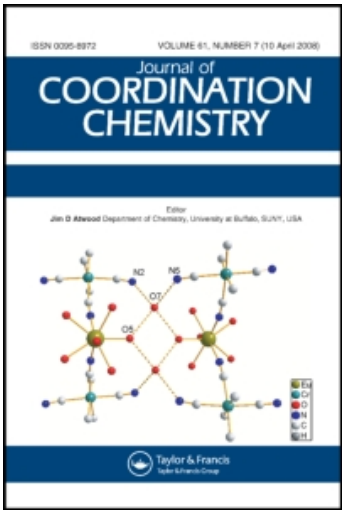

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HIGH FIELD NMR STUDY OF THE BINDING OF LEAD(II) TO CYSTEINE AND GLUTATHIONE

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HIGH FIELD NMR STUDY OF THE BINDING OF LEAD(II) TO CYSTEINE AND GLUTATHIONE

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High field proton and carbon-13 NMR spectroscopy has been used to study the interaction of lead(II) with both cysteine and the tripeptide glutathione in D_2O over a wide pD range. No binding of lead(II) to either biological ligand was observed in acid solution. In alkaline solution, PbL and/or PbL_2 complexes were formed with cysteine, depending on the $[Pb^{2+}]$:[cysteine] ratio.

Chemical shifts experienced by the cysteine protons in the 1:1 complex, together with calculated rotamer populations, indicate a mixture of terdentate (NH_2, COO^-, S^-) and bidentate coordination with binding through the sulfur and carboxylate groups favoured in the bidentate case. For the PbL_2 complex, only limited chemical shift *vs* pD data could be obtained due to precipitation problems between pD 3.4 and 9.8. However, in alkaline solution, coordination of Pb^{2+} through the S^- donor was again confirmed. With glutathione, both PbL and PbL_2 complexes were also formed in alkaline solution. Proton chemical shift data are inconsistent with a previously proposed tetradentate binding mode of glutathione. For the PbL_2 complex no change in the partial rotamer populations of the cysteinyl residue occurs upon complexation, indicating monodentate coordination only through the S^- group. Chemical shift data also support monodentate coordination through the S^- group and monodentate coordination through the S^- group of glutathione in the 1:1 PbL complex.

Keywords: Lead(II), cysteine, glutathione, NMR

INTRODUCTION

Lead toxocity is generally considered to be initiated by coordination of lead(II) to appropriate functional groups on amino acids and proteins. However, there is currently little detailed information at the molecular level as to the nature of these interactions. Only one X-ray crystal structure has been reported[1] for a lead-amino acid complex, namely that of *D*-penicillaminatolead(II). In the latter species, *D*-penicillamine acts as a terdentate ligand, forming strong bonds to lead(II) *via* amine, carboxyl and sulfide groups.

In solution, NMR spectroscopy has been shown to be a powerful tool for elucidating the nature of metal binding sites to biological ligands. For example, the binding of lead(II) to the amino acids cysteine,[2] *S*-methylcysteine,[2] cysteine methyl ester[2] and acetyl glycine[3] has been studied at 60 MHz. Low field proton NMR spectroscopic studies have also been carried out on the interaction of lead(II) with the polyglycine peptides diglycine, triglycine, and tetraglycine.[4] Extension to more complex peptides

is restricted with low field NMR spectroscopy because of the complexity of the spectra. However, a ^{13}C NMR study has been made[5] of the lead-glutathione complex.

In the present study, high field proton (400 MHz) and carbon-13 (100 MHz) NMR spectroscopy has been used to investigate the interaction of lead(II) with the potential target amino acid cysteine and the tripeptide glutathione, which is the most abundant intracellar non-protein thiol. The results provide powerful evidence as to the complex stoichiometries at various Pb(II)-ligand ratios and pH regimes, the ligand donor sites employed, and in some instances the population of the various rotamer complexes.

EXPERIMENTAL

Materials

L-cysteine and glutathione were obtained from Sigma Chemicals, and stored in a desiccator under refrigeration. The lead salt used in all cases was analytical reagent grade lead nitrate (BDH).

D_2O (99.8%) and NaOD (9.8 M) were obtained from Nova Chem, and working solutions of the latter were prepared as needed. The 7% DNO_3 solution employed was prepared from 70% nitric acid (AnalaR) as follows: a 0.5 cm^3 aliquot of concentrated HNO_3 acid was made up to 5 cm^3 with D_2O. The solution was evaporated to near dryness under partial vacuum and the remaining liquid made up to 5 cm^3 with D_2O.

The internal reference compounds *t*-butyl alcohol and 1,4-dioxane were obtained from BDH.

NMR studies

All NMR spectra were recorded on a Jeol GX400 FT spectrometer at 27°C. For ^1H NMR spectra, 40 scans were typically acquired using a resolution of 0.155 Hz. Carbon-13 NMR spectra typically involved 2000 scans and a resolution of 0.61 Hz.

Proton NMR spectra of the free amino acids and glutathione were recorded using a ligand concentration of 0.01 mol dm^{-3}. Lead-ligand solutions also employed a 0.01 mol dm^{-3} ligand concentration with the requisite amount of lead(II) added to achieve the desired ligand:lead ratio. All solutions were prepared in D_2O and pD adjustments made by the dropwise addition of either 2 M NaOD or 7% DNO_3. Solutions for ^{13}C NMR spectra employed [Pb(II)] = [ligand] = 0.1 mol dm^{-3}, pD adjustments being made by adding 7% DNO_3.

pD measurements

All pD measurements were made with an Activon 109 pH meter fitted with an Activon BJ331 NMR micro pH electrode, standardised using Merck buffers (4.00 and 9.00). Accurate pD values were obtained from equation (*1*) of Glascoe and Long.[6]

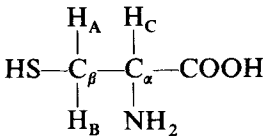
$$pD = \text{scale reading} + 0.4 \qquad (1)$$

RESULTS AND DISCUSSION

(*A*) *Cysteine*

Assignment of spectra

The ^1H NMR spectrum of free cysteine was assigned using the proton labelling system shown below. In keeping with Natusch and Porter,[2] the proton resonance at lowest field is attributed to proton C (attached to the alpha carbon), while the highest field signal is assigned to proton B (one of the magnetically non-equivalent protons attached to the beta carbon). This order appears to be valid over the entire pH range studied.



Cysteine

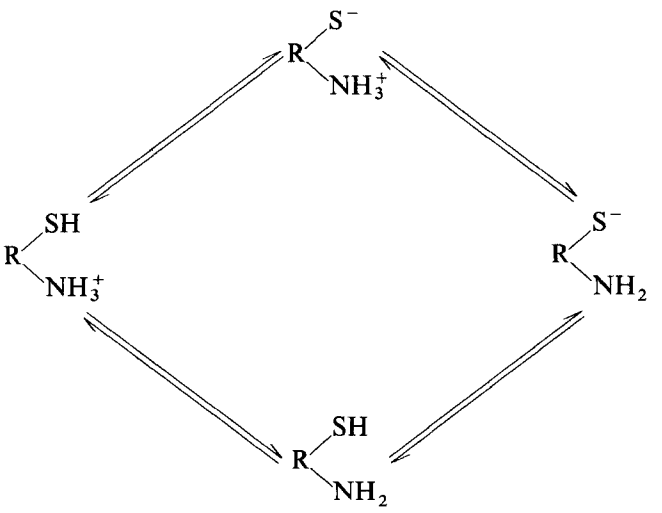
As expected, the ^1H NMR spectrum of free cysteine at 400 MHz exhibits an ABC character in alkaline and acid pD regions. For example, at pD = 12.9 a well-resolved 12-line ABC spectrum of three double doublets is observed. However, at pD *ca* 8.4, the spectrum acquires A_2B character, presumably due to a fortuitous equivalence of protons A and B.

The ^{13}C NMR spectrum of free cysteine at pD = 1.9 shows three well-separated resonances. Our assignments follow those of Jung *et al.*,[7] in which the signal due to the carboxyl carbon is assumed to be the furthest downfield (171.4 ppm) and that due to the β carbon at highest field (24.8 ppm); the α carbon is at intermediate field (55.7 ppm).

pH dependence of the free cysteine spectrum

The pH dependence of the ^1H NMR spectrum of *L*-cysteine has previously been reported at 60 MHz.[8–11] These studies lacked definitive information in the lower pH regions, primarily due to the inability to resolve the signals of protons A and B. This problem is overcome at 400 MHz, and the pD profile of the chemical shifts of each of the protons A, B, C of free cysteine are shown in Figure 1. The pD dependence of the associated coupling constants is summarised in Table I. The coupling constants obtained at pD = 12.9 agree well with previous data obtained by others[2,8–11] in alkaline solution.

The proton chemical shifts in Figure 1 exhibit two distinct regions of change. Between pD 1.8–4, the chemical shift of proton C is shifted upfield by 0.3 ppm, while protons A and B are only slightly affected. This behaviour can be ascribed to the deprotonation of the carboxylic acid group, for which the reported pKa is 1.88 ± 0.02.[12] Between pD 7.5–12.5, the marked changes in chemical shifts for protons B and C may be attributed to deprotonation of the protonated amino group and the sulfhydryl group, as shown in the microscopic dissociation Scheme 1 ($R = CH_2CHCOO^-$).



II

Scheme 1.

Cysteine rotamer populations

There are three possible staggered rotamers for cysteine arising from rotation about the C_α–C_β axis, as shown in Figure 2. The *t* rotamer has the carboxylate and thiolate groups *trans* to each other, while there are two *gauche* configurations (*g*, *h*).

The rapid interconversion of the rotamers gives rise to averaged vicinal coupling constants across the C–C bonds, weighted in proportion to the mole fractions of *t*, *g* and *h*. The relative rotamer populations of cysteine at any particular pH may be

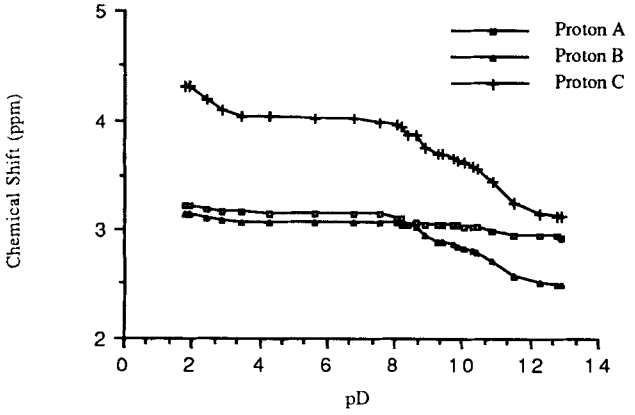


FIGURE 1 Plot of proton chemical shift *vs* pD for free cysteine

TABLE I
Proton coupling constants (Hz) for free cysteine at various pD values

pD	J_{AB}	J_{BC}	J_{AC}
12.92	12.58	9.65	3.40
12.77	12.63	9.73	3.43
12.29	12.66	9.61	3.51
11.50	12.89	9.34	3.62
10.84	13.28	8.77	3.97
10.43	13.54	8.40	4.24
10.29	13.62	8.35	4.20
10.05	13.67	8.17	4.20
9.71	13.89	8.09	4.27
9.40	13.89	7.78	4.27
9.29	14.00	7.74	4.27
8.91	14.15	7.29	4.27
8.39	–	5.34	5.34
8.19	14.61	4.54	5.64
8.03	14.80	4.28	5.80
7.56	14.80	4.12	5.73
6.75	14.99	4.18	5.65
5.61	14.96	4.12	5.65
4.28	14.96	4.08	5.65
3.46	14.99	4.06	5.75
2.89	14.99	4.19	5.48
2.42	15.03	4.20	5.57
1.80	15.10	4.27	5.57

calculated[2,10–13] using the relationships (*2–4*),

$$t = (J_{BC} - J_g)/(J_t - J_g) \quad (2)$$

$$g = (J_{AC} - J_g)/(J_t - J_g) \quad (3)$$

$$h = (J_t + J_g - 2J_{ave})/(J_t - J_g) \quad (4)$$

where J_{AC} and J_{AB} are the observed vicinal coupling constants and $2J_{ave} = J_{AC} + J_{BC}$; J_t is the vicinal coupling constant when H_c is *trans* to H_A or H_B, and J_g is the value for the *gauche* rotamers. We have used values estimated[13] for simple amino acids of $J_t = 13.6$ Hz and $J_g = 2.6$ Hz. It has also been assumed that deviations from dihedral

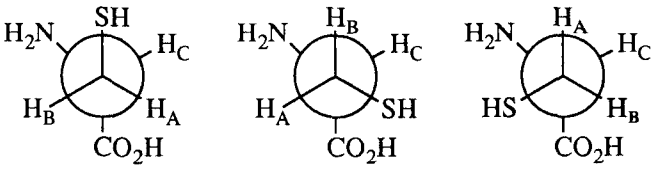


FIGURE 2 Newman projection of the 3 rotamers of cysteine.

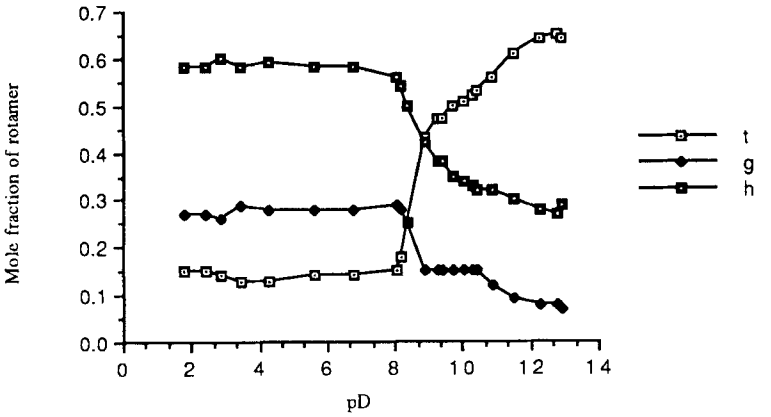


FIGURE 3 Rotamer populations of free cysteine at various pD values.

angles of 60° and 180° are negligible, and that J_t and J_g are independent of the ionic form of cysteine.

Using coupling constants data from Table I, rotamer populations of free cysteine have thus been calculated over a wide pD range (Figure 3). It is seen that the *t* rotamer is strongly favoured in alkaline solution (mole fraction > 0.6 at pH > 11). This may be rationalised in terms of the need to minimise both electrostatic and steric repulsion between the COO^- and S^- groups in fully deprotonated cysteine (L^{2-}). Qualitatively similar results were reported by Natusch and Porter[2] in alkaline solution (using slightly different J_t and J_g values).

However, unlike these earlier workers, we have been able to obtain comprehensive rotamer population data in acidic solution. As pD decreases, the two *gauche* rotamers are seen to be favoured at the expense of the *t* rotamer (Figure 3). Interestingly, after the LH_2 species is produced at pD 8, no further significant changes in rotamer populations occur (down to pD 1.8).

Cysteine-lead(II) complexes

The previous study by Natusch and Porter[2] of the interaction of lead(II) with cysteine, using 220 MHz ^1H NMR spectrometry, employed a large excess of lead(II) ($[Pb^{2+}]/[\text{cysteine}] = 5$). Under these conditions, only a 1:1 lead/cysteine complex was noted in alkaline solution (with bidentate coordination through the amino and sulfur groups), while no evidence was found for complex formation in acid solution.

In order to examine the possible formation of 2:1 or higher cysteine/lead complexes, we have varied the initial $[Pb^{2+}]/[\text{cysteine}]$ ratio over the range 4.0 to 0.16. Well-resolved 400 MHz ^1H NMR spectra were obtained over two pD regions, namely pD 1.8–3.4 and pD 9.8–12.9. Precipitation problems between pD 3.4 and 9.8 limited the amount of useful data in this region. Complex formation causes the signals for each of the cysteine protons A, B and C to move downfield. Since lead(II)-ligand exchange processes are known to be extremely rapid, the observed proton chemical shifts and coupling constants in the lead(II)-containing solutions are average values arising from the populations of free and complexed rotamers in the equilibrium mixtures.

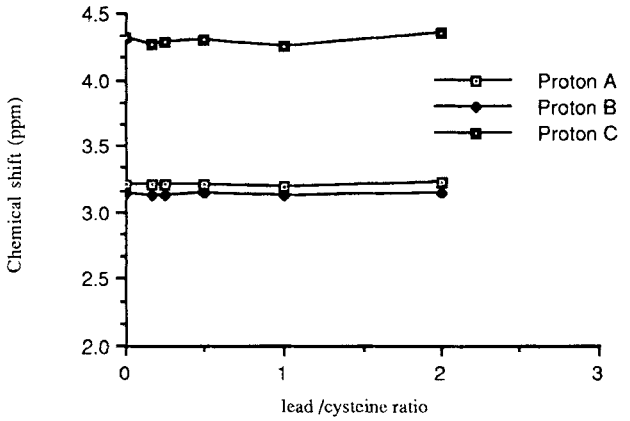


FIGURE 4 Proton chemical shift values at various Pb^{2+}/cysteine ratios (pD = 1.8 ± 0.1).

Acid solution

Figure 4 shows a plot of chemical shift *vs* $[Pb^{2+}]/[\text{cysteine}]$ ratio for each of the cysteine protons at pD 1.9 ± 0.1. At this pD all possible ligand binding sites in free cysteine are protonated. The presence of lead(II) is seen to have minimal effect on the chemical shifts of each of the cysteine protons over the $[Pb^{2+}]/[\text{cysteine}]$ range of 0.16 to 2.0. Similar results were obtained at pD = 2.4 and pD = 3.4 using a fixed $[Pb^{2+}]/[\text{cysteine}]$ ratio of 1:2. In addition, no significant changes were observed in the coupling constants and relative rotamer populations between free cysteine and cysteine in the presence of lead(II) over the pD range 1.8 to 2.4. These observations indicate no significant lead(II)-cysteine complex formation in this acid region.

This conclusion is supported by ^{13}C NMR spectral data at pD 1.9. The chemical shifts for each of the COOH, α-CH, and β-CH_2 carbons of cysteine are insensitive to the presence of lead(II). However, an interesting feature of the ^{13}C NMR spectrum of the lead-containing solution is the appearance of the α-carbon as an apparent triplet. This suggests that partial deuterium exchange has occurred at the α-carbon during the long spectrum accumulation time. Complete deuteration would give rise to a triplet (rel. intensities 1:1:1) for the α-carbon atom. It therefore seems probable that, in common with some other metal ions,[14] lead(II) catalyses hydrogen exchange at the α-carbon of amino acids, albeit through very weak interactions, in acid solution.

Alkaline solution

In contrast, in alkaline solution (pD 12.9), addition of lead(II) has a marked effect on the chemical shifts of the cysteine protons (Figure 5). At this pD the free ligand exists as the fully ionised cysteinate anion L^{2-}. Three regimes are apparent from Figure 5:

(i) where $[\text{cysteine}]/[Pb^{2+}] > 2$, the 2:1 complex $[PbL_2]$ and free L^{2-} are present,
(ii) where $[\text{cysteine}]/[Pb^{2+}]$ is between 2 and 1, $[PbL_2]$ and the 1:1 complex [PbL] coexist, and
(iii) where $[\text{cysteine}]/[Pb^{2+}] < 1$, [PbL] is the only complex present.

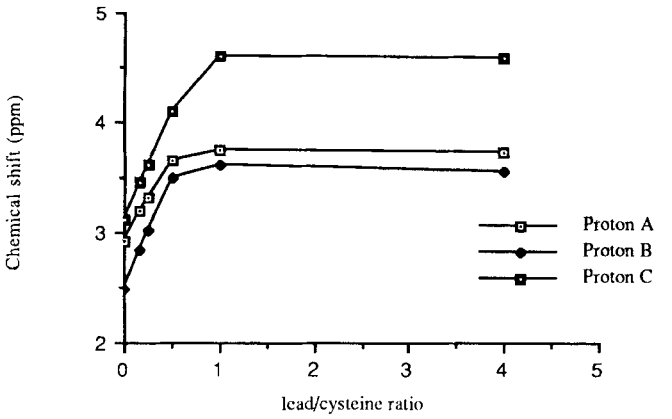


FIGURE 5 Proton chemical shift values at various Pb^{2+}/cysteine ratios (pD = 12.9).

These conclusions are consistent with published stepwise formation constants for the Pb(II) cysteine system at 25°C ($pK_1 = 17.4$, $pK_2 = 27.3$).[14]

The large increase in chemical shift experienced by each of the cysteine protons upon complexation (Figure 5) suggests that all three possible binding sites (NH_2, COO^-, S^-) are involved in the lead-cysteine binding.

Rotamer populations for complexed cysteine (Table II), calculated from coupling constant data in each of the regimes in Figure 5, throw further light on the nature of the lead(II)-cysteine interactions.

1:1 Complex

Formation of a 1:1 (PbL) complex causes a dramatic decrease in the *t* rotamer population compared to free cysteine, with a concurrent increase in the *g* and *h* populations (Table II). Molecular model studies, using ligand-metal bond lengths similar to those reported in the solid *D*-penicillaminatolead(II) complex and an octahedral lead geometry, indicate that the *t* and *g* rotamers (Figure 2) are restricted to bidentate coordination. However, the *h* rotamer of cysteine allows terdentate coordination through each of the N, S and O sites. The resulting geometry of the lead-cysteine bonding is trigonal pyramidal with the lead atom at the apex of the pyramid (Figure 6); bidentate coordination with the *h* rotamer of cysteine is considered unlikely on steric grounds, since it has all three bulky groups on the same

TABLE II
Mole fraction (±0.02) of rotamers *t*, *g* and *h* at various cysteine/lead ratios; pD = 12.9

[cysteine]/[Pb]	Species	*t*	*g*	*h*
∞	L^{2-}	0.64	0.07	0.29
6:1	$(PbL_2) + L^{2-}$	0.64	0.07	0.29
4:1	$(PbL_2) + L^{2-}$	0.53	0.06	0.41
2:1	(PbL_2)	0.41	0.08	0.51
1:1	(PbL)	0.07	0.30	0.63
1:2	(PbL)	0.08	0.24	0.68

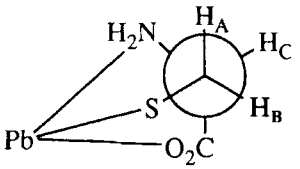


FIGURE 6 Suggested structure for the tridentate lead-cysteine (1:1) complex.

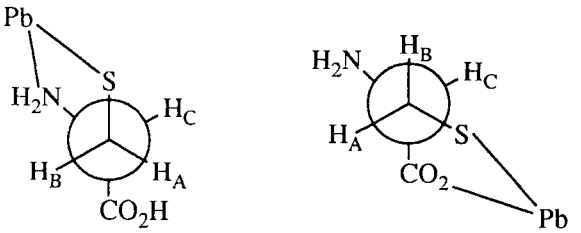
side of the molecule. Since the *h* rotamer accounts for 63 ± 2% of the total rotamer population in the 1:1 complex (Table II), a maximum of 63% of the cysteine may be concluded to be tridentate in the [PbL] complex.

If one assumes that S is the most preferred lead binding site on cysteine (supported by studies with glutathione, *vide infra*) then the relative *t* and *g* rotamer populations provide a means by which the preference of lead(II) for binding through either the amine or carboxylate group in [PbL] may be determined. The *t* rotamer can only permit binding through the sulfur and amine groups, simultaneous coordination of S and COO^- being impossible because of their mutual *trans* location (Figure 7a). The *g* rotamer on the other hand can only bind through the sulfur and carboxylate groups, because of the *trans* location of the S and NH_2 groups (Figure 7b). The relative populations of the *t* (7 ± 2%) and *g* (30 ± 2%) rotamers in the [PbL] complex (Table II) therefore indicate that binding through the sulfur and carboxylate groups (Figure 7b) is the preferred bidentate coordination mode in this 1:1 complex.

It should be noted that the rotamer populations calculated here for the 1:1 complex (Table II) are considerably different to those reported previously by Natusch and Porter.[2] In their study, using a large excess of lead(II) ([cysteine]/[Pb] = 1/5), the *t* rotamer was favoured over the *h* rotamer. The chemical shifts and coupling constants of alpha amino acids have been shown to be independent of ionic strength[15] and metal ion and ligand concentrations (for a given metal ion:ligand ratio).[2] Data from the present study confirm this for ligand concentrations of 0.01 M. The differences between our results and those of Natusch and Porter[2] may arise from the very large excess of lead(II) employed in the earlier study, although the mechanism of this interference is uncertain.

2:1 Complex

Inspection of Figure 5 shows that addition of one further equivalent of cysteine to the 1:1 [PbL] complex, forming the 2:1 species [PbL_2], causes a marked change



(a) (b)

FIGURE 7 Suggested structure for bidentate lead/cysteine (1:1) complexes: (a) binding to the *t* rotamer of cysteine; (b) binding to the *g* rotamer of cysteine.

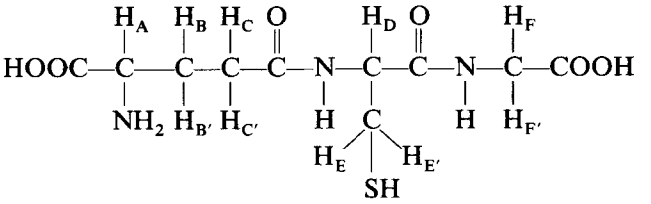
in the chemical shift of proton C while protons A and B are only slightly affected. This suggests that the S group is coordinated to the lead in both the [PbL] and [PbL_2] complexes, whereas the mode of coordination of the NH_2 and COO^- functional groups is different in the two complexes.

As noted earlier, only limited chemical shift *vs* pD data could be obtained for 2:1 cysteine:lead(II) mixtures because of precipitation problems between pD 3.4 and 9.8. Interestingly, data collected over the pD region 9.8 to 12.95 for the 2:1 [PbL_2] complex reveal almost no dependence of the coordinated cysteine proton chemical shifts on pD. This contrasts with free cysteine where deprotonation of the NH_3^+ and SH groups occurs (see above). Presumably the electron withdrawing influence of the lead(II) causes a lowering of the associated pKa's of the coordinated species into the pD region masked by precipitation.

(*B*) *Glutathione*

Assignment of spectra

The ^1H NMR spectrum of free glutathione was assigned from spin decoupling experiments, using the labelling system shown below.



Glutathione

In alkaline solution, the signal due to proton D occurs at lowest field. This is followed by the signals due to protons F, F′, A, E, E′, C, C′, and B and B′ at the highest field. In acid solution the signals due to E and E′ merge to form a multiplet, as do the signals for B and B′. The signals due to F, F′ collapse to a singlet and the signal for proton A exhibits a significant downfield shift caused by protonation of the adjacent carboxylate and amine groups. The order of proton signals seen in acid solution from lowest to highest is D, A, FF′, EE′, C, BB′. Protons B and B′ on the glutamic acid residue also show two well-separated multiplets in alkaline solution (pD = 12.9). These signals merge at pD < 11.2 and appear as a multiplet over the remaining pD range studied. Signals due to protons C, C′ appear as two overlapping multiplets over the entire pD range.

pD dependence of free glutathione spectrum

Previous studies of glutathione[16] report the following pKa values: $pK_{a1} = 2.3$, $pK_{a2} = 3.3$, $pK_{a3} = 9.05$, $pK_{a4} = 9.5$. These values can be attributed to the dissociation of the two terminal carboxylic acid groups, the glutamyl amino group and the cysteinyl sulfhydryl group. As in the case of cysteine, this occurs *via* a series of microscopic ionisation schemes arising from the near simultaneous ionization of two or more functional groups. In the glutathione case the terminal carboxylic acid groups dissociate at a similar pK_a value to the sulfhydryl and protonated amine group.

As each of the functional groups involved in dissociation are well-separated in glutathione, the ionisation of each species can be identified from the chemical shift

vs pD profile of protons adjacent to the active site. For example, the chemical shift of proton A is affected by both the deprotonation of the glutamyl carboxylic acid group (pD < 1.8–5.2) and the dissociation of the protonated amine group (pD 7.2–11.2) (Figure 8a). Analogous plots for protons B, B^1 and C (Figures 8a and 8b) show similar behaviour, as they too are affected by the dissociation of the abovementioned groups.

The proton chemical shifts of the glycine residue (protons F, F^1) are relatively unaffected by pD changes in the pD region 6–12.9. However, between pD *ca* 2–6 a significant change in chemical shifts is observed, due to the protonation of the glycyl carboxylic acid. Thus, it can be seen from the behaviour of the chemical shifts of protons A and F in the pD range <1.8 to *ca* 6 that the ionization of each carboxylic acid is nearly simultaneous.

The deprotonation of the sulfhydryl group would be expected to affect the chemical shifts of protons E, E′ and D as they are adjacent to the ionization site. This feature is observed for each of these protons between pD *ca* 8 and *ca* 11 (Figures 8a and 8b), indicating that the sulfhydryl group is deprotonated over this pD range.

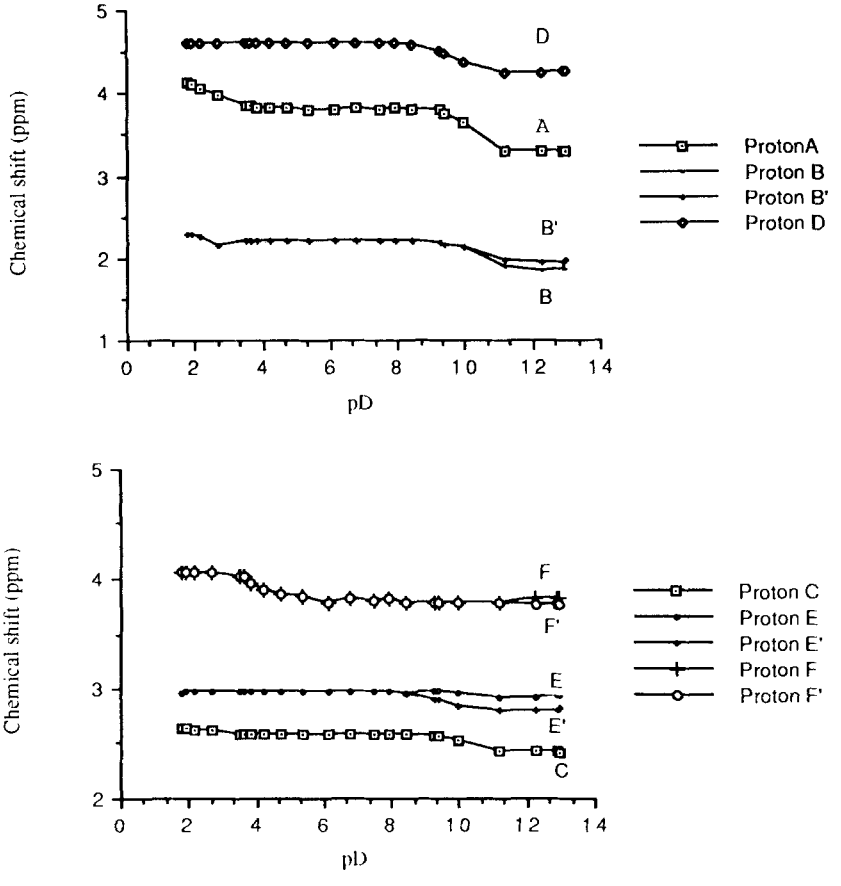


FIGURE 8a,b Plots of chemical shift *vs* pD for various glutathione protons.

Glutathione has a series of partial rotamers involving rotation about both the cysteinyl and glutamyl centres. In this study, the partial rotamer populations arising from rotation about the cysteinyl Cα–Cβ axis were determined using the same equations (*2*)–(*4*) and J_t, J_g values as those used above for cysteine. Results are summarised in Table III. (Unfortunately, no useful rotamer population data could be obtained for the glutamyl residue because of difficulty in obtaining the required coupling constants).

The most stable conformer over the pD range studied is seen from Table III to be the *t* rotamer. This is reasonable on steric grounds, as it places the bulky SH or S^- group *trans* to the peptide carbonyl. The rotamer populations remain relatively constant over the pD range studied (12.8–9.2), although glutathione changes from the L^{3-} to the LH^{2-} form. The effect of further changes in pH could not be studied because of the inability to resolve the signals due to E and E′ at lower pD values. The *h* rotamer population calculated here compares well with that reported by Fujiwara *et al.*[13] They were unable, however, to assign populations to the individual *t* and *g* rotamers.

Glutathione-lead(II) interaction

In a thermodynamic study of lead(II) interaction with several amino acids and peptides, Corrie and Williams[14] suggested the formation of a lead-glutathione complex [PbL], with binding to the lead through the glutamyl carboxylate and amine groups as well as the cysteinyl sulfur and the glycyl carboxylate groups. For a 2:1 glutathione/lead(II) mixture they suggested that the species PbL_2H is formed. Binding in this complex was thought to be through the four donor groups used in the 1:1 complex for one ligand, and only through either the S or the NH_2 for the other ligand.

In contrast, from ^{13}C NMR studies, Fuhr and Rabenstein[5] suggested that in a 2:1 glutathione:lead(II) mixture binding occurs through only the cysteinyl sulfur (between pD 5.4 and 12), and to a lesser extent the glycyl carboxylic acid group (significant at pD < 9). At pD > 9 lead-hydroxy-glutathione mixed complexes were formed. Binding to glutathione in these mixed complexes was thought to be through the cysteinyl sulfur only. They also postulated binding through either terminal carboxylic acid group at pD < 2.

Our present 400 MHz NMR studies provide more definitive information on the mode of attachment of Pb(II) to glutathione.

TABLE III

Mole fraction (±0.02) of the *t*, *g* and *h* rotamers of the cysteinyl residue of glutathione at various pD values

pD	*t*	*g*	*h*
12.80	0.48	0.22	0.30
12.30	0.50	0.22	0.28
11.19	0.50	0.24	0.26
9.97	0.49	0.21	0.30
9.39	0.46	0.21	0.33
9.25	0.45	0.21	0.34

TABLE IV
Chemical shift (ppm) of glutathione protons in the presence of varying amounts of lead; pD = 12.9

Ligand:lead Ratio	Protons								
	A	B	B′	C	D	E	E′	F	F′
Free ligand	3.30	1.87	1.96	2.42	4.26	2.94	2.82	3.83	3.70
2:1	3.32	1.88	1.97	2.42	*	3.17		3.83	
1:1	3.32	1.87	2.00	2.40	*	3.37		3.84	

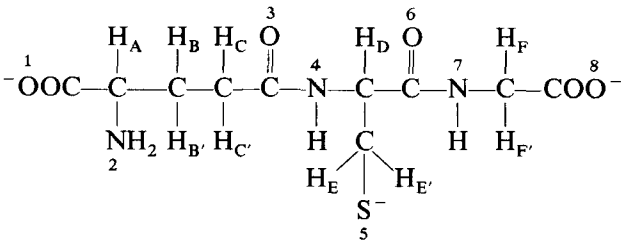
* Signal obscured by HOD resonance (4.87 ± 0.12 ppm).

Acid solution

As with cysteine, the proton and carbon-13 chemical shifts of glutathione are insensitive to the presence of lead(II) in acid solution (pD *ca* 1.9), indicating no significant interaction between lead(II) and glutathione in the low pH region.

Alkaline solution

^{1}H NMR data for glutathione/lead(II) mixtures in alkaline solution were obtained at pD 12.9 ± 0.1. At this pD value, glutathione has 8 possible binding sites (shown below), three of which are ionised. The addition of one equivalent of lead(II) to an alkaline glutathione solution causes a 0.05 ppm downfield shift in the signal for proton F, and a 0.02 ppm downfield shift in the signal for proton F^1 (Table IV). These small shifts are more in keeping with binding to a site further away from F, F′ than either of sites 7 or 8. No shifts in the signals for protons adjacent to sites 1, 2 or 3 were evident. Our ^{1}H NMR data indicating no Pb(II)-binding at sites 1, 2, 3, 7 and 8, are therefore clearly inconsistent with the tetradentate binding mode previously proposed[14] for lead-glutathione complexes.



However, the addition of 0.5 equivalent of lead(II) to an alkaline solution of glutathione causes the resonance signals of protons D, E and E′ to move downfield by 0.15, 0.06 and 0.12 ppm, respectively. The addition of a further 0.5 equivalent of lead(II) to this solution causes protons D and E, E′ (average signal) to move a further 0.23 ppm and 0.14 ppm downfield, respectively. These large shifts indicate attachment of lead(II) to either sites 4, 5 or 6, *i.e.*, the cysteinyl S^- or the nearby peptide CO and/or NH groups. The addition of up to a four-fold excess of lead(II) causes only minimal further changes in the chemical shifts.

Thus, data obtained in alkaline solution indicate the formation of a 2:1 glutathione:lead(II) complex in the presence of excess glutathione. However, when there is a molar excess of lead the 1:1 glutathione:lead(II) complex is favoured. This behaviour is in accordance with published[14] complex stability constant data at 25°C

TABLE V
Mole fraction (± 0.02) of the partial rotamers *t*, *g* and *h* of the cysteine residue of glutathione at various ligand:lead ratios; pD = 12.9

Ligand/lead ratio	*t*	*g*	*h*
Free glutathione	0.48	0.22	0.30
2:1	0.46	0.21	0.33

($I = 3.00\ \text{mol dm}^{-3}$), which report $pK_1 = 16.8$ and $pK_2 = 23.4$ for the stepwise Pb(II)/glutathione formation constant.

2:1 complexes

As stated above, lead(II) binding in the 2:1 complex can be through either the sulphur, peptide carbonyl or peptide amino groups of the cysteine residue. Interestingly, it can be seen in Table V that no changes in the partial rotamer populations of the cysteine residue occur upon complexation. This indicates that glutathione-lead(II) complexation involves monodentate binding only, since bidentate binding would be expected to favour one of the partial rotamers over the others.

Presumably this monodentate binding is through the S^- group, as one would expect the lead(II) ion to have a higher affinity for this "soft" ionised site over the "harder" carbonyl and amino groups. Thus it would appear from our proton NMR data that a 2:1 glutathione:lead complex (Figure 9) exists in alkaline solution, as suggested by Fuhr and Rabenstein.[5]

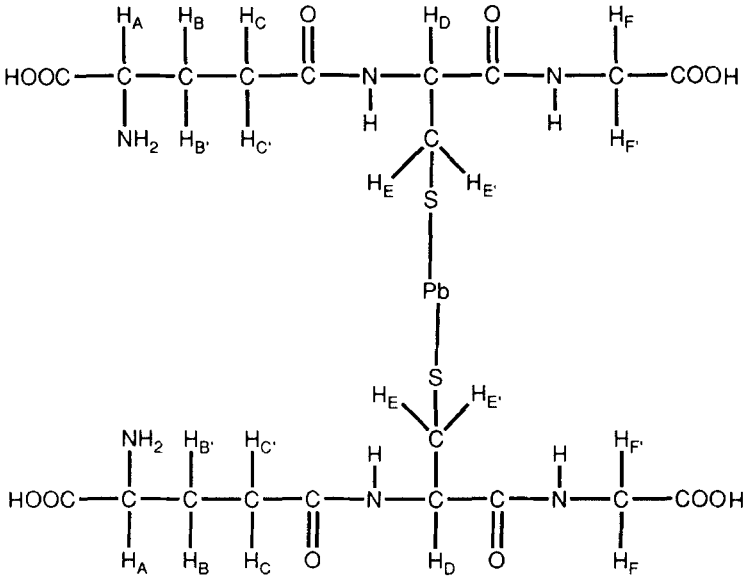


FIGURE 9 Suggested structure for the 2:1 glutathione:lead complex.

1:1 complex

As noted above, the formation of a 1:1 glutathione:lead(II) complex also shifts the signals of protons D, E and C^1, indicating that binding occurs at site(s) adjacent to these protons (*i.e.*, sites 4, 5 or 6). Due to the complexity of the resulting NMR spectra, we were unable to determine the coupling constants necessary for calculation of the populations of the partial rotamers of the glutathione cysteinyl residue. However, by analogy with the 2:1 complex, binding through only the S^- group might be expected.

pD profile of a 2:1 glutathione:lead(II) mixture

The chemical shift *vs* pD profile of a 2:1 glutathione:lead(II) mixture is shown in Figures 10a and 10b. The amount of useful data was limited by precipitation problems between pD 2.9 and 9.0 and overlap of the resonance signals of several protons. It is evident, however, that the protons most affected by pD changes are those adjacent to the S^- binding site, namely protons D, E and E'. Their resonances move downfield

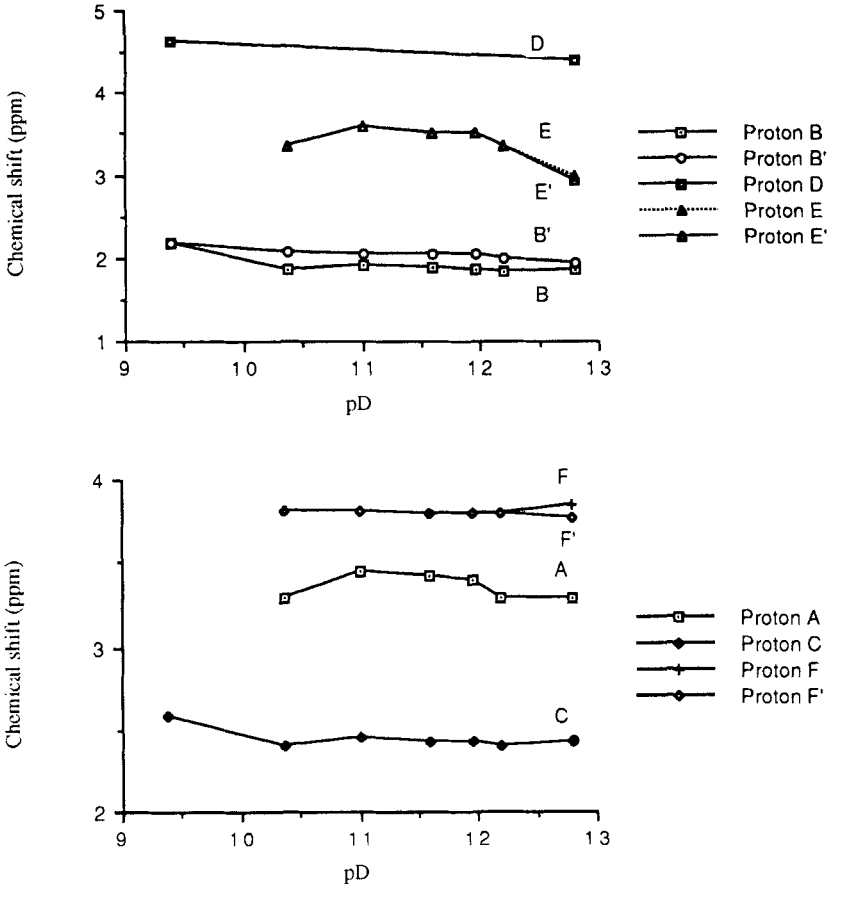


FIGURE 10a,b Plots of proton chemical shift *vs* pD for a 2:1 glutathione:lead mixture.

with decreasing pD. Over the pD range 9.4 to 12.8 these protons always resonate downfield compared to the free ligand at the same pD, indicating that the S^- remains bound to lead(II) over this region.

The signals of protons B, B′, C, F and F′ are relatively unaffected by lead(II) coordination to glutathione. Although the pD profile of proton A is slightly affected by Pb(II) coordination, these results again indicate attachment to the S site of glutathione only.

In the pD region 3.4 to 2.6 the chemical shifts of each of the glutathione protons are similar to that observed for the free ligand at the corresponding pD. This indicates no lead-glutathione interaction in this pD region.

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