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Nuclear Magnetic Resonance Studies of the Complex Formation of Mercuric Chloride with L-Cysteine and with Glutathione

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ABSTRACT: The binding of mercuric chloride, $HgCl_2$, with an amino acid, L-cysteine, and with a tripeptide, glutathione, as a model for sulfhydryl proteins, has been investigated by proton and carbon-13 magnetic resonance spectroscopy. Binding to the active coordination sites was monitored, while observing the chemical shifts of protons and carbon nuclei of the ligand in various conditions of the molar ratio ($HgCl_2$/ligand). The results indicate that mercuric chloride binds exclusively to the sulfhydryl group both in L-cysteine and glutathione. A weak secondary binding may also take place with the carboxylic acid group to some extent in the former and with the cysteinyl carbonyl group in the latter, respectively, up to equimolar point. It is also concluded that the stability of the binding of mercuric chloride depends on the pD of the solution. The results are discussed in terms of possible complex structure.

KEY WORDS ^{13}C-NMR / ^{1}H-NMR / L-Cysteine / Glutathione / Mercuric Chloride / Sulfhydryl Group / Coordination /

The binding of metal ions with peptides and proteins are of fundamental interest in view of the important roles of metal ions in biological systems. Peptides and proteins are composed of a number of functional groups, many of which are active coordination sites as shown in the studies on metal binding with simple amino acids and other compounds.[1-3] Proton magnetic resonance (PMR) spectroscopy has proven useful for elucidating at the molecular level, the binding of selected metal ions with amino acids and simple peptides.[4-8] However, the application of PMR is limited to relatively simple amino acids and simple peptides because of the requirement for distinct well-resolved resonances for monitoring interactions at the active binding sites. Carbon-13 magnetic resonance (CMR) spectroscopy should be more useful than PMR for elucidating the binding of metal ions with peptides and proteins; single lines are obtained for each of the non-equivalent carbons in proton-decoupled CMR spectra and the range of chemical shifts is at least one order of magnitude greater than that in PMR. Thus, by CMR it may be possible to elucidate the nature of metal binding with larger peptides and proteins which contain amino acid residues because there is no proton—proton couplings as seen in PMR. In addition, the fact that carbon atoms are bound directly to the active binding sites makes CMR potentially more sensitive as a probe for the determination of binding mechanism at molecular level. In this paper, we report the results of PMR and CMR studies of the binding of mercuric chloride with an amino acid, L-cysteine and with a tripeptide, glutathione (γ-L-glutamyl-L-cysteinylglycine). Mercury(II) compounds are of great environmental concern in view of their occurrence in many biological samples. Many mercury compounds form a stable Hg—S bond to sulfur-containing amino acid residues and are thus rather tightly bound to proteins in living systems. L-Cysteine, which is a well known amino acid containing the sulfhydryl group, may be compared to the sulfhydryl group of serum albumin in its reaction with the mercuric ion. Glutathione, a true peptide containing the sulfhydryl group,

is found in the erythrocytes of whole blood and has a number of functions including the protection of hemoglobin against oxidation of hydrogen peroxide. It is also believed that glutathione acts as a "sulfhydryl-preserver" in maintaining certain proteins (as sulfhydryl-containing enzymes) in the reduced state which is essential for their activity. Although the binding of mercury(II) to L-cysteine and glutathione has been given considerable investigation,[4-11] there is no final agreement on the coordination sites in binding to the mercuric ion as yet. Furthermore, most enzyme-constituting proteins contain cysteine residue in the polypeptide chain, and the sulfhydryl group of the cysteine residue often plays an important role in the appearance of the enzymic activity and/or in the formation of higher order structure. So, L-cysteine and glutathione are expected to serve as a model for most of sulfhydyl proteins.

EXPERIMENTAL

Materials

All materials, L-cysteine (CySH), L-cystine (CySSCy), reduced glutathione (GSH), oxidized glutathione (GSSG), mercuric chloride ($HgCl_2$), heavy water (D_2O), and hydrochloric acid-d_1 (DCl) were purchased from Tokyo Kasei Co. and used as received.

Methods

pD measurements were made at 25°C with a Toa pH meter HM-7A equipped with a standard glass electrode. Saturated phosphate and oxalate solutions were used to standardize the pH meter. The meter readings were converted to pD values using the expression of Glascoe and Long.[12]

PMR spectra were observed using a JEOL PS-100 spectrometer operated at 100 MHz. Tetramethylsilane (TMS) was used to provide an internal lock signal and to measure chemical shift as an external reference. CMR spectra were observed using a JEOL PS-100 spectrometer operating at 25.15 MHz and equipped with a JEOL EC-6 computer. The Fourier transform mode was used in decoupling with protons. The deuterium resonance from the D_2O was used for an NMR internal lock. Chemical shift measurements were made with respect to dioxane, added as an internal reference. CMR chemical shifts are reported in parts per million relative to dioxane, and the positive sign means chemical shifts corresponding to carbon nuclei less shielded than those of dioxane. The carbon-13 nuclei of dioxane are at 67.4 ppm less shielded than those of TMS.[11]

The initial ligand concentration was 0.5 *M* in DCl—D_2O solution. Many samples having different concentrations of metal salt, $HgCl_2$, were prepared by a gradual addition of about 0.05 mol salt.

RESULTS AND DISCUSSION

PMR and CMR Measurement of L-Cysteine (CySH) and L-Cystine (CySSCy), and the Possible Structure of the Complex

Figure 1 shows the PMR spectra change of CySH as a function of the molar ratio ($HgCl_2$/CySH). The profiles of the α-CH and β-CH_2 proton spectra of CySH change from the A_2X type to the ABX type with an increase in the molar ratio, indicating that the initial equivalent two β-protons change to unequivalent ones (H_A and H_B) with an increase in the molar ratio. The chemical shift data of CySH and CySSCy are plotted in Figures 2 and 3, respectively, against the molar ratio ($HgCl_2$/CySH and $HgCl_2$/CySSCy). As shown in Figure 2, the chemical shifts of the H_A, H_B, and α-proton (hereafter, this proton will be referred as H_C) move continuously to lower field (total shift of 0.98 ppm in H_A, 0.79 ppm in H_B, and 0.33 ppm in H_C, respectively) as the ratio goes from 0 to 2.0. The facts that the chemical shift changes of β-protons are twice as large as the α-proton and that the constancy of the chemical shift of protons in CySSCy, as shown in Figure 3, indicate that the binding occurs almost exclusively at the sulfhydryl group.

Natusch and Porter[4] have reported that $HgCl_2$ forms both 1 : 1 and 1 : 2 (ratio) complexes with CySH. Each of the curves in Figure 2 is composed not of three straight lines, but only of two in which the discontinuity of the slope is 0.87 (molar ratio) in H_A and 1.0 in H_B, respectively. So, it can be concluded that the major complex between $HgCl_2$ and CySH is 1 : 1 one.

Figure 4 is the CMR spectra of CySH and CySSCy in a DCl—D_2O solution at pD 1.0. The assignment for every peak is based on a paper

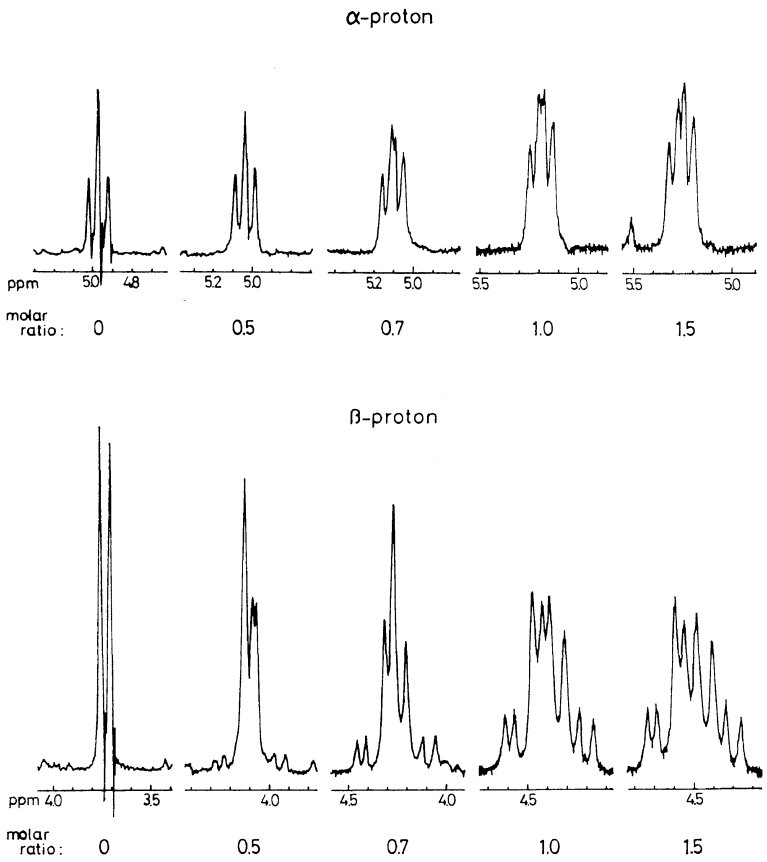


Figure 1. The proton magnetic resonance spectra change of the α- and β-protons of L-cysteine (CySH) as a function of the molar ratio ($HgCl_2$/CySH) in DCl—D_2O solution at pD 1.0.

submitted by Jung.[13] The addition of $HgCl_2$ to a solution of CySH induces the change of chemical shifts as shown in Figure 5, indicating that CMR is of use in the study of metal binding. The chemical shift of the carbons of CySH is plotted against the molar ratio ($HgCl_2$/CySH) in Figure 6. The chemical shift of the C_β carbon moves continuously to the lower field (a total shift of 7.5 ppm) as the ratio goes from 0 to 1.2, and then levels off at a constant values (−36.0 ppm). The C_α carbon indicates a small downfield shift of about 0.8 ppm as the ratio proceeds to 0.5, and an opposite change is observed as the ratio goes from 0.5 to 1.0. The peak for C=O carbon indicates a small upfield shift (total shift of 0.5 ppm) as the ratio goes from 0 to 1.2 and then levels off at a constant value (104.5 ppm). As shown in Figure 7, a slight linear change in chemical shift of the carbons of CySSCy as a function of the molar ratio ($HgCl_2$/CySSCy) does not indicate any significant coordination. Compared with the data in Figures 6 and 7, a large chemical shift change in the C_β carbon in CySH can be interpreted as binding occuring almost exclusively at the sulfhydryl group, because the C_β carbon is the adjacent atom of the sulfhydryl group. A small chemical shift change in the C_α carbon in Figure 6 can be also explained as the C_α carbon's being affected by the binding of $HgCl_2$ to the sulfhydryl group, because the C_α carbon is the second atom from the sulfhydryl group. The interpretation for the small upfield shift in C=O carbon is that weak coordination may occur at the carboxylic acid group. If so, we can well explain the results of our experiment; that is, the weak binding at

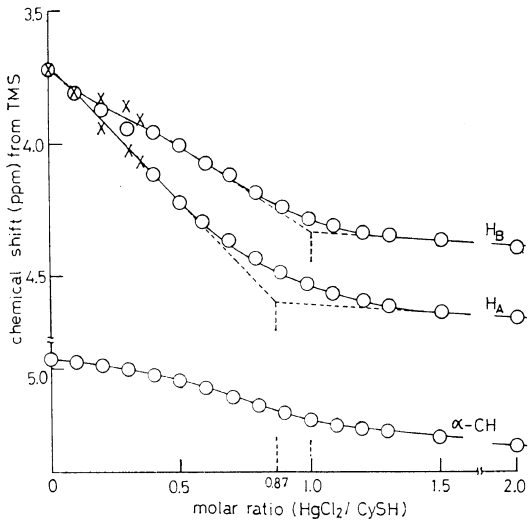


Figure 2. The change in chemical shift of the three protons of L-cysteine (CySH) as a function of the molar ratio ($HgCl_2$/CySH) at pD 1.0. (*x*; 32 scans)

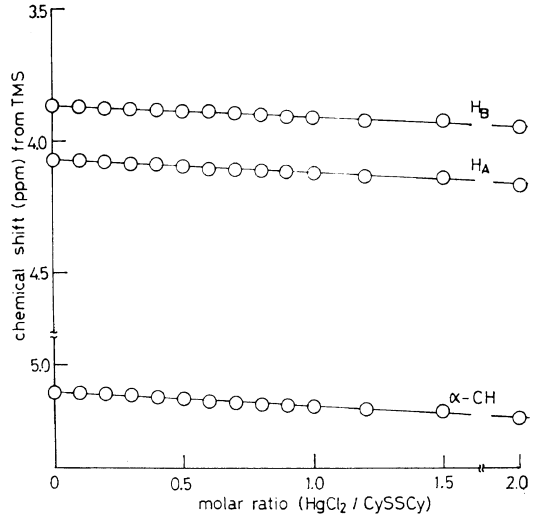


Figure 3. The change in chemical shift of the three protons of L-cystine (CySSCy) as a function of the molar ratio ($HgCl_2$/CySSCy) at pD 1.0.

the carboxylic acid group causes a small upfield shift in C=O carbon as shown in the following inductive effect proposed by Morishima and his co-workers.[14]

$$Hg^{2+} \leftarrow\leftarrow O^{\delta+}=C^{\delta\delta-}(OH)-; \text{ CySH carboxylic acid group}$$

However, as shown in Figure 7, the slight linear change in chemical shift of the C=O carbon of CySSCy suggests that the binding of $HgCl_2$ to the sulfhydryl group is a prerequisite for the binding to the carboxylic acid group of CySH. As shown in Figure 6, the molar ratio at the discontinuity of the C_β curves is about 0.85, which is compatible with our result for PMR measurement, indicating a 1 : 1 complex formation between $HgCl_2$ and CySH. The following equimolar complex with the sulfhydryl and carboxylic acid groups as the coordination site may be the major species in solution under this condition.

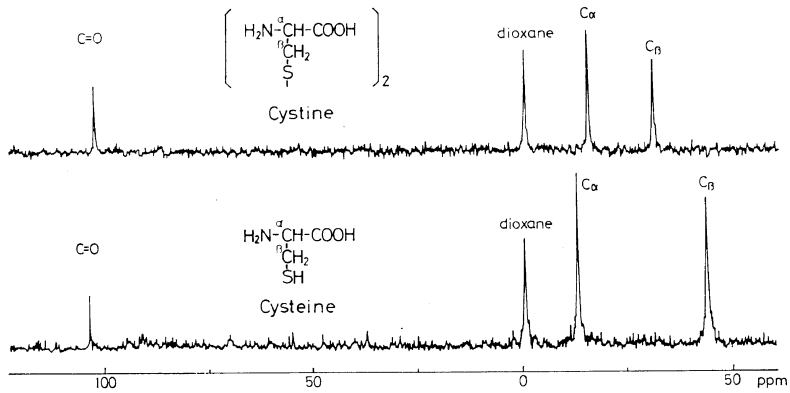


Figure 4. The carbon-13 magnetic resonance spectra of L-cysteine (CySH) and L-cystine (CySSCy) in DCl—D_2O solution at pD 1.0.

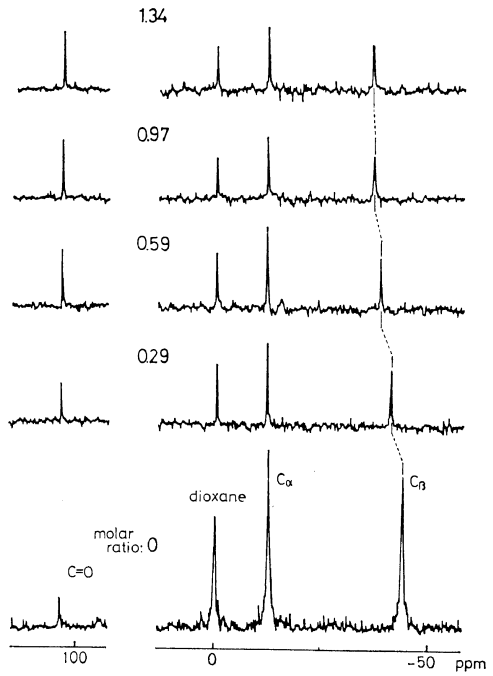


Figure 5. The carbon-13 magnetic resonance spectra change of L-cysteine (CySH) as a function of the molar ratio ($HgCl_2$/CySH) at pD 1.0.

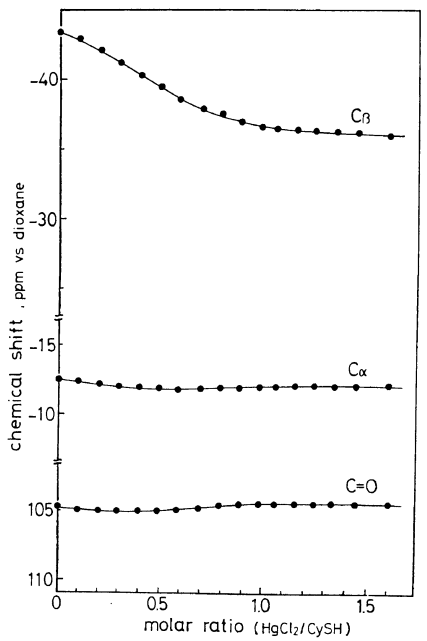


Figure 6. The change in chemical shift of the three carbons of L-cysteine (CySH) as a function of the molar ratio ($HgCl_2$/CySH) at pD 1.0.

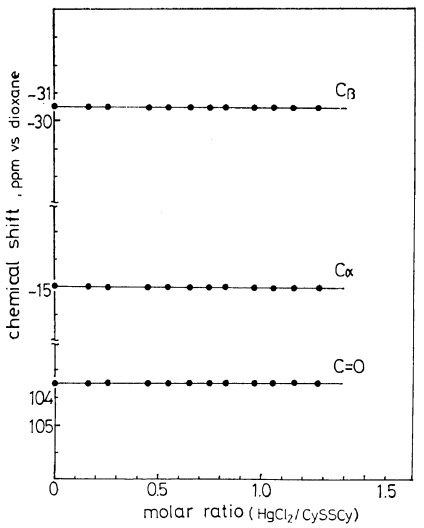
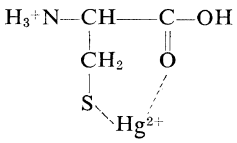


Figure 7. The change in chemical shift of the three carbons of L-cystine (CySSCy) as a function of the molar ratio ($HgCl_2$/CySSCy) at pD 1.0.



CMR Measurement of Reduced Glutathione (GSH) and Oxidized Glutathione (GSSG), and the Possible Structure of the Complex

CMR spectra of GSH and GSSG in DCl—D_2O solution at pD 1.0 are shown in Figure 8. The assignments were made through comparison with the chemical shifts of the constituent amino acids and from the dependence of the chemical shift of each resonance on pD. The assignments obtained are identical with those reported by Jung and his co-workers.[15] The addition of $HgCl_2$ to a solution of GSH induces chemical shift changes in the three peaks, as shown in Figure 9. These three peaks originate from Cys—C_α, Cys—C_β and Cys—CONH. The chemical shift of the carbons of GSH is plotted against the molar ratio ($HgCl_2$/GSH) in Figure 10. Among these, the chemical shift of the Cys—C_β carbon moves continuously to the lower field (total shift of 8.5 ppm) as the ratio goes from 0 to 1.0 and then levels off at a constant value of −33 ppm. The Cys—C_α carbon indicates a small downfield shift as the ratio goes from 0

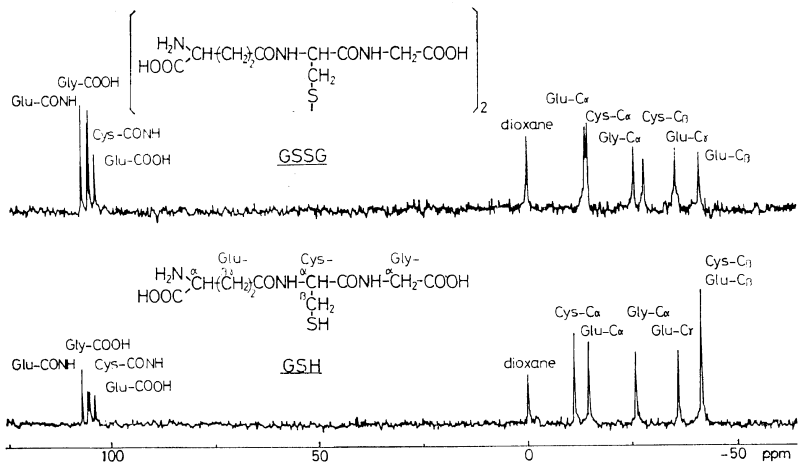


Figure 8. The carbon-13 magnetic resonance spectra of reduced and oxidized glutathione (GSH and GSSG, respectively) in DCl—D_2O solution at pD 1.0.

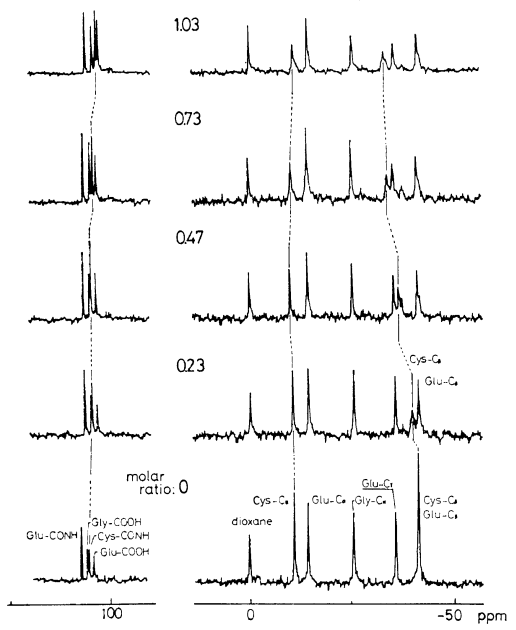


Figure 9. The carbon-13 magnetic resonance spectra change of reduced glutathione (GSH) as a function of the molar ratio ($HgCl_2$/GSH) at pD 1.0.

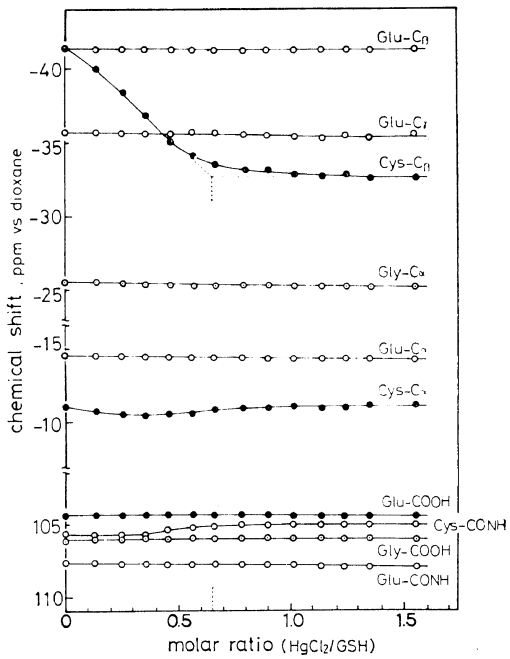


Figure 10. The change in chemical shift of the carbons of reduced glutathione (GSH) as a function of the molar ratio ($HgCl_2$/GSH) at pD 1.0.

to 0.4, and the opposite change can be observed at the ratios greater than 0.4. The Cys—CONH carbon shows a small upfield shift (total shift of 0.9 ppm) as the ratio goes from 0 to 1.0 and then levels off at a constant value of 105 ppm at ratios greater than unity. The chemical shift of the carbons of GSSG is plotted against the molar ratio ($HgCl_2$/GSSG) in Figure 11, indicating to significant coordination. The curves in Figures 10 and 11 indicate that the binding occurs almost exclusively at the sulf-

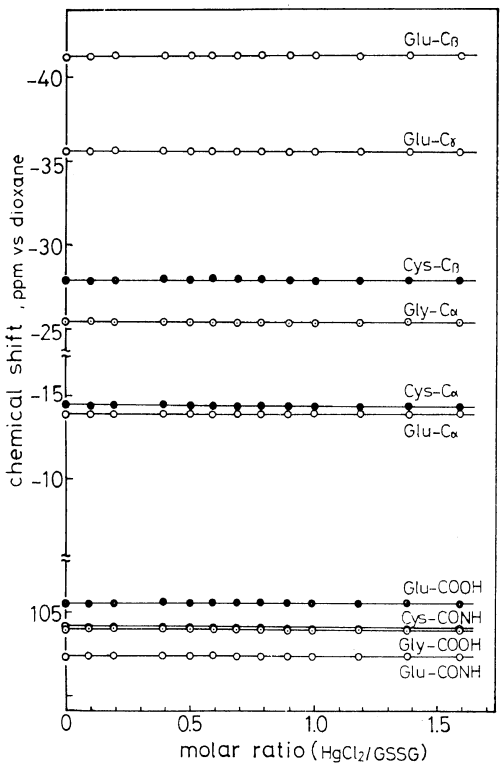


Figure 11. The change in chemical shift of the carbons of oxidized glutathione (GSSG) as a function of the molar ratio ($HgCl_2$/GSSG) at pD 1.0.

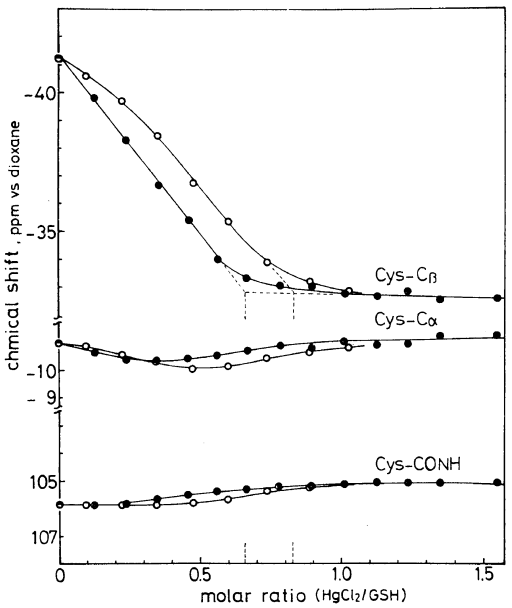


Figure 12. The change in chemical shift of the selected three carbons of reduced glutathione (GSH) as a function of the molar ratio ($HgCl_2$/GSH) at pD 0.6 (●) and pD 1.0 (○).

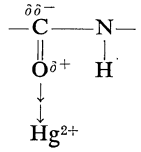
hydryl group and a weak secondary binding may occur at the cysteinyl carbonyl group to some extent at pD 1.0. According to the resonance structure of *N*-methyl acetamide in very acidic solutions,[16] we can estimate that the weak binding occurs at the cysteinyl carbonyl group.

In both series of experiments at pD 0.6 and 1.0, the chemical shifts of the Cys—C_α, Cys—C_β, and Cys—CONH carbons of GSH change as the ratio goes from 0 to 1.0, while those of the remaining carbons of GSH are kept constant. As shown in Figure 12, the change in chemical shifts of the above three carbons of GSH at pD 0.6 indicates a more rapid change than that at pD 1.0, suggesting that the interaction between GSH and $HgCl_2$ depends on the pD of the solution.

It may be concluded from the CMR measurement that, in a strongly acid solution, the exchange of GSH between the free and complexed forms is induced by proton-assisted dissociation of the complex through competition of the proton with Hg^{2+} for the sulfhydryl group, that is, the protonation of the Hg^{2+}-complexed sulfur,

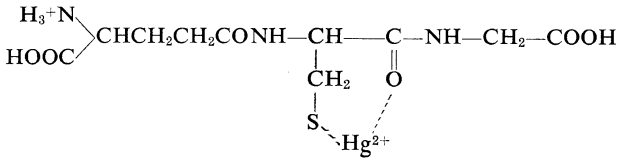
$$-SHg^+ + H^+ \rightleftharpoons -SH + Hg^{2+}$$

Therefore, the lability of sulfhydryl-complexed Hg^{2+} enables a binding with the Cys—CONH group. As shown in Figure 12, the small upfield shift in the Cys-CONH resonance over the molar ratio range 0 to 1.0 is consistent with a small amount of binding to the cysteinyl carbonyl group. This upfield shift in the Cys-CONH resonance can be explained by an inductive effect[14] similarly to the CySH previously described, that is



As shown in Figure 11, the chemical shift of the Cys—CONH carbon of GSSG remains constant in the change of molar ratio. Therefore,

the binding of $HgCl_2$ to the Cys—CONH of GSH is presumably preceded by the binding to the sulfhydryl group. Thus, a 1 : 1 complex with the sulfhydryl and Cys—CONH groups as the coordination site, illustrated below, may be the major species in a strongly acid solution, and the results of our experiment can be well explained by this structure.



In both L-cysteine and glutathione, the bindings occur simultaneously at the sulfhydryl and carboxylic acid groups of L-cysteine or the carbonyl group of cysteine residue, suggesting that similar binding may occur in the sulfhydryl-containing polypeptide. As the reactivity of the sulfhydryl group in polypeptide depends on its various conformational conditions, it may be different from those of simple amino acids or oligopeptides. However, we believe that, at the present, the study of the binding of metal ions by these amino acids and oligopeptides will serve as a model for the elucidation of the effect of metal ions on biological systems.

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