mol dm^{-3}) + histidine (0.20 mol dm^{-3}) + en (0.10 mol dm^{-3}) solutions

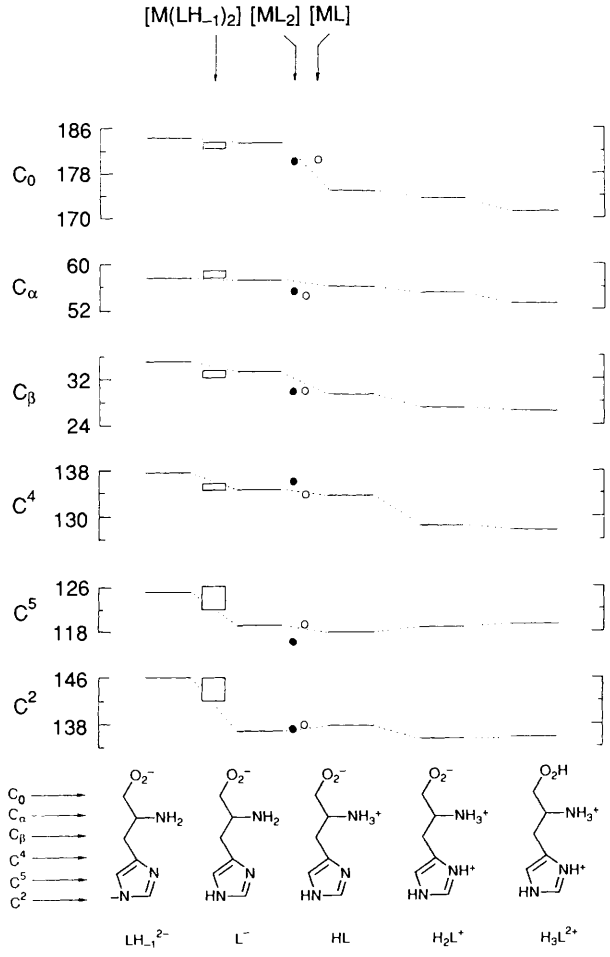


Fig. 5 Summary of ^{13}C NMR chemical shifts for histidine species (see text)

Fig. 2. The titration profile over the pD range 2.5–9 is consistent with the formation of the two documented species [ML] and $[ML_2]$, using the respective (logarithmic) stability constants 6.70 and 5.60, but above pD 9 it would continue as shown by the dotted line if no 'high-pH' complex were formed. The dot-dashed line represents the titration of a single proton in $[ML_2]$ (with $pK^c_D = 11.15$) while the solid line above pD 9 represents the titration of two protons (second $pK^c_D = 11.93$). The solid line (± 0.011 cm^3 standard deviation in terms of volume added on 31 points; estimated errors in $\log K_1 \pm 0.3$, $\log K_2 \pm 0.2$, first pK^c_D of $[ML_2] \pm 0.3$, second pK^c_D of $[ML_2] \pm 0.5$) can be seen to represent the experimental data well.

Although it is not possible to rationalize the high-pD behaviour in Figs. 1 and 2 in terms of the deprotonation of [ML] alone, it does appear from the δ values recorded for a 1.1:1 solution at pD *ca.* 12.5 (see SUP 56859) that $[MLH_{-1}]$ is formed in very alkaline solution. In view of the problems of working with this L:M ratio and with accurate pD measurement in this region, this aspect was not investigated further.

The Ternary Systems.—The measured chemical shifts of the ligand C atoms in the following mixtures at different pD are given in SUP 56859: Zn (0.10 mol dm^{-3}) + histidine (0.20 mol dm^{-3}) + en, pn or gly (0.10 mol dm^{-3}). The spectral lines due to ligand X (en, pn or gly) were consistently sharp while those due to histidine were similar in appearance to those observed in the binary systems. Consequently, all the chemical shift data were analysed on the assumption of rapid exchange.

In the light of the report by Sovago *et al.*[5] that $[Zn(His)]^+$ forms ternary complexes of the form [ML(A)] with en and glyO, we tried to analyse our chemical shifts for the ligand X in terms of parameters for the formation of the species [ML(A)] and [ML(HA)] (see also refs. 9–13). However, these parameters could not be refined successfully to give even moderate agreement between computed and experimental δ values. On the other hand, the assumption that the principal ternary complex formed is $[ML_2(A)]$ (*cf.* ref. 11) produced a fairly good agreement between computed and experimental pD profiles and this was improved by the addition of two further complexes, $[ML_2(HA)]$ and $[ML(LH_{-1})A]$, to the refinement procedure. The experimental data and 'best fit' curves computed using the parameters listed in Table 2 are shown in Fig. 3; the dashed lines (see refs. 9 and 16) indicate the computed behaviour of the appropriate free ligand X. A representative distribution diagram for the en system is given in Fig. 4.

Discussion

Formation of [ML] *and* $[ML_2]$.—Despite the difference in experimental method used for their determination, there is good agreement between our stability constants (Table 2) and those reported previously for the zinc–histidine system. Our values of 6.90 and 5.30, respectively, for $\log K_1$ and $\log K_2$ are well in line with those of Williams[3] (7.07 and 5.67; 25 °C, I = 3.0 mol dm^{-3}) and the average values (6.64 and 5.28) from the four earlier determinations[24,31–33] which used temperatures and ionic strengths similar to our own. More recent values by Daniele and Ostacoli[34] (6.53 and 5.39; 25 °C, I = 0.1 mol dm^{-3}), Pettit and Swash[4] (6.48 and 5.60; 25 °C, I = 0.1 mol dm^{-3}), Sovago *et al.*[5] (6.31 and 5.53; 25 °C, I = 0.2 mol dm^{-3}) and Nair *et al.*[6] (6.41 and 5.33; 37 °C, I = 0.15 mol dm^{-3}) are also acceptably close.

Chemical Shifts.—To rationalize the ^{13}C NMR chemical shifts of the various zinc complexes of histidine (Table 2) it is helpful first to look at the changes in δ value accompanying the protonation and deprotonation of the free ligand, Table 1. The relevant data and those for three of the complexes have also been plotted in Fig. 5 using the same scale for each C atom.

(*i*) *Free histidine.* Quirt *et al.*[25] studied the titration of a number of amino acids by ^{13}C NMR spectroscopy and found that the ionization of remote as well as neighbouring groups can usually be monitored at each carbon atom. They also found that the δ value titration shifts (Δδ) for the carboxylate and α-amino groups are characteristic for each of the three C atoms in the common part of the molecule (*i.e.* C_α, C_β and C_0). The present results reflect both these generalizations. For example, the protonation of the carboxylate group (*i.e.* $H_2L^+ \longrightarrow H_3L^{2+}$) produces upfield shifts in the resonance due to C_α, C_β and C_0 of, respectively, 1.69, 0.78 and 2.50 ppm (compared with the averages of Quirt *et al.* of 1.9 ± 0.2, 0.85 ± 0.15 and 2.6 ± 0.3 ppm) and is also 'seen' by the ring carbons. As for the imidazole ring, the removal of a proton from it (*i.e.* $L^- \longrightarrow LH_{-1}^{2-}$) and the addition of one to it (*i.e.* $HL \longrightarrow H_2L^+$) are both accompanied by changes in δ value (Δδ) at C^2 and C^5 which are similar to those found[35] with the parent compound: for C^2, Δδ = 9.36 and −2.45 compared with 8.9 and −1.6, respectively, and for C^5, Δδ = 5.64 and 0.98 compared with 4.5 and −0.2. Both ionizations are also picked up in the other parts of the molecule.

In attempting a more detailed analysis of these changes it must be remembered that each of the three intermediate forms of histidine exists as a mixture of microstates, each with its own micro-pK_a values (*cf.* ref. 36). For H_2L^+ the principal form can be assumed to be that shown in Fig. 5 but for L^- or HL there is no generally accepted view of the distributions between the microstates. For L^- a value close to 50:50 has been determined[19] between the N^1H tautomer (shown in Fig. 5) and the N^3H tautomer (not shown), as must be the case for imidazole itself. On the other hand, it has also been argued[38] that in L^- the N^1H tautomer is stabilized relative to the N^3H form to the extent of about 2:1 owing to the electronic or steric consequences of substitution at C^4. With the zwitterion HL much of the evidence favours the N^1H form to the extent of about 8:1 and this imbalance is thought[37,38] to be at least partly the result of hydrogen bonding between the NH_3^+ group and N^3.

Returning to the protonation and deprotonation shifts at the individual C atoms, we find that the largest Δδ generally occur when the carbon concerned is β to the atom receiving or losing the proton if it is outside the ring or α to it if it is inside the ring. Thus, for C_0 and C_β the largest Δδ values accompany the ionization of the amino group ($L^- \rightleftharpoons HL$), while for C_α the largest Δδ occurs on ionization of the carboxylate group ($H_2L^+ \rightleftharpoons H_3L^{2+}$). The situation in the ring is complicated by the tautomerism outlined above but one notable feature is the large downfield shift occurring at C^2 and C^5 on forming LH_{-1}^{2-} from L^-.

(*ii*) [ML] *and* [ML_2]. The δ values for [ML] and [ML_2] (Table 2 and Fig. 5) are generally similar. With the aliphatic polyamines we found[8,11–13] that the change in chemical shift (Δδ) for a particular C atom on forming a zinc complex from the deprotonated form of the free ligand L was typically about half that seen on fully protonating it. In line with this, the two carbons in histidine (C_0 and C_β) which have their largest protonation shifts accompanying the ionization of the amino group have δ values for [ML] and [ML_2] which are midway between those for L^- and HL. (For C_α the protonation shifts are comparatively small and conformational effects are likely to be relatively important; nonetheless, δ_{ML} does lie between δ_{H_2L} and δ_{H_3L}.) For the ring carbons, the observed similarity of most of the δ_{ML}, δ_{ML_2} values to the corresponding value for HL is to be expected if, as mentioned above, internal hydrogen bonding between NH_3^+ and N^3 is important in HL.

(*iii*) [ML_2H_{-1}] *and* [ML_2H_{-2}]. A significant feature of the zinc–histidine results is the large downfield shifts of the C^2 and C^5 resonances accompanying the deprotonation of [ML_2]. The δ values for [ML_2H_{-2}] are shown as hollow rectangles in Fig. 5, the height indicating the estimated reliability. Although the errors for C^2 and C^5 are comparatively large, the reason for this (a substantial differential line broadening) is in itself good supporting evidence for there being abnormally large downfield shifts at these two carbons. In view of their similarity to the large deprotonation shifts experienced by C^2 and C^5 in L^- (see above), we attribute them also to the loss of the α-protons (N^1H).

The formation of a ring-deprotonated complex {actually [$M(LH_{-1})$]} was postulated in 1966 by Carlson and Brown.[7] Of the potentiometric studies on the zinc–histidine system (refs. 2–6, 24 and 31–34), only that by Sovago *et al.*[5] refers to the formation of a 'high-pH' complex but these authors prefer a formulation involving one or more zinc-bound hydroxo groups. While not excluding the latter possibility, our results support pyrrole-nitrogen deprotonation since we have already shown[10] that [contrary to the situation[39] with diamagnetic transition-metal ions such as cobalt(III)] large 'through bonds' effects on ^{13}C NMR chemical shifts do not appear to be transmitted between the zinc atom and its ligands: without such through-metal transmission the large downfield shifts we observe at C^2 and C^5 could not originate in the deprotonation of zinc-bound water molecules (of which the stoichiometry indicates that there must be two).

Further evidence against the hydroxo-complex model comes from a consideration of the structure of the bis complex.

Structure of [ML_2].—Only two single-crystal X-ray structure determinations have been published[40,41] on zinc–histidine complexes (one of which[41] has been re-refined subsequently[42]) and they relate to different hydrates of [ML_2]. While the detailed dispositions of the ligands differ, in each case the co-ordination geometry at the metal is described as being 'distorted tetrahedral' since there are four 'normal' Zn–N contacts of 2.00–2.05 Å (involving the amino nitrogen and ring N^3 atoms) and the N–Zn–N angles are all in the range 96–120°. However, in both cases the two carboxylate groups are bent back so as to provide two additional contacts for each zinc atom (Zn–O 2.8–2.9 Å) at the van der Waals distance,[43] which means that it is also possible to describe the complex as having 'distorted octahedral' geometry. The difference between these two viewpoints is more than a matter of semantics since, if the same ligand disposition is retained in solution, the presence of the carboxylate groups would tend to *exclude* a third ligand from the inner co-ordination sphere of the metal (in particular, a hydroxo group or water molecule), even though the oxygens are not 'properly' bound.

Two literature reports support the view that this essentially octahedral arrangement is retained in solution: (*i*) the entropy of formation of $[Zn(His)_2]_{aq}$ is similar[44] to those of octahedral bis(histidine) complexes [$M(His)_2$] (M = Mn^{II}, Fe^{II}, Co^{II} or Ni^{II}) but different from that of the square-planar copper(II) complex; (*ii*) like the corresponding cobalt(II) and nickel(II) complexes but unlike those of cadmium(II) (presumed to be tetrahedral) and copper(II), the zinc complex is formed stereoselectively.[45]

The loss of protons from the imidazole ring rather than from bound water molecules would be a surprising result if [ML_2] did, in fact, contain bound water since Demoulin *et al.*[46] have shown by *ab initio* calculation that a zinc-bound imidazole loses a proton less readily than a zinc-bound water (even though for the free molecules the acidity order is reversed). The implication must be that [ML_2] does not contain zinc-bound water.

Ternary Complexes.—The similarity of histidine to dien[11] in forming ternary complexes involving the bis rather than the mono Zn–L complex has already been noted. In this, histidine and dien differ from the two other terdentate ligands we

have studied, dipropylenetriamine (dpt) and *N*-(2-aminoethyl)-propane-1,3-diamine (aepn), which both form ternary zinc complexes[13] of the general formula [ML(X)] with glycine and other potentially bidentate ligands. The different formulations of these bis-L ternary complexes {[$Zn(His)_2X$] as against [$Zn(Hdien)_2A$][11] can be seen as reflecting the fact that the displaced arm of L is much more basic for dien ($-NH_2$) than for histidine ($-CO_2^-$).

Conclusion

The present results are largely consistent with previous reports on the zinc–histidine system but the use of a higher pH range than usual has enabled us to obtain direct information on two of the deprotonated complexes. We have argued that [$Zn(His)_2H_{-1}$] and [$Zn(His)_2H_{-2}$] are both formed by the loss of a proton from a pyrrole nitrogen of histidine rather than from a metal-bound H_2O, in line with the proposal of Carlson and Brown[7] but contrary to that of Sovago *et al.*[5] It is unfortunate, in view of the interest in establishing the relative acidities of zinc-co-ordinated water and the pyrrole N of a co-ordinated histidine (for example, in connection[1] with the mechanism of carbonic anhydrase and carboxypeptidase), that neither the latter authors nor we were able to obtain quantitative information about $[Zn(His)]^+$, which contains both moieties.

Recently, Christiansen and Alexander[47] identified a frequently recurring triad, carboxylate–His–Zn, at the catalytic site of several zinc enzymes which may play a crucial part in substrate hydrolysis. In this there is hydrogen bonding between the CO_2^- side chain of a neighbouring amino acid residue and N^1H of the zinc-bound histidine ring. Gas-phase calculations by Nakagawa *et al.*[48] have indicated that the binding of Zn^{2+} to N^3 in histidine increases the acidity of N^1H, thereby facilitating the transfer of this proton from histidine to CO_2^-. Our finding that the pK_a of histidine N^1H is at least two units lower in [ML_2] than in the free ligand provides experimental evidence that this is the case in solution also. It also provides an interesting postscript to the discussion between Appleton and Sarkar[49] and Martin[50] about the possible effect on the pK_a of N^1H in an imidazole ring of binding to zinc at N^3.

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