num hydride in 150 ml. of ether was added dropwise during 10 min. with stirring a solution of 4.7 g. of IV, m.p. 172–175°, in 90 ml. of dry benzene. An insoluble white precipitate formed immediately. The mixture was refluxed for 30 min., then carefully treated with water. Isolation in the usual way afforded 4.2 g. (89%) of crude diol V, m.p. 159–183°, and 0.3 g. (7%) of diol, m.p. 150–182°, in two crops from benzene. The crude diol (undoubtedly a mixture of isomers) was used directly for dehydration and aromatization as described below. There was no carbonyl peak in the infrared.

A dicarboethoxy derivative was prepared by treating a solution of 2.0 g. of crude diol, m.p. 159–183°, in 40 ml. of dry pyridine at 0° with 6 ml. of ethyl chloroformate (Eastman White Label). After standing at room temperature for 5 hr. and refluxing for 1 hr., the mixture was poured onto ice. The yellow solid which formed was collected after standing at 0° for 12 hr. and recrystallized from ethanol to yield 1.48 g. (48%) of almost colorless solid. Two recrystallizations from ethanol afforded dicarboethoxy derivative of V as colorless needles, m.p. 147–148° (5.8 μ).

Anal. Calcd. for $C_{25}H_{27}FO_6$: C, 67.9; H, 6.2; F, 4.3. Found[8]: C, 67.5; H, 6.3; F, 4.3.

1-Fluoro-12-methylbenzo[*c*]phenanthrene (VI).—A solution of 4.25 g. of diol V, m.p. 152–178°, 13 ml. of xylene, and 18 mg. of iodine was heated to reflux. Within a few minutes the iodine color disappeared and a pale yellow color was noted. After 24 hr., 8 mg. of iodine was added. The iodine color again rapidly disappeared. After a total of 100 hr. of reflux the yellow solution was cooled, diluted with ether, washed with sodium bisulfite solution, and worked up as usual to yield a brown gummy residue. Chromatography over alumina yielded 1.55 g. of a colorless oil (ultraviolet max. at 289 mμ, ϵ 47,800) which was rich in VI. Crystallization from absolute ethanol (saturated with nitrogen) yielded 1.4 g. (39%) of colorless VI, m.p. 62–64°. Recrystallization from alcohol yielded pure VI, m.p. 66–67°, with little loss. In other runs similar or lower yields were obtained. Crystallization to yield sharp melting VI was frequently difficult.

Anal. Calcd. for $C_{19}H_{13}F$: C, 87.7; H, 5.0; F, 7.3. Found[8]: C, 87.5; H, 5.0; F, 7.4.

The dark red 2,4,5,7-tetranitrofluorenone[10] complex, m.p. 200–201°, was prepared in glacial acetic acid.

Anal. Calcd. for $C_{32}H_{17}FN_4O_9$: C, 61.9; H, 2.7; F, 3.1; N, 9.0. Found[8]: C, 61.8; H, 2.8; F, 3.1; N, 8.9.

Resolution of VI.—Finding the right conditions for crystallization of a complex of VI with optically active α-(2,4,5,7-tetranitro-9-fluorenylideneaminooxy)propionic acid[10] (TAPA) was difficult. Attempted repetitions of successful experiments often failed for unknown reasons. It was found advantageous to saturate all solvents with nitrogen. A solution of 2.0 g. of VI and 1.5 g. of (−)-TAPA in 15 ml. of chloroform was concentrated to 10 ml. and allowed to stand overnight at room temperature. Filtration afforded 1.6 g. of red solid. This was dissolved in ether–benzene and extracted with aqueous sodium bicarbonate. The organic layer was concentrated to yield 0.63 g. of pale red oil. This was chromatographed over alumina using 4:1 Skellysolve B–chloroform. The colorless oil thus obtained (0.58 g.) had $[\alpha]^{21}D$ −323° in chloroform (*c* 0.016 g./ml.). A second similar treatment with (−)-TAPA afforded crystals, m.p. 64–67°, $[\alpha]^{21}D$ −510°. Recrystallization from acetic acid then afforded 0.12 g. of VI, m.p. 66–67°, $[\alpha]^{20}D$ −580 ± 15° in chloroform, *c* 0.005 g./ml. In a similar way, when the original complex formation between (−)-TAPA was carried out in acetic acid, the (+)-VI which was obtained had a rotation of +573 ± 15°.

Rate of Racemization Experiments.—Since it proved so difficult to obtain large amounts of completely resolved VI, the rate of racemization experiments were carried out on partly resolved material ($[\alpha]D$ 300–400°). In a typical run, a weighed amount, 0.1570 g. of VI, $[\alpha]^{25}D$ −323°, was dissolved in *o*-dichlorobenzene (single peak by v.p.c. analysis) and made up to 12.00 ml. Ten vials were filled with 1.20 ml. of this solution and were sealed up. The sealed vials were weighed before and after heating to ensure that no loss had occurred. These vials were placed in a glycerine bath in an apparatus arranged so that the bath was heated with a constant boiling liquid. The temperatures recorded by the same thermometer (corrected, standardized) were constant to ±0.1° during each run. At the indicated time intervals, a vial was removed and cooled. The rotation was then determined at 25° with the sodium D line in a Rudolph polarimeter. The rate constants were shown to be first order with respect to VI by the fact that straight lines were obtained on plotting log α *vs.* time (in min.) until material initially about 50–60% resolved was almost completely racemized. The values of the rate constants were obtained by using the intercepts on the axes of the above plots and substituting in eq. 1.[21] The rate constants are listed in Table II.

$$\ln(\alpha_1/\alpha_2) = 2k(t_1 - t_2) \quad (1)$$

where α = observed rotation and t = time.

TABLE II

RATES OF RACEMIZATION OF (*l*)-1-FLUORO-12-METHYLBENZO[*c*]PHENANTHRENE

Temp., °C.	*k*, sec.$^{-1}$
130.6 ± 0.1	7.90×10^{-5}
130.4 ± .1	7.73×10^{-5}
109.7 ± .1	9.52×10^{-6}
109.7 ± .1	9.45×10^{-6}
91.7 ± .1[a]	1.24×10^{-6}
92.0 ± .1	$1.30 \quad 10^{-6}$

[a] See text near ref. 22 for explanation of temperature variation.

The temperature variation for the two runs at about 92 and at 130° was caused by the fact that the atmospheric pressure was different on different days. The temperature near 92° was obtained with the water–butanol azeotrope.[22]

The values of log *k vs.* $1/T$ were plotted to yield a straight line. From the slope obtained by drawing the best line through the points, the activation energy was calculated to be 31.3 kcal./mole. The statistical error was calculated to be 0.2 kcal./mole.[23]

(21) For derivation see D. Smith, *J. Am. Chem. Soc.*, **49**, 43 (1927).
(22) Lange's "Handbook of Chemistry," 9th Ed., Sandusky, Ohio, p. 1484.
(23) R. W. Taft, Jr., and C. DeFazio, *J. Am. Chem Soc.*, **77**, 837 (1955).

[CONTRIBUTION FROM THE GERONTOLOGY BRANCH, NATIONAL HEART INSTITUTE, NATIONAL INSTITUTES OF HEALTH, BETHESDA, MD., AND BALTIMORE CITY HOSPITALS, BALTIMORE, MD.]

The Reaction of Mercury(II) with Nucleosides

BY GUNTHER L. EICHHORN AND PATRICIA CLARK

RECEIVED JULY 1, 1963

The attachment of mercury to the nucleosides has been elucidated by a variety of techniques. Titration studies reveal that binding occurs at the N_1O^6 grouping of uridine and guanosine. Experiments in which the amino group is masked by reaction with formaldehyde show that mercury is bound to the amino group in adenosine and cytidine. These results are confirmed by spectrophotometric observations. Support for the mercury–DNA structure proposed by Katz is obtained from titration data with mercury–DNA solutions.

The importance of an understanding of the interaction of metal ions with the nucleic acids becomes evident when it is realized that metals of various kinds, under various conditions, can stabilize the secondary and tertiary macromolecular structures,[1] or labilize these structures,[2] or even bring about the decomposition of the primary structure.[3,4] Of fundamental importance to such an understanding is the knowledge of whether the metal ions bind to the phosphate groups on the polynucleotide chain, or whether they bind to electron donors on the purine or pyrimidine bases.

The discovery of the reaction of mercury(II) ion with DNA by Katz[5] was followed by the demonstration of

(1) J. Shack, R. J. Jenkins, and J. M. Thompsett, *J. Biol. Chem.*, **203**, 373 (1953).
(2) G. L. Eichhorn, *Nature*, **194**, 474 (1962).
(3) E. Bamann, H. Trapmann, and F. Fischler, *Biochem. Z.*, **328**, 89 (1954).
(4) G. L. Eichhorn and J. J. Butzow, in preparation.
(5) S. Katz, *J. Am. Chem. Soc.*, **74**, 2238 (1952).

Thomas[6] that the mercury is attached to the bases, and led, more recently, to further investigation on the binding of mercury with the nucleosides.[7,8] These studies have not exactly defined the position of attachment of the mercury to the various nucleoside molecules. Because it is certain that mercury(II) does react with the purine and pyrimidine bases, the mercury reaction can serve as a model for the reactions of other metal ions, and, by comparison, it should be possible to determine for any metal whether reaction with the bases does indeed occur. Although such was the primary consideration in the undertaking of this work, the techniques that were used were able to characterize the nature of the attachment of mercury to the individual nucleosides in a manner that had not been achieved in previous investigations. During the course of this study we learned that Dr. Richard Simpson[9] was also carrying out some very different experiments that also were designed to elucidate the mercury attachment. The two studies are complementary, and serve to confirm each other's findings.

The present investigation consists essentially of three types of experiments. The first is the titration of the mercury nucleoside complexes and a comparison of the titration curves with those of the components of the complexes. The second is the effect of pH on the spectra of the mercury nucleoside complexes, compared to the nucleosides alone. Finally, the amino groups on the nucleosides were masked by reaction with formaldehyde, and the mercuration studied spectrophotometrically in both the presence and the absence of the formaldehyde.

Previous studies on the mercury nucleoside complex had been carried out in the acidic range in order to avoid precipitation in the more concentrated solutions. The spectrophotometry reported here was carried out in alkaline solution at concentrations at which no precipitation occurs. Precipitates did form during the titrations, but there is no fundamental objection to precipitation during titration, as long as the reaction mixtures are permitted to reach equilibrium throughout the process.

Experimental

The nucleosides and the DNA were obtained from the Sigma Chemical Co.; the inorganic salts were reagent grade from Fisher or Baker, and 37% formaldehyde was from Mallinckrodt. The sodium hydroxide used in the titrations was standardized *vs.* potassium acid phthalate, and the nitric acid was standardized *vs.* the sodium hydroxide.

The titrations were conducted under nitrogen with a Type SBR2 Radiometer titrigraph. Spectra were obtained with a Cary No. 14 recording spectrophotometer. The formaldehyde-containing solutions had a 16% formaldehyde content.

Results and Discussion

Titration.—In Fig. 1 the titration curves of the nucleosides in the absence of metal ions have been compared with the titration curves for mercury alone and those for the mercury and copper complexes of the nucleosides.

The results with uridine (Fig. 1D) are quite informative. In the titration of uridine alone the proton from the N_1O^6 group[10] is removed in the region about pH 8. The curve for copper(II) uridine follows the pattern to be expected if there is weak interaction between metal and nucleoside. Below pH 8 the curve is identical with the curve for the titration of hydrated copper ion, 2 moles of base being required between pH 6 and 7 to remove the protons from two of the water molecules coordinated to the copper. Following this reaction, above pH 8, the N_1O^6 proton is removed as before, and the titration curve runs parallel to the curve for the titration of uridine alone.

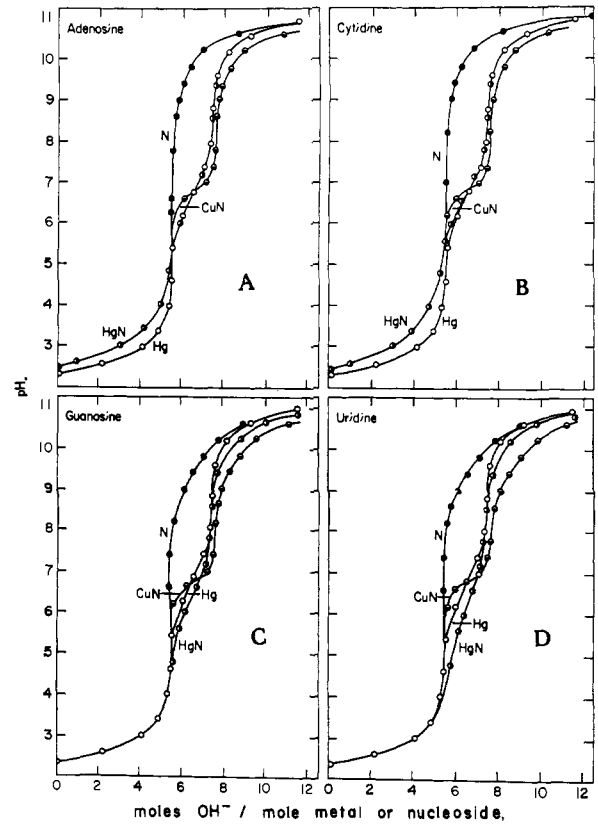

Fig. 1.—Curves for the titrations of the nucleosides and mercury(II) chloride compared with curves for the titrations of 1:1 mixtures of mercury(II) and the nucleosides and 1:1 mixtures of copper(II) and the nucleosides. Nucleoside and metal solutions were 0.98×10^{-3} *M* and contained 5.2×10^{-3} *M* nitric acid at the beginning of the titration with 0.0103 *N* sodium hydroxide, at 25°, under nitrogen atmosphere: ●, nucleoside, N; ○, mercury(II) chloride, Hg; ◑, mercury(II) nucleoside, HgN; ◒, copper(II) nucleoside, CuN; A, adenosine; B, cytidine; C, guanosine; D, uridine.

The titration curve for the hydrated mercury ion resembles somewhat that for the hydrated copper ion, except that the 2 moles of base react with the coordinated water molecules over a longer range, approximately from pH 5 to 8. When uridine is added to the mercury, in contrast to the effect of adding uridine to copper, the titration curve is not superimposable on the mercury titration curve in the low pH range. For any given amount of added sodium hydroxide the pH is considerably lower for mercury–uridine than for mercury alone. In the high pH range, on the other hand, the mercury–uridine curve is practically superimposable on the mercury curve, and does not run parallel to the uridine curve.

It is concluded that, in the presence of mercury, the N_1O^6 group is no longer titrated above pH 8, and must therefore have been titrated in the low pH range, thus explaining the lowered pH throughout that range. Of the 2 moles of base utilized in the mercury–uridine titration, 1 mole must therefore have been used in the titration of the N_1O^6 group and the second for the removal of a proton from a coordinated water molecule. Thus the mercury is bound to the N_1O^6 group of uridine.

(6) C. A. Thomas, *J. Am. Chem. Soc.*, **76**, 6032 (1954).

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

(8) R. Ferreira, E. Benzvi, T. Yamane, J. Vasilevskis, and N. Davidson, "Advances in the Chemistry of the Coordination Compounds," S. Kirschner, Ed., The Macmillan Co., New York, N. Y., 1961, p. 457.

(9) R. Simpson, private communication.

(10) Since two different numbering systems are in use for the pyrimidines, it should be pointed out that in this paper the pyrimidines are numbered in the same manner as the six-membered ring of the purines; O^6 refers to the oxygen atom on the 6-carbon atom.

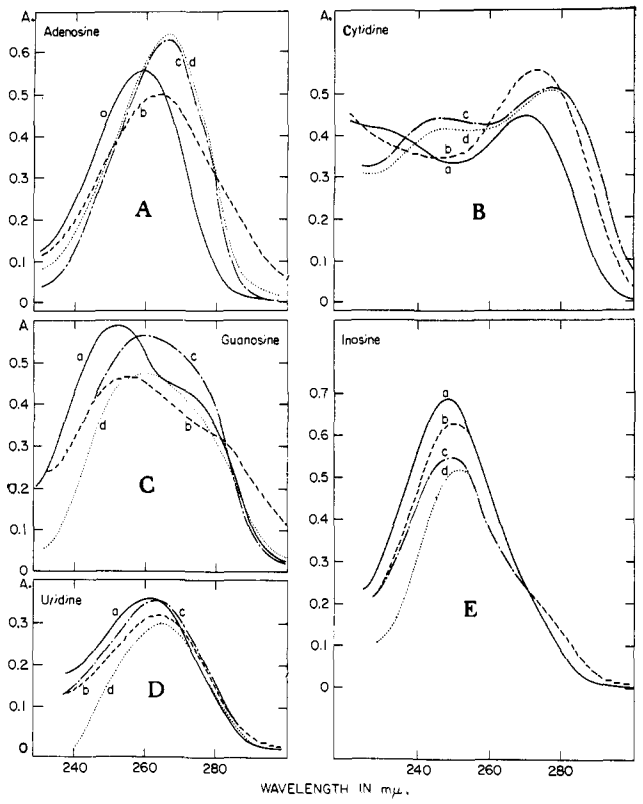

Fig. 2.—Effect of formaldehyde and mercury(II) on the spectra of nucleosides at pH 9: ———, nucleoside alone (a); – – – –, with mercury(II) (b); —·—, with formaldehyde (c); . . . ., with both formaldehyde and mercury (d); A, adenosine, 5.6×10^{-5} *M*; B, cytidine, 5.0×10^{-5} *M*; C, guanosine, 4.7×10^{-5} *M*; D, uridine, 3.6×10^{-5} *M*; E, inosine, 5.7×10^{-5} *M*. All solutions with mercury contained a 32-fold excess of mercury added as $HgCl_2$.

The titration curves involving guanosine can be interpreted in the same way as the curves involving uridine, except that the effects observed are not as great and, therefore, the resulting conclusions perhaps less evident. In Fig. 1C, the mercury–guanosine curve is below the mercury curve at low pH, although not as much below it as the mercury–uridine curve. The mercury–guanosine curve is well above the copper–guanosine curve at high pH. It is concluded that the mercury is attached to the N_1O^6 group of guanosine.

The titration curves of mercury–adenosine (Fig. 1A) and mercury–cytidine (Fig. 1B) are similar to the mercury curve, except for the region below pH 5 where the amino groups of the nucleosides are titrated. These curves therefore yield no information about the nature of the mercury binding to adenosine and cytidine.

The Reaction of Mercury with Nucleosides in the Presence of Formaldehyde.—Fortunately this reaction is most instructive about the attachment of mercury to those nucleosides for which the titrations yield the least amount of information. It has been shown that formaldehyde reacts with the amino groups of the bases in adenosine, cytidine, and guanosine.[11,12] The product of the reaction between formaldehyde and the amino groups in DNA is stable enough to prevent hydrogen bond reformation under conditions that would otherwise bring about the renaturation of denatured DNA.[13] In the present study formaldehyde has been employed as a masking agent to block the amino group from reaction with mercury.

(11) H. Fraenkel-Conrat, *Biochim. Biophys. Acta*, **15**, 307 (1954).

(12) M. Ya. Feldman, *Biokhimiya*, **25**, 563 (1960); Engl. trans. (Consultants Bureau, Inc.), **25**, 432 (1960).

(13) R. Haselkorn and P. Doty, *J. Biol. Chem.*, **236**, 2738 (1961).

The results with adenosine are shown in Fig. 2A. At pH 9 the addition of mercury to adenosine causes a bathochromic shift in the spectrum, just as in acid pH.[7] The absorption intensity, however, is lowered, in contrast to the effect in acid solution. The addition of formaldehyde to adenosine also brings about a bathochromic shift, as had been previously noted. When mercury is added to adenosine in the presence of formaldehyde, the resulting spectrum is virtually identical with that of adenosine in the presence of formaldehyde and the absence of mercury. It is concluded that formaldehyde prevents the reaction of mercury with adenosine and, since the formaldehyde had reacted with the amino group, the mercury must also be attached at the amino group.

The situation with cytidine, Fig. 2B, is very similar to that with adenosine. In the absence of formaldehyde mercury has a profound effect on the cytidine spectrum in the vicinity of the 270-mμ peak. In the presence of formaldehyde, mercury has virtually no effect. Similarly, mercury erases the shoulder in the spectrum of cytidine without formaldehyde, but the addition of mercury causes the 245-mμ peak of the formaldehyde–cytidine spectrum to be retained. It is concluded, therefore, that mercury binds to the amino group in cytidine, as well as in adenosine.

Uridine does not contain an amino group, and therefore it can be predicted that formaldehyde should not be able to prevent the mercuration reaction. Figure 2D does indeed reveal that mercury brings about a very similar change in the uridine spectrum in the presence or absence of formaldehyde. The titration data have already shown that the mercury is attached to the N_1O^6 group. The results of the formaldehyde reaction with uridine thus appear to strengthen the conclusions reached with adenosine and cytidine.

Guanosine does contain an amino group, and thus might be expected to behave toward formaldehyde and mercury in the same manner as adenosine and cytidine. Actually, Fig. 2C reveals that guanosine behaves like uridine, *i.e.*, the formaldehyde does not prevent the mercuration reaction. It thus appears that mercury must react with a group other than the amino group of guanosine, in line with the result of the titration that mercury reacts with the N_1O^6 group. It is evident from Fig. 2E that the spectral changes due to the addition of mercury, or formaldehyde, or both, upon inosine, are similar to those upon guanosine. Since inosine is like guanosine, with the exception that it does not contain an amino group, the reaction of mercury with a nonamino site on guanosine is thus further confirmed. Of course, these data do not preclude the interaction of mercury on the guanosine amino group as a second site. They do show that, whereas mercury at pH 9 reacts only with an amino group in adenosine and cytidine, it reacts with something other than an amino group in guanosine and uridine.

Effect of pH on the Spectra of the Mercury–Nucleoside Complexes.—Confirmation of the results from the titration studies and the formaldehyde reaction can be obtained by comparing the effect of pH on the mercury nucleoside complexes with the effect on the nucleosides alone.

The absorption decrease of uridine between pH 6 and 9 is attributed to the loss of the N_1O^6 proton. From Fig. 1D it was inferred that this same proton is removed at much lower pH in the presence of mercury. Thus, it is possible to explain the fact (Fig. 3D) that there is no substantial change in the absorption of the mercury–uridine complex between pH 6 and 9. Since the N_1O^6 proton has already been removed in the acidic region, the

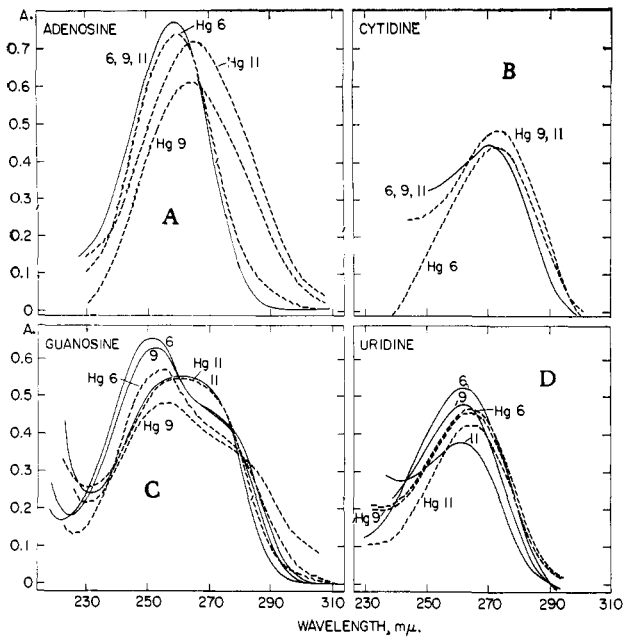

Fig. 3.—Effect of pH on spectra of mercury(II) nucleoside complexes. Curves with numbers only (solid lines) are spectra of nucleosides alone and the numbers represent the pH. Curves labeled with "Hg" and numbers (broken lines) represent spectra of the mercury complexes at the pH's indicated. The nucleoside concentration is 5×10^{-5} *M*, and the mercury(II) concentration is 1.25×10^{-3} *M*: A, adenosine; B, cytidine; C, guanosine; D, uridine.

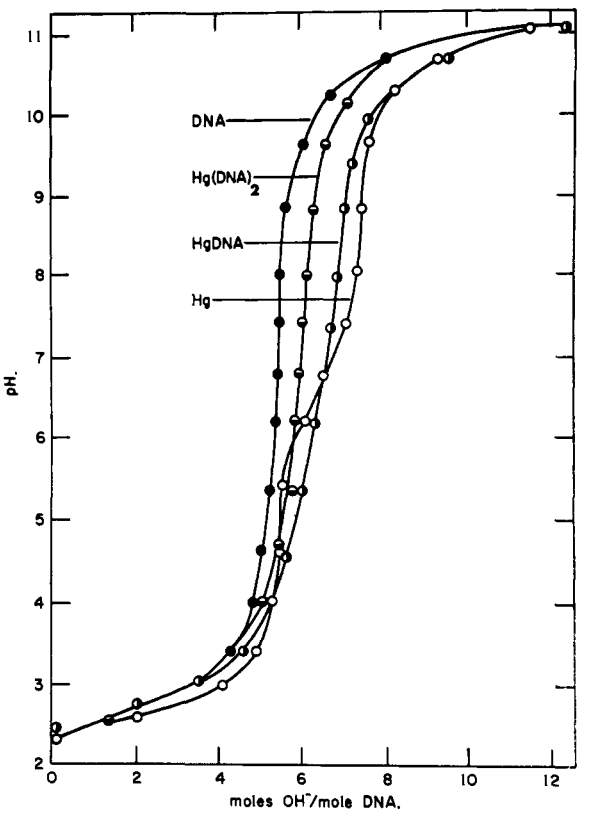

Fig. 4.—Titration curves for solutions containing: ●, DNA, 0.98×10^{-3} *M*; ○, mercury(II) chloride, 0.98×10^{-3} *M*; ◑, DNA, 0.98×10^{-3} *M*, and mercury(II) chloride, 0.98×10^{-3} *M*; ⊖, DNA, 0.98×10^{-3} *M*, and mercury(II) chloride, 0.49×10^{-3} *M*.

spectrum at pH 6 is presumably already the spectrum of the deprotonated complex.

Unmercurated guanosine undergoes little spectral change between pH 6 and 9, but the mercury–guanosine complex changes quite considerably. In this instance there is presumably little deprotonated nucleoside present at pH 9 in the absence of mercury and at pH 6 in the presence of mercury, yet at pH 9 in the presence of mercury a portion of the N_1O^6 is deprotonated. A comparison of the acid region of the curves of Fig. 1C and 1D would lead one to anticipate less deprotonation below pH 9 for guanosine than for uridine. It is interesting that at pH 11 there is virtually no difference between the guanosine spectra with or without mercury.

The spectra of adenosine and cytidine do not vary between pH 6 and 11, since no protons that are detachable within this range exist in these molecules. However, the spectra of the mercury complexes of these nucleosides do vary quite considerably with pH. It is evident from the results of the formaldehyde treatment that the mercury is bound to the amino group of adenosine and cytidine. There are two possible explanations for the pH variations in the spectra of these mercury complexes. One is that the amino-bound mercury is also coordinated to a water molecule which loses a proton to form a hydroxide ion; possibly the hydroxo complex would exhibit a spectrum differing from that of the aquo complex. Figures 1A and 1B show that the water protons coordinated to the mercury are titrated between pH 5 and 8. The mercury cytidine spectra (Fig. 3B) would indeed be explained in this manner, since they vary between pH 6 and 9, but not between pH 9 and 11. However, the mercury adenosine spectra (Fig. 3A) cannot be interpreted in this fashion, since these do exhibit substantial change between pH 9 and 11. The second explanation was suggested by Simpson[9]; in order to account for the lack of pH dependence of the amino–mercury binding, he proposed that the amino group loses a proton and becomes an imino group as a consequence of the mercuration. Figure 3A appears to confirm this proposal for adenosine and, in view of Simpson's work, the loss of an amino proton is also considered to be the explanation of the spectra of the mercury–cytidine complexes.

The Reaction of Mercury with DNA.—Katz[14] has recently proposed a formulation for the structure of the mercury–DNA complex, in which he assumes that each mercury is attached to two purine or pyrimidine bases, on two polynucleotide chains. Figure 4 demonstrates that the titration of a 1:1 (mercury per DNA phosphate) complex takes up only 1.5 moles of sodium hydroxide in the pH region in which mercury alone takes up 2 moles. This phenomenon can be explained by the Katz structure. Since two DNA nucleoside bases are attached to one mercury, only half the mercury can be attached to the DNA in the manner described by Katz,[14] and each mercury thus attached accounts for the removal of only one proton, since only half the nucleosides (guanosine and uridine) lose protons at low pH. The other half of the mercury atoms may or may not be bound to the DNA in some other fashion, *e.g.*, to the phosphates. Mercury bound in such a manner would presumably still be water-coordinated and require 2 moles of base in the titration. Thus, the average number of protons lost per mole of mercury should be 1.5 moles. When a solution containing 2 moles of DNA per mole of mercury was titrated, only 0.5 mole of base was needed per mole of DNA, or 1 mole of base per mole of mercury. These experiments thus appear to confirm the recent ideas of Katz on the structure of the mercury–DNA complex.

Conclusions.—The titration curves of Fig. 1 indicate that mercury is bound to the N_1O^6 group in guanoside and uridine. The formaldehyde reactions

(14) S. Katz, *Nature*, **194**, 569 (1962).

of Fig. 2 indicate that mercury is bound, at pH 9, to the amino group of adenosine and cytidine. The spectra of Fig. 3 are in accord with the data of Fig. 1 and 2.

The data revealing the attachment of mercury to the N_1O^6 site have been deliberately interpreted as such, without any attempt to specify that the mercury must be on either the nitrogen or oxygen atom. It has been suggested elsewhere that, because of the generally greater tendency of mercury to coordinate with nitrogen rather than oxygen atoms, the mercury should not be expected to complex with the hydroxyl group, but should prefer the nitrogen donors instead.[7] However, the nitrogen atoms in the purine and pyrimidine rings and the oxygen atoms on these rings do not have the electronic structure of amino or alcoholic nitrogen and oxygen atoms, and it is believed that there is no *a priori* basis for ruling out binding of mercury to these oxygen atoms on such a basis. Neither are the structures of the uncoordinated nucleosides any help in this matter. Uridine[15] and guanosine[16] do, indeed, have ketonic structures, thus placing the protons on the N_1 atoms. Removal of the proton at high pH, however, brings about conversion to the enolate ion. Whether mercuration results in retention of the protonated structure (nitrogen binding) or addition to the unprotonated structure (oxygen binding) appears to remain an open question.

Previous discussions of the probable orientation of the mercury bonds in these complexes have stressed the tendency of mercury to form two colinear sp-bonds and the relatively low degree of chelate stabilization of mercury complexes compared to some other metal complexes.[7] We should like to point out, nevertheless, that the tetrahedral configuration has been established for many mercury complexes, and that chelate stabilization does occur with mercury. Thus there is a possibility that mercury bound to the amino and hydroxyl groups, respectively, in adenosine and guanosine is also bound to the N_7 position. Attempts to prove or disprove such chelated structures by spectrophotometry and conductometric techniques were fruitless.

Acknowledgment.—The authors wish to thank Miss Barbara Randall and Mr. Edward Tarien for technical assistance, and are grateful to Dr. Richard Simpson for the discussion of his manuscript prior to publication.

(15) H. T. Miles, *Biochim. Biophys. Acta*, **22**, 247 (1956).
(16) H. T. Miles, private communication.

[CONTRIBUTION FROM THE DIVISION OF NUCLEOPROTEIN CHEMISTRY, SLOAN-KETTERING INSTITUTE FOR CANCER RESEARCH, SLOAN-KETTERING DIVISION OF CORNELL UNIVERSITY MEDICAL COLLEGE, NEW YORK 21, N. Y.]

Spectrophotometric Studies of Nucleic Acid Derivatives and Related Compounds. V. On the Structure of 3-Methylcytosine[1]

BY TOHRU UEDA AND JACK J. FOX

RECEIVED JULY 29, 1963

Several 2,3-dihydroimidazo[1,2-*c*]pyrimidines were synthesized by reaction of 4-thiouracil or 4-methylthio-2-pyrimidinone or 1-methyl-4-methylthio-2-pyrimidinone with amino alcohols followed by chlorination and ring closure to condensed ring systems. The absorption spectra of these compounds were determined and their dissociation constants measured spectrally. Spectral comparisons of appropriate molecular species showed that the structure of 3-methylcytosine (neutral species) is of the 4-amino-2-oxo form. 3-Methylcytosine exhibits a hitherto unreported second dissociation (as demonstrated spectrally) in the high alkaline region attributable to proton removal from the 4-amino group. The difference in pK_{a1} values between 1-alkylated and 3-alkylated cytosines is explained by the difference in basicity of their site of protonation. A 1,2,3,4-tetrahydropyrimido[1,2-*c*]pyrimidine (XVI), a new ring system, was also synthesized.

Introduction

The structure of cytosine in aqueous solution has been established as 4-amino-2(1H)-oxopyrimidine (I, R = H).[2,3] The structure of cytidine and 2′-deoxycytidine has also been assigned structure I (R = β-D-ribofuranosyl or 2′-deoxy-β-D-ribofuranosyl) by several investigators[4-6] on the basis of ultraviolet, infrared, and n.m.r. spectral studies.

Brookes and Lawley[7] have recently reported the synthesis of 3-methylcytosine and 3-methylcytidine. They compared the ultraviolet spectrum and pK_a of 3-methylcytosine (7.4)[7] and 3-methylcytidine (8.7)[7] with those for cytosine (4.61)[8] and 1,3-dimethylcytosine (9.3)[9] (see Table I). From these comparisons they implied the 4-amino-2-oxo structure to 3-methylcytosine (II, see Fig. 1).

(1) This investigation was supported in part by funds from the National Cancer Institute, National Institutes of Health, U. S. Public Health Service (Grant No. CA 03190-07). For part IV in this series see J. J. Fox, N. Yung, and I. Wempen, *Biochim. Biophys. Acta*, **23**, 295 (1957).
(2) A. R. Katritzky and A. J. Waring, *Chem. Ind.* (London), 695 (1962).
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TABLE I
APPARENT pK_a VALUES OF VARIOUS CYTOSINES[a]

	pK_{a1}	pK_{a2}
Cytosine	4.61[8]	12.2[14]
1-Methylcytosine	4.55[4]	..
Cytidine	4.1[4]	..[d]
N,N-Dimethylcytosine	4.25[8]	12.3[8]
3-Methylcytosine	7.38[b]	13–14
3-Methylcytidine	8.73[c]	..[d]
1,3-Dimethylcytosine	9.3,[9] 9.4[7]	..
VIa, R = H	6.99	12.6
VIb, R = CH_3	7.01	..
VII	8.08	..
XVI	7.97	~14

[a] The pK_a values are spectrophotometrically determined and are accurate to ±0.05 pH unit unless otherwise indicated. [b] Brookes and Lawley[7] find 7.4. [c] Ref. 7 gives 8.7. [d] A dissociation is spectrophotometrically evident in the pH 12–14 region owing to ionization of the sugar moiety (see text).

Brookes and Lawley explained the rather high pK of 7.4 for II as *vs.* 4.61 for cytosine by the absence of

the acidic (–N–C–) grouping in II. This argument is not convincing, since 1-methylcytosine (I, R = CH_3),

in which the –N–C– acidic grouping is also absent,