7701.
Pratt, W. B., and Ishii, D. N. (1972), *Biochemistry 11,* 1401.
Rousseau, G. G., Baxter, J. D., and Tomkins, G. M. (1972), *J. Mol. Biol. 67,* 99.
Scatchard, G. (1949), *Ann. N.Y. Acad. Sci. 51,* 660.
Schaumberg, B. P. (1972), *Biochim. Biophys. Acta 261,* 219.
Schaumberg, B. P., and Crone, M. (1971), *Biochim. Biophys. Acta 237,* 494.
Shyamala, G. (1973), *Biochemistry 12,* 3085.
Shyamala, G. (1974), *J. Biol. Chem. 249,* 2160.
Stockdale, F. E., Juergens, W. G., and Topper, Y. J. (1966), *Dev. Biol. 13,* 266.
Thatcher, W. W., and Tucker, H. A. (1970), *Endocrinology 86,* 237.
Toft, D., and Chytil, F. (1973), *Arch. Biochem. Biophys. 157,* 464.
Tucker, H. A., Larson, B. L., and Gorski, J. (1971), *Endocrinology 89,* 152.
Turnell, R. W., Beers, P. C., and Wittliff, J. L. (1974), *Endocrinology 95,* 1770.
Turnell, R. W., Kaiser, N., Milholland, R. J., and Rosen, F. (1974), *J. Biol. Chem. 249,* 1133.

Heavy Metal–Nucleoside Interactions. Binding of Methylmercury(II) to Inosine and Catalysis of the Isotopic Exchange of the C-8 Hydrogen Studied by ^1H Nuclear Magnetic Resonance and Raman Difference Spectrophotometry†

Samir Mansy and R. Stuart Tobias*

ABSTRACT: Raman difference spectrophotometry reveals that CH_3Hg^{II} binds quantitatively to N(1) of inosine at pH 8, substituting for the proton. When N(1) is saturated, binding occurs at a second site. Measurements of the ^1H nuclear magnetic resonance spectra of both inosine and of CH_3Hg^{II} are in agreement with the N(1) binding and indicate that the second site for mercuriation is N(7). This second binding reaction is observed to increase the rate of exchange of the C(8) hydrogen with solvent, consistent with results observed for alkylation at N(7). Coordination of the electrophilic CH_3Hg^{II} to N(7) increases the acidity of H(8), facilitating OH^--catalyzed proton abstraction and reprotonation by the medium. For comparison, the reaction of CH_3Hg^{II} with [8-^2H]inosine has been studied. Displacement of the N(1) hydrogen upon mercuriation of inosine causes a significant electron delocalization into the ring, increasing the basicity of N(7), and accounting for the synergic effect in metal binding observed originally by Simpson. In contrast, 1-methylinosine interacts only slightly with CH_3Hg^{II} at pH 8. Coordination appears to be at N(7), since H(8) again is observed to exchange rapidly with solvent protons. In acidic solution, pH <2, binding to inosine is almost quantitative and exclusively to N(7). The behavior of CH_3Hg^{II} is compared with that of Pt(II) and with Ni(II), Co(II), and Zn(II). A brief comparison is made among ultraviolet absorption spectrophotometry, nuclear magnetic resonance (NMR), and Raman difference spectrophotometry for studying reactions of nucleosides and nucleotides.

Recently, we used Raman difference spectrophotometry to study the binding of CH_3Hg^{II} to GMP in H_2O and D_2O at pH 2 and 8.5 (Mansy and Tobias, 1974a). The methylmercury(II) cation makes an ideal probe for determining the binding sites of heavy metals. At pH 8.5, a quantitative substitution of the proton bound to N(1) occurred up to a 1:1 metal:base mole ratio, and at higher ratios a second, almost quantitative reaction occurred with additional binding of CH_3Hg^{II} to the base moiety. There was no indication of any coordination to the phosphate. The changes in the GMP vibrational spectrum suggested that the electron distributions of the base in [$CH_3HgGuoH_{-1}$-5′-P] and [$GuoH_{-1}$-5′-P]$^-$ [1] were quite similar, i.e. that binding of CH_3Hg^+ does not prevent extensive delocalization of the lone electron pair into the ring system. The second binding site could not be assigned definitely, although Simpson (1964) had suggested that binding could occur at N(1), N(7), and the C(2) NH_2 group by analogy with protonation. A recent Raman study of the binding of CH_3Hg^{II} to the amino groups of cytidine and adenosine (Mansy et al., 1975) indicates that this type of reaction will be unimportant under the conditions of the GMP binding studies. At low pH, the Raman spectra suggested that binding occurred with displacement of the N(7) proton of [GuoH-5′-P]$^+$ which is the predominant species at pH 2.

† From the Department of Chemistry, Purdue University, West Lafayette, Indiana 47907. *Received December 17, 1974.* This work was supported by Public Health Service Grant No. AM-16101 from the National Institute for Arthritis, Metabolism, and Digestive Diseases and by National Science Foundation Grant No. GP-4002X.

[1] Since Guo refers to the neutral molecule which is a dibasic acid, we have used $GuoH_{-1}$ to describe the conjugate base which has lost the N(1) hydrogen. The methylmercury complex of this ligand then is written $CH_3HgGuoH_{-1}$. Mercuriation at N(7) of guanosine that is protonated at N(1) gives CH_3HgGuo^+. This emphasizes the pH dependence of the formation of these complexes.

In order to study further the binding of heavy metal ions to purine nucleosides, we decided to investigate the reactions of CH_3Hg^{II} with Ino using Raman difference and ^1H nuclear magnetic resonance (NMR) spectroscopy. Some of the results of the ^1H NMR studies have been reported in a preliminary communication (Mansy and Tobias, 1974a). Since the gel formation that takes place at moderate concentrations with GMP does not occur with inosine, the nucleoside could be used. Simpson (1964) also studied the reactions of CH_3Hg^{II} with inosine using ultraviolet (uv) spectrophotometry. Binding at N(7) was reported for pH 2–4 and at both N(1) and N(7) at pH 5–9. One puzzling feature of the binding reactions was that mercuriation at one site seemed to enhance mercuriation at the other site in the molecule.

Although mercury binding to nucleosides and nucleotides has been studied more thoroughly than the reactions of any other heavy metal, more extensive studies have been carried out with first row transition metal ions, especially Cu(II). This work has been reviewed recently by Eichhorn (1973). Ino proved to be remarkably versatile in its binding of Cu^{2+} (Berger and Eichhorn, 1971a,b), and at low pH coordination occurs almost exclusively at N(7), while at pH 5–6 the Cu^{2+} is distributed almost equally between N(1)···O(6) and N(7). Paramagnetic metal ion broadening of the H(2) and H(8) nuclear magnetic resonances with 0.1 *M* Ino was used to assign binding sites. Similar studies with Ino-5′-P (Berger and Eichhorn, 1972) indicate that N(7) binding is stabilized by phosphate, suggesting that there is cooperative binding between one phosphate and N(7) of another nucleotide. In contrast to the behavior of Cu^{2+}, Mn^{2+} shows no N(1) binding. Recently it was observed that ^{13}C NMR of ca. 0.5 *M* IMP solutions at pD 7.4 to which $CuCl_2$ was added showed preferential broadening of the C(4), C(5), and C(8) resonances indicating that Cu^{2+} is attached mainly at N(7) (Kotowycz and Suzuki, 1973).

With first row transition metal ions such as Mn^{2+} and Cu^{2+}, the binding is mainly to the phosphate moiety, and coordination to the base then may be facilitated by the chelate effect (Anderson et al., 1971). Line broadening measurements of H(8) and H(1)′ resonances of GMP by Mn^{2+} (Anderson et al., 1971) show that this ion does interact with the base rings in the presence of the phosphate groups.

Recently ^1H NMR spectra have been reported for substitutionally inert complexes of diammineplatinum(II) and ethylenediamineplatinum(II) with inosine (Kong and Theophanides, 1974). Coupling of the ^{195}Pt (spin ½) with H(8) in these diamagnetic complexes indicated only binding to N(7). The results of the CH_3Hg^{II} binding studies reported here can be compared with the detailed data available for Cu^{2+} as well as with data for another heavy metal, Pt(II).

Experimental Procedures

Inosine and 1-methylinosine were obtained from Sigma Chemical Co., St. Louis, Mo., and were used without further purification. [8-^2H]Inosine was prepared by exchanging the C(8) hydrogen in D_2O at 95° (Bullock and Jardetzky, 1964). Methylmercury perchlorate was prepared as described earlier (Mansy et al., 1975). Stock solutions of the nucleosides were prepared by dissolving weighed quantities in doubly distilled, deionized H_2O or 99.8% D_2O (Columbia Organic Chemicals, Columbia, S.C.). The pH values (pD values) of the solutions were adjusted with $HClO_4$ ($DClO_4$) or NaOH (NaOD) solutions using a Radiometer PHM-4 meter. For the D_2O solutions, a standard glass electrode was used, and the meter reading was corrected (Glascoe and Long, 1960).

Table I: Equilibrium Constants[a] (25°) Used in the Description of the CH_3Hg^{II}–Inosine System (Previously Assigned Sites of Binding Are in Parentheses).

Reaction	μ	Log K_{eq}
CH_3Hg^{II}		
$CH_3Hg^+ + H_2O \rightleftharpoons CH_3HgOH + H^+$	0.1	−4.59
$CH_3HgOH + CH_3Hg^+ \rightleftharpoons (CH_3Hg)_2OH^+$	0.1	2.37
Ino		
$InoH_{-1}^- + H^+ \rightleftharpoons Ino(N_1)$	0.1	8.9
$Ino + H^+ \rightleftharpoons InoH^+(N_7)$	Var	~1.5
$InoH_{-1}^- + CH_3Hg^+ \rightleftharpoons InoH_{-1}HgCH_3(N_1)$	Var	8.2
$Ino + CH_3Hg^+ \rightleftharpoons InoHgCH_3^+(N_7)$	Var	3.7
$InoH_{-1}^- + 2CH_3Hg^+ \rightleftharpoons InoH_{-1}(HgCH_3)_2^+(N_1,N_7)$	Var	12.5

[a] Dissociation of the protons from the 2′- and/or 3′-OH groups for which p*K* is ca. 12.4 is ignored.

The Raman difference instrument has been described (Amy et al., 1974), and the general procedure for obtaining difference spectra for metal ion–nucleoside solutions also has been described (Mansy et al., 1974). All spectra were excited with the 514.5-nm line of an Ar^+ laser at a power of ca. 800 mW. Unless otherwise specified, the photon counting times were 10 sec per step, and data were collected at 0.25-Å (ca. 1 cm^{-1}) intervals. The monochromator slits were 300 × 300 × 300 μ (ca. 5.4 cm^{-1}). The sample temperature was 25 ± 2°. For the Raman spectra all solutions were 0.100 *M* in ClO_4^- as an internal intensity and frequency standard.

Nuclear magnetic resonance spectra were obtained with a Varian XL-100 spectrometer. All spectra, unless otherwise noted, were obtained with the same radiofrequency field and spectrum amplitude. The standard sweep width was 1000 Hz, and the sweep time was 250 sec. For the more dilute solutions, the spectrum amplitude was reduced, and 64 scans were accumulated. With the D_2O solutions, the solvent provided the lock signal; with H_2O solutions an external lock was provided by D_2O in a capillary. Chemical shifts were measured from an internal standard of $[N(CH_3)_4]^+NO_3^-$ obtained by neutralizing a D_2O solution of $[N(CH_3)_4]OH{\cdot}5H_2O$ (Sigma Chemical Co.) with DNO_3 solutions. The usual sodium 3-(trimethylsilyl)-1-propanesulfonate reference was not employed since the sulfonate group would compete to some extent with inosine for CH_3Hg^{II}. Since the solubility of $[N(CH_3)_4]ClO_4$ is low, nitrate was used as the anion for the NMR experiments. The solutions were maintained at 25 ± 2° except when the spectra were being measured, and the probe temperature was ca. 40°.

Results

Species Distribution. As in our previous work (Mansy et al., 1975; Mansy and Tobias, 1974a), we have used literature values of available equilibrium constants to form a model of the system. Equilibrium constants of Schwarzenbach and Schellenberg (1965) were used to describe the hydrolysis of CH_3Hg^{II}, proton equilibrium constants for inosine were taken from the tables of Izatt et al. (1971), and the values of Simpson (1964) for the CH_3Hg^{II}–inosine reactions were taken as a first approximation. The values used are collected in Table I. Species distributions were comput-

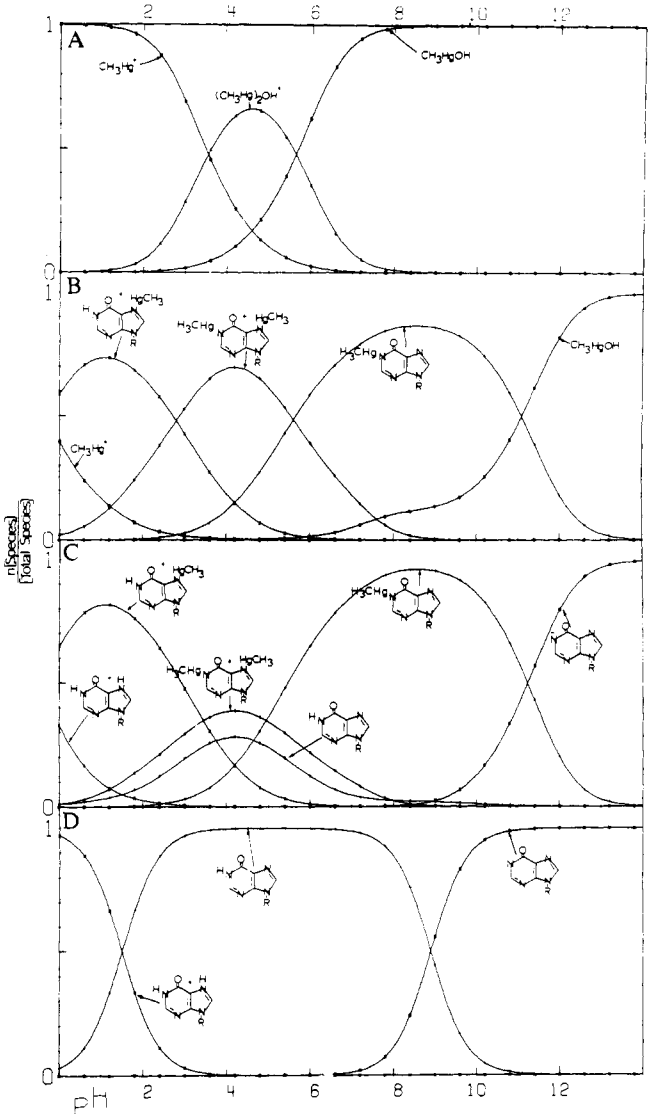


FIGURE 1: Species distribution in the CH_3Hg^+–inosine system computed from a model based on Simpson's (1964) equilibrium constants. Proton transfer from ribose is ignored: (A) 50 m*M* CH_3Hg^+; (B) 50 m*M* CH_3Hg^+ + 50 m*M* Ino, metal distribution; (C) 50 m*M* CH_3Hg^+ + 50 m*M* Ino, Ino distribution; (D) 50 m*M* Ino.

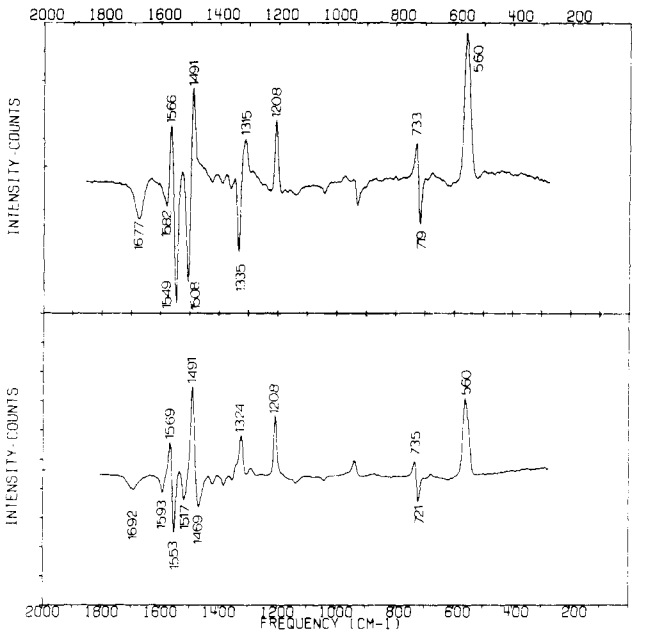


FIGURE 2: Raman difference spectra, 50 m*M* Ino + 50 m*M* CH_3Hg^{II} vs. 50 m*M* Ino: (top) D_2O, pD 7.8; (bottom) H_2O, pH 7.5. All solutions 0.100 *M* in ClO_4^-.

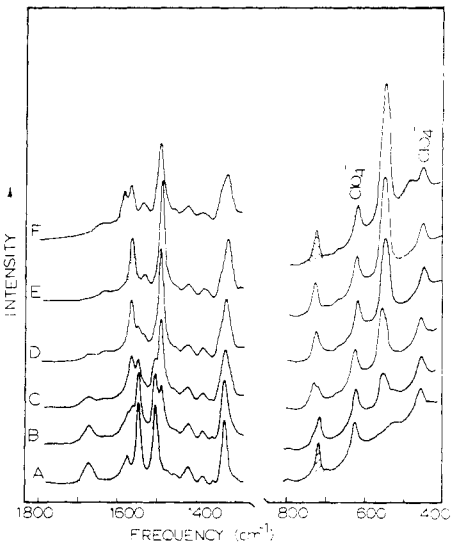


FIGURE 3: Raman spectrophotometric titration of 25 m*M* inosine in D_2O (pD 8.0): (A) 0; (B) 6.25; (C) 12.5; (D) 18.75; (E) 25.0; (F) 50 m*M* CH_3Hg^{II}. All solutions 0.100 *M* in ClO_4^-.

ed as described previously (Mansy et al., 1974), and these are illustrated in Figure 1.

On the basis of the model, it was decided to make measurements at pH ~8 where the system should consist mainly of CH_3HgOH, $CH_3HgInoH_{-1}$, and Ino, and at pH ~1.5, where $CH_3HgOH_2^+$, CH_3HgIno^+, $InoH^+$, and Ino should be present. These spectra should be adequate to establish the binding sites.

Raman Spectra. The Raman spectra of 0.25 *M* inosine and 1-methylinosine solutions in H_2O and D_2O have been reported by Medeiros and Thomas (1971). In order to examine the reactions between CH_3Hg^{II} and Ino at pH ~8, Raman difference spectra were obtained for both H_2O and D_2O solutions 50 m*M* each in inosine and CH_3Hg^{II} from 300 to 1800 cm^{-1}, and these are illustrated in Figure 2. The parent spectra and tabulated frequency and intensity data are available in the microfilm edition (see Supplementary Material Available paragraph at end of paper). All of the frequencies of inosine bands with relative intensities of 2 or larger (10 = maximum intensity) for both H_2O and D_2O solutions agree with the values of Medeiros and Thomas within ±2 cm^{-1}. Both the H_2O and D_2O solutions show a large decrease in the intensity of the highest frequency band (1692, H_2O; 1677, D_2O) when CH_3Hg^{II} reacts with inosine. Since this band is relatively isolated from other inosine bands, it appeared that it would be useful for determining the stoichiometry of the binding reaction. For this purpose D_2O solutions have an advantage, since there is no significant scattering in this region.

A Raman spectrophotometric titration of 25 m*M* inosine with CH_3Hg^{II} was carried out with D_2O solutions (pD 8.0). The spectra are illustrated in Figure 3. Bands which change significantly as the $[CH_3Hg^{II}]$:[Ino] ratio increases are shaded. Up to a ratio of 1:1, the spectra appear to be a superposition of the spectrum of inosine itself and the spectrum of a single CH_3Hg^{II}–Ino complex.

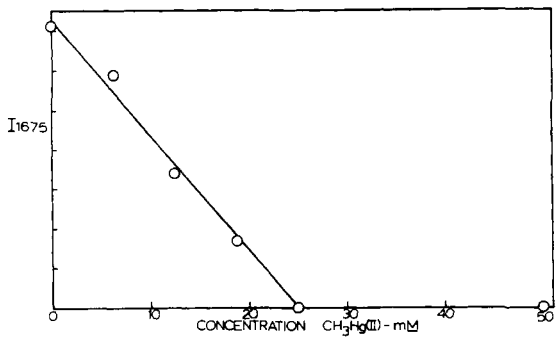


FIGURE 4: Variation of the integrated intensity of the 1675-cm^{-1} inosine band as a function of the total CH_3Hg^{II} concentration in D_2O; [Ino] = 25 m*M*.

To ascertain the stoichiometry of this reaction, the integrated intensity of the inosine band at ca. 1675 cm^{-1} was determined and plotted as a function of the CH_3Hg^{II} total concentration. This is illustrated in Figure 4. Clearly the reaction that leads to the disappearance of the 1675-cm^{-1} inosine vibration has 1:1 stoichiometry. The integration of the band was achieved by plotting the spectra illustrated in Figure 3 in a 20 × 40 in. format and measuring the band area with a Gelman planimeter. Although PROGRAM RAMAN used to process the raw digital data (Lundeen, 1974; Amy et al., 1974) has provisions for determining integrated band intensities, the planimeter integration is as accurate in this case. Very weak scattering grows in with increasing [CH_3Hg^{II}]:[Ino] over the 1600–1650-cm^{-1} region, and a smooth base line must be drawn through the 1600–1700-cm^{-1} range in order to determine the integrated intensity of the 1675-cm^{-1} band. This is done most easily with the large scale plots.

The disappearance of the 1675-cm^{-1} band (D_2O) is similar to the change observed when inosine is titrated with NaOH to pH 13 where it exists as $InoH_{-1}^-$. In D_2O, pD >13, only very weak, broad scattering at 1636 cm^{-1} is observed (Medeiros and Thomas, 1971). Very similar changes are observed with GMP (Mansy and Tobias, 1974a), Urd (Mansy et al., 1974), and dTMP (Chrisman et al., 1974). Substitution of the N(1) proton of inosine by CH_3Hg^{II} allows almost as much delocalization of the lone-pair electron density into the σ and π systems as occurs with the simple conjugate base, $InoH_{-1}^-$. Previously, on the basis of Raman spectral changes, we have assigned binding of CH_3Hg^{II} to the ring nitrogen, N(1) of GMP and N(3) of uridine. This is supported by the recently published structure of $Hg(1\text{-}MeThyH_{-1})_2$ (Kosturko et al., 1974) which shows mercury coordinated to N(3) with a rather short bond distance, d(Hg–N(3)) = 2.04 Å.

With [CH_3Hg^{II}]:[Ino] ratios greater than 1.0, a second reaction occurs at pD 8 as indicated by the shift of the band at 1555 to 1584 cm^{-1} (Figure 3). Only very minor changes occur in the rest of the inosine spectrum. This reaction is not quite quantitative at pD 8, because the 2:1 solution shows weak scattering at ca. 496 cm^{-1} characteristic of undissociated CH_3HgOD (Mansy et al., 1974). The inosine band at 720 which shifts to 733 cm^{-1} with the 1:1 complex still is found at 733 cm^{-1} with the 2:1 solution. The shift of the 1584-cm^{-1} band to higher frequency suggests a decrease in electron delocalization as might be expected to result from binding of a second CH_3Hg^{II}, but it is not possible to establish the second binding site from the spectrophotometric titration data alone.

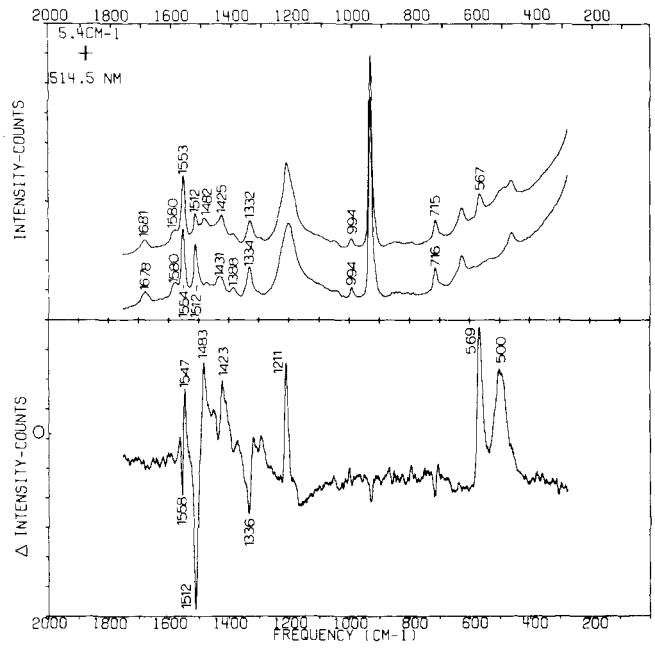


FIGURE 5: Raman difference spectrum, 25 m*M* 1-MeIno + 25 m*M* CH_3Hg^{II} vs. 25 m*M* 1-MeIno; D_2O (pD 8.0): (top) parent spectra; (bottom) complex–ligand difference spectrum, ordinate expanded 3×. All solutions 0.100 *M* in ClO_4.

To test the conclusion from the Raman spectrophotometric titration of inosine with CH_3Hg^{II} that the initial binding is to N(1), a Raman difference spectrum was obtained for a D_2O solution 25 m*M* in CH_3Hg^{II} and 25 m*M* in 1-methylinosine. This spectrum is illustrated in Figure 5. In this case, CH_3Hg^{II} cannot bind at N(1), and as expected there is no change in intensity of the 1-methylinosine band at 1678 cm^{-1} in the solution containing CH_3Hg^{II}. This confirms that the 1:1 reaction described above for inosine does involve binding at N(1). The CH_3Hg^{II}–1-MeIno vs. 1-MeIno difference spectrum does show that some interaction occurs. If no reaction at all took place, the difference spectrum would simply be the spectrum of CH_3HgOD with bands at 496, 569, and 1211 cm^{-1} (Mansy et al., 1974). Small changes occur in the 1-methylinosine modes in the 1300–1600-cm^{-1} range. In addition, the relative intensity of the ca. 500-cm^{-1} band due mainly to (Hg–O) stretching compared to the 569-cm^{-1} (Hg–C) stretching band indicates some substitution of bound hydroxo groups by nucleoside. The (Hg–C) stretching frequency of 569 cm^{-1} observed in the difference spectrum is the value of CH_3HgOD, indicating relatively little reaction with the nucleoside has occurred. Clearly a reaction takes place, but only to a small extent, and it probably involves CH_3Hg^{II} coordination to N(3) or N(7) of the 1-methylinosine. At this point, a ^1H NMR study of the CH_3Hg^{II}–Ino and 1-MeIno solutions was undertaken to attempt to establish the nature of this second reaction.

Proton Magnetic Resonance Spectra. The ^1H NMR spectra of inosine have been studied by Broom et al. (1967) and 1-methylinosine spectra have been studied by Ts'o et al. (1969). Since CH_3Hg^{II} is diamagnetic, its addition to a solution of inosine in D_2O causes only small changes in the inosine chemical shifts. Figure 6 shows spectra of 25 m*M* inosine (pD 8.0) to which varying amounts of CH_3Hg^{II} have been added. The inosine H(2) and H(8) signals show no evidence of coupling with ^{199}Hg (spin ½), because exchange of CH_3Hg^{II} is very fast on the NMR time scale (Simpson,

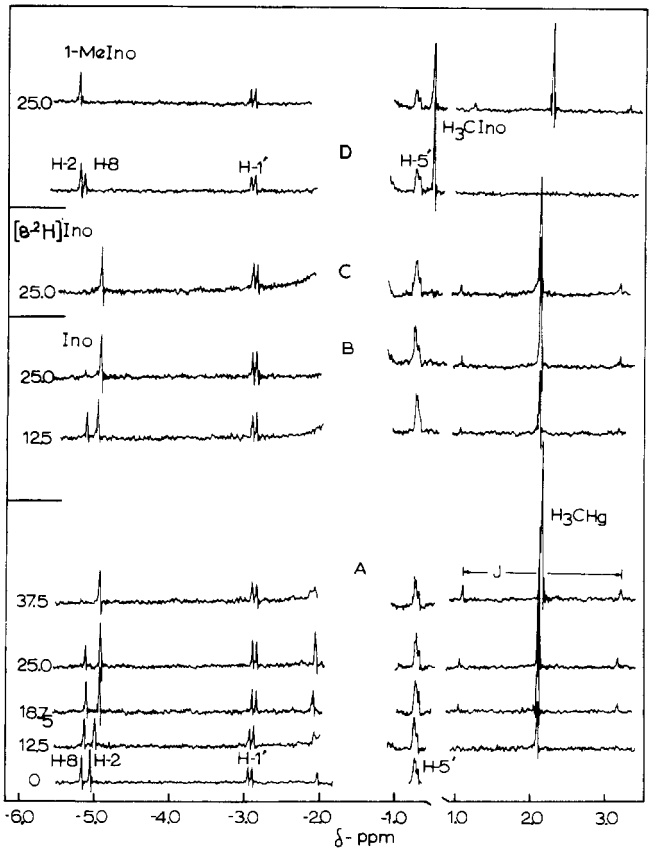


FIGURE 6: Proton magnetic resonance spectra of D_2O solutions containing inosine or 1-MeIno and CH_3Hg^{II} (pD 8.0): (A) 25 m*M* Ino + varying concentrations of CH_3Hg^{II}; (B) Ino-CH_3Hg^{II} solutions after 1 week; (C) 25 m*M* [8-^{2}H]Ino + 25 m*M* CH_3Hg^{II}; (D) 25 m*M* 1-MeIno and 25 m*M* 1-MeIno + 25 m*M* CH_3Hg^{II}.

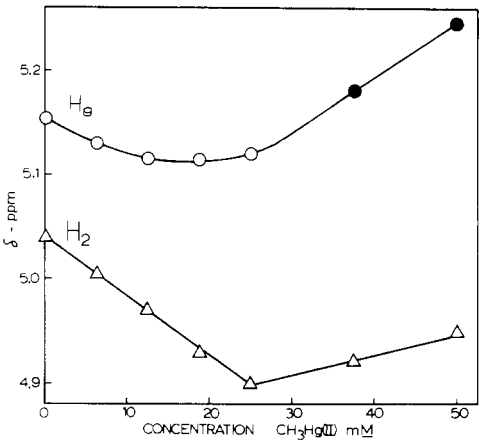


FIGURE 7: Proton magnetic resonance titration of 25 m*M* inosine with CH_3Hg^{II} in D_2O (pD 8). Chemical shifts of H(2) and H(8) relative to $N(CH_3)_4^+$. Solid points indicate stoichiometries for which complete exchange of H(8) with solvent deuterons occurs.

1967; Geier and Erni, 1973). The slight broadening of the inosine H(8) resonance probably is caused by traces of paramagnetic metal ion impurities. When the solutions containing 25 m*M* or higher CH_3Hg^{II} concentrations, stored at 25°, were examined a week later, the chemical shifts were unaltered, but the resonance assigned to H(8) had disappeared completely. This slower reaction will be considered below, but let us first examine the NMR spectral changes that accompany the fast complexation reactions.

Data for an NMR titration of inosine with CH_3Hg^{II} in D_2O (pD 8.0) corresponding to the Raman spectrophotometric titration illustrated in Figures 3 and 4 are illustrated in Figure 7. The chemical shifts of H(8) and H(2) change as the concentration of CH_3Hg^{II} increases. Initially the H(2) resonance shifts most, but at [CH_3Hg]:[Ino] ratios above 1.0, the H(8) resonance changes more. This is what would be expected for binding first at N(1) by a quantitative reaction followed by binding at a second site. The larger effect on the H(8) resonance at ratios above 1.0 suggests binding is to N(7). These reactions are fast, and the chemical shifts of the proton resonances measured beginning only a few minutes after sample preparation show no changes with time. Since the chemical shifts are only ca. 0.15 ppm for binding at an adjacent nitrogen and ca. 0.05 for binding at the more distant site, ^{1}H NMR pmr is not a very sensitive method for studying these reactions.

The solid points in Figure 7 refer to solutions for which the H(8) resonance disappears completely within a few days at 25° (pD 8.0). These chemical shifts were measured within a few minutes after preparation of the solutions. For a solution 25 m*M* in inosine and 25 m*M* in CH_3Hg^{II} (pD 8.0), exchange was essentially complete in 2 weeks at 25°. With higher [CH_3Hg^{II}]:[Ino] ratios, exchange was faster. Under the same conditions, a solution of inosine itself as well as solutions containing CH_3Hg^{II} with [CH_3Hg^{II}]:[Ino] ratios of less than 1.0 showed little exchange. Spectra in H_2O showed both signals even after prolonged periods of time indicating that a simple deuterium exchange is responsible for the disappearance of the H(8) resonance. The spectrum of a freshly prepared solution 25 m*M* in [8-^{2}H]inosine and 25 m*M* in CH_3Hg^{II} (pD 8) was identical with that of the corresponding solution prepared with inosine after the H(8) resonance had disappeared; see Figure 6. No changes in any other resonances were observed for the D_2O solutions. Clearly the binding reaction which occurs up to a [CH_3Hg^{II}]:[Ino] ratio of 1.0 has little effect on the C(8)-H exchange rate, while at higher ratios CH_3Hg^{II} catalyzes the exchange process. A similar effect is observed with Guo-5'-P solutions containing CH_3Hg^{II}.

In a separate NMR experiment, 25 m*M* CH_3HgOD was titrated with Ino in D_2O at pD 8.0. The methyl proton shift and the ^{199}Hg-proton coupling constant are illustrated in Figures 8 and 9. Because of the rapid exchange of CH_3Hg^{II}, again only a single resonance is observed. For CH_3HgOD, the chemical shift δ is 2.35 ppm relative to $N(CH_3)_4^+$ and the coupling constant $^2J(^{199}Hg-H)$ is 202 Hz, in good agreement with the values reported by Libich and Rabenstein (1973) of 2.34 ppm and 203.0 Hz, respectively. At low inosine concentrations, both [$H_3CHgInoH_{-1}$] and [$(H_3CHg)_2InoH_{-1}]^+$ will be produced, so the chemical shift and especially the coupling constant change rapidly from the values characteristic of CH_3HgOD. If a quantitative 1:1 reaction were occurring, the plots of δ and J vs. the inosine concentration would be linear. The values for the 1:1 complex [$H_3CHgInoH_{-1}$] are δ 2.15 ppm and J = 211 Hz.

The ^{1}H NMR spectrum of a 25 m*M* CH_3Hg^{II}-25 m*M* 1-MeIno solution (pD 8) also was obtained together with the spectrum of 1-methylinosine as a control. This is illustrated in Figure 6. The spectrum of the ligand has been discussed by Ts'o et al. (1969) and by Berger and Eichhorn (1971a,b). The H(2) resonance appeared to be unshifted in the solution containing CH_3Hg^{II} compared to 1-methylinosine alone. The CH_3Hg^{II} resonance shifted downfield by 0.05 ppm, and $^2J(^{199}Hg-H)$ increased to 207 Hz. These are

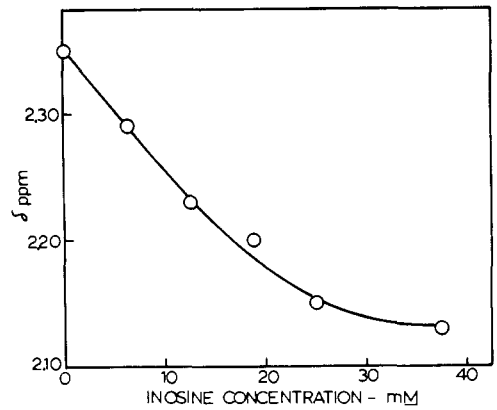


FIGURE 8: Variation of the chemical shift of the CH_3Hg^{II} protons with inosine concentration in D_2O (pD 8); $[CH_3Hg^{II}] = 25$ mM.

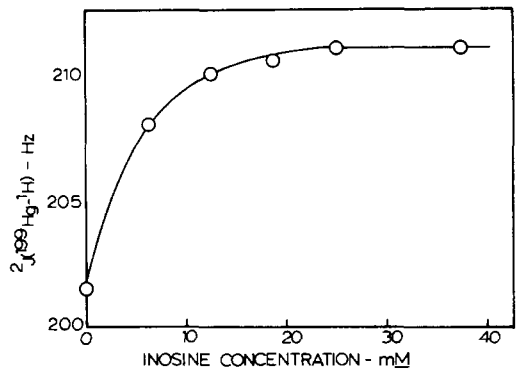


FIGURE 9: Variation of $^2J(^{199}Hg-^1H)$ with inosine concentration in D_2O (pD 8); $[CH_3Hg^{II}] = 25$ mM.

the changes expected for a system containing both CH_3HgOD and $[CH_3Hg\text{-}1\text{-}MeIno]^+$, bonded through N(7). The H(8) resonance, however, disappeared completely.

From the NMR data, it can be concluded that coordination of CH_3Hg^{II} to N(1) of inosine has little effect on the rate of exchange of either H(2) or H(8) with solvent, but coordination of CH_3Hg^{II} to the second binding site of inosine or the binding site of 1-methylinosine markedly increases the rate of exchange of H(8).

Raman Spectra of [8-^{2}H]Inosine and Its Complex with CH_3Hg^{II}. In order to determine if any of the shifts in the inosine Raman spectra for D_2O solutions upon the addition of CH_3Hg^{II}, e.g. the experiments illustrated in Figure 3, were caused by exchange of H(8), we have examined the effect of deuteration on the spectrum. The difference spectrum [8-^{2}H]inosine vs. inosine is illustrated in Figure 10 and frequency–intensity data for [8-^{2}H]inosine are tabulated in the microfilm edition (see paragraph at end of paper regarding supplementary material). The only large effect is the disappearance of the inosine band at 1507 cm^{-1} and increases in scattering at 1485 and 1546 cm^{-1} with [8-^{2}H]inosine. In studies of base stacking interactions, Medeiros and Thomas (1971) noted that heating inosine to 85° in D_2O induced exchange of H(8), and they followed the exchange using the Raman spectrum. The spectrum reported, however, only showed a small amount of exchange.

Figure 11 compares the spectra of the 1:1 CH_3Hg^{II} complexes with inosine in D_2O (measured directly following sample preparation) and with [8-^{2}H]inosine in D_2O. The shifts caused by deuteration of $[H_3CHgInoH_{-1}]$ at C(8)

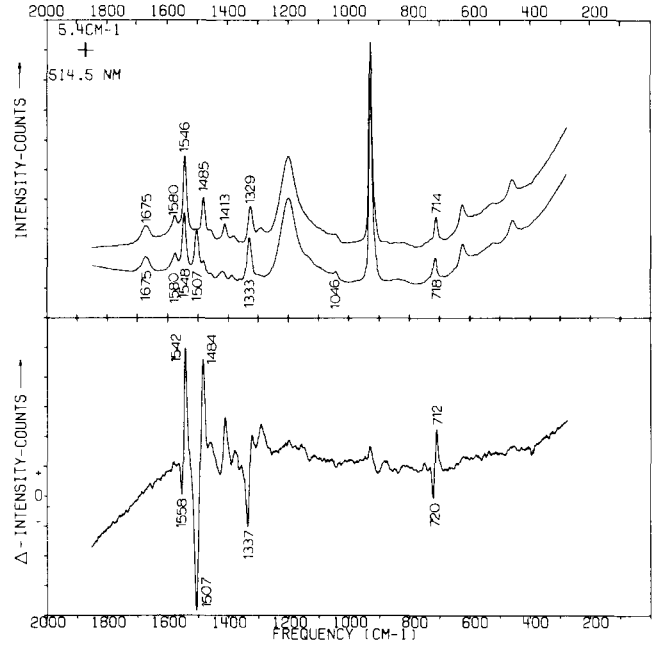


FIGURE 10: Raman difference spectra, [8-^{2}H]Ino vs. Ino in D_2O (pD 8): (top) 25 mM [8-^{2}H]Ino + 0.100 M $NaClO_4$; (middle) 25 mM Ino + 0.100 M $NaClO_4$; (botom) (A − B), ordinate expanded 2×.

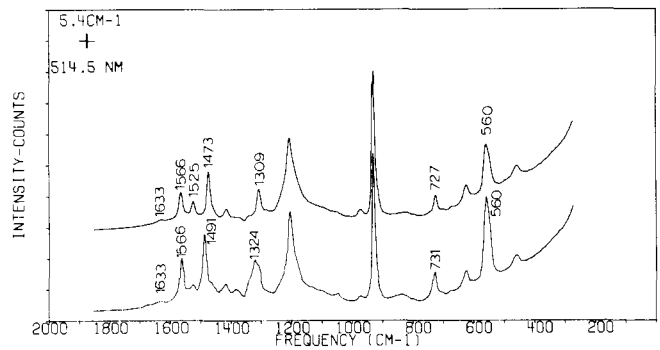


FIGURE 11: Raman spectra of 25 mM $[CH_3Hg[8\text{-}^2H]InoH_{-1}]$ (top) and of 25 mM $[CH_3HgInoH_{-1}]$ (bottom) in D_2O at pD 8. Both solutions are 0.100 M in ClO_4^-.

are very similar to those which occur when inosine itself is deuterated. Exchange was minimal within the time required to obtain the Raman spectrum as indicated by the absence of the 1473-cm^{-1} band due to coordinated [8-^{2}H]inosine in the $[CH_3HgInoH_{-1}]$ spectrum. The Raman difference spectrum of 25 mM CH_3Hg^{II} + 25 mM [8-^{2}H]Ino vs. 25 mM [8-^{2}H]Ino in D_2O (pD 8) illustrated in the microfilm edition is qualitatively very similar to the corresponding difference spectrum in Figure 2. The positive feature at 1491 shifts to 1472 and the feature at 733 shifts to 729 cm^{-1} in the 8-^{2}H-labeled complex, effects which are similar to those observed in the spectra of [8-^{2}H]inosine and inosine alone. These spectra show that the CH_3Hg^{II} cannot be bound to C(8), since there would be no deuterium isotope effect on the complex in that case.

Reaction of CH_3Hg^{II} with Inosine at Low pH. A high hydrogen ion concentration should block coordination of CH_3Hg^{II} to N(1) effectively. Figure 12 shows the Raman difference spectrum of 25 mM inosine + 25 mM CH_3Hg^{II} vs. 25 mM inosine for D_2O solutions at pD 1.6. Frequency–intensity data for the complex are tabulated in the microfilm edition. The reference solution at this pD value should consist of a mixture of Ino and $InoH^+$ according to Figure

1. Comparison with spectra for D_2O solutions, pD <1 and 7, reported by Medeiros and Thomas (1971) clearly shows this to be the case. The reference spectrum has strong bands both at 1507, 1549 (Ino), and 1564 cm^{-1} ($InoH^+$), and the relative intensities suggest that inosine predominates. The vibrations involving predominantly (C=O) stretching are observed at 1677 (Ino) and 1699 cm^{-1} ($InoH^+$). Addition of CH_3Hg^{II} causes these two bands to disappear, and a single band is observed at 1691 cm^{-1}. Clearly, this reaction does not lead to displacement of the proton bound to N(1) as occurs at pH 8. The increase in frequency of the (C=O) vibration relative to that for inosine and the small decrease relative to $InoH^+$ are consistent with coordination to N(7) with a concomitant reduction in electron delocalization compared to inosine and a slight increase in delocalization compared to $InoH^+$. The other major changes upon complex formation are disappearance of the intense bands at 1570 and 1549 cm^{-1} characteristic of inosine and the appearance of a single intense band at 1562 cm^{-1}. The resulting spectrum is quite similar to $InoH^+$, protonated on N(7). The appearance of a sharp (Hg–C) stretching band at 561 cm^{-1} which is characteristic of methylmercury bound to a nucleoside at a nitrogen donor site (Mansy et al., 1974; Mansy and Tobias, 1974a) indicates that reaction is virtually quantitative.

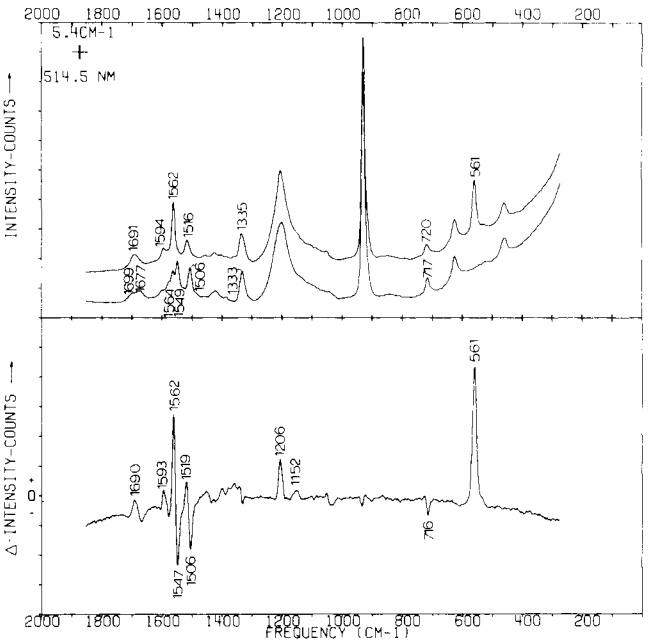
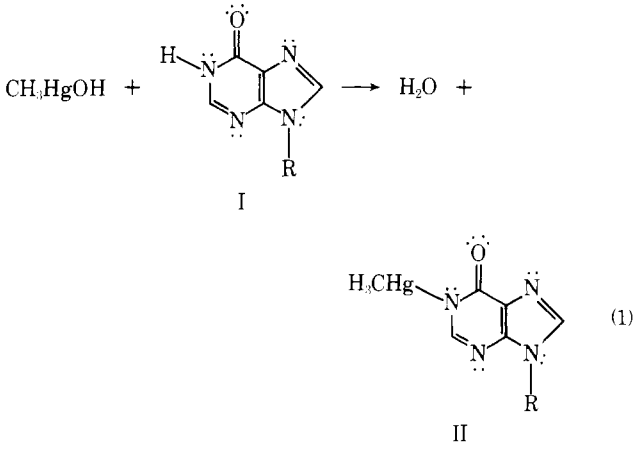


FIGURE 12: Raman difference spectra, 25 m*M* Ino + 25 m*M* CH_3Hg^{II} vs. 25 m*M* Ino in D_2O (pD 1.6): (top) parent spectra; (bottom) complex–ligand difference spectrum; ordinate expanded 1.5×.

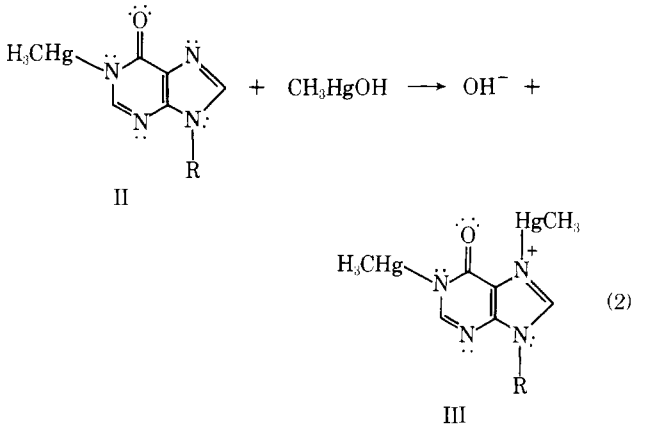
Discussion

The results of the Raman studies on inosine and 1-methylinosine may be summarized as follows. At pH 8, CH_3HgOH reacts quantitatively with inosine (I) displacing N(1)–H and binding to N(1) according to eq 1. The spectra



clearly show there is extensive delocalization of electron density into the ring system of II. The spectrum of II compared to I shows changes which are similar to those which occur when H(1) of inosine is removed at high pH. The effect is very much like that observed with uridine (Mansy et al., 1974; Mansy and Tobias, 1975), deoxythymidine (Chrisman et al., 1974), and guanosine (Mansy and Tobias, 1974a). With uridine and deoxythymidine, the CH_3Hg^{II} complexes also have spectra similar to the conjugate bases produced at high pH. LCAO-SCF calculations on the thymine conjugate base, $ThyH_{-1}^-$ (Snyder et al., 1970), indicate delocalization of ca. ¾ of an electron into the σ and π systems, and it may be reasoned that similar delocalization occurs in the CH_3Hg^{II} complex (Mansy and Tobias, 1975).

The increased electron delocalization in II compared to I would account for Simpson's (1964) observation that mercuriation at one site enhanced mercuriation at the second site. The Raman spectrophotometric titration clearly shows that mercuriation at pH 8 occurs exclusively at N(1) until this site is saturated when reaction 2 with binding at N(7) occurs, almost quantitatively. Examination of the data reported previously for GMP (Mansy and Tobias, 1974a) indiciates that the binding reactions are the same as with inosine. In the Guo-5′-P study, the second site could not be assigned with complete confidence. Apparatus modifications have improved the quality of the spectra in this work, the Raman titration procedure is more useful than the continuous variation method used previously, and the NMR data help in the assignment.



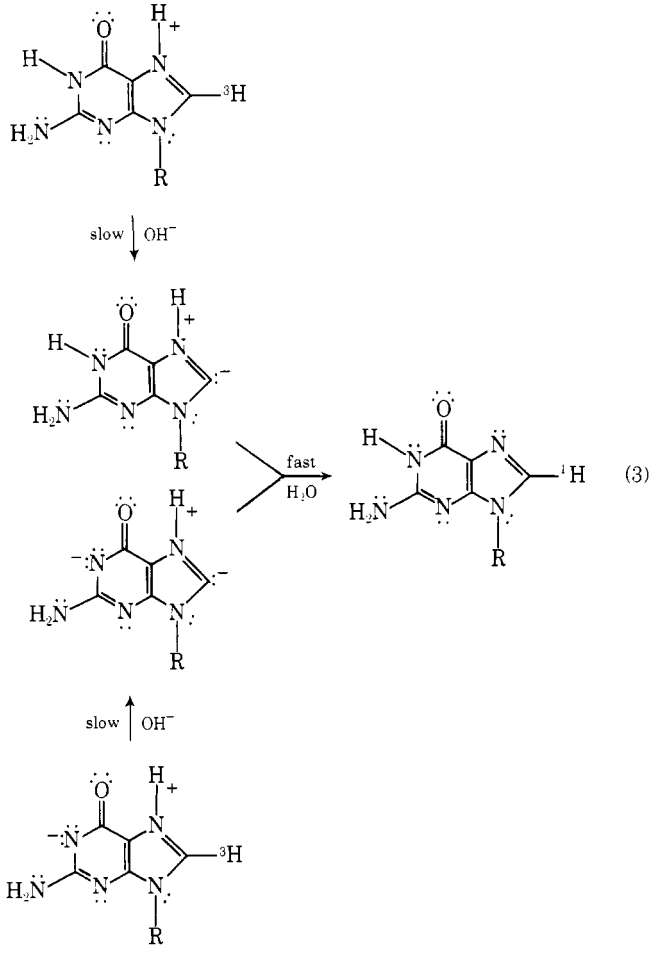
Enhancement of the basicity of a ligand for a second metal ion by coordination of the first is rare in coordination chemistry and only will occur where there is extensive electron delocalization. Ford et al. (1968) found that coordination of pentaammineruthenium(II) to pyrazine increased the proton basicity of the second nitrogen by two orders of magnitude, and Creutz and Taube (1973) found that the binuclear complex, $[((NH_3)_5Ru)_2(C_4H_4N_2)]^{4+}$ was synthesized easily. In this case, there appears to be charge transfer from the ruthenium(II) center to pyrazine, while in the CH_3Hg^{II}–inosine system the methylmercury(II) cation is just less able to localize charge than the proton.

The equilibrium constants estimated from the uv absorption spectra (Simpson, 1964) are only very approximate values because of the similarity in the spectra of the different species. For example, with the solutions giving the data in Figure 3, they lead to the prediction that only 12% of the inosine will be mercuriated in both positions, while the Raman spectra show that the reaction is nearly complete.

The increase from the highest frequency band of II with binding of the second CH_3Hg^{II} suggests a decrease in electron delocalization occurs. This electron withdrawing effect also is indicated by the increase in the rate of exchange of C(8)-H with solvent protons which occurs when N(7) is mercuriated both with inosine and Guo-5′-P. The effect is very similar to what is observed upon alkylation at N(7). For example, 7-methylinosine exchanges C(8)-H with solvent deuterons in D_2O very rapidly (Ts'o et al., 1969).

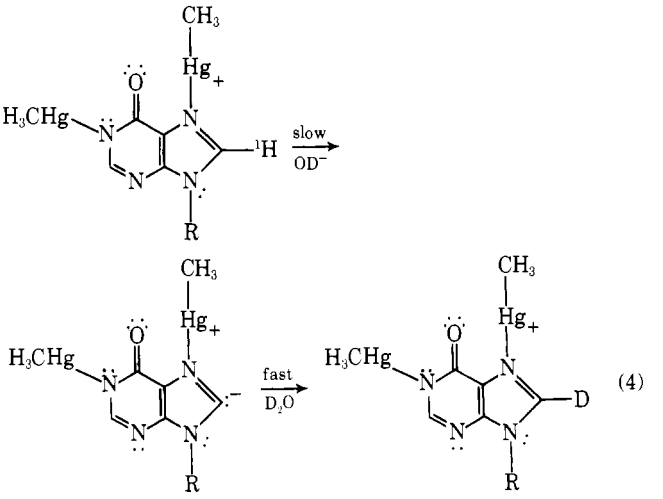
The changes in the spectra upon mercuriation appear to result largely from electronic effects, i.e. there is very little mixing of the (Hg-N) coordinate into the normal modes. This is to be expected because of "energy factoring" as a consequence of the large mass of mercury.

The isotopic exchange of C(8)-H of guanosine was studied by Tomasz et al. (1972) using tritium labeling. The mechanism suggested involved OH-catalyzed abstraction of C(8)-H from the 7-protonated form giving rise to a ylide type intermediate which is then reprotonated at C(8) by the medium. A second pathway involving participation of a new tautomeric form of guanosine, the zwitterion, also was proposed (eq 3). Coordination of CH_3Hg^+ to N(7) of GMP or



(3)

inosine then has a similar effect to protonation, and the kinetics should be first order in base (eq 4). The exchange indicates that the second binding site with both inosine and GMP must be N(7). The kinetics of these exchange processes are being investigated in more detail.

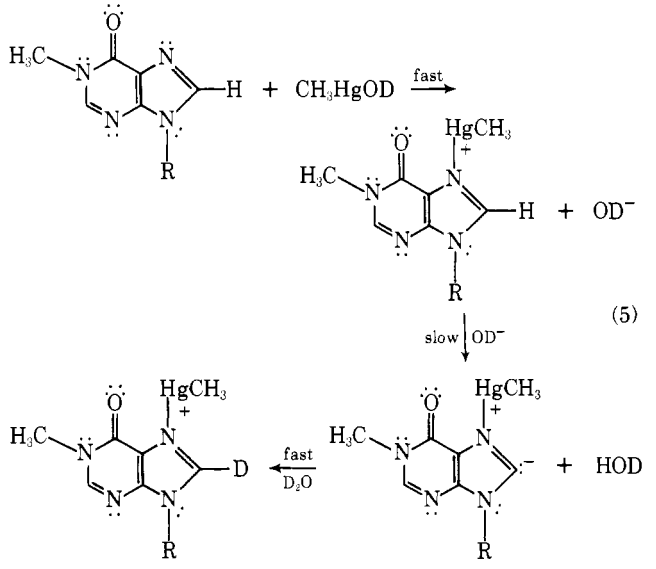


(4)

No tendency was observed for CH_3Hg^{II} to stabilize the ylide form of inosine, i.e. for III to isomerize to give C(8)- rather than N(7)-bound mercury. The high stability of mercury–carbon bonds suggests that such a reaction might occur; however, as noted by Pearson (1973), the CH_3^- ligand has a strong antisymbiotic effect, and the affinity of CH_3Hg^{II} for soft bases is considerably less than that of Hg(II).

Although the Raman spectrophotometric titration of inosine with CH_3Hg^{II} in D_2O at pD 8 was done with freshly prepared solutions, there was a question concerning the extent of exchange of H(8) and its effect on the spectra. Deuteration at C(8) was observed to cause significant changes in the Raman spectrum of inosine itself as well as in its complex with CH_3Hg^{II}. Comparison of the spectra of the complexes between inosine and [8-^{2}H]inosine with CH_3Hg^{II} in D_2O at pD 8 shows that no significant exchange occurred during the time required for Raman data collection.

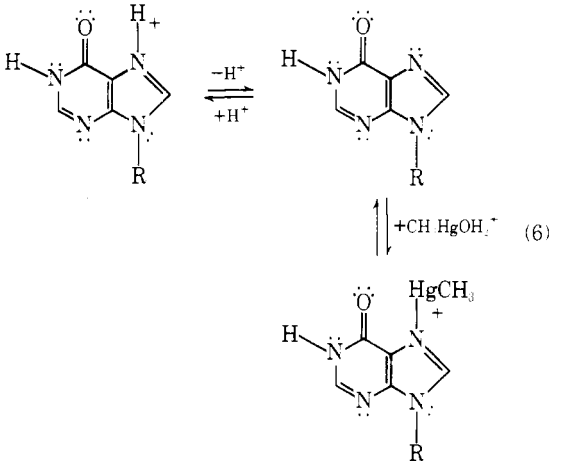
As expected, 1-methylinosine reacts very differently at pH 8 than does inosine. Binding at N(7) occurs, but the extent of reaction is very much less than is the case with $CH_3HgInoH_{-1}$. Clearly the methyl group at N(1) blocks mercuriation at that site and consequently the increased delocalization of electron density into the ring system, and 1-methylinosine is a much poorer ligand for N(7) binding



(5)

than is $CH_3HgInoH_{-1}$. At pH 8, hydroxide is able to compete very effectively for CH_3Hg^{II} with N(7) of 1-methylinosine. Coordination of CH_3Hg^{II} at N(7) again enhances the rate of exchange of H(8) of 1-methylinosine, and complete exchange was observed with the D_2O solvent (reaction 5).

At low pH, reaction of CH_3Hg^{II} with N(1) of inosine is minimized, but the nucleoside is still a good ligand for the more reactive $CH_3HgOH_2^+$, and the reaction for binding at N(7) (eq 6) is almost quantitative even at pD 1.6.



The reactions of CH_3Hg^{II} with inosine and 1-methylinosine are not unlike those found for Cu^{2+}. At pH 3, NMR line broadening experiments (Berger and Eichhorn, 1971a,b) indicated binding at N(7), while at pH 7 and above, coordination at the N(1)···O(6) site was observed. In the intermediate pH range, there appeared to be interaction of Cu^{2+} with both sites. Since these experiments were done with rather high inosine concentration (0.1 *M*) and low Cu^{2+} concentration ($\leq 10^{-4}$ *M*) only the 1:1 interaction was observable. At pD 7.8, 1-methylinosine interacted with Cu^{2+} at N(7), since N(1) is blocked.

IMP behaves somewhat differently. First row transition metal ions interact to give quite stable complexes with the phosphate moiety. At pD 7.5, Cu^{2+} broadens both H(2) and H(8) of IMP indicating that it interacts with both sites. Recent ^{13}C NMR measurements (Kotowycz and Suzuki, 1973) suggest that binding is primarily to N(7) with D_2O solutions, pD 7.4. A crystal structure has been reported for $[Zn(IMP)]\cdot H_2O$ (DeMeester et al., 1974). Each Zn(II) is bound both to the N(7) position of the base and to phosphate oxygens from three other nucleotides. The structures of $[Ni(IMP)(H_2O)_5]\cdot 2H_2O$ (Clark and Orbell, 1974) and $[Co(IMP)(H_2O)_5]\cdot 2H_2O$ (DeMeester et al., 1974) exhibit a somewhat different geometry in which the metal ion is coordinated only to N(7) of the IMP but not to the phosphate moiety. These compounds generally are crystallized from acidic aqueous solution (pH ~4), and this as well as solubility considerations probably govern the nature of the metal ligand binding in the crystals.

It is clear from the behavior of CH_3Hg^{II} with uridine, deoxythymidine, GMP, and inosine that this heavy metal binds preferentially at N(1) of the purines and N(3) of the pyrimidines substituting for a proton. With first row transition metal ions, N(7) binding seems relatively much more favorable. The only other reactions of inosine with a heavy metal which have been reported are those with Pt(II) (Kong and Theophanides, 1974). The compounds *cis*-$[Pt(NH_3)_2Ino_2]Cl_2$ and $[Pt(en)Ino_2]Cl_2$ (en = ethylenediamine), when dissolved in D_2O, gave ^1H NMR spectra showing a significant downfield shift of H(8) together with ^{195}Pt coupling to this proton, consistent with N(7) binding. We have observed similar spectra for solutions of $[Pt^{II}en(OH_2)_2]^{2+}$ and inosine at pD 7. The reason for the apparent absence of N(1) binding is not clear, particularly since hydroxide should not compete as effectively for Pt(II) as for CH_3Hg^{II}. With $[Pt(NH_3)_2(OH_2)_2]^{2+}$ the acid dissociation constants are $pK = 5.66$ and $pK_2 = 7.32$ (Sillen and Martell, 1964) compared to those with CH_3Hg^{II}, $pK_1 = 4.59$; see Table I. The conditions under which the syntheses of these substitutionally inert platinum complexes are carried out may be quite important, since considerable time is required for equilibration.

Comparison of Ultraviolet Absorption Spectrophotometry, Nmr, and Raman Difference Spectrophotometry for Studying Metal Ion Binding. The study of the shifts in electronic transitions of the base moieties upon metalation is the classical method for studying metal binding; ^1H and more recently ^{13}C NMR have been used extensively in the past 5 years, and Raman difference spectrophotometry has been used only recently. The advantage of uv absorption measurements lies in their sensitivity which permits the use of 10^{-4} to 10^{-5} *M* solutions. The main disadvantage is that metalation at different sites gives very similar shifts in the spectra, and the different bases all absorb in the same region of the spectrum. It is easy to tell when a reaction occurs but difficult to tell exactly what it is. NMR is an excellent technique with paramagnetic ions where easily measurable effects such as line broadening at low metal ion concentrations occur. It usually is possible to assign the binding site unambiguously. With diamagnetic metal ions and labile systems, the effects are small. In the case of inosine, both H(2) and H(8) shift as CH_3Hg^{II} binds at N(1), and the same is true for binding to N(7). Because the proton shifts are small, it would be difficult to use these data to assign binding sites. Raman difference spectrophotometry is very sensitive to metalation, and metalation at different sites generally gives different shifts in the spectra. Coordination by displacement of a proton from a ring nitrogen is distinguished easily from binding to a nonprotonated site. Solutions in the 5-50 m*M* range can be studied fairly easily. The interpretation of the spectral changes is less obvious than in the case of NMR, but enough data are available now to permit unequivocal assignments of binding sites in most cases.

Supplementary Material Available

Raman spectra will appear following these pages in the microfilm edition of this volume of the journal. Photocopies of the supplementary material from this paper only or microfiche (105 × 148 mm, 24× reduction, negatives) containing all of the supplementary material for the papers in this issue may be obtained from the Journals Department, American Chemical Society, 1155 16th St., N.W., Washington, D.C. 20036. Remit check or money order for $4.00 for photocopy or $2.50 for microfiche, referring to code number BIO-75-2952.

References

Amy, J., Chrisman, R. W., Lundeen, J. W., Ridley, T. Y., Sprowles, J. C., and Tobias, R. S. (1974), *Appl. Spectrosc. 28*, 262.

Anderson, J. A., Kuntz, G. P. P., Evans, H. H., and Swift, T. J. (1971), *Biochemistry 10,* 4638.
Berger, N. A., and Eichhorn, G. L. (1971a) *Biochemistry 10,* 1857.
Berger, N. A., and Eichhorn, G. L. (1971b), *J. Am. Chem. Soc.* 93, 7062.
Berger, N. A., and Eichhorn, G. L. (1972), 164th National Meeting of the American Chemical Society, New York, N.Y., Abstract INORG-76.
Broom, A. D., Schweizer, M. P., and Ts'o, P. O. P. (1967), *J. Am. Chem. Soc. 89,* 3612.
Bullock, F. J., and Jardetzky, O. (1964), *J. Org. Chem., 29,* 1988.
Chrisman, R. W., Mansy, S., and Tobias, R. S. (1974), unpublished research.
Clark, G. R., and Orbell, G. R. (1974), *J. Chem. Soc., Chem. Commun.,* 139.
Creutz, C., and Taube, H. (1973), *J. Am. Chem. Soc. 95,* 1086.
DeMeester, P., Goodgame, D. M. L., Jones, T. J., and Skapski, A. C. (1974), *Biochim. Biophys. Acta 353,* 392.
Eichhorn, G. L. (1973), in Inorganic Biochemistry, Eichhorn, G. L., Ed., New York, N.Y., Elsevier, Chapter 33.
Ford, P. C., Rodd, D. F. P., Gaunder, R. G., and Taube, H. (1968), *J. Am. Chem. Soc. 90,* 1187.
Geier, G., and Erni, I. W. (1973), *Chemia 27,* 635.
Glascoe, P. K., and Long, F. H. (1960), *J. Phys. Chem. 64,* 188.
Izatt, R. M., Christensen, J. J., and Rytting, J. H. (1971), *Chem. Rev. 71,* 439.
Kong, P-C., and Theophanides, T. (1974), *Inorg. Chem. 13,* 1167.
Kosturko, L. D., Folzer, C., and Stewart, R. F. (1974), *Biochemistry 13,* 3949.
Kotowycz, G., and Suzuki, O. (1973), *Biochemistry 12,* 5325.
Libich, S., and Rabenstein, D. L. (1973), *Anal. Chem. 45,* 118.
Lundeen, J. W. (1974), Ph.D. Thesis, Purdue University, Lafayette, Ind.
Mansy, S., and Tobias, R. S. (1974a), *J. Am. Chem. Soc. 96,* 6874.
Mansy, S., and Tobias, R. S. (1974b), *J. Chem. Soc., Chem. Commun.* 957.
Mansy, S., and Tobias, R. S. (1975), *Inorg. Chem. 14,* 287.
Mansy, S., Frick, J. P., and Tobias, R. S. (1975), *Biochim. Biophys. Acta 378,* 319.
Mansy, S., Wood, T. E., Sprowles, J. C., and Tobias, R. S. (1974), *J. Am. Chem. Soc. 96,* 1762.
Medeiros, G. C., and Thomas, G. J. (1971), *Biochim. Biophys. Acta 247,* 449.
Pearson, R. G. (1973), *Inorg. Chem. 12,* 712.
Schwarzenbach, G., and Schellenberg, M. (1965), *Helv. Chim. Acta 48,* 28.
Sillén, L. G., and Martell, AE. (1964), *Chem. Soc., Spec. Publ. No. 17.*
Simpson, R. B. (1964), *J. Am. Chem. Soc. 86,* 2959.
Simpson, R. B. (1967), *J. Chem. Phys. 46,* 4775.
Snyder, L. C., Shulman, R. G., and Neumann, D. B. (1970), *J. Chem. Phys. 53,* 256.
Tomasz, M., Olson, J., and Mercado, C. M. (1972), *Biochemistry 11,* 1235.
Ts'o, P. O. P., Kondo, N. S., Robins, R. K., and Broom, A. D. (1969), *J. Am. Chem. Soc. 91,* 5625.