existence of stable intermediate forms between native and unfolded protein molecules must be a general phenomenon. Of the proteins for which calculations have been made, only β-lactoglobulin could possibly be considered as unfolding by means of an all-or-none reaction in which intermediate states have only a transient existence. And even for β-lactoglobulin, this possibility exists only if the minimum α_i values are assigned.

It is worthwhile at this point to note that values of α_i smaller than those represented by the top lines of Tables II to IV are considered improbable when urea is the unfolding agent. Smaller values of the α_i parameters would require that the final state of unfolding attained in urea is one in which the major part of the molecule is in fact still tightly folded. This would mean, in a molecule of the size of serum albumin or fragment I of γ-globulin, that folded regions as large as the entire ribonuclease or β-lactoglobulin molecule could still exist. If this were so, then the optical rotatory properties (for example) of unfolded proteins would be expected to vary from one protein to another by as much as the similar properties of the smaller native proteins vary from each other. Moreover, inaccessible disulfide bonds or anomalous tyrosyl residues would be expected. In fact, urea-unfolded molecules have not been found to exhibit such variations, except in instances where the changes produced by urea clearly have not been completed at the highest experimentally attainable urea concentrations.

Small values of α_i would also result if most of the peptide groups and side chains of the native molecule, in its equilibrium conformation, were freely accessible to solvent. This possibility is considered highly improbable.

It should be noted in conclusion that there is evidence to suggest that guanidine resembles urea as an unfolding agent, except that it is effective at lower concentrations. Thus the theoretical treatment of this paper could be applied equally well to unfolding by guanidine, if the requisite solubility data were available. It is possible, however, that other frequently used "denaturing agents" such as acids, bases, ethanol, etc., may have a significantly different mode of action. It is quite likely that the final product obtained by reaction with these reagents is an incompletely unfolded molecule, with specific local intramolecular interactions maintained. If a reagent is to behave similarly to urea, it must solubilize both hydrophobic and peptide groups, and must also maintain a high dielectric constant so as to avoid intramolecular ion-pair formation.

[CONTRIBUTION FROM THE NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES, NATIONAL INSTITUTES OF HEALTH, PUBLIC HEALTH SERVICE, U. S. DEPARTMENT OF HEALTH, EDUCATION AND WELFARE, BETHESDA, MARYLAND]

Association Constants of Methylmercuric and Mercuric Ions with Nucleosides

BY RICHARD B. SIMPSON

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Spectra of methylmercuric hydroxide or mercuric hydroxide with nucleosides at various pH values were used to determine the sites of mercury binding and the association constants. In addition to the expected binding to the nitrogen sites having titratable hydrogens, there is binding to the primary amine nitrogens, as deduced independently by Eichhorn and Clark. Guanosine and inorganic mercury evidently polymerize slowly. The affinity of calf thymus DNA for inorganic mercury is at least an order of magnitude greater than that of the nucleosides.

In 1952 Katz[1] added mercuric chloride to a nucleic acid solution and discovered a remarkable reversible decrease in the viscosity, as well as changes in other properties, indicating some kind of configurational rearrangement. The molecular structural changes are still a subject of investigation and conjecture. At Katz's suggestion, we decided to determine the sites of binding and association constants of methylmercuric and mercuric ions with the nucleosides, since this information might prove useful in explaining the nucleic acid reaction. A study had already been made by Davidson and co-workers[2,3] at an acid pH. The present investigation covers the whole pH range.

Experimental

A 15% solution of methylmercuric hydroxide was a gift from the Morton Chemical Co. of Woodstock, Illinois. 7-Methylguanosine was a product of Cyclo Chemical Co. Spectra were measured on a Cary recording spectrophotometer with 1 mm., 1 cm., and 5 cm. cells (the shortest cells for the highest methylmercuric or mercuric concentrations and *vice versa*). Spectra of each nucleoside were recorded at several values of pH with various concentrations of methylmercuric hydroxide or mercuric nitrate. Usually, only perchloric acid or sodium hydroxide was added, but sometimes low concentrations of such weakly complexing buffers as fluoride, acetate, phosphate, or borate were used.

Although methylmercuric or mercuric compounds were added to the "blank" cells or, in some difference spectra, to one of a pair of extra cells in the light paths, the absorbance of concentrated solutions of mercury or methylmercury is so high at short wave lengths that this region of the nucleoside difference spectra is less reliable for calculations. The molar absorption coefficient (ϵ_M) of CH_3HgOH is approximately 1.8 at 250 mμ and 0.35 at 260 mμ, and ϵ_M of CH_3HgClO_4 is about twice as large as that of CH_3HgOH.

Results

Sites of Binding.—The recorded spectra, in general, represent absorption by molecules with partial mercuration at one or more sites. After some trial and error, it was found that on the basis of a hypothetical but chemically reasonable set of sites the spectra could be analyzed into component difference spectra, each of which corresponded to mercuration at one site only.

Some of the difference spectra may be identified with nitrogen sites of known pK by means of the pH dependence. The effect of pH on the mercuration at a particular site, *i.e.*, on the height of the difference spec-

(1) S. Katz, *J. Am. Chem. Soc.*, **74**, 2238 (1952).

(2) T. Yamane and N. Davidson, *ibid.*, **83**, 2599 (1961).

(3) R. Ferreira, E. Ben-Zvi, T. Yamane, J. Vasilevskis, and N. Davidson, Advances in the Chemistry of the Coordination Compounds," Macmillan Co., New York, N. Y., 1961, pp. 457–462.

trum, will depend on whether the site is protonated and whether CH_3HgOH or CH_3Hg^+ predominates in the pH range considered.

$$CH_3HgOH + NH = CH_3HgN + H_2O \quad \text{no pH dependence} \quad (1)$$

$$CH_3HgOH + N = CH_3HgN^+ + OH^- \quad \text{more mercuration as pH decreases} \quad (2)$$

$$CH_3Hg^+ + NH = CH_3HgN + H^+ \quad \text{more mercuration as pH increases} \quad (3)$$

$$CH_3Hg^+ + N = CH_3HgN^- \quad \text{no pH dependence} \quad (4)$$

Equations 1 and 4 show that the ratio of mercurated form to unmercurated form is proportional to $[CH_3HgOH]$ below the pK of the base and proportional to $[CH_3Hg^+]$ above this pK. Quantitatively

$$\frac{[CH_3HgN^+]}{[N] + [NH^+]} = K \frac{[CH_3Hg^+]}{1 + [H^+]10^{pK}} \quad (5)$$

where K is the association constant of CH_3Hg^+ with the nonprotonated base N, and 10^{pK} is the hydrogen ion association constant of the base N. If no other anions besides hydroxyl are present to complex the mercury, then substitution of the hydrogen ion association constant[4] of CH_3HgOH

$$\frac{[CH_3Hg^+][H_2O]}{[CH_3HgOH][H^+]} = 10^{4.5} \quad (6)$$

where $[H_2O]$ is taken as 1, gives

$$\frac{[CH_3HgN^+]}{[N] + [NH^+]} = K \frac{1}{1 + [H^+]10^{pK}} \frac{[H^+]10^{4.5}[\text{free } CH_3Hg]}{1 + [H^+]10^{4.5}} \quad (7)$$

where [free CH_3Hg] = $[CH_3HgOH] + (CH_3Hg^+]$ = methylmercury not combined with the base N.

In addition to the difference spectra which may be readily identified with nitrogens of known pK, high CH_3HgOH concentrations reveal changes in spectra which, for the following reasons, must be due to mercuration of the primary amines with displacement of a proton or protons.

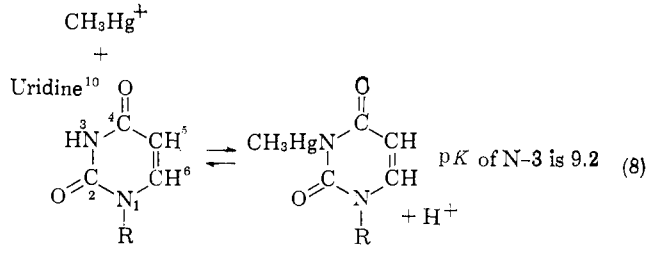
(1) Nucleosides having primary amine groups, *i.e.*, adenosine, cytidine, guanosine, and 7-methylguanosine, show the weak binding, while inosine and uridine do not.

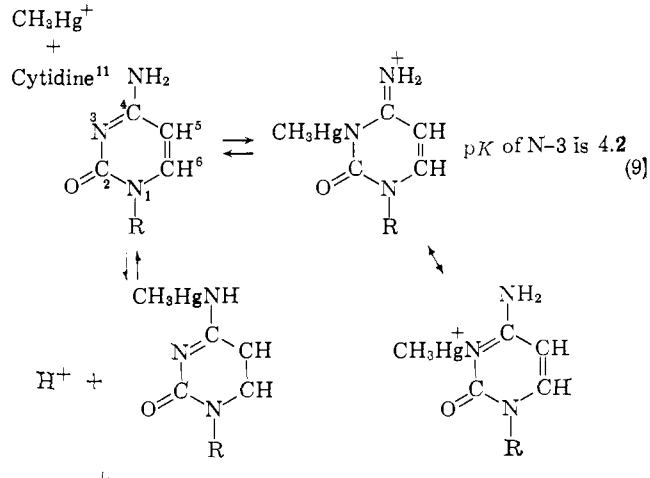
(2) This weak binding of methylmercuric ions from methylmercuric hydroxide is independent of pH above pH 5, indicating that a proton is displaced (eq. 1). This eliminates as possible sites of binding the other ring nitrogens because they are not protonated.

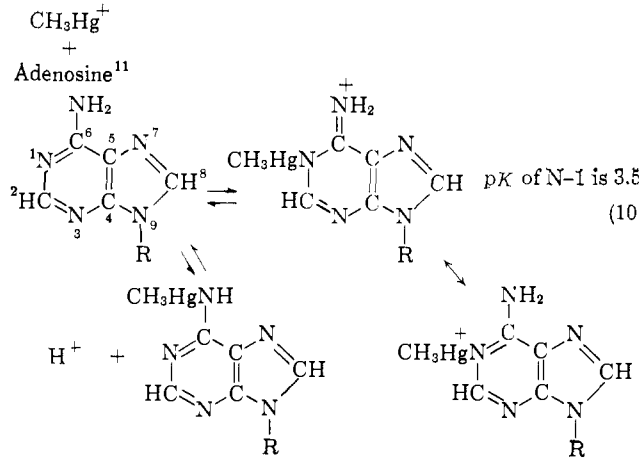
(3) Eichhorn and Clark had concluded independently from competition of formaldehyde with mercury for nucleosides[5] that the primary amines bind mercury.

(4) A similar reaction is known for gold. In the complex of ethylenediamine with gold, the gold replaces a proton of the amine.[6]

The assumed sites of binding are shown below. Since the relative affinity of mercury (compared to protons) is considerably greater for nitrogen than for oxygen,[7-9]







it seems very likely that, if protonation of a nucleoside occurs at a nitrogen rather than an oxygen, mercuration which displaces the proton will also be on the nitrogen. Therefore, the tautomers of the mercurated forms are assumed to be analogous to those of the protonated forms identified by Miles from infrared spectra.[10-13] To correlate the sites of mercury binding with the familiar hydrogen ionization constants, the pK of the hydrogen ion dissociation is given opposite the corresponding mercury reaction; $\leftrightarrow$ is used to indicate resonance between two forms.

Notation.—One system will be used in Fig. 1a–5a (molar absorption *vs.* wave length) and in expressions for concentration of a species. The first letter of the name of the nucleoside will indicate the neutral form of the nucleoside. A nucleoside with mercuration at certain sites will be denoted by the sum of the symbols for those sites, *e.g.*, (NH_2 + N-1) denotes a nucleoside mercurated at NH_2 and N-1.

(4) T. D. Waugh, R. E. Walton, and J. A. Laswick, *J. Phys. Chem.*, **59**, 395 (1955).

(5) G. Eichhorn and P. Clark, *J. Am. Chem. Soc.*, **85**, 4020 (1963).

(6) B. P. Block and J. C. Bailar, *ibid.*, **73**, 4722 (1951).

(7) N. V. Sidgwick, "The Chemical Elements and Their Compounds," Vol. I, Oxford University Press, London, 1950, p. 300.

(8) R. B. Simpson, *J. Am. Chem. Soc.*, **83**, 4711 (1961), Table III.

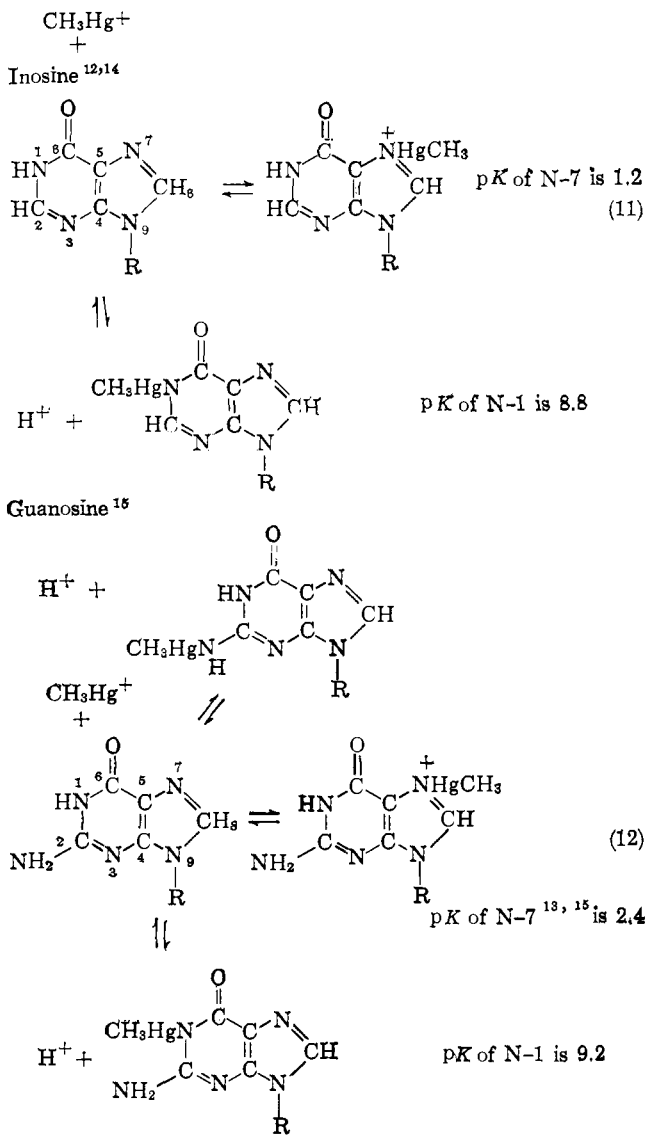
(9) E. S. Gould, "Inorganic Reactions and Structure," Rev. Ed., Holt, Rinehart and Winston, New York, N. Y., 1962, p. 203.

(10) H. T. Miles, *Biochim. Biophys. Acta*, **30**, 324 (1958).

(11) H. T. Miles, *ibid.*, **27**, 46 (1958); H. T. Miles, R. B. Bradley, and E. D. Becker, *Science*, **142**, 1569 (1963).

(12) H. T. Miles, *Proc. Natl. Acad. Sci. U. S.*, **47**, 791 (1961).

(13) H. T. Miles, F. B. Howard, and J. Frazier, *Science*, **142**, 1458 (1963).



Another system will be used to designate the process of mercuration at a particular site, as in Table I and

TABLE I

ASSOCIATION CONSTANTS OF METHYLMERCURIC IONS AND MERCURY(II) WITH NUCLEOSIDES[a]

Nucleoside	Site of mercuration	Hydrogen pK	Log K	Log K_{CH_3HgOH}	Log $K_{Hg(OH)_2}$	Exptl. pH range
U	*N-3*	9.2	9.0	4.3	5.1	6–12
C	*N-3*	4.2	4.6	9.1*	9.5*	2–4
C	NH_2			0.8		10–11
C	NH_2(*N-3*)			1.7	3.6	2–6
A	*N-1*	3.5	~3.0	~7.5*	~8.7*	2–4
A	NH_2			1.1		10–11
A	NH_2(*N-1*)			1.9	~2.8	2–6
I	*N-7*	~1.2	3.7	8.2*		2–4
I	*N-1*	8.8	8.2	3.7	4.3	7–9
I	*N-7*(*N-1*)	~1.2	4.3	8.8*	7.9*	5–9
I	*N-1*(*N-7*)	8.8	8.8	4.5		2–6
G	*N-7*[b]	~2.4	4.5	~9.0*	~9.0*	2–4
G	*N-1*	9.2	~8.1	~3.4	~4.0	9–12
G	NH_2(*N-7, N-1*)			~0.8		5–8
7MG[c]	*N-1*			3.9	4.1	3–6
7MG	NH_2(*N-1*)			0.9		4–6

[a] No corrections were made for activity coefficients. [b] The constants for *N-7*(*N-1*) and *N-1*(*N-7*) of guanosine are nearly the same as those for *N-7* and *N-1*, respectively. [c] 7MG denotes 7-methylguanosine.

(14) The low pK site of protonation has not been proven to be N-7, but has been assumed to be there by analogy with guanosine.

(15) C. D. Jardetzky and O. Jardetzky, *J. Am. Chem. Soc.*, **82**, 222 (1960).

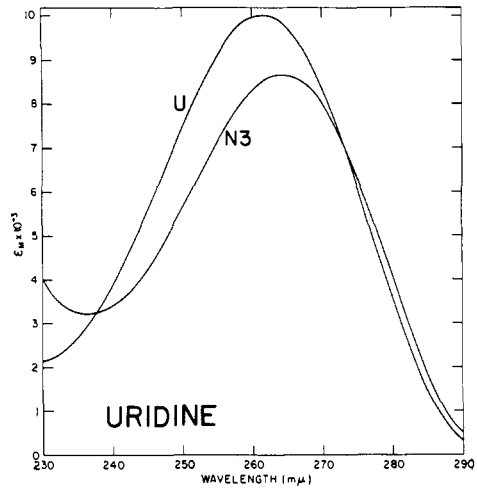


Fig. 1a.—U, uridine at pH 7; N-3, uridine in 0.044 *M* CH_3HgOH at pH 8.

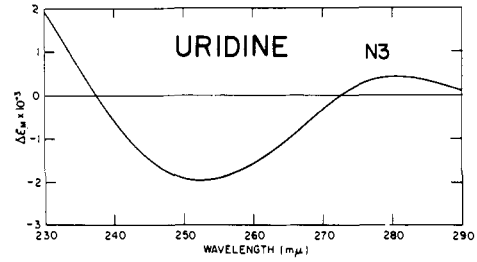


Fig. 1b.—*N-3*, mercuration at N-3.

the component difference spectra (Fig. 1b–5b, changes in molar absorption coefficient *vs.* wave length on complete methylmercuration at a site, unless otherwise indicated). The site of mercuration under consideration is given first, followed (if necessary) by parentheses containing the other sites already mercurated. Thus NH_2(*N-1*) denotes the process of mercuration at NH_2 when N-1 is already mercurated.

Definition of Constants in Table I.—*K* is the same as in eq. 5 and 7, *i.e.*, the association constant of CH_3Hg^+ with the nonprotonated nitrogen site.

A more useful constant at pH 5 to 9 is K_{CH_3HgOH}, the constant for an equilibrium among species predominant in neutral solution

$$K_{CH_3HgOH}(\text{unstarred}) = \frac{[\text{mercurated form}][H_2O]}{[\text{unmercurated form}][CH_3HgOH]} \quad (13)$$

if the nitrogen site is protonated in neutral solution, and

$$K_{CH_3HgOH}(\text{starred}) = \frac{[\text{mercurated form}][H_2O]}{[\text{unmercurated form}][CH_3HgOH][H^+]} \quad (14)$$

if the nitrogen site is nonprotonated in neutral solution. *E.g.*, for NH_2(*N-1*) of adenosine, the constant for reaction of CH_3HgOH with the primary amine when N-1 is already mercurated is

$$K_{CH_3HgOH} = \frac{[(NH_2 + \text{N-1})^+][H_2O]}{[\text{N-1}^+][CH_3HgOH]} \quad (15)$$

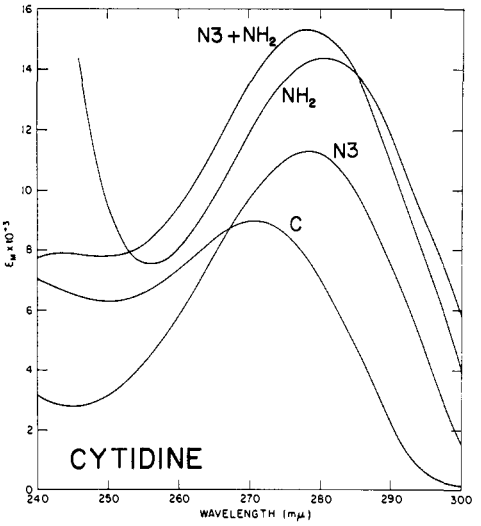


Fig. 2a.—C, cytidine at pH 7; N-3, cytidine in 0.0075 *M* methylmercury at pH 2.55 (approx. 87% mercuration at N-3 and 0.5% at amine); NH_2, cytidine in 0.88 *M* CH_3HgOH at pH 11.5 (approx. 86% mercuration at amine); (N-3 + NH_2), cytidine in 0.88 *M* CH_3HgOH at pH 6.

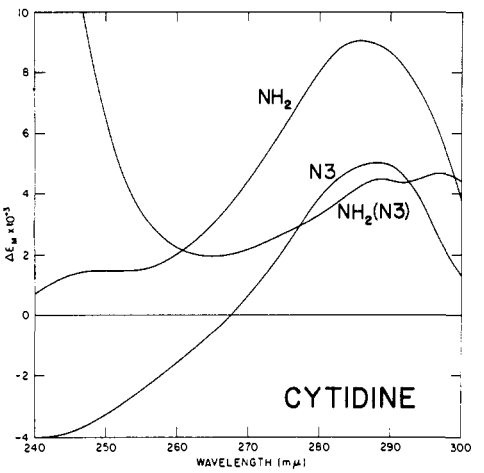


Fig. 2b.—*N-3*, mercuration at N-3 of cytidine; *NH_2*, approx. 86% mercuration at amine; *NH_2(N-3)*, mercuration at amine when N-3 is already mercurated.

The definitions of $K_{Hg(OH)_2}$ are similar, with [$Hg(OH)_2$] in place of [CH_3HgOH]. In the complex, one hydroxyl is assumed to be still attached to the mercury. [H_2O] is taken as 1, and the mercury or methylmercury is assumed to replace only one proton of the amine.

Spectra and Association Constants.—In Fig. 1a–5a are examples of recorded spectra with methylmercury. For these examples we have chosen spectra which we believe represent almost exclusively a single species, *i.e.*, in which mercuration at any site is either nearly complete or nearly zero.

The method of obtaining the constants in Table I and the difference spectra in Fig. 1b–5b may be illustrated with adenosine. A series of spectra with varying concentrations of CH_3HgOH are taken near pH 11, where very little mercuration at N-1 ($pK = 3.5$) would be expected. The change in absorption at the wave length of maximum change (282 mμ) is plotted against log [CH_3HgOH] and a sigmoid titration curve is fitted to the points. At the midpoint of this curve [NH_2] = [A], so that K_{CH_3HgOH} is equal to the reciprocal of the CH_3HgOH concentration (eq. 13).

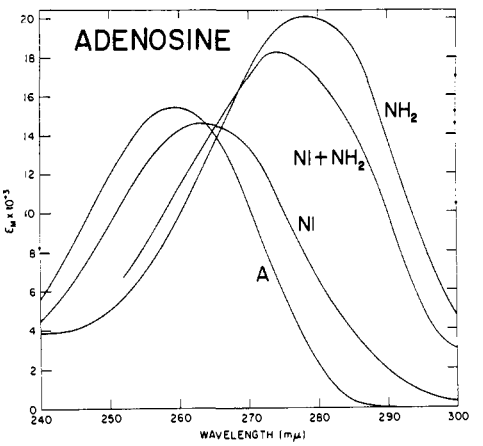


Fig. 3a.—A, adenosine at pH 7; N-1, adenosine in 0.044 *M* methylmercury at pH 2.7 (approx. 85% mercuration at N-1 and 21% at amine); NH_2, adenosine in 0.88 *M* CH_3HgOH at pH 11 (approx. 92% mercuration at amine); (N-1 + NH_2), adenosine in 0.88 *M* CH_3HgOH at pH 5.5.

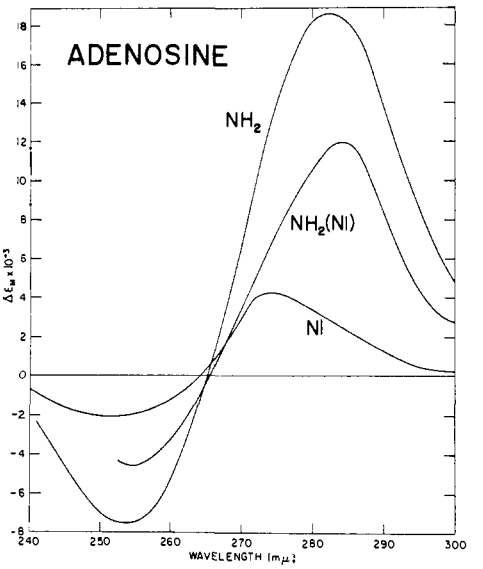


Fig. 3b.—*N-1*, mercuration at N-1 of adenosine; *NH_2*, approx. 92% mercuration at amine; *NH_2(N-1)*, mercuration at amine when N-1 is already mercurated.

Then in a series of spectra at low pH (2 to 6) it is noted that around pH 4 to 5.5 with high methylmercury concentrations the maximum change in absorption with change in [CH_3HgOH] is at 284 mμ. Since N-1 already is largely mercurated under these latter conditions, $\Delta\epsilon_{284}$ is tentatively taken as a measure of *NH_2(N-1)* and a plot of $\Delta\epsilon_{284}$ against log [CH_3HgOH] allows calculation of a tentative value of the constant for *NH_2(N-1)*.

With low methylmercury concentrations at pH 3, the greatest change (after appropriate corrections for the spectrum of the acid form of adenosine) from the spectrum of neutral adenosine is at 272 mμ. Since [CH_3HgOH] and therefore mercuration at NH_2 are small under these conditions, $\Delta\epsilon_{272}$ is used for measuring mercuration at N-1. $\Delta\epsilon_{272}$ will, however, contain a contribution from *NH_2(N-1)*. This contribution is the product of the experimentally measured $\Delta\epsilon_{284}$ and the ratio $\Delta\epsilon_{272}/\Delta\epsilon_{284}$ for *NH_2(N-1)*. The ratio is obtained from the difference spectra at pH 4 to 6 which have the smallest value of this ratio. After using the corrected $\Delta\epsilon_{272}$ to obtain a value of the con-

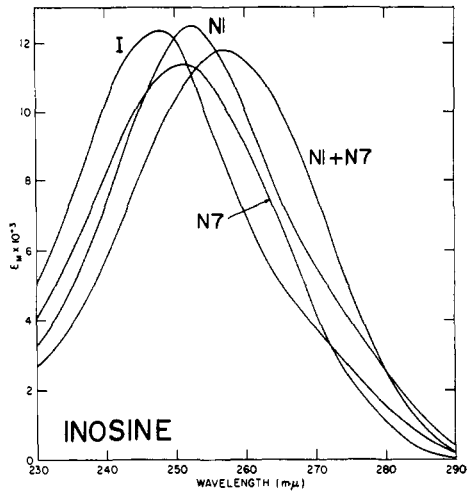


Fig. 4a.—I, inosine at pH 7; N-7, inosine in 0.012 *M* methylmercury at pH 0.7; N-1, inosine in 0.0087 *M* CH_3HgOH at pH 7.6; (N-1 + N-7), inosine in 0.0086 *M* methylmercury at pH 4.3.

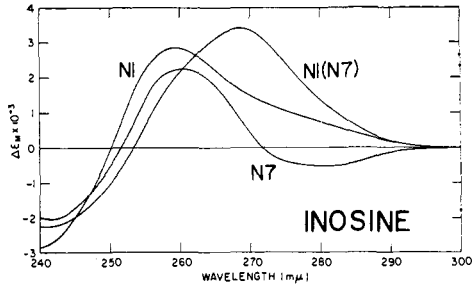


Fig. 4b.—*N-7*, mercuration at N-7 of inosine; *N-1*, mercuration at N-1; *N-1(N-7)*, mercuration at N-1 when N-7 is already mercurated.

stant for mercuration at N-1, we have enough data to calculate the contribution of *N-1* to $\Delta\epsilon_{284}$ (which was initially assumed to be entirely due to $NH_2(N\text{-}1)$). The correction process is repeated until a set of difference spectra and association constants are obtained which fit fairly well all the recorded spectra.

In Fig. 1b to 5b are the difference spectra for complete methylmercuration at a single site, except that, for the primary amines in basic solution, the largest difference spectrum recorded is given, rather than a calculated difference spectrum to be expected on complete mercuration.

It should be noted that all the difference spectra of the singly mercurated species are with respect to the neutral nucleoside.

$NH_2(N\text{-}1)$ of Guanosine.—The difference spectrum and the association constant for $NH_2(N\text{-}1)$ of guanosine are difficult to obtain because of the weak binding. The difference spectrum is very similar in shape to that of *N-1*. If it is assumed that $K_{CH_3HgOH} = 10^{0.5}$ for $NH_2(N\text{-}1)$, a value about half that for $NH_2(N\text{-}1, N\text{-}7)$, then for complete mercuration of $NH_2(N\text{-}1)$, $\Delta\epsilon_M \times 10^{-3} \cong 7$ at the 290 mμ peak. That is, the difference spectrum for $NH_2(N\text{-}1)$ would be about five and a half times that for *N-1*.

7-Methylguanosine, with or without mercury, shows spectral changes in alkaline solution due to decomposition,[16] so binding experiments were confined to a pH range below 6.5.

(16) L. B. Townsend and R. K. Robins, *J. Am. Chem. Soc.*, **85**, 242 (1963).

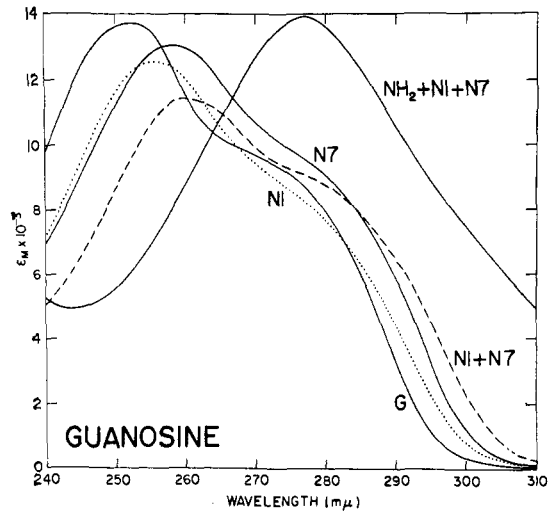


Fig. 5a.—G, guanosine at pH 7; N-7, guanosine in 0.0072 *M* methylmercury at pH 2.4 (approx. 93% mercuration at N-7 and 10% mercuration at N-1); N-1, guanosine in 0.0072 *M* CH_3HgOH at pH 8.6 (approx. 90% mercuration at N-1 and 4% mercuration at NH_2); N-1 + N-7, guanosine in 0.0058 *M* CH_3HgOH at pH 6.0 (approx. 90% mercuration at both N-1 and N-7 and 3% mercuration at NH_2); NH_2 + N-1 + N-7, guanosine in 0.88 *M* CH_3HgOH at pH 8 (approx. 84% mercuration at amine and complete mercuration at N-1 and N-7).

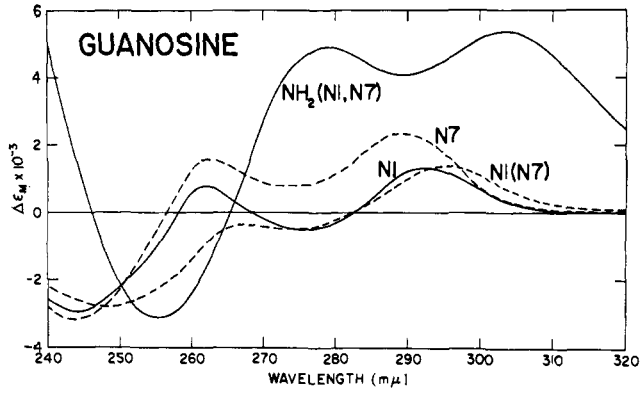


Fig. 5b.—*N-7*, mercuration at N-7 of gunaosine; *N-1*, mercuration at N-1; *N-1(N-7)*, mercuration at N-1 when N-7 is already mercurated; $NH_2(N\text{-}1, N\text{-}7)$, approx. 84% mercuration at NH_2 when N-1 and N-7 are already mercurated.

Methylmercury.—In general, except for the primary amines (see Discussion), association constants calculated at two wave lengths agreed closely. The precision of measurement of the constants is about 0.1 in log units except for *N-1* of adenosine and the constants for guanosine which are less precise.

It may be noted that in compounds with multiple equilibria, not all of the constants are independent. For cytidine and adenosine, the constant not determined experimentally may be calculated from the other three. For the N-1 and N-7 sites of inosine and guanosine, the four constants were determined and agreed fairly well. For these sites, the values given in Table I are chosen from those within the range of experimental error to satisfy the criterion of interdependence.

Similarly, the difference spectra of the sets mentioned above are interdependent, so that a fourth spectrum may be calculated from three of a set in each of Fig. 2b–5b.

Figure 6 shows the effect of pH on the mercuration, as calculated from the constants in Table I. It should

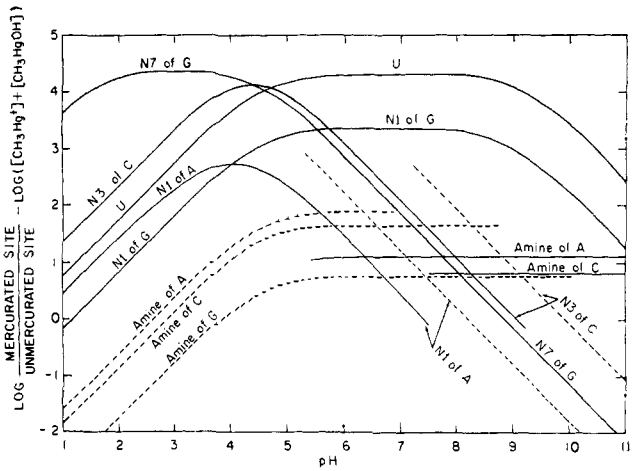


Fig. 6.—Effect of pH on mercuration with CH_3Hg. Solid lines refer to mercuration at the site indicated when no other site is mercurated. The dashed lines refer to mercuration at the site indicated when another site is mercurated.

be emphasized that if anions are present which complex mercury strongly, then $[CH_3Hg^+] + [CH_3HgOH]$ will, at least in the acid region, be much less than the total concentration of methylmercury.

Inorganic Mercury.—The solubility of mercuric oxide is too small to permit complete mercuration of the primary amines and good determinations of these association constants, but all the nucleosides with primary amines showed spectral changes attributable to mercuration there. For all sites, the data obtainable indicate that the difference spectra on complete mercuration with Hg^{+2} are about the same as with CH_3Hg^+. With uridine, which may be completely mercurated, these spectra are practically identical. With other nucleosides the spectra are nearly consistent with this hypothesis.

For uridine, our results agree very well with those of Ferreira, *et al.*,[3] for thymidine; the first association constant of Hg^{+2} with U or T is about $10^{1.5}$ times that of CH_3Hg^+ with U or T.

$$Hg^{+2} + T \longrightarrow HgT^+ + H^+ \quad K = 10^{1.3} \qquad (16)$$

where HgT^+ denotes the complex of Hg^{+2} with the thymidine anion. In chloride solutions near neutral pH it was possible to measure

$$HgCl_2 + U \longrightarrow ClHgU + H^+ + Cl^- \quad K = 10^{-5.4} \qquad (17)$$

where ClHgU represents the complex with the uridine anion.

An experiment was performed to decide between opposite assumptions in the literature[17,18] as to whether the spectrum of HgU_2 should differ from that of 2UHgOH. The spectrum of uridine with just enough mercury to form HgU_2 was found to be the same as that of uridine in a large excess of mercuric hydroxide (except for the small absorption of mercuric hydroxide) indicating that the reaction

$$HgU_2 + Hg(OH)_2 \longrightarrow 2UHgOH$$

results in no appreciable spectral change.

For the other nucleosides, the determination of the association constants is complicated by the possibility that the nucleoside with two sites mercurated may have two mercury atoms (like the methylmercury complexes) or just one mercury atom per nucleoside (if there is chelation or bridging between nucleosides).

At pH values below neutrality, the cytidine difference spectra show a peak at 290 mμ in low mercuric hydroxide concentrations and a peak at 300 mμ in high concentrations. The 300 mμ peak appears to be sharper than that in Fig. 3b. The occurrence of two peaks in different regions of mercury concentration shows that the N-3 form as well as the N-3 + NH_2 form exists, *i.e.*, that the mercurated cytidine cannot be all chelated or bridged.

The adenosine difference spectra with mercury are very similar to those with methylmercury, and are also incompatible with complete chelation or bridging. If it is assumed that the difference spectra of the completely mercurated forms have the same extinction coefficient as those for methylmercury, there can be very little chelation. The constants in Table I were calculated on the assumption of no chelation or preferential bridging.

In the case of inosine, the *N-1* difference spectrum with inorganic mercury is only about 0.6 the height of that with methylmercury, but the *N-7(N-1)* difference spectra for these two kinds of mercury are nearly identical.

Guanosine is unique among the nucleosides studied in showing a change in spectrum with time after adding inorganic mercury. On adding mercuric hydroxide to a neutral solution of guanosine and comparing it with guanosine alone, the difference spectrum shows a maximum at 297 mμ and a minimum at 250 mμ, and the differences increase with time. Although the rates and relative changes at 297 and 250 mμ were not very reproducible, the following characteristics were noted (in studies with 5×10^{-4} Hg).

There was a very rapid change at first followed by a gradually decreasing rate of change. With 10^{-4} *M* guanosine at pH 8.5, the decrease at 250 mμ was virtually complete in a couple of days, with a half-time of much less than a day, whereas the absorption at 297 mμ showed a further slow increase for weeks. With 2×10^{-5} *M* guanosine, the half-time for the decrease at 250 mμ was about a week.

Even after many days the spectrum could be immediately reversed to that of guanosine by the addition of 5 mmoles of cyanide per liter.

The rate of change was slower at 40° and faster at 15° than at room temperature.

The rate of change was several times faster at pH 8.5 than at pH 4.5.

On cooling to 0° a solution of 10^{-4} *M* guanosine and 5×10^{-4} *M* mercuric hydroxide in pH 8.5 borate buffer, a precipitate appeared, while a solution identical except for the absence of guanosine remained clear. With 10^{-3} *M* guanosine and 10^{-3} *M* mercuric hydroxide, a precipitate formed at room temperature, and removal of the precipitate removed most of the ultraviolet absorbing material from solution.

To avoid complications due to the time dependent reactions, spectra of low concentrations of guanosine (2×10^{-5} *M*) recorded soon after adding mercuric hydroxide were used to determine the association constants in Table I. Under these conditions the spectra were similar to those of the corresponding methylmercuric complexes.

(17) T. Yamane and N. Davidson, *Biochim. Biophys. Acta*, **55**, 780 (1962).
(18) Y. Kawade, *Biochem. Biophys. Res. Commun.*, **10**, 204 (1963).

The constants for mercury generally are less reliable than those for methylmercury for several reasons.

(1) Because of the higher values of the association constants, lower mercury concentrations are used in the experiments, so that the corrections for nucleoside-bound mercury are a larger fraction of the total mercury.

(2) Replacement of hydroxyl by nucleoside on one valence of mercury may change the competition between hydroxyl and nucleoside at the other valence.

(3) The insolubility of mercuric oxide limits the range of mercuric hydroxide concentrations that may be used.

Discussion

Comparison of columns 3 and 4 in Table I shows that, in general, the association constants determined here do not deviate too far from what we liked to think of as a general rule—that the affinity of methylmercury is about the same as the affinity of a proton for a nitrogen site. One may calculate from column 5, however, that for the primary amine nitrogens of the nucleosides, the affinity of methylmercuric ion is from $10^{-2.6}$–$10^{-3.7}$ of the affinity of a proton. Comparison of columns 5 and 6 shows that the constants for inorganic mercury are higher than for methylmercury, with the striking exception of the *N*-7 site of inosine, and perhaps of guanosine.

It seems somewhat strange that the presence of mercury nearby should increase the affinity of mercury (compared to the affinity of a proton) for the amine nitrogen of adenosine or cytidine by a factor of nearly ten, and even stranger that a distant mercury should enhance mercuration at the other site in inosine. Nevertheless, we believe the phenomena are real, for the differences in association constants are considerably larger than our precision.

From our data we cannot make a decision as to whether methylmercury replaces one or both protons of the primary amines. A titration curve in which the reaction with a second proton differs from that with the first only by a statistical factor, *i.e.*, $K_2 = K_1/4$, fits our data about as well as the curve for a single reaction. In a series of difference spectra for mercuration at an amine, the wave length of the maximum remains the same, but the shape of the curve does change somewhat. *E.g.*, with adenosine at high pH, the ratio of $\Delta\epsilon$ at 292 mμ to $\Delta\epsilon$ at 272 mμ changes from 0.83 at 0.065 *M* CH_3HgOH to 0.85 at 0.11 *M* CH_3HgOH and to 1.14 at 0.88 *M* CH_3HgOH. We do not believe this is conclusive evidence for two reactions, however, for the change is not very large except at high CH_3HgOH concentrations where it might be due to solvent effects, inasmuch as 0.88 *M* CH_3HgOH is 12% CH_3HgOH.

The time dependent spectral changes of guanosine on the addition of inorganic mercury are almost certainly due to polymerization, probably involving hydrogen bonds as well as mercuric linkages. The hydrogen bonded polymerization of guanylic acid[19] is favored by lower temperatures, so the negative temperature coefficient of the rate of polymerization of guanosine with mercury suggests hydrogen bonding. From the facts that the spectrum of inosine plus inorganic mercury does not change with time, and that, at the mercury concentrations which cause the polymerization of guanosine, very little mercuration of the primary amine group would be expected, one may surmise that the primary amine is involved in hydrogen bonding in the polymerization.

Polynucleotides.—It was hoped that the difference spectra of polynucleotides with mercury could be explained as a sum of the difference spectra of an equivalent mixture of the component nucleosides, plus a contribution from a structural change in the polynucleotide, *i.e.*, loss of hypochromism. This hope seems unattainable for two reasons.

(1) With one mercury for two bases at pH 5.6, 4×10^{-3} *M* chloride[20] is required to remove half the mercury from calf thymus DNA, whereas only 4×10^{-4} *M* chloride will remove half the mercury from uridine or thymidine under the same conditions (eq. 17). Since the other nucleosides at pH 5.6 have even less affinity for mercury, it is evident that the structure and charge of DNA account for a greater than tenfold increase in the affinity and could therefore affect the relative mercuration at various sites.

(2) With a slight excess of mercuric hydroxide at neutral pH and room temperature the difference spectrum[17,18] of polyU is about one and a half times that of an equivalent amount of mercurated uridine (Fig. 1a and 1b).

Nevertheless some observations on the literature may be made. Our constants for the nucleosides are consistent with the deduction made by Katz[21] from Yamane and Davidson's hydrogen ion release experiments[2] that at pH 5.6 uridine or thymidine has a greater effective affinity for mercury than any other site on the polynucleotides.

On the other hand, the mercuration of the primary amines compared to mercuration at other sites appears to be greater in the polynucleotides than in the nucleosides. Yamane and Davidson's hydrogen ion release experiments with polynucleotides show that well over[2] one hydrogen is released when one mercury is added per base pair. Our eq. 8–12 show that at neutral pH mercuration of sites other than the primary amines cannot release more than one proton per base pair, *i.e.*, one from U or T and one from G. This indicates that there is considerable mercuration at the primary amines of the polynucleotides despite the low values of the association constants found for these sites in the nucleosides.

(19) M. Gellert, M. N. Lipsett, and D. R. Davies, *Proc. Natl. Acad. Sci. U. S.*, **48**, 2013 (1962).
(20) Ref. 2, Fig. 5.
(21) S. Katz, *Nature*, **194**, 569 (1962).