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Separation of Deoxyribonucleic Acids by Hg(II) Binding and Cs_2SO_4 Density-Gradient Centrifugation*

Uma Shankar Nandi,† James C. Wang, and Norman Davidson

ABSTRACT: Native deoxyribonucleic acid (DNA) binds mercuric ion reversibly at *p*H 9. There is one kind of a complex up to a bound mercury to nucleotide ratio (r_b) of 0.5. Two protons are displaced per mercury ion bound. Synthetic or natural (crab) deoxyadenosylthymidine alternating copolymer is somewhat different in that the first complex saturates at one mercury per four nucleotides. The equilibrium concentration of Hg^{2+} as a function of r_b has been measured. Denatured or single-strand DNA binds more strongly than native; for native DNA's, the binding strength increases with increasing thymidine content. The binding is somewhat cooperative.

The buoyant density of DNA in a Cs_2SO_4 density gradient increases greatly on binding Hg^{2+}; the quantitative relation $d\rho/dr_b \approx 0.4$ g/ml^{-1} ($0 \leq r_b \leq 0.50$). The selective binding reaction can be used to create large buoyant density differences between single- and double-stranded DNA, or between DNA's with different base compositions. Various separations, including the separation of the two halves of λb^+ DNA, are demonstrated. The natural native DNA's readily come to equilibrium with Hg^{2+}. Care must be taken to ensure good mixing on adding Hg^{2+} to synthetic dAT; otherwise, nonequilibrium, nonhomogeneous mercury complexes form. Mercury ion can be removed from DNA by adding a sufficiently strong complexing agent. The DNA is recovered in its original form, with no deterioration in its structure or biological activity. Several alternate structures for the DNA-Hg^{2+} complexes, all involving Hg^{2+} cross-links between complementary strands, are considered.

The complexing of calf thymus deoxyribonucleic acid (DNA) by mercuric ion was first studied by Katz (1952) and then by Thomas (1954). Yamane and Davidson (1961) investigated the binding of Hg(II) by a number of DNA's with different base compositions at *p*H 5.6. For our present purposes, the pertinent results of these several investigations are: (a) As mercury(II) is added to DNA, there is one type of complex which forms up to a mercury to nucleotide ratio of 0.5, irrespective of the base composition of the DNA; and a second type of complex forms up to a 1:1 ratio [deoxyadenylylthymidine alternating copolymer (dAT[1]), synthetic or natural, is an exception to this rule; Katz (1963); Davidson *et al.* (1965)]. (b) The binding is reversible; on addition of a sufficiently strong complexing agent for Hg^{2+}, such as Cl^- or CN^-, the original DNA is recovered. Indeed, the transforming activity of native pneumococcal DNA is not affected by such a cycle of mercuration and demercuration (Dove and Yamane, 1960). (c) The binding is selective; AT-rich DNA's bind more strongly than do GC-rich ones, and denatured DNA more strongly than native. (d) When Hg^{2+} adds to DNA, protons are displaced. Thus it is expected that the amount of binding at equilibrium can be manipulated by changing the *p*H. (e) All the evidence

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† Present address: Department of Physical Chemistry, Indian Association for Cultivation of Science. Calcutta 32, India.

[1] Abbreviations used in this work: A, adenosine; T, thymidine; G, guanidine; C, cytidine; dAT, deoxyadenosylthymidine alternating copolymer.

indicates that the mercuric ion is bound to the base moieties rather than to the phosphates.

It was previously suggested (Yamane and Davidson, 1961) that, in view of the selective nature of the binding, the mercury complexes might be useful for the fractionation of DNA samples. Because of the large atomic weight of mercury, one would expect a considerable change in the buoyant density of a DNA on binding Hg^{2+}. The present communication presents a detailed description of such a fractionation method, based on the large increase in buoyant density of DNA in Cs_2SO_4 density-gradient ultracentrifugation when Hg^{2+} is bound. The application of this technique for the separation of crab dAT from the main component of crab DNA has already been briefly described (Davidson *et al.*, 1965). In addition, further studies of the binding equilibria and of the proton release at the *p*H (9) used in the centrifuge experiments are presented.

Experimental

Materials. Native calf thymus DNA was purchased from the Worthington Biochemicals Corp. T4 phage was a gift from Professor W. Dreyer, and T4 DNA was prepared by a phenol extraction procedure (Mandel and Hershey, 1960). *Escherichia coli* and *Micrococcus lysodeikticus* DNA's were prepared by a phenol extraction procedure which included an RNAase treatment and several stages of alcohol precipitation. These DNA's contained less than 5% RNA (Schmidt-Tannhauser test) and less than 5% protein (Lowry test). Details are given elsewhere (Jensen, 1965). Whole and half molecules of λb^+ DNA, the latter prepared by hydrodynamic shear in a Vertis homogenizer, were a gift from Dr. Peter Davisson. ϕX DNA was a gift from Dr. R. Sinsheimer. The procedure used in the isolation of crab dAT from crab DNA has been reported recently (Davidson *et al.*, 1965). Thermal denaturations of *E. coli* DNA, T4 DNA, and calf thymus DNA were done by heating the DNA stock solutions (in 0.1 M Na_2SO_4, neutral *p*H) to 95° for 10 minutes followed by quick chilling to 0°. Cs_2SO_4 stock solution was prepared by concentrating a 60% solution supplied by the Gallard-Schlesinger Corp. ("special grade for ultracentrifugation separation") to saturation (density ≈ 1.99 g/ml) in a stream of dry nitrogen. This material when diluted to $\rho = 1.50$ g/ml^{-1} has an A_{260} of about 0.07, and A_{230} of 0.14. CsCl stock solution was prepared from optical grade CsCl (Harshaw) and buffered with 0.02 M Tris buffer (Sigma Chemical Co.), *p*H 8.5. All other chemicals used were of reagent grade, and doubly distilled water was used in the preparation of all the solutions.

CsCl *Analytical Ultracentrifugation.* Analytical ultracentrifugation experiments were carried out in a Spinco Model E ultracentrifuge at 44,770 rpm and 25°. Two and four degree Kel-F center pieces were used. *M. lysodeikticus* DNA was used as a marker in determining the buoyant density of any other DNA, using the equation:

$$\rho_0 = 1.731 - 0.00918(r_0^2 - r^2) \text{ g/ml} \quad (1)$$

where ρ_0 is the buoyant density of the DNA in question, and r_0 and r the distances from the center of rotation of the *M. lysodeikticus* DNA band and the DNA band in question, respectively (Schildkraut *et al.*, 1962).

When a DNA-Hg(II) complex is banded in CsCl at any *p*H below 9.5, the chloride ion concentration is sufficiently high (a solution of density 1.70 g/ml is 5.7 M in CsCl) to complex all the Hg(II) ions and therefore the buoyant density of the DNA itself rather than that of the Hg(II) complex is obtained.

Cs_2SO_4 *Analytical Ultracentrifugation.* The buoyant density experiments for DNA-Hg(II) complexes in Cs_2SO_4 were performed in the following manner. Calculated quantities of Cs_2SO_4 stock solution (ρ = 1.99 g/ml), borate buffer (0.1 M $Na_2B_4O_7$, *p*H 9.2), DNA stock solution (usually in 0.1 M Na_2SO_4), $HgCl_2$ solution (1–10 × 10^{-4} M), and water were mixed to give a final solution containing 0.005 M borate buffer, DNA at an A_{260} of approximately 0.1, with the desired mercury to DNA ratio and the desired initial density. The reagents were usually mixed in the order listed. While adding the $HgCl_2$, gentle stirring was accomplished by motion of the micropipet. As reported below, the conditions of mixing do not appear to be critical for the native natural DNA's. A better mixing procedure may be desirable for denatured DNA's, and is necessary for synthetic dAT. The density of the solution was predicted by assuming no volume change on mixing. The actual value of the density was calculated from the measured refractive index by the equation $\rho^{25°} = 13.6986 n_D^{25} - 17.3233$ (Vinograd and Hearst, 1962). The apparent *p*H of such a solution, as measured by a Beckman general purpose semimicro glass electrode and a semimicro Ag–AgCl reference electrode, was usually in the range 9.5–9.8. No significant change in buoyant density was observed due to this small variation in *p*H. In general, the solutions used for centrifugation should not contain any species which complexes Hg^{2+}, such as EDTA, chloride ion, or Tris buffer, at concentrations sufficiently high to interfere with the quantitative binding of the Hg^{2+} by the DNA. Chloride ion at concentrations of the order of 0.1 M and Tris buffer at concentrations of the order of 0.001 M do not reverse the binding at *p*H 9. A Joyce-Loebl recording microdensitometer was used to trace the ultraviolet absorption photographs taken after centrifuging for 16–18 hours at 44,770 rpm.

Buoyant densities were calculated from the equation $\rho_0 = \rho_e + (\alpha\omega^2/2)(r^2 - r_e^2)$, where r_e is the limiting isoconcentration distance, r the position of band center, and ρ_e the density of the initial solution (Vinograd and Hearst, 1962).

For the buoyant density gradient in the neighborhood of ρ_0 = 1.42 g/ml, Erikson and Szybalski (1964) give an α value of 1.40 × 10^{-9} for *E. coli* DNA. We estimated the compositional density gradient from two preparative runs with ρ_e = 1.482 and 1.629 g/ml, respectively, at 31,000 rpm and 25° in the same manner as reported by Ifft *et al.* (1961). Results from the drippings are displayed in Figure 1. The α values obtained from these two samples were identical

within experimental error, and an average value 1.38 $\times$ 10^{-9} cgs unit was taken. Since α obtained in this manner is likely to be a lower limit, we believe that for Cs_2SO_4 the buoyant density gradient is approximately equal to the compositional density gradient. Thus the value $\alpha = 1.40 \times 10^{-9}$ was used in calculating the buoyant densities of DNA-Hg(II) complexes in the density range 1.4–1.8 g/ml.

In different series of runs, the precision obtained for the measured buoyant densities in Hg^{2+}, Cs_2SO_4 experiments varied between 0.005 and 0.015 density unit. This is probably mainly due to pipetting error which affects the Hg(II) to DNA ratio. Because of the large density changes produced by the mercury binding, it was not always convenient to band the DNA close to the isoconcentration point of the Cs_2SO_4 gradient. Therefore the accuracy of these measurements may also be affected by imperfect knowledge of the density gradient. It is not possible to add a reference marker DNA of known density, because it too can bind Hg^{2+}. Density differences in the CsCl experiment were reproducible to ± 0.0003 unit.

Preparative Ultracentrifugation. Solutions used in the preparative ultracentrifugation were made up in the same manner as those for analytical ultracentrifugation except higher DNA concentrations, such as A_{260} = 0.2–5, were used. Usually 2 ml of solution covered by 3 ml of hydrocarbon (Bayol, from the Humble Oil and Refining Co.) in each SW 39 centrifuge tube was spun in a Spinco Model L ultracentrifuge for 40–70 hours at 31,000 rpm and 25°. After braking to a halt, samples were immediately dripped. Usually drops were collected into vials each containing 0.20–0.50 ml of water, and the spectrum of each fraction was measured by a Cary Model 14 spectrophotometer using a microcell. For samples with low DNA concentrations, a 0–0.1 absorbance unit slide wire was used for the spectrophotometer.

Binding of Hg(II) *Ions by DNA at* pH *9.* The equilibrium degree of binding as a function of the concentration of Hg^{2+} at a fixed *p*H was measured by making spectrophotometric observations of the equilibrium in the reaction:

$$y\mathrm{Br}^- + \text{DNA-Hg} \rightleftarrows \text{DNA} + \mathrm{HgBr}_y^{(y-2)-} \quad (2 \leq y \leq 4) \quad (2)$$

The absorption spectrum of the DNA is a function of the degree of mercuration (Thomas, 1954; Yamane and Davidson, 1961); the $HgBr_y^{(y-2)-}$ also absorbs in the same wavelength region. Because there are three important species, $HgBr_2$, $HgBr_3^-$, and $HgBr_4^{2-}$, the average molar absorptivity of $HgBr_y^{(y-2)-}$ is itself a function of free bromide ion concentration. The experiments were carried out at room temperature (approximately 27°) in 0.5 M $NaClO_4$ and 0.01 M $Na_2B_4O_7$ (*p*H 8.95), using a Cary Model 14 spectrophotometer. One to two ml of a DNA solution (A_{260} = 0.5–1.0) was added to a 3-ml quartz cell. Aliquots of 5–10 μl of a $HgCl_2$ solution of appropriate concentration were then added to the DNA solution such that each addition would increase r_f (r_f is defined as the molar ratio of total Hg(II) to total DNA phosphate) by 0.05–0.1. A small glass-coated magnet was placed in the quartz cell and a magnetic stirrer was used to provide proper mixing during the addition of titrant. Micropipets were used in measuring solutions of μliter quantities. The spectrum of the DNA solutions was recorded after each addition of $HgCl_2$. After r_f increased to approximately 0.6 (or 0.3 if the low r_f region is of interest), NaBr (1 or 2 M) was added in 5–10 μl aliquots and the spectrum recorded after each addition. At any wavelength > 235 mμ (below this wavelength the salt background absorbs strongly),

$$A_T = A_{\text{DNA-Hg}} + A_{\text{Hg-Br}} = A_{\text{DNA-Hg}} + \bar{\epsilon}[\mathrm{Hg}_{T,\mathrm{Br}}] \quad (3)$$

or

$$[\mathrm{Hg}_{T,\mathrm{Br}}] = (A_T - A_{\text{DNA-Hg}})/\bar{\epsilon} \quad (4)$$

where A_T is the absorbance of the solution, $A_{\text{DNA-Hg}}$ the absorbance of the DNA-Hg(II) complex, $A_{\text{Hg-Br}}$

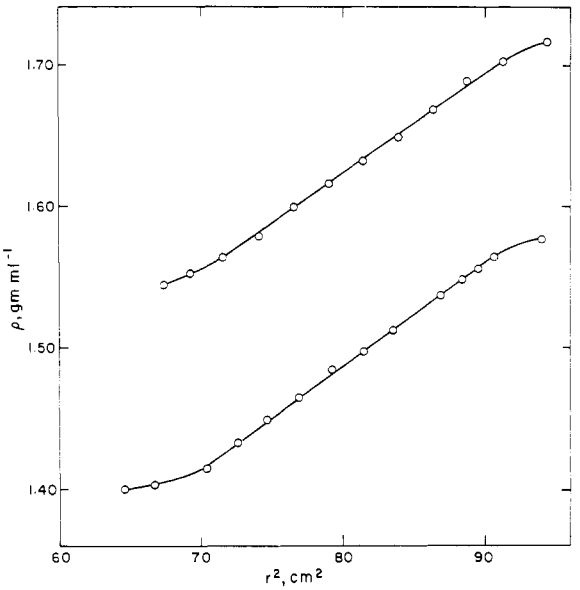


FIGURE 1: Compositional density gradient of Cs_2SO_4. Samples were dripped after 45 hours at 31,000 rpm and 25°. ρ_e is the initial density of the solution, r is the distance from the center of rotation. The limiting isoconcentration distance r_e is calculated from the equation $r_e^2 = (r_a^2 + r_a r_b + r_b^2)/3$, where r_a and r_b are the distances from the center of rotation of the meniscus and bottom of the Cs_2SO_4 solution. The slopes of the linear portion of the two curves are 7.15×10^{-3} (upper curve) and 7.32×10^{-3} (lower curve). The corresponding values of α are 1.36×10^{-9} and 1.39×10^{-9} g ml^{-1} radians^{-2} sec^2 cm^{-2}. The values of ρ_e and r_e^2 (calcd) for the upper curve are 1.629 and 80.6; for the lower curve they are 1.486 and 79.1.

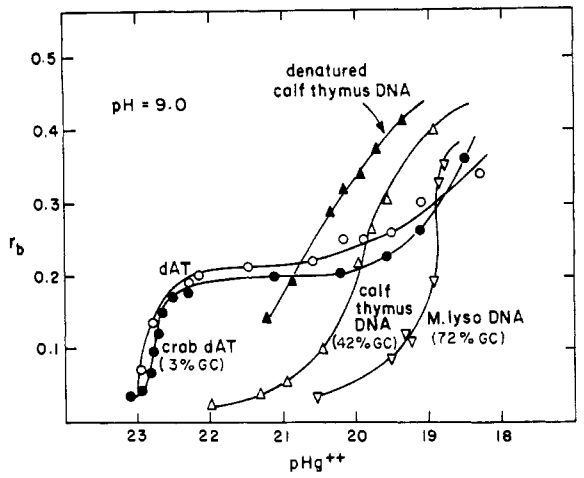


FIGURE 2: Binding curves for the complexing of DNA by Hg(II) at 27° in 0.5 M $NaClO_4$ and 0.01 M $Na_2B_4O_7$, *p*H 8.95. (○), synthetic dAT; (●), crab dAT; (△), calf thymus DNA; (▲), denatured calf thymus DNA; (▽), *M. lysodeikticus* DNA.

the absorbance of all Hg(II) complexes of Br^-, $\bar{\epsilon}$ the average molar absorptivity of the bromo complexes, and $[Hg_{T,Br}]$ the total molar concentration of all the bromo complexes of Hg(II). The quantity $\bar{\epsilon}$ is not a constant and depends on $[Br^-]$, the free bromide concentration. In the present studies, the total bromide ion concentration, $[Br^-]_T$, was at least 100 times higher than the total Hg(II) concentration, $[Hg]_T$. Thus as a good approximation, $[Br^-] = [Br^-]_T$. Therefore $\bar{\epsilon}$ can be determined experimentally from the spectrum of a solution of the same $[Br^-]_T$ and known total Hg(II) concentration but no DNA. For $[Br^-]_T$ = 0.05–0.2 *M*, $\bar{\epsilon}_{240}$ increases from approximately 10^4 to 3×10^4.

To calculate $[Hg_{T,Br}]$ from equation (4), it is convenient to select an isosbestic wavelength of the DNA-Hg(II) complex so that A_{DNA-Hg} remains constant and is independent of r_f (after proper correction for dilution). For *M. lysodeikticus* DNA, spectra of samples with r_f 0.068, 0.12, 0.25, 0.37, and 0.50 showed two sharp isosbestic points at 241 and 258 mμ. The DNA itself ($r_f = 0$) absorbs somewhat higher (approximately 3%) at these wavelengths. For calf thymus DNA with $0.10 \leq r_f \leq 0.5$, two isosbestic points at 242 and 259 mμ were observed. It appeared that the spectrum of the sample with $r_f = 0$ had a slightly higher absorbance at 259 mμ than the others. Denatured calf thymus DNA with $0.10 \leq r_f \leq 0.5$ exhibited a sharp isosbestic point at 238 mμ. The second isosbestic point at *ca.* 262 mμ was less sharp, and the sample with $r_f = 0$ had considerably higher absorbance than the others at this wavelength. The absorption spectra of the Hg(II) complexes of synthetic dAT are shown in Figure 3. Spectra of crab dAT-Hg(II) are similar to those of synthetic dAT-Hg(II).

The following equations were used to calculate r_b, the number of moles of Hg(II) bound to DNA per mole of DNA phosphate, and $[Hg^{2+}]$, the free mercuric ion concentration:

$$r_b = ([Hg]_T - [Hg_{T,Br}] - [Hg(OH)_2])/[DNA]_T$$
$$= ([Hg]_T - [Hg_{T,Br}] - K[Hg^{2+}]/[H^+]^2)/[DNA]_T \quad (5)$$

$$[Hg^{2+}] = [Hg_{T,Br}]/(1 + \beta_1[Br^-] + \beta_2[Br^-]^2 + \beta_3[Br^-]^3 + \beta_4[Br^-]^4) \quad (6)$$

where *K*, the equilibrium constant for the hydrolysis of Hg^{2+}, is taken as $10^{-6.3}$. The formation constants for the bromo complexes $HgBr^+$, $HgBr_2$, $HgBr_3^-$, and $HgBr_4^{2-}$ are taken to be $\log \beta_1 = 9.05$, $\log \beta_2 = 17.33$, $\log \beta_3 = 19.74$, and $\log \beta_4 = 21.0$, respectively (Bjerrum *et al.*, 1958). The molar concentrations of total DNA-P, $[DNA]_T$, were calculated from the absorbance of the DNA's at 260 mμ using the following molar absorptivities: *M. lysodeikticus* DNA 6920, calf thymus DNA 6600; crab dAT and synthetic dAT 6650 liters (mole DNA-P)$^{-1}$ cm^{-1}. For the concentration range of Br^- used in these studies, $1 + \beta_1[Br^-]$ is negligible compared with the other terms, and $[Hg(OH)_2]$ is no more than a few per cent of $[Hg]_T$.

Proton-Release Experiments. The number of protons released per Hg(II) bound at 25° in a medium of 0.1 M Na_2SO_4 at *p*H 9 for calf thymus DNA and *M. lysodeikticus* DNA was determined by *p*H-stat titrations in the same way reported previously (Yamane and Davidson, 1961).

Results

Proton Release and Equilibrium Binding. In the absence of DNA, when $HgCl_2$ is added to a solution at *p*H 9, the reaction

$$HgCl_2 + 2H_2O \longrightarrow Hg(OH_2) + 2H^+ + 2Cl^- \quad (7)$$

takes place quantitatively. In fact, in the presence of DNA, up to $r_f = 0.50$, it is observed that two protons (1.90 ± 0.10) are released per $HgCl_2$ molecule added. Thus could be due to reaction (7) above with no reaction with the DNA or to the reaction

$$HgCl_2 + H_2B_2 \rightleftharpoons HgB_2 + 2H^+ + 2Cl^- \quad (8)$$

where H_2B_2 represents one base pair of DNA and B_2 is the base pair *sans* two protons. Since the equilibrium studies reported below show that the added mercury was completely bound under the conditions of the *p*H-stat titrations (a conclusion which was confirmed by direct spectrophotometric observation), we conclude that equation (8) is correct and that 2.0 protons are displaced per Hg^{2+} added for $r_b \leq 0.5$. These experiments were performed with native calf thymus DNA and with *M. lysodeikticus* DNA.

Figure 2 displays r_b as a function of the free Hg(II) concentration for a number of DNA's. The relative order of the strength of complexing agrees in general

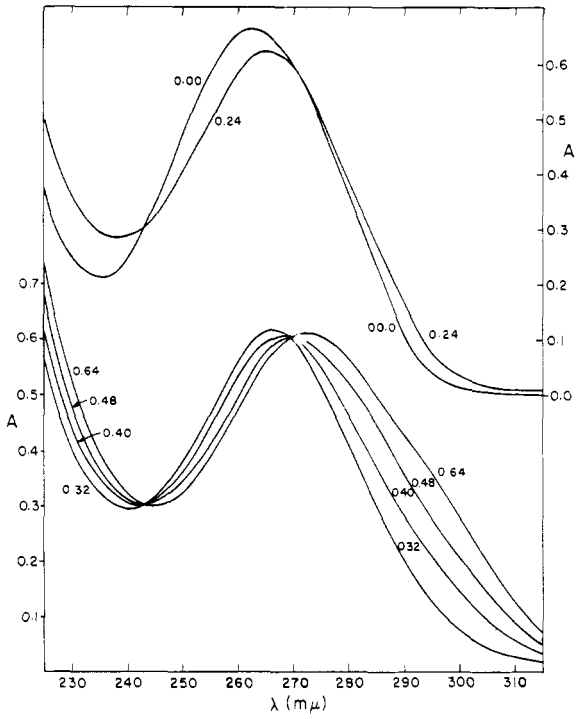


FIGURE 3: Ultraviolet absorption spectra of synthetic dAT-Hg(II). Numbers indicated are the r_f values of the samples. The ordinate for the upper two curves is on the right-hand side of the figure and that for the lower curves is on the left-hand side.

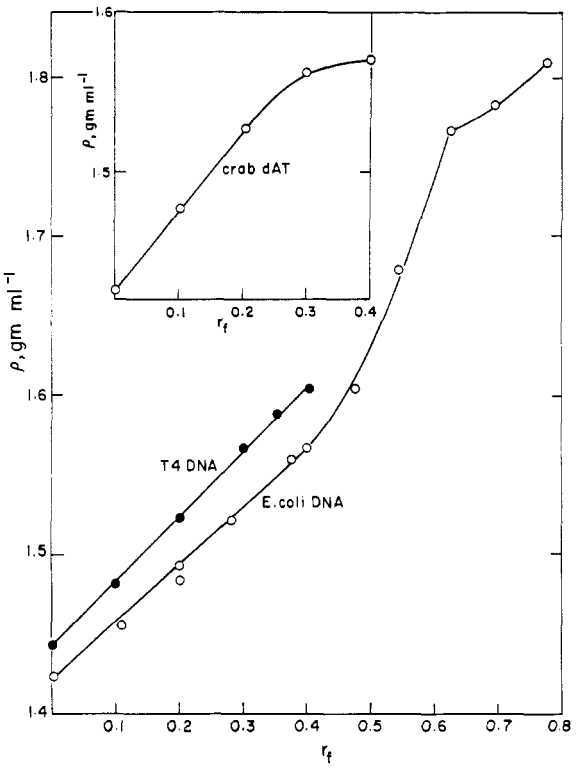


FIGURE 4: Buoyant densities of the Hg(II) complexes of *E. coli* DNA, T4 DNA, and crab dAT as a function of r_f = ($HgCl_2$ added)/(DNA phosphate).

with that observed at *p*H 5.6 (Yamane and Davidson, 1961). Calf thymus DNA (42% GC) binds Hg(II) more strongly then *M. lysodeikticus* DNA (72% GC), and denatured calf thymus DNA binds Hg(II) more strongly than native calf thymus DNA.

The plateau region at r_b = *ca.* 0.2 exhibited by the binding curves of both the synthetic dAT and crab dAT (the latter has 3% GC) is atypical compared with binding curves of other DNA's.

The spectral changes on binding Hg^{2+} for the ordinary DNA's (calf thymus *E. coli*, T-4 phage, *M. lysodeikticus*) are similar and almost identical with those reported previously (Thomas, 1954; Yamane and Davidson, 1961), and are not reproduced here. For $0.05 \leq r_b \leq 0.50$, there is one kind of spectral change, with several isosbestic points. A second kind of spectrophotometric complex forms for $r_b > 0.5$. The situation is different with dAT, either synthetic or crab [the latter contains about 3% GC base pairs (Swartz, *et. al.*, 1962)]. The spectra (Figure 3) agree with the binding studies and the density gradient studies (*vide infra*) in indicating one kind of a complex for $0 \leq r_b \leq 0.25$, and a second complex for $r_b \leq 0.25$. (Incidentally, the spectral changes for the ordinary DNA's for $0.05 \leq r_b \leq 0.50$ are rather similar to those observed for dAT for $0.25 \leq r_b$ (Figure 3).)

Effect of Hg(II) *Binding on the Buoyant Density of DNA.* In view of the large atomic weight of mercury, it was to be expected that mercury binding would markedly increase the buoyant density of DNA. It is to be noted that one possible complicating factor is not important. Since 2.0 protons are released per mercury ion bound, there is no change in the formal charge of the macroanion on binding. Therefore there is no change in the number of cesium ions which move with the DNA in the centrifugal field to preserve electroneutrality. A change in the number of associated Cs^+ ions would contribute significantly to buoyant density changes (Vinograd *et al.*, 1963; Vinograd and Hearst, 1962). The experimental results presented in Figure 4 show that very large changes in the buoyant density of a DNA are caused by the addition of Hg^{2+}.

The horizontal coordinate in Figure 4 is r_f, the ratio of added Hg^{2+} to DNA phosphate. It is necessary to consider the relation between this quantity and r_b, the ratio of bound Hg^{2+} to DNA phosphate. The difference between r_f and r_b can be estimated as follows. At alkaline *p*H values, the over-all equation for the binding of Hg(II) by DNA can be written as:

$$Hg(OH)_2 + DNA = (DNA - 2H^+)Hg + 2H_2O \quad (9)$$

since Hg(II) not bound to DNA is primarily in the form $Hg(OH)_2$. The notation $-2H^+$ is meant to indicate the release of two protons from the DNA per Hg(II) bound. Then

$$(r_f - r_b)[\text{DNA}]_T$$

$$= \text{concentration of Hg(II) not bound to DNA}$$

$$= [\text{Hg(OH)}_2] = K[\text{Hg}^{2+}]/[\text{H}^+]^2 \qquad (10)$$

where $K = 10^{-6.3}$. For a given r_b, the free mercuric ion concentration is given in Figure 2. Clearly, $r_f - r_b$ increases with decreasing DNA concentration. For calf thymus DNA with an A_{260} of 0.1 (1.5×10^{-5} M in DNA-P), r_f and r_b are approximately equal for $r_b \leq 0.4$ (at $r_b = 0.4$, $\log [\text{Hg}^{2+}] = -18$ and $r_f - r_b$ is calculated to be 0.03). It is noted that the reaction shown above is *p*H independent; thus $r_f - r_b$ calculated from the binding curve (measured at *p*H 9 and in 0.5 M $NaClO_4$) will not vary with moderate variations of *p*H. The effect of the ionic medium—0.50 M $NaClO_4$ for the experiments of Figure 2 and 2 M Cs_2SO_4 in the centrifuge experiments—or the effect of the high pressure in the lower part of the centrifuge tube cannot be predicted from data at hand, but the general concordance of all our results suggest that these effects are small.

The break point at $r_f \approx 0.62$ for *E. coli* DNA (Figure 4) probably corresponds to an r_b of 0.5; that is, there is a significant difference between r_f and r_b at this high r_b. The sudden decrease of the slope of the ρ_0 versus r_f curve above this point may be attributed to (a) much weaker binding and/or (b) the number of protons released per Hg(II) bound being less than 2, thus decreasing the number of Cs^+ ions bound to the macroanion and resulting in a smaller increase in buoyant density per Hg(II) bound.

The buoyant density is approximately a linear function of r_f (and therefore of r_b) for both *E. coli* DNA and T4 phage DNA for $0 \leq r_f \leq 0.4$, with slopes of *ca.* 0.36 and 0.40 g ml^{-1}, respectively. The *E. coli* curve was extended beyond this point and shows an upward curvature until the break point at $r_f \approx 0.6$ is reached. The significance of this curvature in ρ_0 is not clear due to the following reason. The buoyant density of the polymer is affected by the change in the specific volume of the unsolvated polymer and by any change in the extent of solvation. The latter depends strongly on the water activity of the solution in equilibrium with the polymer (Hearst and Vinograd, 1961). Thus two buoyant bands with different r_b values not only differ in the specific volumes of the unhydrated macromolecules but also differ in the extent of hydration. Therefore the buoyant density may be a composite function of r_b.

The buoyant density data for crab dAT-Hg(II) are also plotted in Figure 4. Below $r_f \approx 0.2$, ρ_0 increases linearly with r_f with a slope of 0.50 g/ml. Above $r_f = 0.2$, ρ_0 increases much less sharply. This is in complete agreement with what would be predicted from the binding curve of crab dAT, since for $r_f > 0.2$, the difference between r_f and r_b is large.

The buoyant density of denatured DNA-Hg(II) is less reproducible at a given r_f compared with that of the Hg(II) complex of native DNA. This may be dependent on the extent of renaturation of the DNA. The values of $d\rho/dr_f$ for denatured DNA were similar to those for native DNA. With denatured T4 DNA, we frequently observed two bands; for example, with $r_f = 0.25$, fairly well-resolved bands at $\rho = 1.579$ and 1.6405 were observed. This is possibly due to nonequilibrium phenomena as described below for dAT, possibly to partial renaturation, or possibly to a base composition heterogeneity in the denatured DNA. However, the phenomenon was not studied further.

Fractionation of DNA's by Hg(II) *Binding in* Cs_2SO_4 *Density-Gradient Centrifugation.* Suppose a mixture of equal amounts of calf thymus DNA and *M. lysodeikticus* DNA in Cs_2SO_4 with an average r_b value ($\bar{r}_b$) of 0.2 is centrifuged. Neglecting possible effects of a centrifugal field, the r_b values for the two DNA's should adjust such that the DNA molecules are at equilibrium with the same free Hg^{2+} concentration.[2] From the binding curves in Figure 2 it can be estimated that the individual r_b values at equilibrium are approximately 0.31 and 0.09, respectively, for calf thymus DNA and *M. lysodeikticus* DNA. Assume $d\rho_0/dr_b$ is a constant and equals 0.36 g/ml for the two DNA's; then for calf thymus DNA, ρ_0 ($r_b = 0.31$) = 1.424 + (0.36 × 0.31) = 1.54 g/ml and for *M. lysodeikticus* DNA, ρ_0 ($r_b = 0.09$) = 1.435 + (0.36 × 0.09) = 1.47 g/ml. Thus the addition of Hg(II) to $\bar{r}_b = 0.2$ would result in a difference in buoyant densities of 0.07 g/ml. Such a difference corresponds to a separation of 0.54 cm for the two buoyant bands in a Cs_2SO_4 density gradient at 31,000 rpm, if an SW 39 rotor is used. Some analytical ultracentrifugation results are displayed in Figure 5. T4 DNA, with 67% AT, binds Hg(II) more strongly than *E. coli* DNA, with 50% AT. Thus at $\bar{r}_f = 0.15$, the T4 band corresponds to an r_b of 0.19 and the *E. coli* band corresponds to an r_b of 0.09 (calculated from the buoyant densities of the two bands and Figure 4). Similarly, *E. coli* DNA binds Hg(II) more strongly than *M. lysodeikticus* DNA (28% AT); thus at $\bar{r}_f = 0.22$, the r_b value of the former is equal to 0.30 while that of the latter is approximately 0.1.

Since denatured DNA binds Hg(II) more strongly than native DNA, the difference in buoyant densities between denatured and native DNA can be greatly increased upon adding Hg(II). Figure 5c shows the buoyant patterns of a mixture of equal amounts of native and heat-denatured T4 DNA with $\bar{r}_f = 0$ and 0.25.

The single stranded ϕX DNA (30% T) is expected to behave like denatured T4 DNA in complexing Hg(II), and therefore to bind Hg(II) more strongly than native T4 DNA. Indeed at $\bar{r}_f = 0.21$, a mixture of native T4 DNA and ϕX DNA resolved into two clearly separated bands (Figure 5d).

It should be mentioned that in the case of *E. coli* DNA and T4 DNA (Figure 5a) a preparative run was

[2] We believe that the centrifugal field will affect the distribution of Hg(II) between the two DNA's in a way which depends on the sedimentation equilibrium for $Hg(OH)_2$. This effect, which has not yet been investigated, will probably act to enhance the density gradient separations.

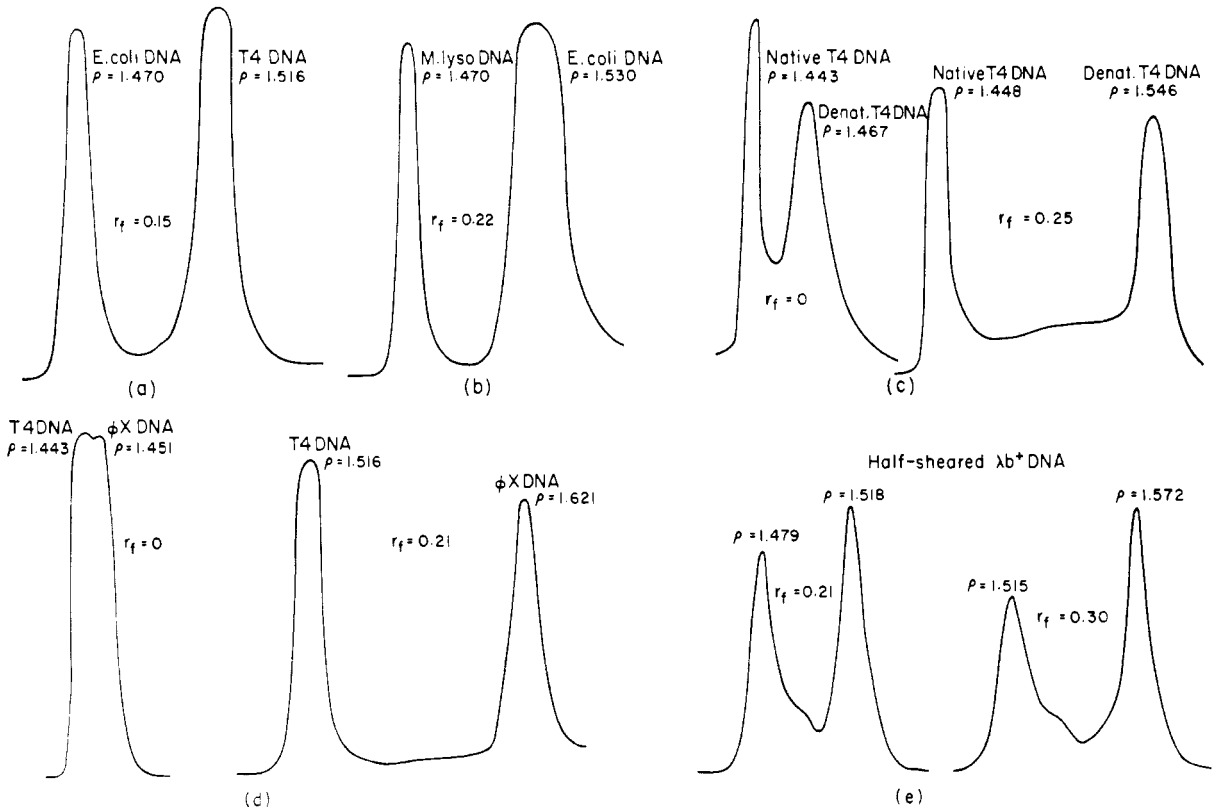


FIGURE 5: Densitometer tracings of some analytical ultracentrifugation experiments. A_{260} for the samples used were: (a) *E. coli* DNA, 0.10; T4 DNA, 0.18; (b) *M. lysodeikticus* DNA, 0.11; *E. coli* DNA, 0.25; (c) molar ratio of T4 DNA to denatured T4 DNA = 1; total A_{260} = 0.3; (d) T4 DNA, 0.18; φX DNA, 0.11; (e) λb⁺ DNA half molecules, obtained by shear breakage, A_{260} = 0.10.

carried out and the two separated buoyant fractions were identified by their buoyant densities in CsCl. In the other cases assignment of bands were made by comparing the relative absorbance of the two bands from the densitometer tracing to the molar ratio of the two DNA's (adjusted to be about 2 usually) in the initial solution.

Finally, Figure 5e shows that when a preparation of the half-molecules of λb⁺ DNA, obtained by shear breakage of whole molecules, is banded in the presence of an optimum amount of Hg^{2+} ($r_f \approx 0.2$–0.4), an excellent separation into two bands is obtained. That the two bands contain the separated "left" and "right" halves of the λ DNA is demonstrated for the case of λdg DNA in the following paper. The GC contents of the two halves of λb⁺ are 54 and 45% (Hershey, 1964).

An important consideration in these competitive binding experiments is whether or not the mercury ions reach their equilibrium distribution between the several kinds of DNA molecules. That this is typically the case is indicated by the following experiment. The dripping patterns of two samples from a preparative ultracentrifugation were examined. Sample A was prepared by mixing equal amounts of T4 DNA and *E. coli* DNA followed by adding Hg(II) to an r_f of 0.1. Sample B was identical in composition as compared with sample A, but *E. coli* DNA and Hg(II) were mixed prior to the addition of T4 DNA. The samples were centrifuged for 45 hours at 31,000 rpm at 25°. Identical dripping patterns were obtained with results similar to those shown in Figure 5a. Thus the equilibrium amount of mercury was transferred from the *E. coli* to the T4 DNA. It may be concluded that the system was at equilibrium. The bands were well resolved, showing that there is a negligible amount of cross-linking of different DNA molecules by Hg^{2+} ions.

Buoyant Density Behavior of the Mercury(II) Complex of Synthetic dAT. When synthetic dAT was banded according to our usual procedure with $0.08 < r_f < 0.18$, two or three bands were observed. There was always a light band with a density close to 1.43, corresponding to $r_b = 0.0 \pm 0.05$, and a heavy band with a density of 1.56, corresponding to $r_b \approx 0.20$–0.25. There was sometimes a small band of intermediate density. The DNA of both bands was pure dAT, as shown by various tests. They both had a buoyant density of 1.679 in CsCl, as expected for dAT. When the light band was isolated from a preparative run, treated with additional Hg^{2+} to give $r_f = 0.10$, and rebanded in

Cs_2SO_4, it gave two bands with ρ = 1.435 and 1.562.

The two bands are due to a rate phenomenon and a failure to achieve an equilibrium distribution of Hg^{2+} among the various molecules. For if equal volumes of dAT in Cs_2SO_4 (with borate buffer) and $HgCl_2$ in Cs_2SO_4 were mixed with fast stirring and then centrifuged, only one band with a buoyant density of 1.487 g/ml was observed for a sample with r_f = 0.125. On the other hand, if known ratios of two dAT solutions with r_f values of 0 and 0.25, respectively, were mixed and centrifuged, densitometer tracings of the ultraviolet absorption photographs showed two bands for each sample with buoyant densities (1.43 and 1.56) corresponding to r_f = 0 and 0.25, respectively. The ratio of the area under the two peaks agreed with the known molar ratio of the two dAT solutions used, indicating no exchange in Hg(II) had occurred.

It was mentioned previously that, in mixtures of T4 and *E. coli* DNA, the Hg^{2+} does come to its equilibrium distribution. The very steep cooperative nature of the binding curve for dAT (Figure 2) favors the initial formation of a nonuniform distribution of mercury among the various DNA molecules on mixing; the low concentration of free Hg^{2+} (or $Hg(OH)_2$) causes the re-equilibration reactions to be slow.

We should like to mention that with a very steep, cooperative binding curve it is theoretically possible for a mercury-DNA complex with, e.g., r_b = 0.10 to undergo an equilibrium disproportionation to give two bands with r_b close to zero and close to saturation (0.25 for dAT, 0.50 for ordinary DNA's). The centrifugal field is the driving force for the disproportionation. This theoretical analysis, which may or may not be of purely academic interest, will be presented separately.

Biological Assays. An important consideration bearing on the practical utility of the separation method described here is that of the effects of the $HgCl_2$, Cs_2SO_4 treatment on the biological activity of the DNA. In the riginal experiments by Dove and Yamane (1960), pneumococcal transforming DNA was exposed at *p*H 6.8 in 0.1 M $NaClO_4$ at 4° to mercuric chloride at r_f = 6.0. Cysteine was added about 5–10 minutes after adding the $HgCl_2$ to complex the Hg^{2+}, and, after suitable dialysis, transformation assays were performed. Within experimental accuracy (±10%), there was no loss of biological activity.

It is conceivable that under the somewhat different conditions used here (*p*H 9, Cs_2SO_4 solution, 30–60 hour exposure, room temperature) the results would be different; therefore additional tests of the effect of Hg(II) complexing on the biological activity of several DNA's were performed.

Infectivity assays for ϕX 174 DNA were kindly performed by Dr. Alice Burton. The method was that of Guthrie and Sinsheimer (1960). All samples contained ϕX 174 DNA at a concentration of 3.5 μg ml^{-1}. The solutions were as follows: control no. 1 (Cl), 0.01 M Na_2SO_4, 0.01 M borate, *p*H 9; control no. 2 (C2), Cs_2SO_4 solution with ρ = 1.50 g/ml^{-1}, 0.01 M borate, *p*H 9; sample no 1. (S1), same as C2 with $HgCl_2$ added to r_f = 0.2. All solutions were allowed to stand for 2 days at room temperature and then assayed. The relative infectivities of the samples were: C1, 1.00; C2, 1.41; S1, 1.55.

Infectivity assays for λdg DNA were kindly performed by Mr. Elton T. Young. The assay method was that described by Kaiser (1962). All samples contained λdg DNA at a concentration of 2.7 μg ml^{-1}. The solutions were as follows: control no. 1 (C1), 0.1 M Na_2SO_4, 0.01 M borate buffer, *p*H 9; control no. 2 (C2), ρ = 1.50 g ml^{-1} Cs_2SO_4, 0.01 M borate; samples no. 1, 2, and 3 (S1, S2, and S3), the same as C2 but with $HgCl_2$ added to r_f = 0.2, 0.4, 0.6, respectively. After standing at room temperature for 2 days, the solutions were dialyzed against three changes of 1 M NaCl, 0.01 M Tris buffer, *p*H 7, at 4°, and then assayed. The relative biological activities of the several samples in two duplicate assays were: C1, 1.00, 1.00; C2, 0.29, 0.45; S1, 0.72, 0.67; S2, 1.05, 0.97; S3, 0.56, 0.84.

The results for the several experiments show somewhat more variability than that encountered by experienced workers in the field. This is possibly due to our inexperience with the techniques necessary to avoid contamination and inactivation of the DNA's. Nevertheless, the results rather clearly indicate that the addition and subsequent removal of mercuric ion has no deleterious effects on the biological activity of the DNA's studied.

(Professor David Hogness has pointed out that some of the variability in the λdg assays may be due to a certain amount of circle formation or end-to-end aggregation of the λDNA through linkage by its cohesive sites on storage, either in the Cs_2SO_4 solution or the 1 M NaCl solution. It is known that the λDNA circles are inactive in this assay (D. Hogness, A. D. Kaiser, and R. B. Inman, private communications).)

Krueger and Baldwin (1934) report that staphylococcus phage K is completely inactivated after standing for a few days in 2.8% $HgCl_2$ (~0.1 M). The phages are completely reactivated by treatment with H_2S. We do not know that the $HgCl_2$ actually penetrated the phage coat and complexed the DNA. We suspect however that it did, because it has been observed in our laboratory that $HgCl_2$ reacts with T4 and ϕX 174 phages to give the expected shifts in absorption spectra for quantitative reaction with the DNA. The reactions take about 20 minutes. The mercury can be removed by addition of excess chloride. Electron microscopy revealed that intact phages were still present, but no biological assays were performed. Other examples of the inactivation of phages by $HgCl_2$ are reported by Adams (1959), but reversibility studies were not made.

Thus all the evidence indicates that the addition and removal of mercuric ion causes no denaturation, depurination, or other deleterious effects on DNA. We feel, however, that in view of the limited number of studies which have been made it would be prudent for investigators applying the Hg(II), Cs_2SO_4 technique to biologically active DNA's to run suitable tests of their own to examine this point.

Discussion

The discussion logically falls into two parts: a consideration of the ultracentrifuge results and a discussion of the structure of the complexes and the interpretation of the equilibrium binding and proton displacement measurements.

The ultracentrifuge separations, although probably the most important contribution of this work, do not require much comment. The separation between the two halves of λb^+ DNA in Figure 5e for $r_f = 0.3$ is 0.057 density unit; in CsCl it is 0.009 (Hershey and Burgi, 1965). The value of α (where $d\rho/dr = \alpha\omega^2 r$) is 1.40×10^{-5} for Cs_2SO_4 and 8.4×10^{-10} for CsCl. Thus the separation between these two DNA's is six times as large in density units and four times in distance at a given ω for the Hg^{2+}, Cs_2SO_4 system as for the CsCl system. The bands are usually somewhat broader in Hg^{2+}, Cs_2SO_4 than in CsCl (which may itself be an indication of some unknown heterogeneity), so that the improvement in separation factor is not quite as good as suggested by the above numbers. The important practical argument seems to be that in the two cases we have studied (crab dAT and λdg halves), the Hg, Cs_2SO_4 procedure has proved advantageous. Hershey *et al.* (1965) have recently described a partial preparative separation of the two halves of λb^+ in CsCl using an angle rotor for generating a shallower density gradient and thus increasing the distance of separation of the two bands. The same trick could be used with Hg, Cs_2SO_4 centrifugation.

All of the centrifuge investigations reported here were made in a borate buffer at *p*H 9.0. Some preliminary investigations were made in cacodylate buffer at *p*H = 6.2–7.0. Buoyant density increases were observed, but the results were not reproducible; multiple bands and bands which migrated with time were sometimes observed. The reasons for this are not known.

At *p*H 9, with two protons released per mercury ion added to DNA, the equilibrium

$$Hg(OH)_2 + DNA \rightleftarrows Hg(DNA-2H^+) \quad (11)$$

is *p*H independent. If one were to add sufficient bromide or chloride (X^-) to reverse the binding reaction, the effective reaction would be

$$HgX_{y+2}^{y-} + DNA \rightleftarrows Hg(DNA\text{-}2H^+) + 2H^+ + (y+2)X^- \quad (2 \leq y \leq 4) \quad (12)$$

Thus the degree of binding could be controlled by controlling both *p*H and X^- concentration.

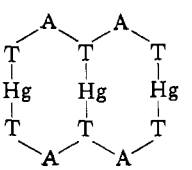
We may ask whether the value of the buoyant density change on binding Hg^{2+} is reasonable. This question is complicated because of the change in degree of hydration of DNA with water activity (Hearst and Vinograd, 1961) and therefore with Cs_2SO_4 concentration. The buoyant densities of T4 DNA in Cs_2SO_4 at $r_b = 0$ and 0.3 are 1.443 and 1.566 (Figure 4). According to Hearst and Vinograd (1961), the corresponding water activities are 0.935 and 0.916, and the hydration of DNA is 19.0 and 14.4 moles of water per DNA-P, respectively. We take 472 daltons for the residue weight of cesium T4 DNA and 2.12 for its dry density (Hearst, 1962). If the hydration of mercurated DNA ($r_b = 0.3$) is taken as 14.4, we may calculate the molar volume increase per mercury, ΔV, due to binding mercury from the equation

$$\rho = \frac{472 + 201r_b + 18(14.4)}{(472/2.12) + r_b\Delta V + 18(14.4)} \quad (13)$$

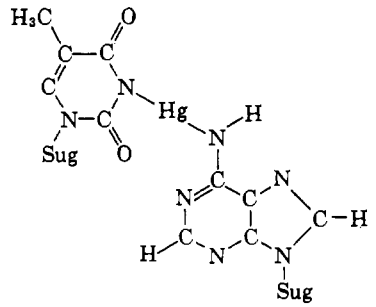
The result is $\Delta V = 11$ cc mole^{-1}. This result is reasonable (the molar volume of elemental mercury is 14.8 cc).

The binding curves in Figure 2 reveal not only that DNA has a high affinity for Hg^{2+} but that the binding is a cooperative phenomenon. For crab dAT, the change in $p(Hg^{2+})$ between one-third and two-thirds saturation of the binding sites ($r_b = 0.067$ to $r_b = 0.134$) is 0.16; for *M. lysodeikticus* DNA, the $\Delta p(Hg^{2+})$ between one-third and two-thirds saturation of the sites ($r_b = 0.167$ to $r_b = 0.333$) is 0.19. For calf thymus DNA, $\Delta p(Hg^{2+}) = 0.64$. For homogeneous, independent binding sites, one expects $\Delta p(Hg^{2+}) = 0.60$. It is clear that when one mercury ion enters the DNA helix of dAT or *M. lysodeikticus* DNA, the affinity for the next mercury ion is increased. The same may well be true for calf thymus DNA; the width of the titration curve may be mainly due to base composition heterogeneity.

The proton release data and the saturation of the binding curve at $r_b = 0.20$–0.21, as well as the steepness of the binding curves, for synthetic and crab dAT are strong evidence for the structure proposed by Katz (1963) for this complex. According to this theory, the formation of the mercury complex requires a chain slippage; the first stage in the complexing of dAT by Hg^{2+} is complete with the formation of the structure



The Yamane and Davidson (1961) results at *p*H 5.6 for the natural DNA's are that the number of protons released per mercury ion bound, $\Delta H^+/\Delta r_b$, decreases from 1.5–2.0 for $r_b = 0$ to $\Delta H^+/\Delta r_b \approx 1$ for $r_b = 0.5$. Katz proposed that for the DNA's, too, the first reaction was chain slippage with formation of T-Hg-T dimers; other cross-linking reactions, with and without proton displacement, followed when all the available T,T sites were complexed. At *p*H 9, two protons are displaced uniformly and the binding curves of Figure 2 seem to represent a single process. A possible alternative to the chain slippage mechanism is one in which the mercury ions cross-link the original complementary base pairs to form structures like



Note that a hydrogen has been displaced from the amino group of adenine as well as from the NH group of thymine. It is known that silver and mercury can displace hydrogens of the amino groups of purines and pyrimidines (Simpson, 1964; Gillen, *et al.*, 1964). A similar structure is possible for a GC-base pair. This structure (as well as Katz's) is consistent with the decreased viscosity and the retention of hypochromicity by mercurated DNA, as well as the reversibility of the reaction. We are not aware of any reliable method for deducing the relative amounts of mercuration by chain slippage and by the mechanism proposed above (at *p*H 9).

With denatured DNA, we suppose that base-mercury-base bonds are formed in a less specific way. A large number of such cross-linked structures are possible, and it is probable that many metastable products are formed, rather than the most stable equilibrium state.

Acknowledgments

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