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Nuclear Magnetic Resonance Studies of the Complexation of Trimethyllead by Glutathione in Aqueous Solution and in Intact Human Erythrocytes

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Abstract: The complexation of trimethyllead (TML), $(CH_3)_3Pb^{IV}$, by glutathione (γ-L-glutamyl-L-cysteinylglycine (GSH)) has been studied in aqueous solution and in intact human erythrocytes by nuclear magnetic resonance spectroscopy. The deprotonated sulfhydryl group is shown to be the strongest binding site for TML. Formation constants, including microscopic formation constants for sulfhydryl complexes in which the amino group is protonated and deprotonated, have been determined from the dependence of the chemical shift of the exchange-averaged ^1H resonance for the methyl protons of TML on solution conditions. To determine if TML is also complexed by GSH in a more complex biological system, we have measured ^1H spin–echo Fourier transform (SEFT) NMR spectra for intact erythrocytes to which TML has been added. Resonances are observed for the naturally occurring GSH and several other small molecules, including glycine, alanine, ergothionine, creatine, and lactic acid. Of these potential ligands, TML is found to be complexed only by the intracellular GSH, and the complex is identical with that which forms in much simpler aqueous solutions. The NMR results on the erythrocytes also indicate that TML, added as trimethyllead acetate, rapidly crosses the erythrocyte membrane and that the TML-poisoned erythrocytes continue to metabolize glucose. Measurements have also been made on the competitive complexation of TML in intact erythrocytes by the naturally present GSH and added penicillamine, a molecule which has been tried as a treatment for alkyllead poisoning. The results obtained in this study demonstrate that detailed information about the complexation of metals in intact cellular systems such as erythrocytes can be obtained by ^1H NMR spectroscopy.

There is presently considerable interest in the aqueous chemistry of organometallic forms of the metals, particularly as it relates to their environmental and toxicological behavior.[1] Of the various organometallic forms of lead, the trialkyllead(IV) species are of particular interest; inhalation or absorption of tetraalkyllead compounds results in lead in the fluids and tissues of the body, primarily as trialkyllead(IV)salts.[2,3]

In previous studies, we have shown that trimethyllead (TML) reacts as a one-coordinate species in aqueous solution and that, of the potential binding sites in amino acids, the deprotonated sulfhydryl group binds TML most strongly at physiological pH.[4] Of the various sulfhydryl-containing biological molecules, glutathione (GSH, γ-L-glutamyl-L-cysteinylglycine) is among the most abundant. For example, in human erythrocytes, GSH is typically present at the 2 mM level.[5,6] Because of the high affinity of TML for sulfhydryl groups and the abundance of GSH in nature, we have studied the complexation of TML by GSH. The complexation in aqueous solutions has been characterized by ^1H and ^{13}C NMR spectroscopy. Formation constants, including microscopic formation constants for the binding of TML to the deprotonated sulfhydryl group of the amino protonated and deprotonated forms of GSH,[7] have been derived from the dependence of the exchange-averaged chemical shifts of the $(CH_3)_3Pb^{IV}$ protons on solution composition and pH. To determine if TML is also complexed by GSH in a more complicated biological system, we have made ^1H spin–echo Fourier transform NMR measurements on the GSH in intact human erythrocytes to which TML has been added. We also have studied the competitive complexation of TML by GSH and penicillamine in intact human erythrocytes. Penicillamine is a ligand which has been found to be effective in the treatment of several forms of heavy-metal poisoning.[8,9] These studies on intact human erythrocytes clearly demonstrate the power of NMR methods for the elucidation of the coordination chemistry of metal ions in complex biological systems.

Experimental Section

Chemicals. Trimethyllead acetate (Alfa Inorganics) was used as the source of TML. Because acetate forms a complex with $(CH_3)_3Pb^+$ in aqueous solution, it was converted to a stock solution of trimethyllead perchlorate by an ion-exchange procedure described previously.[10] The stock solution was standardized by potentiometric titration. A pH 7.0 solution of trimethyllead acetate was used in the studies of intact erythrocytes.

The glutathione (Sigma) was used as received. The purity of the GSH in terms of the sulfhydryl group was checked by titration with coulometrically generated iodine in acetate buffer using biamperometric endpoint detection.[11]

Sample Preparation. All solutions in the formation constant studies were prepared with doubly distilled H_2O which had been freshly boiled and cooled under a stream of argon. The solutions were prepared from the stock trimethyllead perchlorate solution and solid ligand. The ionic strength was adjusted to 0.3 M with $NaClO_4$ for the ^1H NMR studies and 0.4 M for the ^{13}C NMR studies. *tert*-butyl alcohol was added as a chemical shift reference for the ^1H measurements and 1,4-dioxane for the ^{13}C measurements. The solutions were usually made acidic (pH ~2.5) with $HClO_4$, and then NaOH was added and NMR samples withdrawn at pH intervals of ~0.4 pH unit up to pH ~12.5. Samples

(1) Brinkman, F. E., Bellama, J. M., Eds. *ACS Symp. Ser.* **1978**.
(2) Shapiro, H.; Frey, F. W. "The Organic Compounds of Lead"; Interscience: New York, 1968; p 17.
(3) Cremer, J. E. *Br. J. Ind. Med.* **1959**, *16*, 191.
(4) Backs, S. J.; Rabenstein, D. L. *Inorg. Chem.* **1981**, *20*, 410.
(5) Beutler, E.; Duron, O.; Kelley, B. M. *J. Lab. Clin. Med.* **1963**, *61*, 882.
(6) Rabenstein, D. L.; Saetre, R. *Clin. Chem. (Winston-Salem, N. C.)* **1978**, *24*, 1140.
(7) Rabenstein, D. L. *J. Am. Chem. Soc.* **1973**, *95*, 2797.
(8) Brugsh, H. G. *J. Occupat. Med.* **1965**, *7*, 394.
(9) Selander, S.; Cramer, K.; Hallberg, L. *Br. J. Ind. Med.* **1966**, *23*, 282.
(10) Sayer, T. L.; Backs, S.; Evans, C. A.; Millar, E. K.; Rabenstein, D. L. *Can. J. Chem.* **1977**, *55*, 3255.
(11) Kreshkov, A. P.; Oganesyan, L. B. *J. Anal. Chem. USSR (Engl. Transl.)* **1971**, *26*, 534.

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were prepared under a stream of argon and were run immediately after preparation to minimize air oxidation.[12]

Erythrocyte Preparation. Intact human erythrocytes were obtained from whole venous blood which was drawn into EDTA-containing vacutainers (Becton, Dickinson and Co.). The whole blood was centrifuged at 5000 rpm for 15 min. The plasma was drawn off, and the cells were then washed two or three times by suspending them in a D_2O solution of isotonic saline–glucose.

NMR Measurements. In the formation constant studies, ^1H NMR spectra were obtained on a Varian A-60-D spectrometer at a probe temperature of 25 ± 1 °C. ^{13}C NMR spectra were obtained at 25.1 MHz and 25 ± 1 °C on a Varian HA-100 spectrometer equipped with a Digilab FTS/NMR-3 data system. The pulsed Fourier transform mode was used with proton decoupling. For each free induction decay signal, 8K data points were collected in the computer, and 500 transients were accumulated. For measurement of the ^{207}Pb–^{13}C coupling constant, 4000 transients were accumulated. The deuterium resonance from C_6D_6 in a coaxial capillary served as the lock signal.

In the red-blood cell studies, ^1H NMR spectra were measured at 400 MHz on a Bruker WH-400/DS spectrometer operating in the pulsed Fourier transform mode. Spectra were measured at 25 °C on 0.4–0.5 mL of packed cells or on 0.4 mL of packed cells suspended in 0.04 mL of saline glucose solution in 5-mm o.d. NMR tubes. The free induction decay was collected in 8K of data points with an acquisition time of 0.819 s. Quadrature detection was used with spectral widths of 5000 Hz, and from 100 to 300 transients were collected for each spectrum. Chemical shifts are reported relative to the methyl resonance of sodium 4,4-dimethyl-4-silapentanesulfonic acid (DSS), on the basis of the resonance for the CH_2 protons of the glycine residue of GSH having a chemical shift of 3.76 ppm.

The ^1H NMR spectrum for packed erythrocytes, as measured by the standard single pulse sequence, consists of a broad, rather featureless envelope from the carbon-bonded protons of hemoglobin, with resonances from the less abundant cellular constituents, including those from GSH, buried in the hemoglobin envelope.[13] The spin–echo Fourier transform (SEFT) technique was used to completely eliminate the interfering hemoglobin resonances. The hemoglobin resonances can be selectively eliminated with the spin–echo pulse sequence ($90° - \tau_2 - 180° - \tau_2$ acquisition) because their spin–spin (T_2) relaxation times are considerably shorter than those of resonances from the smaller, more mobile intracellular compounds.[13-16]

Results

Characterization of the Complexation of TML by GSH. GSH has several potential coordination sites for TML, including two carboxylate groups[10] and the sulfhydryl group.[4] To identify the sites to which TML binds, we measured ^{13}C chemical shifts as a function of pH for GSH solutions containing TML. The chemical shift data for several of the ^{13}C resonances of GSH are presented in Figure 1.[17] The carbon atoms are identified by Glu, Cys, or Gly to indicate the amino acid residue in which the carbon is located and C_α or C_β to indicate the particular carbon of that residue. The GSH resonances omitted from Figure 1 do not differ significantly in their pH vs. chemical shift behavior from those of GSH in solutions containing no complexing metal ion.

Above pH 3, the curves for Cys–C_α and Cys–C_β are displaced from those of free GSH, indicating complexation at the sulfhydryl group even at fairly low pH values. The curves for the Glu–COOH, Glu–C_α, Gly–COOH, and Gly–C_α carbon atoms are not displaced significantly from those of free GSH in the pH 2–6 region where maximum carboxylate complexation is expected to occur,[10] indicating little binding to the carboxylate groups. The coincidence of the Glu–C_α curve also indicates no significant binding to the amino group of the Glu residue. A value of 308.8 Hz was obtained for the ^{207}Pb–^{13}C coupling constant of TML in a pH 5.0 solution containing 0.095 M TML and 0.146 M GSH. This value is in the region of coupling constants reported for a number of similar trialkyllead compounds under a variety of conditions.[18]

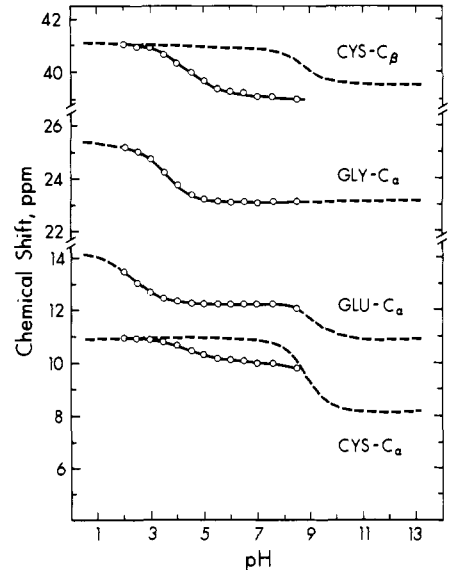

Figure 1. pH dependence of the ^{13}C chemical shifts of selected carbons of GSH at 25 °C in a solution of 0.21 M GSH only (dashed line) and in a solution of 0.146 M GSH and 0.0947 M TML (circles). Chemical shifts are relative to dioxane; positive shifts correspond to resonances upfield from dioxane.

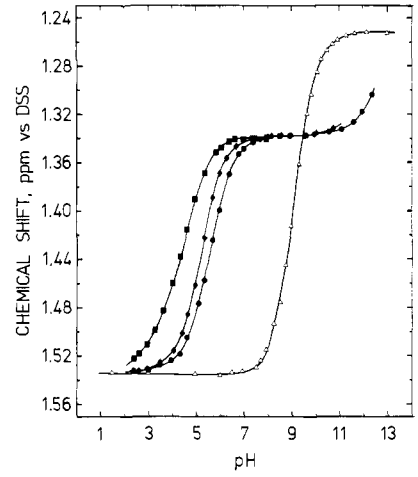

Figure 2. pH dependence of the ^1H chemical shift of TML at 25 °C and 0.3 M ionic strength in solutions of 0.0100 M TML only (△), 0.005 01 M TML and 0.009 73 M GSH (●), 0.005 01 M TML and 0.0194 M GSH (♦), and 0.0749 M TML and 0.146 M GSH (■).

Formation Constant Studies. Formation constants for the complexation of TML by GSH were determined from the dependence of the chemical shift of the ^1H resonance for TML on solution composition and pH. The pH dependence at three different TML and GSH concentrations is shown in Figure 2. Also shown is the pH dependence of the chemical shift for a solution of trimethyllead perchlorate titrated with NaOH. For all solutions studied in this work, the exchange of TML between its free and complexed forms was fast on the NMR time scale. Formation

(12) Trimethyllead acetate is reported to react slowly with L-cysteine and D,L-penicillamine in basic solution to give polymeric dimethyllead(IV) cysteinate or penicillaminate: Carty, A. J. *ACS Symp. Ser.* **1978**. In the ^{13}C NMR study presented in Figure 1, a small quantity of white precipitate was observed at pH >6 in the TML-containing solutions of GSH. No attempt was made to characterize the precipitate. To avoid problems from the possible conversion of TML to dimethyllead(IV) in the formation constant studies, we used much lower TML and GSH concentrations and we obtained NMR spectra immediately after sample preparation. There was no detectable decrease in the intensity of the TML resonance relative to the resonance from *tert*-butyl alcohol which was present at a constant concentration throughout the entire pH range in each experiment, both in these studies and in those involving L-cysteine and D,L-penicillamine.[4]

(13) Brown, F. F.; Campbell, I. D.; Kuchel, P. W.; Rabenstein, D. L. *FEBS Lett.* **1977**, *82*, 12.

(14) Rabenstein, D. L.; Isab, A. A. *J. Magn. Reson.* **1979**, *36*, 281.

(15) Rabenstein, D. L.; Nakashima, T. T. *Anal. Chem.* **1979**, *51*, 1465A.

(16) Rabenstein, D. L. *Anal. Chem.* **1978**, *50*, 1265A.

(17) The ^{13}C spectrum of GSH has been assigned and the pH dependence of the resonances has been reported by: Jung, G; Breitmaier, E.; Voelter, W. *Eur. J. Biochem.* **1972**, *24*, 438.

(18) Mitchell, T. N.; Gmehling, J.; Huber, F. *J. Chem. Soc., Dalton Trans.* **1978**, 960.

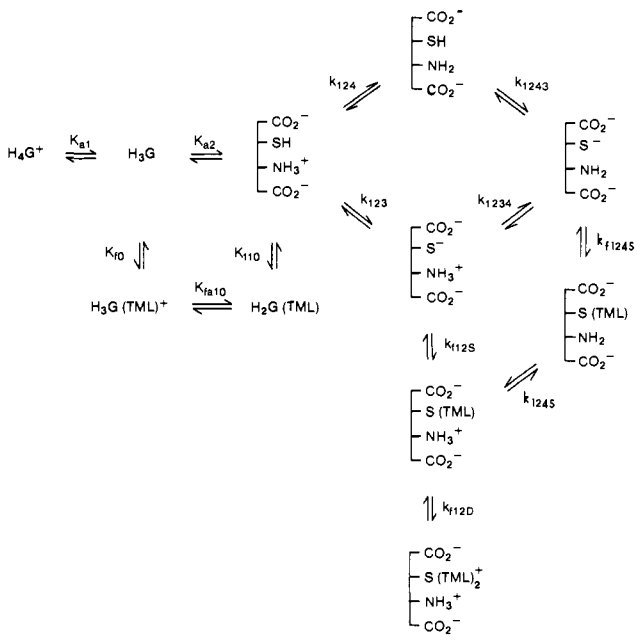

Figure 3. Microscopic acid dissociation and TML complexation scheme for GSH.

Table I. Formation Constants, Acid Dissociation Constants, and Chemical Shifts of $(CH_3)_3Pb^{IV}$ Complexes of GSH[a,b]

constant[c]	value of constant	chemical shift[d]
K_{f_0}	1.2 ± 1.1	1.433 ± 0.076
$K_{f_{10}}$	10.8 ± 1.7	1.455 ± 0.012
$k_{f_{12}S}$	3.47 (±0.05) × 10^5	1.338 ± 0.001
$k_{f_{124}S}$	4.23 (±0.26) × 10^5	1.335 ± 0.001
$k_{f_{12}D}$	17.4 ± 4.9	1.351 ± 0.011
$K_{a_{10}}$[e]	2.9 × 10^{-3}	
$k_{124}S$[f]	5.45 × 10^{-10}	

[a] At 25 °C and 0.3 M ionic strength. [b] Errors are linear estimates of the standard deviation. [c] Defined in Figure 3. [d] Chemical shifts of the TML protons in the complexes formed by complexation reaction defined by the constant in column 1. [e] Calculated from K_{f_0}, $K_{f_{10}}$, and K_{a_2}. [f] Calculated from $k_{f_{12}S}$, $k_{f_{124}S}$, and k_{1234}.

constants for the complexes were determined from the chemical shift of the exchange-averaged TML resonance, which for a given solution is the average of the chemical shifts of the various TML species present in solution, weighted according to their relative populations. The protonation and complexation equilibria are summarized in Figure 3. Formation constants and chemical shifts for the various TML complexes shown in Figure 3 were calculated by procedures described previously.[19] The results are reported in Table I.

The most important complexes are those in which TML is complexed by the deprotonated sulfhydryl group. Microscopic formation constants (k_{f12S} and k_{f124S}) were determined for the complexation of TML by the amino-protonated and amino-deprotonated forms of GSH by using the microscopic acid dissociation constants for GSH.[7] As was found in a previous study of the complexation of TML by sulfhydryl-containing amino acids and related ligands,[4] there is a small amount of complex in which two TML ions are complexed by one sulfhydryl group. The amount of this complex is quite small, and thus its formation constant cannot be determined with much precision. Such a complex forms only in the pH region where the ammonium group is still protonated; at higher pH, competition from hydroxide ion for TML[10] prevents formation of the two-to-one complex. As was also the case with sulfhydryl-containing amino acids,[4] there is no evidence for complexes having a ligand-to-TML ratio greater than one-to-one.

(19) Millar, E. K.; Evans, C. A.; Rabenstein, D. L. *Can. J. Chem.* **1978**, *56*, 3104.

The Complexation of TML by GSH in Human Erythrocytes. The 400-MHz ^1H spin-echo Fourier transform NMR spectrum of intact human erythrocytes is shown in Figure 4. As described in the Experimental Section, the majority of the resonances from the carbon-bonded protons of hemoglobin can be eliminated with the SEFT technique, resulting in considerable spectrum simplification.[13] The sharp resonances[20] in the 1–6.9-ppm region in Figure 4 are due to small molecules in the intracellular region of the erythrocytes, many of which have been assigned.[13,21,22] Those assigned to compounds known to form complexes with metal ions in aqueous solution are identified in Figure 4. Of particular interest here are the resonances for the carbon-bonded protons of GSH. The glycine CH_2 group of GSH gives resonance I at 3.76 ppm, the cysteinyl CH and CH_2 groups give the resonances at 4.55 (H) and 2.92 (J) ppm, respectively, and the glutamyl β- and γ-CH_2 groups give the resonances at 2.15 (L) and 2.55 (K) ppm, respectively.

Figure 5A shows an expanded portion of the 400-MHz ^1H SEFT NMR spectrum for another sample of intact human erythrocytes. In this case, the NMR tube contained 0.40 mL of packed erythrocytes and 0.04 mL of D_2O solution containing isotonic saline and 5 mM glucose. Figure 5B shows the spectrum measured for the same cells immediately after the addition of 10 μL of 0.10 M trimethyllead acetate. The change in the spectral pattern for the β-CH_2 protons of the cysteinyl residue and the decreased intensity of the glycine residue indicate complexation of TML by the intracellular GSH. There are no changes in the resonances for the intracellular glycine, creatine, alanine, ergothioneine, or lactate, indicating no detectable complexation of TML by these ligands. The resonance at 1.91 ppm is from the $CH_3CO_2^-$ added with the TML.

The large decrease in the intensity of the resonance for the glycine CH_2 group of GSH (Figure 5B) is unexpected since the ^{13}C results in Figure 1 indicate the predominant binding site to be the sulfhydryl group.[23] Figure 6 shows, however, that complexation of GSH by TML causes the glycine CH_2 resonance to go from a singlet centered at 3.76 ppm (spectrum 6A) to an AB pattern, also centered at 3.76 ppm (spectrum 6B).[24] The lack of any change in the average chemical shift is consistent with no complexation of TML by the glycine carboxylate group at pD of 7.0.[23] As shown in Figure 7, the intensity of the AB pattern is phase modulated in the SEFT spectrum, which causes the considerable decrease in its intensity in Figure 5B even though complexation is at the relatively distant sulfhydryl group. Figure 7A,B also shows the change in the spin-echo multiplet pattern for the CH_2 protons of the cysteinyl residue of GSH due to complexation of TML by the sulfhydryl group.

The Complexation of TML in Human Erythrocytes by Penicillamine. In Figure 8 are presented a series of spectra which show the effect of penicillamine (PSH) on the complexation of TML by intracellular GSH. Spectrum A was measured immediately after the addition of 10 μL of 0.10 M trimethyllead acetate to an NMR tube containing 0.40 mL of intact human erythrocytes plus 0.04 mL of a D_2O solution containing isotonic saline and 5 mM glucose. Spectrum B was obtained from the same sample 3 h later. Then 10 μL of a 0.10 M PSH solution was added.[25]

(20) In the spin-echo experiment, positive, negative, and out-of-phase signals can be obtained for resonances which are multiplet patterns due to homonuclear spin–spin coupling. The frequency modulation of multiplet patterns in the spin-echo experiment is discussed in ref 15.

(21) Isab, A. A.; Rabenstein, D. L. *FEBS Lett.* **1979**, *106*, 325.

(22) Rabenstein, D. L.; Isab, A. A.; Brown, D. W. *J. Magn. Reson.* **1980**, *41*, 361.

(23) With the formation constants in Table I and the acid dissociation constants for GSH,[7] it can be shown that the ratio of sulfhydryl-complexed to carboxylate-complexed TML at pD 7.4 is 950:1.

(24) The CH_2 protons of the glycine residue of methylmercury(II)-complexed GSH, in which the CH_3Hg^{II} is coordinated only to the sulfhydryl group (Rabenstein, D. L.; Fairhurst, M. T. *J. Am. Chem. Soc.* **1975**, *97*, 2086) and of oxidized glutathione also give AB patterns, indicating that chemical changes at sites somewhat distant from the glycine residue can cause the two protons of the glycine CH_2 group to be nonequivalent.

(25) Penicillamine readily crosses the red cell membrane when red cells are incubated in a saline–glucose solution containing penicillamine (Isab, A. A.; Rabenstein, D. L., unpublished results).

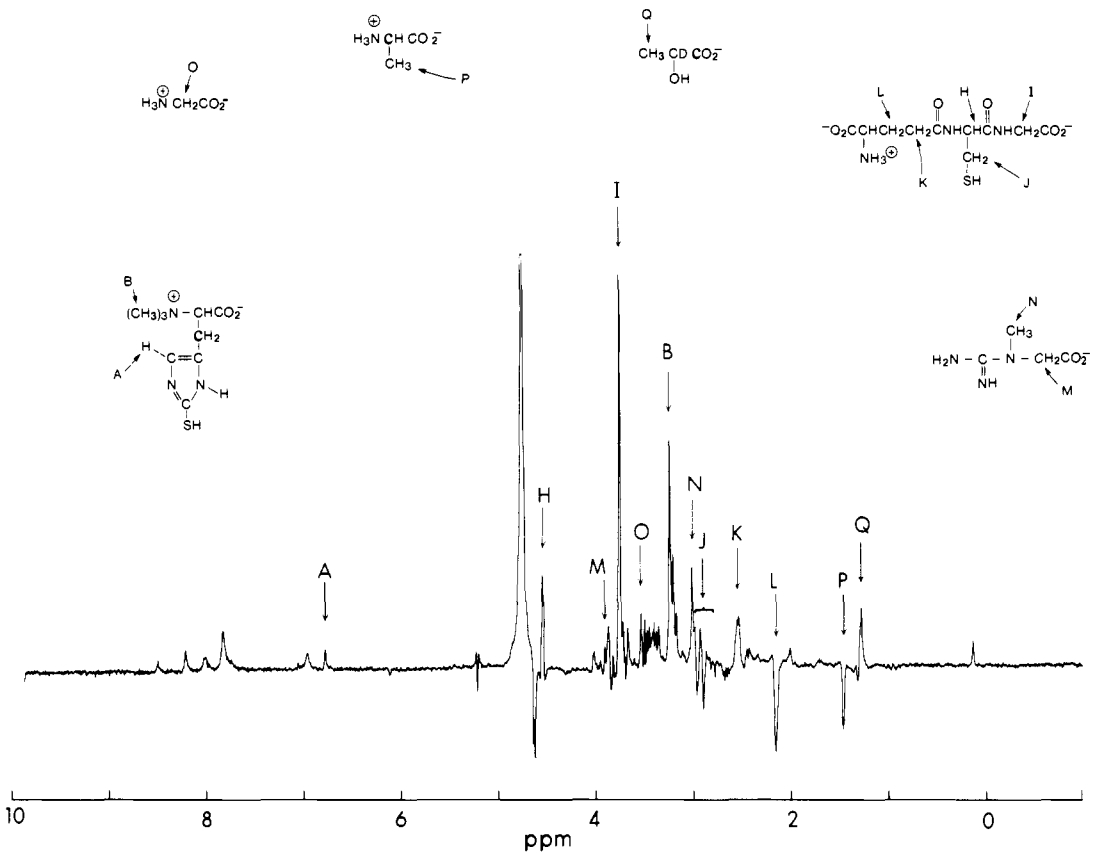

Figure 4. The 400-MHz spin–echo Fourier transform ^1H NMR spectrum of packed intact red blood cells which had been washed three times with a D_2O solution of isotonic saline–glucose. A τ_2 of 0.060 s was used in the spin–echo Fourier transform pulse sequence, and 300 transients were collected.

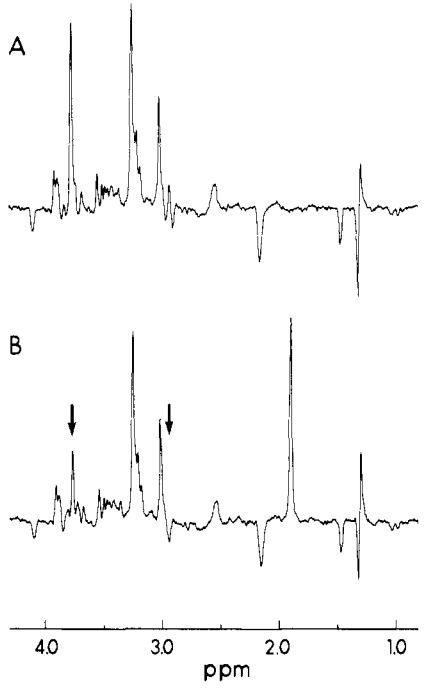

Figure 5. Portions of the 400-MHz spin–echo Fourier transform ^1H NMR spectra of (A) 0.40 mL of packed red cells and 0.040 mL of a D_2O solution containing isotonic saline and 0.005 M glucose and (B) the same sample immediately after the addition of 10 μL of 0.10 M TML acetate. A τ_2 of 0.060 s was used in the spin–echo Fourier transform pulse sequence, and 100 transients were collected.

Spectrum C was obtained within 5 min after the addition of PSH, and spectra D and E were obtained 2 and 12 h later. The two resonances centered at 1.5 ppm are due to the two methyl groups of PSH.

The addition of TML causes the resonance for the glycine CH_2 protons of GSH to decrease in intensity (spectra A and B). With use of the resonance for the CH_3 protons of ergothioneine (resonance B in Figure 4) as an intensity reference, TML causes the ratio of the intensity of the glycine CH_2 resonance to that of the ergothioneine CH_3 resonance to decrease from 0.90 to 0.39. Within 5 min after the addition of the PSH, the ratio has increased to 0.43. Two hours later the ratio has increased further to 0.45 and then 12 h later has increased to 0.51. In a similar experiment with cells from a different donor, the ratio decreased from 1.16 to 0.54 within 5 min after the addition of 10 μL of 0.1 M trimethyllead acetate to an NMR tube containing 0.40 mL of intact erythrocytes and 0.04 mL of a D_2O solution containing isotonic saline and 5 mM glucose. Within 5 min after the addition of 25 mL of 0.1 M PSH, the ratio had increased to 0.70 and then continued to increase further with time, reaching a maximum of 0.77 6 h later.

Discussion

The results of this study show that TML binds strongly to the sulfhydryl group of glutathione, both in relatively simple aqueous solutions and in intact human erythrocytes.

In aqueous solution, the formation constants for the binding of TML by the deprotonated sulfhydryl group of GSH are similar in magnitude to those found for binding to several sulfhydryl-containing amino acids and related molecules.[4] As was also found with the amino acids, the strength of the TML binding shows some dependence on the protonation state of the amino group in the molecule, although the dependence found for GSH is somewhat less than for the amino acids. For example, the formation constant for the binding of TML by cysteine increases from log $K_f = 4.99$ to log $K_f = 5.97$ upon deprotonation of the ammonium group,[4] for the analogous complex of penicillamine the increase is from 4.05 to 5.63, while for GSH the increase is from 5.54 to 5.63. The much larger effect with cysteine and PSH is presumably due to the amino group being closer to the sulfhydryl group. The effect of the protonation state of the amino group on the acid–base

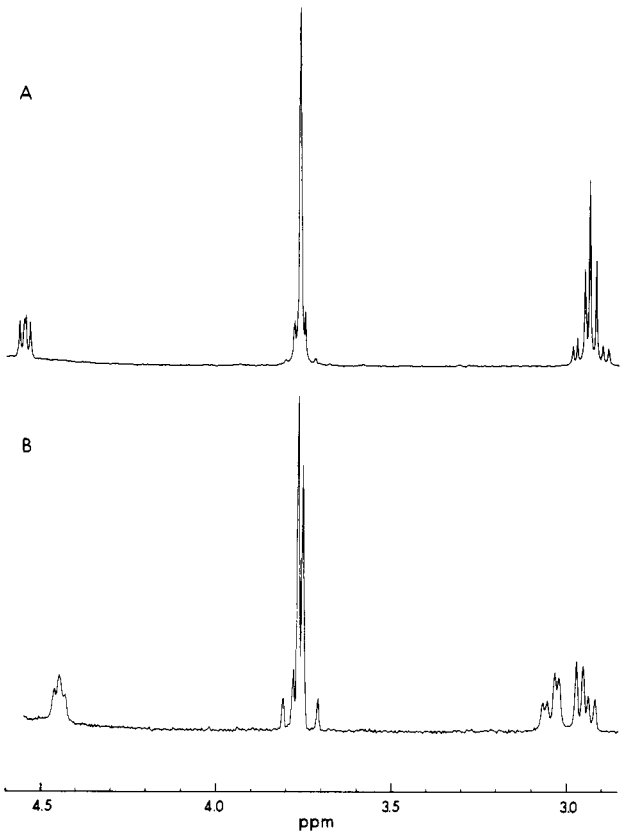

Figure 6. Portions of the 400-MHz ^1H NMR spectra of a pD 7.0 D_2O solution of (A) 0.003 M GSH in 0.154 M NaCl and (B) 0.003 M GSH and 0.003 M TML in 0.154 M NaCl. A total of 40 transients were collected.

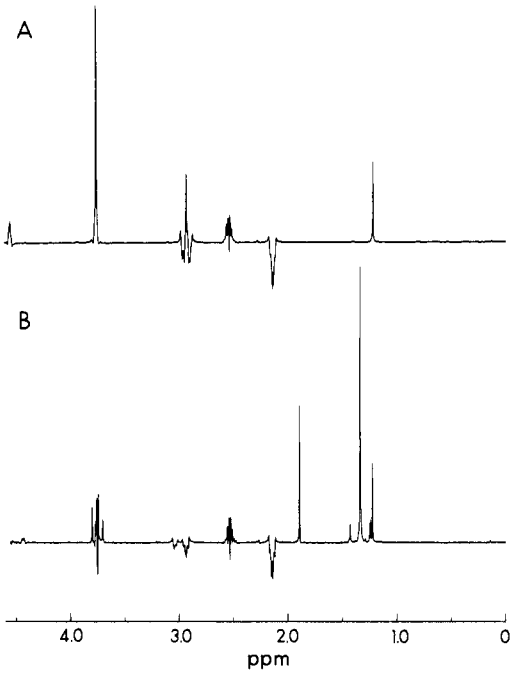

Figure 7. Portions of the 400-MHz spin–echo Fourier transform ^1H NMR spectra of the same solutions as in Figure 6. A τ_2 of 0.060 s was used in the spin–echo Fourier transform pulse sequence, and 40 transients were collected. The resonance at 1.23 ppm is due to *tert*-butyl alcohol added as a chemical shift reference and that at 1.91 ppm is due to the acetate added with the TML.

properties of the sulfhydryl group shows a similar distance dependence, with the microscopic pk_A's for the sulfhydryl group increasing from 8.38 to 9.74, from 8.03 to 9.70, and from 8.97 to 9.08 following deprotonation of the ammonium group of cysteine,[26] PSH,[27] and GSH,[7] respectively.

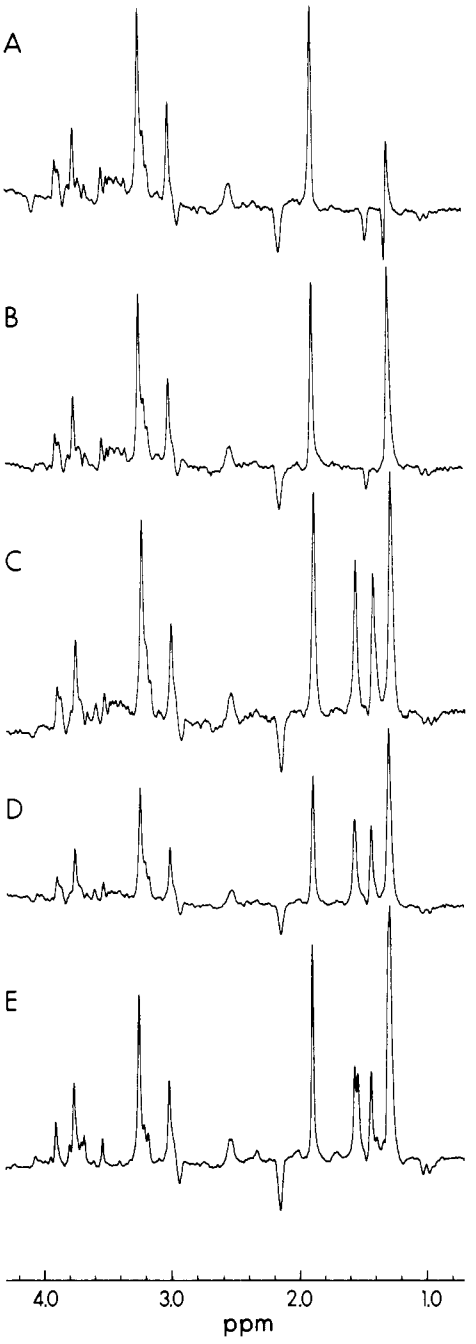

Figure 8. Portions of the 400-MHz spin–echo Fourier transform ^1H NMR spectra of (A) 0.40 mL of packed red blood cells and 0.040 mL of a D_2O solution containing isotonic saline and 0.005 M glucose immediately after the addition of 10 μL of 0.10 M TML acetate, (B) the same sample 3 h later, (C) the same sample as in (B) immediately following the addition of 10 μL of 0.10 M PSH, (D) and (E) the same sample as in (C) but 2 and 12 h following the addition of PSH. A τ_2 of 0.060 s was used in the spin–echo Fourier transform pulse sequence, and 100 transients were collected.

The changes in the resonances for the α-CH and β-CH_2 protons of the cysteine residue of GSH and the CH_2 protons of the glycine residue (Figure 5) indicate that TML is complexed by the intracellular GSH of intact human erythrocytes. There is no detectable change in resonances for other small molecules, including ergothioneine which also has a sulfhydryl group, indicating no

(26) Calculated for an ionic strength of 0.3 M from the values reported by: Wilson, E. W.; Martin, R. B. *Arch. Biochem. Biophys.* **1971**, *142*, 455.
(27) Calculated for an ionic strength of 0.3 M from the values reported by: Coates, E.; Marsden, C. G.; Rigg, B. *Trans. Faraday Soc.* **1969**, *65*, 3032.

detectable binding of TML in the intracellular region by these other molecules. The effect of the binding of TML on the ^1H SEFT NMR spectrum of GSH is identical in aqueous solution and in erythrocytes, suggesting that the complex which forms in erythrocytes is identical with that which we have characterized in detail in aqueous solution. This is in contrast to what we observe for the binding of zinc by GSH in the intracellular region of erythrocytes, where the binding is somewhat different from that in a simple aqueous solution containing zinc and GSH.[28] In the case of zinc, it appears that at least some of the GSH is in a hemoglobin–zinc–GSH mixed complex. In the present study, there is no indication of the formation of the analogous complex with TML. This is consistent with the reaction of TML as a one-coordinate species with sulfhydryl-containing ligands in aqueous solution.[4]

There is indirect evidence, however, that some TML is complexed by hemoglobin, presumably to the sulfhydryl groups of the two cysteine β-93 residues. The evidence is that no resonance is observed in Figures 5B or 8 for the protons of TML, whereas in Figure 7B the TML protons give an intense singlet (1.343 ppm) and two satellites due to coupling to the natural abundance ^{207}Pb (J = 73 Hz). If some TML is bound to hemoglobin, it will have the motional characteristics and thus the short T_2 relaxation times characteristic of the carbon-bonded hemoglobin protons. The results in Figure 2 as well as those in previous studies of the binding of TML by sulfhydryl ligands[4] indicate that exchange of TML among sulfhydryl ligands is fast on the NMR time scale. Thus, if some TML is complexed by hemoglobin, it is expected to be rapidly exchanging with that complexed by GSH. The result is that the exchange-averaged TML resonance will have a T_2 and width which are the weighted averages of those of TML complexed by hemoglobin and by GSH.

The resonances for GSH have changed due to complexation by TML within 5 min after the addition of TML to the NMR tube (Figure 5B), indicating that TML rapidly crosses the cell membrane. Comparison of spectra A and B in Figure 8 shows that there is no further change in the complexation of TML by GSH during the next 3 h.

The experiments represented by spectra C-E in Figure 8 were done to determine the extent to which an added sulfhydryl-containing ligand can extract TML from its complexes with ligands naturally present in erythrocytes. If the added ligand forms a complex with some of the TML, this should be reflected by an increase in the intensity of the resonances for the glycine CH_2 protons of GSH, the size of the increase being in proportion to the fraction of the total GSH released from its TML complex. For this study we chose PSH, a ligand which is used to treat several forms of heavy-metal poisoning. The small increase in the ratio of the intensity of the resonance for the glycine CH_2 protons of GSH to that for the CH_3 protons of ergothioneine within 5 min following the addition of PSH indicates that some PSH rapidly enters the red cell where it binds to a small amount of TML. The further increase in the ratio with time indicates more complexation of TML by PSH, presumably as a result of more PSH having crossed the red cell membrane. However, when the amount of TML complexed by PSH reaches a maximum, there still is a large fraction of the GSH complexed by TML. From the formation constants for the PSH[4] and GSH complexes of TML, conditional formation constants of 1.18×10^3 and 3.63×10^3, respectively, are calculated for pH 7, which predict that binding of TML by GSH is favored over binding by PSH. This is in accord with the observation here that PSH complexes only a relatively small fraction of the intracellular TML. It also should be noted that these PSH concentrations are considerably higher than would be achieved in vivo,[29] and thus it is not surprising that PSH has not been effective as a treatment for trialkyllead poisoning.[2]

The spectra in Figure 8 also contain information about the metabolic activity of the red cells. In spectrum A, most of the resonances between those for the glycine CH_2 of GSH and the CH_3 groups of ergothioneine (resonances I and B in Figure 4) are due to glucose protons. Also, the out-of-phase resonance at $\sim$1.29 ppm is for the methyl protons of lactate, the product of red cell metabolism of glucose. The resonance is out of phase because it results from the partial overlap of a negative doublet and a positive singlet for the methyl protons of lactate in which the C(2) is protonated and deuterated ($CH_3CHODCO_2^-$ and $CH_3CDODCO_2^-$), respectively.[13] The lactate methyl resonance is considerably increased in intensity in spectrum B, indicating that the red cells have continued to metabolize glucose during the time between the recording of spectra A and B, i.e., the TML has not stopped the metabolic activity of the cells. Also, the negative component of the lactate resonance is not present in spectrum B, suggesting that there is little of the $CH_3CHODCO_2^-$ form of lactate left in the cells. This is further indicated by the disappearance of the resonance at 4.09 ppm for the proton on C(2) of $CH_3CHODCO_2^-$. The decreased intensity of the glucose resonances in spectra D and E and the increased intensity of the lactate resonance indicate that the red cells have also continued to metabolize glucose following the addition of PSH.

The spectra in Figures 5 and 8 demonstrate that detailed information about the complexation of a metal ion by specific molecules in an intact cellular system can be obtained directly by ^1H NMR spectroscopy. These results suggest that with ^1H NMR spectroscopy it should be possible to study not only the complexation chemistry of heavy metals in erythrocytes but also their removal from erythrocytes by potential ligands for the treatment of heavy-metal poisoning.

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