Nuclear Magnetic Resonance Studies of the Solution Chemistry of Metal Complexes. 25. $Hg(thiol)_3$ Complexes and Hg(II)-Thiol Ligand Exchange Kinetics

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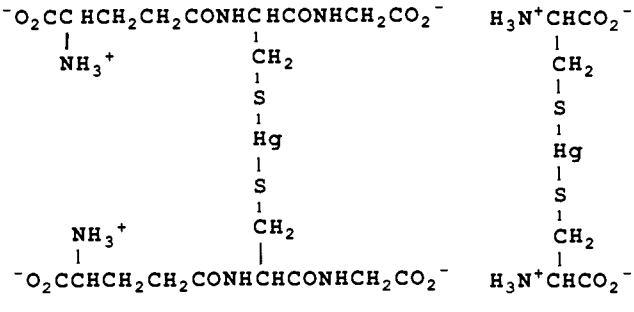
Abstract: The complexation of Hg(II) by the sulfhydryl-containing ligands glutathione, cysteine, and penicillamine has been studied under conditions of excess ligand, with the objective of elucidating the reactions by which Hg(II) is rapidly exchanged among thiol ligands in biological systems. ^{13}C NMR spectra were measured as a function of pH at constant ligand to Hg(II) ratios and as a function of ligand to Hg(II) ratio at constant pH. The results indicate that, even though Hg(II) has a strong tendency to form linear, two-coordinate complexes with sulfhydryl-containing ligands, complexes of the stoichiometry $Hg(SR)_3$ can form when the ligand to Hg(II) ratio is greater than two. Formation constants, which were determined from ^{13}C chemical shift data, indicate that binding of the third ligand to form $Hg(SR)_3$ is much weaker than binding of the two ligands in $Hg(SR)_2$. However, binding is sufficiently strong that a significant fraction of the Hg(II) is present as $Hg(SR)_3$ at physiological pH. Rate constants for exchange of glutathione between its free and Hg(II)-complexed forms were determined from exchange-broadened ^{13}C resonances. The results are discussed in terms of the known highly labile nature of Hg(II) in biological systems.

Sulfhydryl ligands have an extremely high affinity for mercury. For example, the stability constants for the $Hg(glutathione)_2$ (I) and $Hg(cysteine)_2$ (II) complexes at physiological pH and 25 °C



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are reported to be 9×10^{40} and 2×10^{40}, respectively.[1] Consequently, it is thought that all the inorganic mercury, Hg(II), in the blood and tissues of humans is complexed by sulfhydryl groups of cysteine-containing peptides and proteins.[2,3] However, even though the thermodynamic stability of Hg(II)-thiol complexes is high, Hg(II) in biological systems must be labile, exchanging among the multitude of sulfhydryl groups it encounters with some ultimately combining with sulfhydryl groups of enzymes and other molecules which are dependent on free thiol groups for their activity.

Direct evidence for the labile nature of Hg(II)-thiol binding in cells has been obtained from ^1H NMR studies of Hg(II) binding in intact human erythrocytes.[4] Changes in the resonances for intracellular glutathione (GSH) upon addition of $HgCl_2$ to erythrocyte suspensions indicate that some of the added Hg(II) is complexed by GSH and that the lifetime of the complex must be less than 30 s. The labile nature of Hg(II)-thiol binding in biological systems is also evidenced by the fact that sulfhydryl-containing therapeutic agents, for example, 2,3-dimercaptopropanol (British anti-Lewisite), 2,3-dimercaptopropanesulfonic acid, and penicillamine, extract Hg(II) from its thiolate complexes in the fluids and tissues of the body and cause the body burden of Hg(II) to decrease more rapidly than by natural processes.

The lability of Hg(II)-thiol bonding is also evident in the ^{13}C NMR spectroscopy of Hg(II)-thiol complexes.[5] For example, ^{13}C-^{199}Hg coupling has not been detected in ^{13}C NMR spectra of $Hg(thiol)_2$ complexes in aqueous solution. Also, exchange-averaged ^{13}C resonances are observed for all the carbon atoms of glutathione (GSH) in solutions containing Hg(II) and GSH at ratios up to 0.5. Under these conditions, GSH is present in both the free and complexed forms,[6] and the observation of exchange-averaged resonances indicates fast exchange, on the NMR time scale, of GSH between these two forms.

In the present paper, we report the results of ^{13}C NMR studies of the coordination chemistry of Hg(II) with cysteine, penicillamine, and glutathione ligands. The objective of these studies has been to elucidate the reactions which result in the rapid ligand exchange kinetics of $Hg(thiol)_2$ complexes in vivo and in vitro. The results indicate that, in addition to complexes of the stoichiometry $Hg(thiol)_2$, complexes having the stoichiometry $Hg(thiol)_3$ are formed at physiological pH under conditions of thiol:Hg ratio greater than 2:1. Formation constants for the $Hg(thiol)_3$ complexes were determined from ^{13}C chemical shift data. The rapid exchange of thiol between its free and $Hg(thiol)_2$ forms proceeds via a mechanism involving the $Hg(thiol)_3$ complexes, and rate constants were determined for the exchange of GSH between its free and Hg(II)-complexed forms. The kinetics of $Hg(GSH)_2$ ligand exchange are of particular interest because GSH has been identified by ^1H NMR as a major binding site for Hg(II) in intact human erythrocytes,[4] and GSH has been implicated in numerous other studies of the toxicology of Hg(II).[7]

Experimental Section

Chemicals. Glutathione, D,L-penicillamine, and D,L-cysteine (Sigma) were used as received. All thiols were stored under an inert atmosphere below 4 °C. Their purities were determined by procedures described previously.[8,9]

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Solutions of Hg(II) were prepared from analytical grade mercuric nitrate (J.T. Baker Chemical Co.) and were standardized by titration with EDTA. 1,4-Dioxane and D_2O were obtained from BDH Chemicals Ltd. and Stohler Isotope Chemicals, respectively.

Solution Preparation. All solutions were prepared in water which had been purified by distilling it twice and then passing it through a Barnstead D8902 ultrapure mixed bed ion-exchange resin. KNO_3 (0.3 M) was added for ionic strength control. The solvent mixture (5% D_2O/95% H_2O) was deoxygenated by bubbling with argon before addition of thiol to minimize oxidation by dissolved oxygen. Inert gas was also bubbled through the solution during adjustment of pH, and NMR tubes were flushed with the gas before transfer of an aliquot of solution to the tube. Most NMR measurements involved measuring the ^{13}C chemical shifts as a function of pH for a solution containing mercuric nitrate and thiol at a constant ratio. The solutions were prepared by adding stock mercuric nitrate solution to a solution of the thiol. The thiol concentration in the solutions used in the NMR measurements was generally 0.20 M, and the ratio of thiol to mercuric nitrate varied between 2 and 6 but was typically 2, 3, and 4. Samples for NMR measurements were prepared by adjusting the pH of the solutions to the low pH end of the pH range of interest with concentrated nitric acid, and then withdrawing samples at closely spaced pH intervals as the pH was increased by the addition of 2 M sodium hydroxide. The latter was stored in a polyethylene reservoir of a Mettler DV11 autoburet and was delivered to the titration solution through a Pederson 5-μL micropipet, the tip of which was placed in the solution. Drift in pH due to diffusion from the tip was negligible.

pH Measurements. All pH measurements were carried out at 25.0 ± 0.1 °C in a water-jacketed titration cell. The cell was covered with a tightly fitting plastic lid with holes for glass and reference electrodes, a micropipet delivery tube, and argon inlet and outlet tubes. Both buffer and sample solutions were stirred with a magnetic stirrer. pH measurements were made with an Orion Model 701A pH meter equipped with a Philips glass electrode (GAT130) and a Philips glass sleeve double junction Ag/AgCl reference electrode (R44/2-SD/12). KNO_3 (0.3 M) was used as the outer-junction electrolyte to preclude chloride complexation of Hg(II). Freshly prepared N.B.S. pH standard solutions having pH values of 4.008 (phthalate) and 6.865 (phosphate) were used for calibration. For pH measurements made on D_2O solutions, the pH meter was calibrated with aqueous buffers and the pH meter readings were converted to pD values with the relation pD = pH meter reading +0.40.[10]

NMR Measurements. ^{13}C NMR measurements were made at 90.56 MHz and 25 °C on a Bruker WM-360 spectrometer operating in the pulse Fourier transform mode. The sample temperature was maintained at 25 °C by collecting free induction decays under conditions of inverse gated decoupling (NOE suppressed) to minimize heating of the sample, and by blowing chilled air over the NMR tube in the probe. ^{13}C chemical shifts were measured relative to internal 1,4-dioxane, but are reported relative to tetramethylsilane (Me_4Si). The resonance for 1,4-dioxane is at 67.40 ppm to high frequency from Me_4Si.

Results

Mercury(II) Glutathione. The chemical shifts of the cysteinyl carbons of GSH are plotted as a function of pH in Figure 1 for solutions containing only free GSH (A), GSH and $Hg(NO_3)_2$ at a 2:1 mole ratio (B), and GSH and $Hg(NO_3)_2$ in a 3:1 mole ratio (C). The chemical shifts of the other carbons of GSH are not plotted because they are essentially identical for all three solutions.

The changes in the chemical shifts of the cysteinyl-CONH, Cα and Cβ carbons for free GSH over the pH range 7–12 are due primarily to titration of the sulfhydryl group. In solution B, the sulfhydryl groups are coordinated to Hg(II) in the complex $Hg(SG)_2$ and the chemical shifts of the resonances for the cysteinyl carbons are nearly independent of pH over the range 5–12. However, the chemical shifts for solution C are pH dependent because the solution contains both free and complexed GSH, and exchange between the two forms is fast,[5] although the exchange-averaged resonances for the Cys–Cα and Cys–Cβ carbons are broadened over the pH range 4.6 to 8.0. The observed chemical shifts, δ_{obsd}, of the exchange-averaged resonances are the weighted average of the chemical shifts of the various forms:

$$\delta_{obsd} = P_f\delta_f + (1 - P_f)\delta_c \quad (1)$$

where δ_f and δ_c are the chemical shifts and P_f and $(1 - P_f)$ the fractional concentrations of free and complexed GSH. If the only

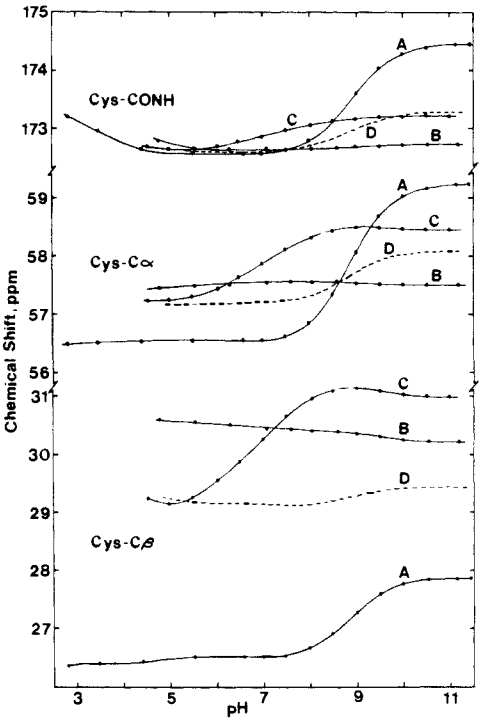

Figure 1. pH dependence of the ^{13}C chemical shifts of the cysteinyl carbons of GSH at 25 °C in solutions containing (A) 0.20 M GSH; (B) 0.20 M GSH and 0.10 M $Hg(NO_3)_2$; (C) 0.20 M GSH and 0.067 M $Hg(NO_3)_2$. The dashed curves (D) represent the chemical shift behavior expected for the three cysteinyl carbons in solution (C) if only free GSH and $Hg(SG)_2$ were present.

forms of GSH present in (C) were free GSH and the linear, two-coordinate complex $Hg(SG)_2$,[6] the observed chemical shifts would follow the dashed curves (D), which are predicted curves calculated using eq 1. At pH 5, the predicted and observed curves are similar, indicating only $Hg(SG)_2$ and free GSH in solution at this pH. However, at higher pH, the observed chemical shifts for all three cysteinyl carbons deviate from the predicted behavior, which indicates the formation of at least one other Hg(II)–GSH complex in addition to $Hg(SG)_2$. Similar results were obtained from chemical shift vs. pH studies at GSH to Hg(II) ratios of 4:1 and 6:1.

To determine the stoichiometry of the additional Hg(II)–GSH complexes, the ^{13}C chemical shifts of GSH were measured as a function of the Hg(II) to GSH ratio at pH values of 6.04, 9.50, and 10.50. Results obtained at pH 9.50 are presented in Figure 2. The dashed lines represent the chemical shifts expected if only free GSH and $Hg(SG)_2$ were present in solution. The Cys–Cβ carbon, in particular, is deshielded continuously (total shift of 3.51 ppm) as the ratio varies from 0 to 0.33, and then is shielded by 0.8 ppm as the ratio increases from 0.33 to 0.5. The deviation between the dashed line and the observed chemical shift reaches a maximum at 0.33, which indicates formation of a complex having a 1:3 Hg(II)–GSH stoichiometry. Also, the chemical shift of the Cys–Cβ carbon shows the largest deviation from the expected shift for a solution containing only GSH and $Hg(SG)_2$, which indicates complexation of the three GSH ligands to Hg(II) through their sulfhydryl groups. The very small variation in the chemical shift of the Gly–COOH carbon atom (total shift of 0.25 ppm up to a ratio of 0.5) in Figure 2 suggests that there is essentially no Hg(II) binding to the glycyl carboxylate groups in either $Hg(SG)_2$ or $Hg(SG)_3$. The difference between the Glu–COOH chemical shift for Hg(II) to GSH at ratios of 0 and 0.5 is 0.28 ppm; however, there is no difference for the Glu–Cα chemical shift which indicates no binding of Hg(II) by the glutamyl $-CO_2^-$ or NH_2 functional groups in $Hg(SG)_2$. However, the chemical shifts of all the glutamyl carbons differ from the expected values at 0 < Hg(II):GSH ratio < 0.5; e.g., at a ratio of 0.33, the differences are Glu–COOH (0.61 ppm), Glu–Cβ (0.29 ppm), Glu–CONH

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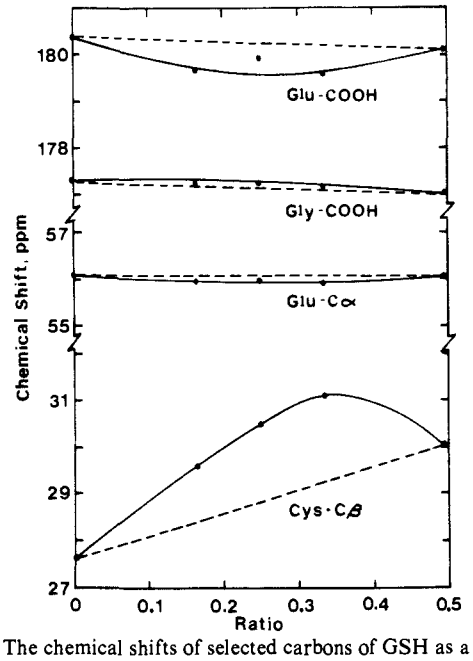

Figure 2. The chemical shifts of selected carbons of GSH as a function of the Hg(II) to GSH ratio at pH 9.50. The dashed lines represent the chemical shifts expected if only free GSH and $Hg(SG)_2$ were present in solution.

(0.19 ppm), Glu-Cα, Glu-Cγ (0.1 ppm). Although these differences might indicate some binding to the glutamyl $-CO_2^-$ and $-NH_2$ groups in the $Hg(SG)_3$ complex, they are more likely due to differences in the acidity of the $-NH_3^+$ group in complexed and free GSH. At pH 9.50, the $-NH_3^+$ group is partially titrated. Because the change in the chemical shifts of the glutamyl carbons upon titration of the Glu-NH_3^+ group in free GSH is large (Glu-COOH, 8.44 ppm; Glu-Cβ, 4.5 ppm; Glu-Cα, 1.35 ppm; Glu-CONH, 1.24 ppm; Glu-Cγ, 0.85 ppm), a small change in the pK_A of the $-NH_3^+$ group upon formation of $Hg(SG)_3$ will cause the chemical shifts of the exchange-averaged resonances to differ from those predicted for free GSH and $Hg(SG)_2$.

To quantitate the effect of complexation of Hg(II) at the sulfhydryl group on the acidity of the ammonium group, acid dissociation constants were determined for the two ammonium groups of the $Hg(SG)_2^{2-}$ complex (I) by pH titration methods. The pK_A values obtained are 8.89 ± 0.02 and 9.50 ± 0.03. For comparison, the pK_A of the ammonium group of free GSH was determined (details below) to be 9.21 ± 0.01. Thus, the differences in the acidity of the ammonium groups of free GSH and $Hg(SG)_2^{2-}$, and probably $Hg(SG)_3$, can account for the small deviations between observed and predicted chemical shifts for the glutamyl carbons over the Hg(II):GSH range of 0 to 0.5 at pH 9.50. At pH 10.50, where the ammonium groups are essentially completely deprotonated and thus a change in the acidity of the ammonium groups will have no effect, the differences between predicted and observed chemical shifts for the glutamyl carbons at a ratio of Hg(II):GSH of 0.33 are much smaller (Glu-COOH, 0.12 ppm; Glu-CONH, 0.11 ppm; Glu-Cα, 0.01 ppm; Glu-Cγ, 0.05 ppm; and Glu-Cβ, 0.01 ppm), consistent with the above interpretation. The chemical shift data also indicates that binding between Hg(II) and GSH in both $Hg(SG)_2$ and $Hg(SG)_3$ occurs exclusively at the sulfhydryl groups.

The formation constant for the $Hg(SG)_3$ complex, as defined by

$$Hg(SG)_2 + GS^- \rightleftarrows Hg(SG)_3 \qquad K_{f3} = \frac{[Hg(SG)_3]}{[Hg(SG)_2][GS^-]} \quad (2)$$

was determined from exchange-averaged chemical shift data for solutions containing GSH and Hg(II) at ratios of 3:1, 4:1, and 6:1. The exchange-averaged chemical shift, δ_{obsd}, is the weighted average of the chemical shifts of the various species present:

$$\delta_{obsd} = P_{GSH}\delta_{GSH} + P_{GS^-}\delta_{GS^-} + P_{1:2}\delta_{1:2} + P_{1:3}\delta_{1:3} \quad (3)$$

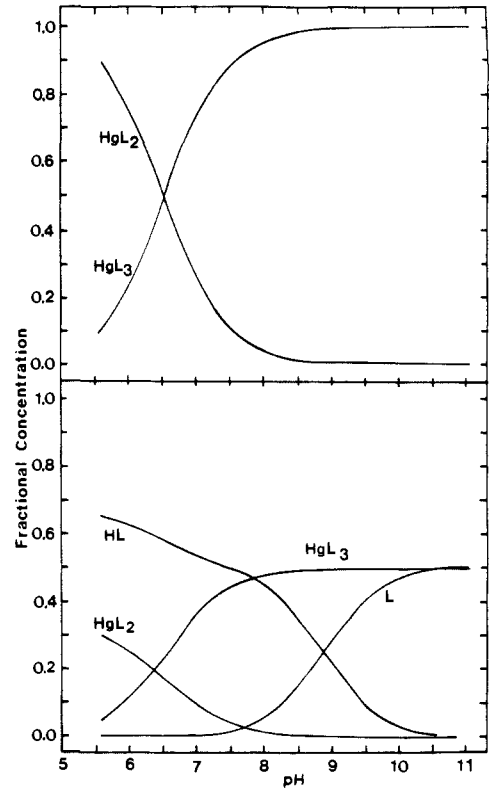

Figure 3. pH dependence of the Hg(II)-containing (upper plot) and the GSH-containing (lower plot) species distribution in a solution containing 0.20 M GSH and 0.033 M $Hg(NO_3)_2$.

where P_{GSH}, P_{GS^-}, $P_{1:2}$, and $P_{1:3}$ represent the fractional concentrations and δ_{GSH}, δ_{GS^-}, $\delta_{1:2}$, and $\delta_{1:3}$ the chemical shifts of glutathione in the sulfhydryl protonated, sulfhydryl ionized, $Hg(SG)_2$, and $Hg(SG)_3$ forms, respectively. By using the Hg(II) and GSH mass balance equations, δ_{obsd} was expressed in terms of the total Hg(II) and GSH concentrations, δ_{GSH}, δ_{GS^-}, $\delta_{1:2}$, $\delta_{1:3}$, $[H^+]$, K_{f3}, and K_{SH}, the acid dissociation constant for the SH group of GSH. The unknowns K_{f3} and $\delta_{1:3}$ were obtained by fitting the observed chemical shift data to the resulting equations. The procedure involved first estimating K_{f3} and $\delta_{1:3}$, and then using these values and the derived equations to predict the chemical shift for the pH and concentrations of each experiment δ_{obsd}. The sum of the squares of the differences between the predicted and observed chemical shifts was calculated, and then the nonlinear least-squares program KINET was used to refine the initial estimates of K_{f3} and $\delta_{1:3}$ to minimize the sum of the square of the residuals.[11] For each of the cysteinyl carbons, the chemical shift data from the experiments at three different GSH:Hg(II) ratios were fitted simultaneously. The values obtained are Cys-Cβ, log K_{f3} = 3.28 ± 0.06; Cys-Cα, log K_{f3} = 3.29 ± 0.05; Cys-CONH, log K_{f3} = 3.09 ± 0.07, where the uncertainties are linear estimates of the standard deviations. In Figure 3 are plotted the fractional concentration vs. pH for the various glutathione (bottom) and Hg(II)-containing (top) species calculated for a solution containing 0.20 M GSH and 0.033 M Hg(II) using the value obtained for K_{f3} from the Cys-Cβ data.

The value used for K_{SH} in the above calculations was determined from ^{13}C chemical shift-pH titration curves for free GSH for the solution conditions used in this study. Dissociation of the sulfhydryl group and the associated acidity constant are represented by equations:

$$GSH \rightleftarrows GS^- + H_3O^+ \quad (4)$$

$$K_{SH} = [GS^-][H_3O^+]/[GSH] \quad (5)$$

Assuming that changes in the chemical shifts of the cysteinyl

(11) Dye, J. L.; Nicely, V. A. *J. Chem. Educ.* **1971**, *48*, 443.

Table I. Acid Dissociation Constants for Thiol Ligands[a-c]

ligand	carbon used for determination	constants[d]
penicillamine	CO_2^-	$pK_2 = 7.97 \pm 0.01$; $pK_3 = 10.59 \pm 0.01$
	CH	$pK_2 = 7.98 \pm 0.01$; $pK_3 = 10.59 \pm 0.01$
	C	$pK_2 = 7.96 \pm 0.01$; $pK_3 = 10.63 \pm 0.01$
cysteine	CO_2^-	$pK_2 = 8.18 \pm 0.01$; $pK_3 = 10.28 \pm 0.01$
	CH	$pK_2 = 8.19 \pm 0.01$; $pK_3 = 10.28 \pm 0.01$
	CH_2	$pK_2 = 8.16 \pm 0.01$; $pK_3 = 10.28 \pm 0.01$
glutathione	Cys-Cβ, Glu-Cα	
	Cys-Cα, Glu-Cβ	$pK_3 = 8.67 \pm 0.01$; $pK_4 = 9.40 \pm 0.01$
	Cys-Cβ	$pK_{SH} = 8.87 \pm 0.01$
	Cys-Cα	$pK_{SH} = 8.88 \pm 0.01$
	Cys-CONH	$pK_{SH} = 8.91 \pm 0.01$
	Glu-Cα	$pK_{NH_3} = 9.21 \pm 0.01$
	Glu-CO_2^-	$pK_{NH_3} = 9.20 \pm 0.01$
	Glu-Cβ	$pK_{NH_3} = 9.20 \pm 0.01$
	Glu-Cγ	$pK_{NH_3} = 9.23 \pm 0.01$

[a] 25 °C, 0.3 M KNO_3. [b] Mixed constants: a_{H^+} (approximated by $10^{-(\text{pH meter reading})}$) and concentration of acid and its conjugate base. [c] Uncertainities are linear estimates of the standard deviations of the best fit values.[11] [d] pK_2 and pK_3 for cysteine and penicillamine and pK_3 and pK_4 for glutathione are composite macroscopic constants for simultaneous dissociation of the SH and NH_3^+ groups.[12]

carbons over the pH range 7–12 are due only to changes in the protonation state of the sulfhydryl group, K_{SH} was determined by fitting the cysteinyl carbon chemical shift–pH titration data to a monoprotic acid model using procedures described previously.[12] The values obtained from the chemical shift data for the three cysteinyl carbons are presented in Table I. Similarly, changes in the chemical shifts of the glutamyl carbons over the pH range 7–12 are assumed to be due only to titration of the ammonium group, and its acid dissociation constant, pK_{NH_3}, was determined from the glutamyl carbon chemical shift–pH titration data. The values obtained for pK_{NH_3} from chemical shift data for the four glutamyl carbons are presented in Table I. Macroscopic acid dissociation constants obtained by combining the chemical shift titration data for the cysteinyl and glutamyl carbons by procedures described previously[12] are also presented in Table I.

As mentioned above, the Cys-Cα and Cys-Cβ resonances are exchange broadened over the pH range 4.6 to 8.0 at GSH:Hg(II) ratios larger than 2. Since the Cys-Cβ resonance is shifted more upon complex formation (Figure 1), it exhibits greater broadening and thus is more sensitive to the rate of exchange. Rate constants for the reactions by which glutathione exchanges between its various free and complexed forms were determined from the exchange broadening for solutions having Hg(II) to GSH ratios of 1:3, 1:4, and 1:6. The procedure involved first determining the mean lifetimes of glutathione in its three environments, free,[13] $Hg(SG)_2$, and $Hg(SG)_3$, from the line broadening. The fractional concentrations of GSH in the free, $Hg(SG)_2$, and $Hg(SG)_3$ forms, P_f, $P_{1:2}$, and $P_{1:3}$, were calculated for each experiment where the Cys-Cβ resonance was exchange broadened using the values determined for K_{f3} and the acid dissociation constants in Table I. These fractional concentrations were then used together with $\delta_{1:2}$ and δ_f to calculate the Cys-Cβ chemical shift for $Hg(SG)_3$ in the absence of exchange, $\delta_{1:3}$, at that pH from:

$$\delta_{obsd} = P_f\delta_f + P_{1:2}\delta_{1:2} + P_{1:3}\delta_{1:3} \quad (6)$$

The lifetimes of glutathione in $Hg(SG)_3$, $\tau_{Hg(SG)_3}$, were then obtained from the line widths of the exchange-broadened Cys-Cβ resonances by matching experimental and computer-simulated spectra.[14] Spectra were simulated using Bloch equations modified

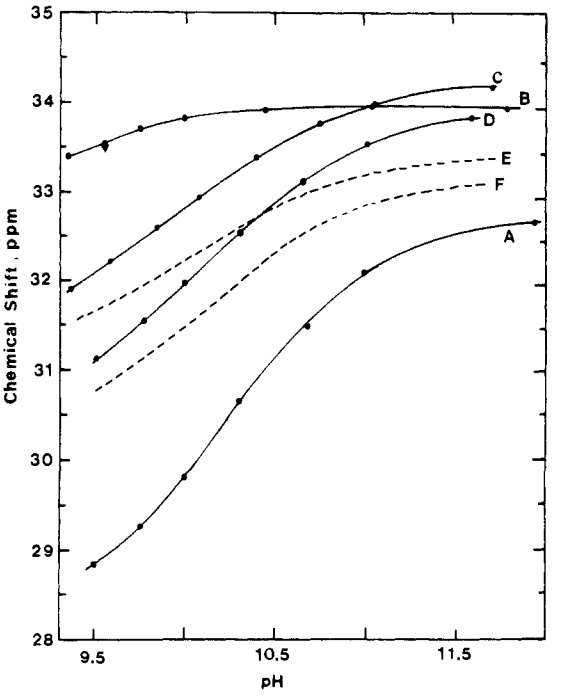

Figure 4. pH dependence of the ^{13}C chemical shift of the Cβ carbon of cysteine at 25 °C in solutions containing (A) 0.20 M CSH; (B) 0.20 M CSH and 0.10 M $Hg(NO_3)_2$; (C) 0.20 M CSH and 0.06 M $Hg(NO_3)_2$; (D) 0.20 M CSH and 0.04 M $Hg(NO_3)_2$. Curves E and F represent the chemical shift behavior expected for solutions C and D, respectively, if only free CSH and $Hg(SC)_2$ were present. The point ▼ at pH 9.54 refers to a solution containing 0.0233 M CSH and 0.0117 M $Hg(NO_3)_2$.

by the approach of McConnell[15] and Meiboom[16] to account for the transfer of magnetization between the three sites by chemical exchange. Spectra were simulated as a function of $\tau_{Hg(SG)_3}$, $\tau_{Hg(SG)_2}$, and τ_{free} until the width at half-height and the chemical shift of the simulated spectrum matched that of the experimental spectrum.[17]

Possible reactions by which glutathione might exchange between its various free and complexed forms are:

$$Hg(SG)_2 + GS^- \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} Hg(SG)_3 \qquad K_{f3} = k_1/k_{-1} \quad (7)$$

$$Hg(SG)_2 + GSH \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} Hg(SG)_3 + H^+ \qquad K'_{f3} = k_2/k_{-2} \quad (8)$$

where GSH and GS^- represent the thiol and thiolate forms of glutathione. The inverse of the mean lifetime of $Hg(SG)_3$ is related to the rate of decrease in its concentration by:

$$\frac{1}{\tau_{Hg(SG)_3}} = -\frac{d[Hg(SG)_3]}{dt}\frac{1}{[Hg(SG)_3]} \quad (9)$$

The rate of decrease in the concentration of $Hg(SG)_3$ is given by:

$$-d[Hg(SG)_3]/dt = k_{-1}[Hg(SG)_3] + k_{-2}[Hg(SG)_3][H^+] \quad (10)$$

Division by $[Hg(SG)_3]$ leads to the following expression for $1/\tau_{Hg(SG)_3}$:

$$1/\tau_{Hg(SG)_3} = k_{-1} + k_{-2}[H^+] \quad (11)$$

(12) Rabenstein, D. L.; Sayer, T. L. *Anal. Chem.* **1976**, *48*, 1141.

(13) The free glutathione is present as GSH and GS^- over the pH range 4.6–8. However, because exchange of glutathione between the GSH and GS^- forms is fast on the NMR time scale, as evidenced by sharp, exchange-averaged resonances for solutions containing only glutathione, the free glutathione is treated as a single exchange site.

(14) Using the program EXCHANGE written by Dr. R. E. D. McClung, Chemistry Department, University of Alberta, Edmonton, Alberta, Canada T6G 2G2 and the program NMRKINETICS written by J. Pleasants, Chemistry Department, University of California, Riverside, Ca 92521.

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Values of k_{-1} and k_{-2} were obtained from the intercept and slope of plots of $1/\tau_{Hg(SG)_3}$ vs. [H$^+$] for 1:3, 1:4, and 1:6 ratios of Hg(II) to GSH. The average of the values obtained for k_{-1} and k_{-2} at 25 °C are 6.3 ± 3.0 × 10^3 s^{-1} and 6.3 ± 1.0 × 10^9 L/mol-s.[18] A value of 1.3 ± 0.6 × 10^7 L/mol-s was calculated for rate constant k_1 from the average value for k_{-1} and K_{f3} (eq 7). In the same way, a value of 1.7 ± 0.3 × 10^4 L/mol-s was calculated for k_2 from the average value for k_{-2} and K_{f3}'.[19]

Mercury(II) Cysteine. ^{13}C NMR spectra were measured as a function of pH for solutions containing 0.20 M cysteine (CSH) and 0.0 (A), 0.10 (B), 0.06 (C), and 0.04 (D) M $Hg(NO_3)_2$. At these concentrations, Hg(II)–cysteine complexes precipitate below pH 9.36, 9.37, and 8.65 for solutions B, C, and D, respectively. The resonances for all carbons of cysteine shift upon complexation, with the resonances for the Cβ carbon experiencing the largest shift. Chemical shift data for the Cβ carbon are plotted in Figure 4. ^{13}C chemical shifts were also measured at pH 2.03 and 9.54 for a solution containing 0.0233 M CSH and 0.0117 M $Hg(NO_3)_2$. The chemical shift of the Cβ carbon in this dilute solution at pH 9.54 (denoted by the ▼ in Figure 4) is the same, within 0.05 ppm, as that observed for the more concentrated 2:1 solution (B).

Cysteine has three potential coordination sites. The resonance for the Cβ carbon is shifted most upon complexation. Also, the pH dependence of the Cβ chemical shift for the 2:1 solution indicates titration of free ammonium groups as the pH is increased up to 10.5, which suggests that the deprotonated sulfhydryl groups are the predominant binding sites in the 2:1 complex. The close agreement between the chemical shifts for the 2:1 solutions at two different concentrations indicate the same complex is present at the two concentrations and that equilibria between complexes of different Hg(II):CSH stoichiometry do not occur for a 2:1 ratio.

The dashed curves labeled E and F in Figure 4 represent the chemical shifts expected for 3.33:1 and 5:1 cysteine:Hg(II) ratios, respectively, if only free cysteine and the 2:1 complex were present in solution. The large differences between C and E and between D and F in Figure 4 indicate that complexes in addition to $Hg(SC)_2$ are formed at cysteine:Hg(II) ratios larger than 2:1. To determine their stoichiometry, chemical shifts were measured as the Hg(II) to cysteine ratio was increased from 0 to 0.5 at pH values of 9.50 and 11.59. The observed chemical shifts for all three carbons of cysteine deviate from those predicted if only free cysteine and the 2:1 complex were present, providing further evidence for an additional complex(es). The resonance for the Cβ carbon shows the largest deviation; the deviation increases as the ratio at pH 11.59 is increased from 0 to 0.3, and then decreases to 0 as the ratio is increased from 0.3 to 0.5, indicating a complex of the stoichiometry $Hg(SC)_3$.

Formation constants for the 1:3 Hg(II)–cysteine complexes, defined by eq 12 to 14, were calculated from the ^{13}C chemical shift vs. pH data at cysteine to Hg(II) ratios of 3.33 to 1 and 5 to 1.

$$HgL_2^{2-} + L^{2-} \rightleftarrows HgL_3^{4-} \qquad K_{f3} = [HgL_3]/[HgL_2][L] \quad (12)$$

$$HgL_2^{2-} + HL^- \rightleftarrows HgL_3H^{3-} \qquad K_{f3H} = [HgL_3H]/[HgL_2][HL] \quad (13)$$

$$HgL_2H^- + HL^- \rightleftarrows HgL_3H_2^{2-} \qquad K_{f3H_2} = [HgL_3H_2]/[HgL_2H][HL] \quad (14)$$

Owing to the proximity of the ammonium group to all three carbons of cysteine, it is necessary to account for the effect of titration of the ammonium groups on their ^{13}C chemical shifts in both the 2:1 and the 3:1 complexes when determining the formation constants. Values determined by potentiometry (pK_3' = 8.17 ± 0.03 and pK_4' = 9.19 ± 0.03)[20] for the ammonium groups of $Hg(SC)_2$ were used in the calculations, whereas those for the ammonium groups of the $Hg(SC)_3$ complex can be derived from the values determined for K_{f3}, K_{f3H}, and K_{f3H_2}. The values obtained by nonlinear least-squares curve fitting of the chemical shift data for the Cβ carbon to a model including the equilibria in eq 12 to 14 and the acid dissociation equilibria for the ammonium groups of $Hg(SC)_2$ and for the sulfhydryl and ammonium groups of free cysteine (Table I)[21] are log K_{f3} = 0.87 ± 0.08, log K_{f3H} = 1.01 ± 0.08, and log K_{f3H_2} = 1.49 ± 0.13. The solid curves drawn through the points for C and D in Figure 4 are theoretical curves calculated using the formation constants and chemical shifts determined as described above.

Mercury(II) Penicillamine. ^{13}C chemical shift data were measured as a function of pH for solutions containing 0.20 M penicillamine and 0 (A), 0.10 (B), 0.067 (C), and 0.05 (D) M $Hg(NO_3)_2$ and as a function of the Hg(II):penicillamine ratio at pH 6.92 and 9.45. The results are similar to those discussed above for the Hg(II)–cysteine system, and indicate the formation of complexes in addition to $Hg(SP)_2$. Chemical shift data were also measured for a solution containing 0.030 M penicillamine and 0.0075 M Hg(II). The chemical shifts of the resonances for the COOH, Cα, and Cβ carbons differed from those observed for the more concentrated 4:1 solution by −0.21, 0.20, and 0.32 ppm at pH 11.53, providing further evidence for the existence of an equilibrium between several complexes having different Hg(II)–penicillamine stoichiometries in solutions with penicillamine to Hg(II) ratios greater than 2:1. Values were determined for K_{f3}, K_{f3H}, and K_{f3H_2} (eq 12 to 14) by nonlinear least-squares curve fitting of the chemical shift versus pH data for the 3:1 and 4:1 solutions. The values obtained from the Cβ carbon data are log K_{f3} = 3.59 ± 0.35, log K_{f3H} = 3.35 ± 0.29, and log K_{f3H_2} = 3.11 ± 0.25. The values used for the acid dissociation constants for the ammonium groups of the $Hg(SP)_2$ complex were determined by potentiometry (pK_3' = 7.41 ± 0.02, pK_4' = 8.99 ± 0.02),[20] and the acid dissociation constants used for free penicillamine were determined from ^{13}C chemical shift data (Table I).[21]

Discussion

The complexation of Hg(II) by sulfhydryl-containing amino acids and peptides has been the subject of a number of studies, including potentiometric studies in which the ligand:Hg(II) ratio was greater than 2:1, to determine the stoichiometry and stability of the complexes.[1-5,22,23] Thus, it is surprising that complexes of the stoichiometry Hg(thiol)$_3$ with sulfhydryl-containing amino acids and peptides have not been detected previously. The ^{13}C chemical shift data clearly indicate their formation in solutions having a ligand to Hg(II) ratio greater than 2:1 and pH greater than ~5. However, evidence for the formation of such complexes with alkanethiol ligands has been obtained from synthetic and structural studies.[24] Vibrational spectroscopy indicates that the centrosymmetric dinuclear anion $[(MeS)_2Hg(\mu\text{-}SMe)_2Hg(SMe)_2]^{2-}$, where MeS^- is methanethiolate, dissociates on dissolution in ethanol to give the mononuclear $[Hg(SMe)_3^-]$ species. The *tert*-butylthiolate complex $[Hg(SBu\text{-}t)_3^-]$ is mononuclear in both the solid state and in ethanol solution. In the synthetic study, the only complexes to crystallize were of the stoichiometry

(18) Uncertainties indicate the range of experimental values.

(19) The value of 2.61 ± 0.42 × 10^{-6} which was used for K_{f3}' (=[Hg(SG)$_3$][H$^+$]/[Hg(SG)$_2$][GSH]) was calculated using the relation $K_{f3}' = K_{f3}K_{SH}$.

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(21) The acid dissocation constants for sulfhydryl and ammonium groups of cysteine and penicillamine were determined for the appropriate experimental conditions by fitting ^{13}C chemical shift data to a diprotic acid model as described previously.[12] Because the chemical shifts of all carbons of cysteine and penicillamine are affected by titration of both acidic groups, only macroscopic acid dissociation constants, which are functions of the microscopic constants for the individual acidic groups, can be determined.

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$Hg(SR)_3$, even though mole ratios of RS^- to $Hg(SR)_2$ in the range 2.0 to 5.0 were used.

The species distribution diagram in Figure 3 shows that, even though the formation constant for addition of the third glutathione ligand to form the $Hg(SG)_3$ complex is much less than those for formation of $Hg(SG)_2$, it is large enough that a significant fraction of the Hg(II) is present as the $Hg(SG)_3$ complex at the concentrations used in the ^{13}C NMR studies. These concentrations are somewhat higher than those used in potentiometric studies;[1,23] however, a significant fraction of the Hg(II) is also present as $Hg(SR)_3$ at the lower concentrations. For example, using the formation constants determined in this work, 16 and 50% of the Hg(II) is calculated to be present as $Hg(SG)_3$ at pH 8 in solutions containing 1×10^{-3} M Hg(II) and 1×10^{-3} M and 7×10^{-3} M GSH, respectively.

The ^{13}C chemical shift data indicates that Hg(II)-ligand binding is exclusively to the deprotonated sulfhydryl groups in the 3:1 complexes of glutathione, cysteine, and penicillamine, with the ammonium groups undergoing titration over essentially the same pH ranges as for the free ligands. However, the ^{13}C NMR results do not provide any information about the geometry of the HgS_3 unit. Vibrational spectroscopy indicates that the mononuclear $Hg(SR)_3$ complexes formed from methanethiolate and *tert*-butylthiolate ligands have a trigonal planar geometry in solution.[24] Assuming this to be the geometry of the $Hg(SR)_3$ complexes of glutathione, cysteine, and penicillamine, the two ligands of $Hg(SR)_2$ and the third ligand to bind become equivalent in the $Hg(SR)_3$ complex and all three ligands have equal probabilities of dissociating to re-form $Hg(SR)_2$ and free ligand. Using the values determined for k_{-1} and k_{-2} for the Hg(II)-glutathione system, the average lifetime of the $Hg(SG)_3$ complex is 7.9×10^{-5} s at pH 6, 1.4×10^{-4} s at pH 7, and 1.57×10^{-4} s at pH 8. Consequently, once formed the complex dissociates very rapidly. Since the three ligands are equivalent in $Hg(SG)_3$ and thus have an equal probability of dissociation, the result is a rapid exchange of free ligand for bound ligand.

In conclusion, it is of interest to use the results of this study to predict the equilibrium and kinetic properties of Hg(II) complexes in vivo. We will take as an example the conditions in human erythrocytes,[25] where the glutathione concentration is $\sim 2.2 \times 10^{-3}$ M and the pH is ~ 7.4. Under these conditions, 9.9, 10.8, and 10.9% of the Hg(II) is predicted to be present as the $Hg(SG)_3$ complex at total Hg(II) concentrations of 1×10^{-4}, 1×10^{-5}, and 1×10^{-6} M, respectively. The average lifetime of the $Hg(SG)_2$ complex before reaction with free glutathione to form $Hg(SG)_3$ is predicted to be 1.1×10^{-3} s while the average lifetime of $Hg(SG)_3$ is predicted to be 1.5×10^{-4} s. Thus, the rate and equilibrium constants determined in this study predict that, even though the stability of $Hg(SG)_2$ is extremely high,[1] exchange of glutathione between its free and Hg(II)-complexed forms will be fast under the conditions present in human erythrocytes, as has been observed experimentally in ^1H NMR studies of Hg(II) binding in intact human erythrocytes.[4] Considering the ubiquity of glutathione in cellular systems,[26] it is likely that the reactions characterized in this work play a major role in the mobility of Hg(II) in biological systems.

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Registry No. Glutathione, 70-18-8; cysteine, 52-90-4; penicillamine, 52-67-5.

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Chlorine NMR Studies of Ionized and Associated Silyl Perchlorates

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Abstract: ^{35}Cl and ^{37}Cl NMR studies have been carried out on triphenylsilyl (sityl) perchlorate and trimethylsilyl perchlorate in sulfolane. Line widths vary from the kilohertz range at 0.1 M concentrations to a few hertz at millimolar concentrations. Chemical shifts vary in an analogous fashion. These observations are consistent with a two-site, fast exchange between associated (e.g., ion-paired) and fully ionized forms of the silyl perchlorate. Quantitative analysis with explicit consideration of the presence of water yielded the equilibrium constant for dissociation of the associated form to free ions and hence percentages of the two forms at each concentration. These results provide unequivocal evidence for the existence of free silylenium ions in dilute sulfolane solution.

Demonstration of the existence of silylenium ions (R_3Si^+) in solution has been difficult over the years[2] and only recently realized.[3-6] Abstraction of hydride from silyl hydrides by triphenylmethyl perchlorate gives triphenylmethane and silyl perchlorates. The reaction is carried out in solvents of high polarity but low nucleophilicity, such as dichloromethane, sulfolane, and acetonitrile. In dilute solution, cryoscopic measurements demonstrated that the silyl perchlorates exist as two particles and conductance measurements showed that they possess an ionic structure, for the cases of tris(alkylthio)silyl [$(RS)_3Si^+$ (R = Me,

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