Binding Kinetics of Mercury(II) to Polyribonucleotides†

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ABSTRACT: Kinetic studies of the interaction of Hg(II) with polyribonucleotides have been used to investigate structural fluctuations of the bases in nucleic acids. The reaction of Hg(II) with poly(A) · poly(U) occurs in two phases which differ in time scale by a factor of about 100. The slow phase is first order and exhibits cooperativity or autocatalytic kinetics. The rate is found to increase as decreasing chain length of poly(U) is used to make the double helical complex. The reaction appears to initiate at the ends of poly(U) strands and may be associated with a molecular rearrangement which results in strand separation with Hg(II) being linked only to uridine. The fast reaction phase is second order and shows little cooperative behavior. Protons are released at this stage indicating alteration of the double helix. The measured second-order rate constant is nearly three orders of magnitude smaller than that found for poly(U) alone. This rate difference suggests that the reactive sites are blocked by double helix formation, and become available for reaction with Hg(II) only through a structural fluctuation. The ratio of rate constants for the reaction of Hg(II) with poly(U) and poly(A) · poly(U) was used to place an upper limit on the equilibrium constant for the structural fluctuation of 2×10^{-3} at 15° and 0.5 *M* $NaClO_4$. The heat of the "breathing" reaction can be estimated to be ~9 kcal/mol from comparison of the temperature coefficient of the reaction with poly(U) to that with poly(A) · poly(U).

The study of the interaction of metal ions with polynucleotides provides structural and biological information about the macromolecules. The effects of metals are diverse. Some stabilize the secondary and tertiary macromolecular structure (Shack et al., 1953) while others destabilize it (Eichhorn, 1962). Others cause decomposition of the primary structure (Barmann et al., 1954). Most metals, such as magnesium(II), manganese(II), and calcium(II), react with the negatively charged phosphoryl groups of polynucleotides (Shack and Bynum, 1959); but copper(II) (Eichhorn and Clark, 1966), silver(I) (Yamane and Davidson, 1962a), and mercury(II) (Yamane and Davidson, 1961) interact to some extent with the nitrogen bases. Mercury(II) provides a better model of base interaction than the other metals since it forms only two complexes, with the first being saturated before the second is initiated (Yamane and Davidson, 1961; Thomas, 1954). The results with mercury may thus be helpful in further elucidating the mechanism of interaction of other metals with the bases.

The study of mercury(II) binding to polynucleotides was initiated by Katz when he observed a drastic decrease in the viscosity of DNA when complexed by mercury(II) (Katz, 1952). The reaction has been found to be completely reversible by a variety of techniques (Katz, 1952; Yamane and Davidson, 1961; Thomas, 1954; Pour-El, 1960) when an excess of a sufficiently strong mercury complexing agent is added. It has been studied spectrally with a large selection of DNAs and RNAs as well as with natural and synthetic polynucleotides, the latter being of particular interest in the work reported here.

All existing evidence indicates that, at the pH's used in the present study, mercury(II) binds to the purine and pyrimidine bases of polynucleotides rather than to the phosphates (Yamane and Davidson, 1961), with N_3 of thymine or uridine being the site with highest affinity for mercury (Simpson, 1964; Ferreira et al., 1961; Eichhorn and Clark, 1963; Mansy et al., 1974; Kosturko et al., 1974). In double-stranded polynucleotides, the final binding site is thought to be the H-bond position, bridging the two strands (Yamane and Davidson, 1961, 1962b; Gruenwedel and Davidson, 1967, 1968). However, the binding of mercury(II) to poly(A) · poly(U) may result in chain separation (Kawade, 1963).

If mercury reacts with N_3 of uridine, the rate of the reaction may be limited by a structural fluctuation of the polynucleotide which exposes the H-bonding nitrogen. Such a "breathing reaction" has been suggested as being required in the biological role of DNA for recognition of its base-pairing characteristics. There is evidence from the study of the intercalation of dyes (Lerman, 1961; Li and Crothers, 1969) and tritium exchange of the hydrogen bonded protons (Von Hippel and Printz, 1965) that structural alterations do occur. In the present study, the kinetics of the binding of mercury have been used to estimate the equilibrium constant and the heat of the "breathing reaction".

Materials and Methods

Poly(U) was purchased from Sigma Chemical Co. and poly(A) from Schwartz Biochemicals. Oligo(U) of estimated chain length 15–20 was obtained from pooled fractions of a TEAE-cellulose fractionation of a partial alkaline hydrolysis of poly(U). All solutions were first dialyzed against 0.5 *M* sodium perchlorate plus ethylenedinitrilotetraacetate and then dialyzed exhaustively into the appropriate buffer. The concentrations of the polynucleotides were calculated using the following extinction coefficients at neutral pH: poly(A), 9.5×10^3 M^{-1} cm^{-1}, at 261 nm (Krakauer, 1968), poly(U), 10.4×10^3 M^{-1} cm^{-1} at 257 nm (Krakauer, 1968), and poly(A) · poly(U) 6.3×10^3 M^{-1} cm^{-1}

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at 257 nm (Yamane and Davidson, 1962). All other chemicals were reagent grade. A Beckman Model DU-2 spectrophotometer and a Cary 14 were used for ultraviolet absorbance measurements.

Preparation of Double-Stranded Polyribonucleotides. Equimolar solutions of poly(A) and poly(U) (or oligo(U)) were combined, heated in an 80° water bath for 2 min, and allowed to anneal slowly at room temperature. The melting curve as monitored at 280 and 260 nm verified that the complex formed was poly(A) · poly(U) and not poly(A) · 2poly(U) under these conditions (Stevens and Felsenfeld, 1964). The optical density of the solution was always recorded before and after any alteration of pH since a change in absorbance would imply a structural change.

Equilibrium Binding of Mercury(II) by Polyribonucleotides. The reaction of Hg(II) with poly(A) · poly(U) may be described as follows

$$\text{poly(A)} \cdot \text{poly(U)} + HgX_2 \rightleftarrows \text{poly(A)} \cdot \text{poly(U)–Hg(II)} + nH^+ + 2X^-$$

where "n" is the number of protons released per mole of mercury bound to 1 mol of polyribonucleotide phosphate and "X^-" is the leaving ligand. Previously, Yamane and Davidson reported that their spectral results, viscosity studies, and proton release determinations were independent of the leaving ligand, suggesting that the complexing species with DNA is Hg^{2+} and not HgX^+ (Yamane and Davidson, 1961).

Since the binding of mercury is accompanied by a spectral shift of the absorbance of the polyribonucleotide, the magnitude of which is a function of the degree of mercuration (Yamane and Davidson, 1962; Kawade, 1963), ultraviolet absorption may be used for studying the formation of the complex.

The experiments were conducted on the DU-2 spectrophotometer at room temperature with λ_1 290 nm and λ_2 260 nm. The solutions were prepared in 0.5 M $NaClO_4$ (pH 5.6) and 0.5 M $NaClO_4$–0.01 M phosphate buffer (pH 7.0); 2 ml of the polyribonucleotide solutions was placed in a quartz cuvet containing a small Teflon stirring bar. Mercuric acetate, 2.50×10^{-3} M in H_2O, was added in microliter quantities with each addition being sufficient to change "r_b", the ratio of moles Hg(II) bound per mole of polyribonucleotide, by 0.01–0.02. Since the binding is very tight, essentially all mercury added is bound in solution of low chloride content. During each addition, a magnetic stirrer was used to ensure complete mixing. The optical density was recorded for $0 < r_b < 0.5$. The reaction was reversed by the addition of 0.5–20.0-μl quantities of 1 M NaCl and the solutions were permitted to stand until no further changes of absorbance occurred.

The following expression describes "r_b" as a function of chloride concentration:

$$r_b = ([Hg]_T - [Hg_{T,Cl}] - [HgClOH] - [Hg(OH)_2])/[\text{poly(A)}\cdot\text{poly(U)}]_T =$$

$$\left([Hg]_T - [Hg_{T,Cl}] - \frac{[HgCl^+][OH^-]}{[Cl^-]}K_{HgClOH} - \frac{[Hg^{2+}]}{[H^+]^2}K_{Hg(OH)_2}\right)/[\text{poly(A)}\cdot\text{poly(U)}]_T$$

where the subscript "T" indicates the total concentration in solution, and $[Hg_{T,Cl}]$ represents the total concentration of mercury bound to chloride. The last two terms in the numerator may be neglected at the pH's and chloride concentrations in these studies when Partridge's values for K_{HgClOH} and $K_{Hg(OH)_2}$ are used (Partridge et al., 1965).

The concentration of free mercuric ion is given by:

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

where formation constants for $HgCl^+$, $HgCl_2$, $HgCl_3^-$, and $HgCl_4^{2-}$ are $\beta_1 = 10^{6.74}$, $\beta_2 = 10^{13.22}$, $\beta_3 = 10^{14.17}$, $\beta_4 = 10^{15.22}$, respectively (Sillen and Martell, 1964).

Thus, $r_b = (Hg_T - Hg_{T,Cl})/[\text{polynucleotide}]_T$ and

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

The binding isotherm of mercury(II) interaction with the polyribonucleotide may be displayed in a plot of "r_b" vs. the negative logarithm of free mercury in solution.

Stopped-Flow Experiments. The rate of reaction of mercuric chloride with polyribonucleotide was followed on a stopped-flow apparatus designed by Sturtevant (Sturtevant, 1964). The rate was monitored both by change of ultraviolet absorbance at 290 nm and by proton release using Chlorophenol Red (CPR)[1] as an indicator. CPR was chosen as the indicator since over a period of several days it formed no detectable complex with poly(A) · poly(U) as monitored by ultraviolet and visible spectroscopy. Since the indicator is negatively charged, it is not expected to associate with the phosphate on the poly(A) · poly(U) backbone, and, as mentioned above, no interaction with the bases was detected. Furthermore, it was presumed that CPR would complex only weakly, if at all, with mercury.

Determination of Kinetic Difference Spectra. The wavelength dependence of the change of absorbance for the reaction of mercury with poly(A) · poly(U) was determined on the Cary 14 spectrophotometer. Sufficient mercuric acetate (10^{-3} M) was mixed with a solution of polyribonucleotide (8.5×10^{-5} M, in 0.01 M phosphate buffer (pH 6.5) with 0.25 M $NaClO_4$) for a ratio of 0.25 mol of Hg^{2+}/mol of phosphate. The reaction was then monitored as a function of time at 25° in the wavelength range 255–285 nm in 5-nm steps. The wavelength dependence was examined at pH 6.5 so that the results would not be complicated by possible formation of the poly(A) acid double helix.

Results

Equilibrium Binding of Mercury(II) to Polynucleotides. The equilibrium spectral changes on binding Hg(II) to poly(A), poly(U), and poly(A) · poly(U) at pH 5.6 and pH 7.0 were found to be qualitatively the same as reported previously (Yamane and Davidson, 1962b; Kawade, 1963). The binding of Hg(II) to poly(A) is accompanied by a small decrease in absorbance at 260 nm and a large increase at 290 nm, while the binding of Hg(II) to poly(U) is accompanied by a large decrease at 260 nm and a small increase at 290 nm. Figures 1 and 2 display very close titration points for the binding of Hg(II) to poly(A) · poly(U). The early increments for $0 \leq r \leq 0.05$ suggest mercury may initially seek the single-stranded regions where there is slight mismatch of poly(A) · poly(U). The region from 0.05 $\leq r \leq$ 0.25 is accompanied by a large hyperchromism at

[1] Abbreviation used is: CPR, Chlorophenol Red.

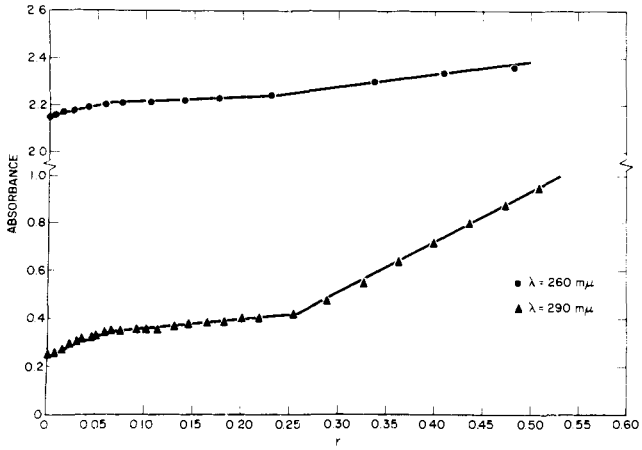

FIGURE 1: Change in absorbance of poly(A) · poly(U) (0.347 m*M* nucleotide phosphate) as a function of the ratio, "*r*", the moles of mercury(II) added/mole of polyribonucleotide phosphate, pH 5.6, 500 m*M* $NaClO_4$.

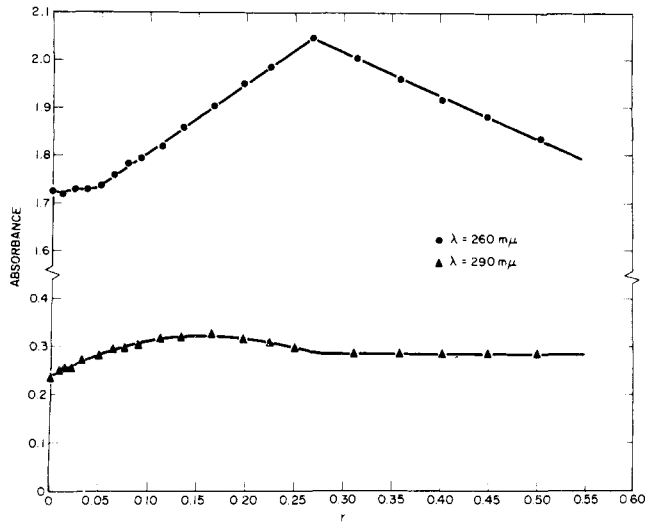

FIGURE 2: Change in absorbance of poly(A) · poly(U) (0.279 m*M* nucleotide phosphate) as a function of "*r*", pH 7.0, 500 m*M* $NaClO_4$, 10 m*M* phosphate buffer.

260 nm in spite of the intrinsic decrease of absorption of both poly(A) and poly(U) upon complexing with mercury (Yamane and Davidson, 1962b; Kawade, 1963). Similar ultraviolet spectral changes occur upon reaction of Hg(II) with poly(A) · oligo(U). Kawade (1963) suggested that at pH 7.0 the hyperchromicity may indicate extensive breakdown of the double helix with possible chain separation. The results at pH 5.6 can be interpreted in the same manner since the anticipated hyperchromism upon binding of Hg(II) to poly(A) · poly(U) would be decreased by formation of the acid double helix of poly(A) upon chain separation (Adler et al., 1969).

It is evident from the binding isotherm in Figure 3 that poly(A) · poly(U) has high affinity for Hg(II). The change between one-third and two-thirds saturation of binding sites is $\Delta p Hg^{2+} = 0.11$; thus, the reaction is cooperative. The binding of Hg(II) to crab dAT is also cooperative (Nandi et al., 1965).

Stopped-Flow Kinetic Studies on the Rapid Phase. (a) RATE AS FUNCTION OF CONCENTRATION OF POLYRIBONUCLEOTIDE. The rate of reaction of mercuric chloride with poly(A), poly(U), and poly(A) · poly(U) was

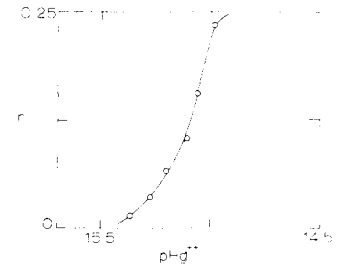

FIGURE 3: Moles of mercury(II) bound per mole of polyribonucleotide, "r_b", as a function of the negative logarithm of free mercury in solution. Conditions: 10 m*M* phosphate buffer, 250 m*M* $NaClO_4$, pH 6.5, temperature, 25°.

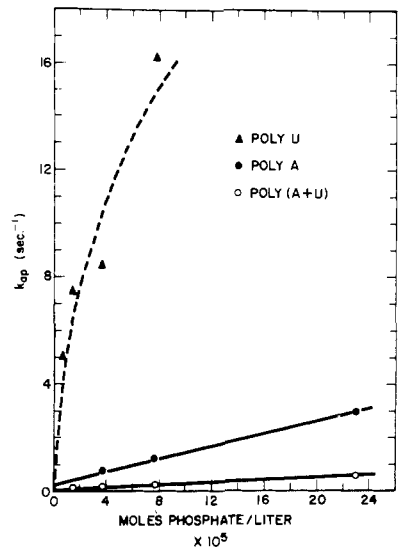

FIGURE 4: Rate of reaction of mercuric chloride with poly(U), poly(A), and poly(A) · poly(U). Conditions: 500 m*M* $NaClO_4$, 15°, 0.05 m*M* indicator, pH 5.6 for poly(U) and poly(A) · poly(U), and increased to pH 6.2 for poly(A) to avoid formation of the acid double helix. No more than 10% of the indicator reacted with H^+ in any experiment. (The presence of poly(A) significantly buffers the proton release.)

followed on the stopped-flow apparatus. The experiments were performed at 15°, pH 5.6, so the results could be compared to those obtained previously with calf thymus DNA (Williams, 1968). In the ultraviolet range, the rapid phase of the reaction with poly(A) · poly(U) and poly(U) was found to be the same in the presence and absence of indicator, CPR, and the rate of proton release exactly corresponded to the rate of change in uv absorption. Thus, proton release and ultraviolet absorption could be used interchangeably for following the course of the reaction. The indicator was employed for low polyribonucleotide concentrations since the color effect is greater than the ultraviolet absorbance change.

Typical kinetic curves for the reaction of poly(A) · poly(U) with mercury were followed over about 30 sec from mixing. The logarithm of the change in absorbance was plotted as a function of time, and found to be linear for over 90% of the reaction, implying apparent first-order kinetics with the first-order rate constant given by the slope of the line. As shown in Figure 4, when the rate constant was determined for a series of concentrations of polyribonucleotide it was found to be dependent on concentration. Thus, the linearity of the first-order plot is a result of "apparent" first-order kinetics, even though the concentration of polyribonucleotide phosphate was only four times that of mercu-

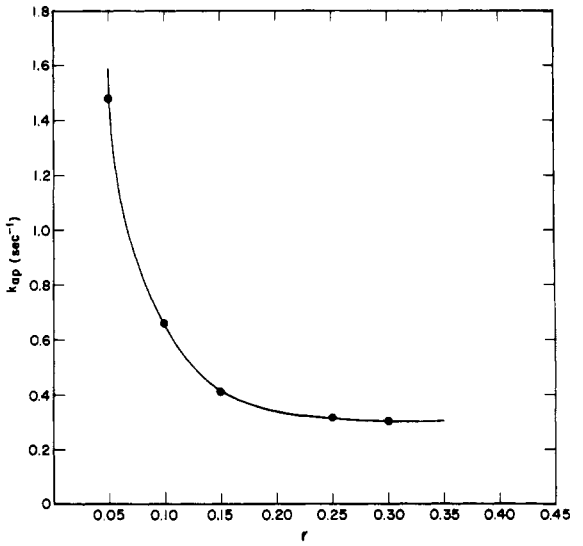

FIGURE 5: Dependence of the apparent first-order rate constant on ratio of moles of mercury added per mole of poly(A) · poly(U). Conditions: [poly(A) · poly(U)] = 0.1 mM, temperature, 15°, 500 mM $NaClO_4$, 0.05 mM indicator, pH 5.6.

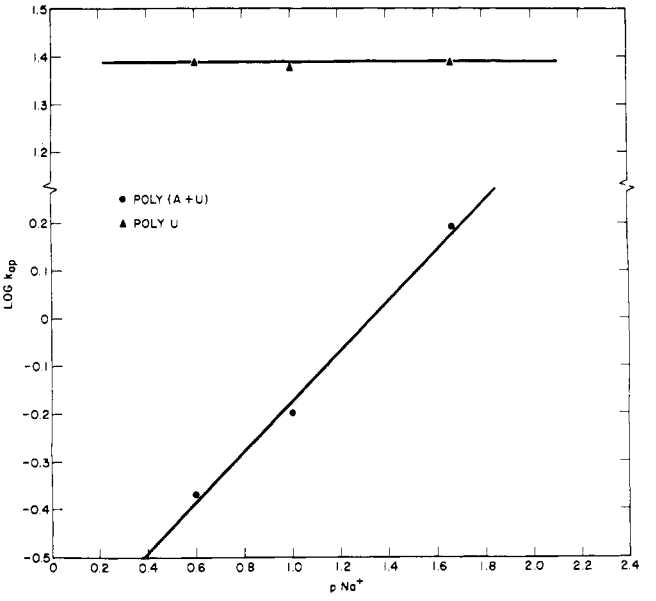

FIGURE 6: Logarithm of the apparent first-order rate constant as a function of sodium perchlorate concentration. Conditions: [poly(A) · poly(U)] = 0.1 mM, [poly(U)] = 0.1 mM, r = 0.25, [indicator] = 0.05 mM, pH 5.6, λ 573 nm, temperature, 15°.

ry. The rate constant determined in this manner will be referred to throughout the present study as "k_{ap}" where

$$k_{ap} = k_{2nd\ order}c$$

and "c" is the concentration of the polyribonucleotide in moles of phosphate. The relative rates of reaction were poly(U) > poly(A) > poly(A) · poly(U) with second-order rate constants of 1.4×10^6, 1.2×10^5, and 2.7×10^3 M^{-1} sec^{-1}, respectively. The second-order rate for poly(U) was determined from a tangent drawn to the initial slope of the curve shown in Figure 4. A double reciprocal plot of the poly(U) data yielded $k_{2nd\ order} = 1.1 \times 10^6$ M^{-1} sec−1. As reflected in these rate constants, the single-stranded polyribonucleotides react more rapidly with Hg(II) than does the double helix, poly(A) · poly(U).

The curvature exhibited in the data for poly(U) seems to be characteristic of mercury reactions and cannot now be explained. Similar behavior is observed in the reaction of Hg(II) with thymidine, and in the hydrolysis of mercuric chloride (Williams, 1968).

(b) RATE AS FUNCTION OF RATIO OF MERCURY(II) TO POLY(A) · POLY(U). As the ratio of moles of Hg(II) to moles of poly(A) · poly(U) phosphate is increased, the rate of reaction of mercuric chloride with poly(A) · poly(U) decreases (Figure 5). At low ratios of mercuric chloride, the rate approaches that of reaction with poly(U). The equilibrium binding curves suggest that mercury may seek the unbonded bases at the ends of the poly(A) · poly(U) or possibly unbonded interior loops. The kinetic data support this conclusion since at low binding ratios the rate approaches that of binding to single-stranded polyribonucleotides.

(c) RATE AS FUNCTION OF THE MOLECULAR WEIGHT OF POLY(U). In order to verify that end effects were not dominating the reaction, the binding of mercury was examined using poly(A) · oligo(U). The rate of the reaction was essentially independent of the chain length of poly(U). There was only a 25% increase in the fast initial rate going from high molecular weight poly(U) to oligo(U). Hence this phase of the reaction exhibits little cooperativity, as judged by the absence of kinetic end effects or autocatalytic kinetics.

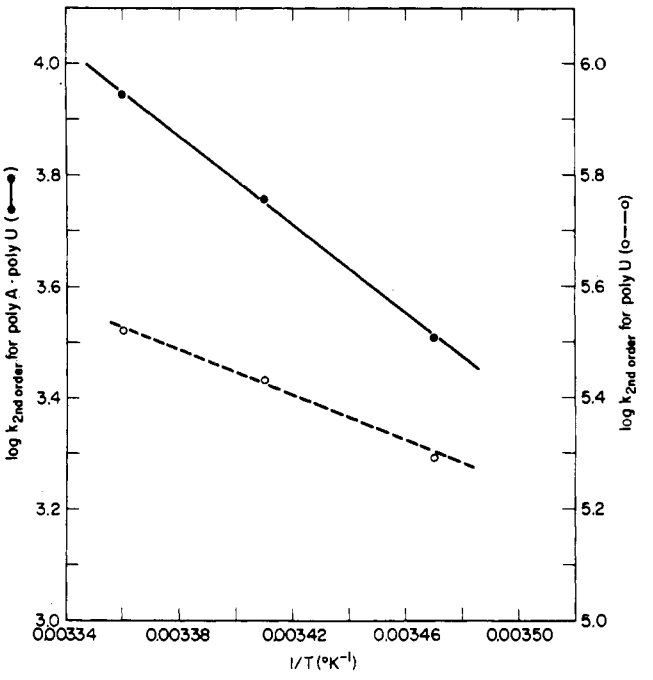

FIGURE 7: Dependence of the second-order rate constant on temperature for reaction of poly(A) · poly(U) and poly(U) with mercuric chloride. Conditions: [poly(A) · poly(U)] = 0.1 mM, r = 0.25, [indicator] = 0.05 mM, 500 mM $NaClO_4$, pH 5.6, λ 573 nm.

(d) RATE AS FUNCTION OF IONIC STRENGTH. The apparent rate constant for the reaction of mercuric chloride with poly(A) · poly(U) decreases as the sodium perchlorate concentration increases, but the apparent rate constant for reaction with poly(U) is independent of the ionic strength of the solution (Figure 6).

(e) RATE AS FUNCTION OF TEMPERATURE. The data in Figure 7 show the dependence of the second-order rate constant on temperature. The activation energy can be determined from the Arrhenius equation: $\ln k = -E_a/RT + C$, or E_a = 18.1 and 9.2 kcal/mol for the reaction with poly(A) · poly(U) and poly(U), respectively. Thus, the

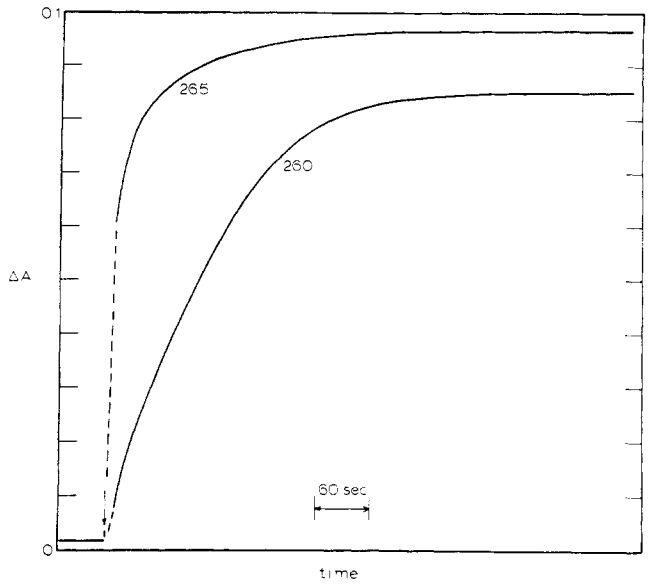

FIGURE 8: Kinetic curves for the reaction of mercuric acetate with poly(A) · poly(U) as monitored at 260 and 265 nm. Conditions: [poly(A) · poly(U)] = 0.0865 m*M* in same buffer as Figure 3.

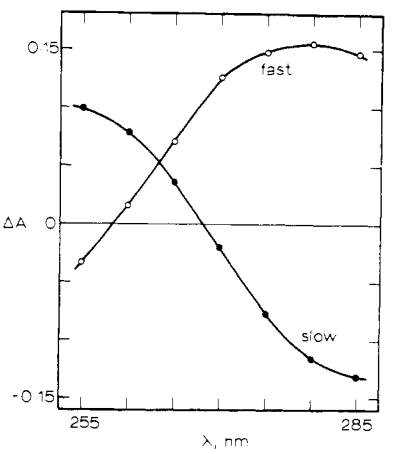

FIGURE 9: Kinetic difference spectrum for binding of mercuric acetate to poly(A) · poly(U), r = 0.25. Conditions same as in Figure 8.

reaction rate of Hg(II) with double helix is more sensitive to both temperature and ionic strength than the rate of Hg(II) with poly(U).

Kinetic Difference Spectra and Analysis of the Slow Reaction Phase. Although the equilibrium binding of Hg(II) to poly(A) · poly(U) exhibits cooperatively, this is not reflected in the rapid phase of the reaction. However, there are slow absorbance changes which are not accompanied by proton release, as shown by the absence of any indicator color change occurring at the slow rate. Hence a careful investigation was made of the slow phase, using the uv absorbance changes. For this study the pH was increased to pH 6.5 to eliminate the added complication of the formation of the acid double helix of poly(A) if chain separation should occur.[2] A fast reaction phase closely analogous to that studied carefully at pH 5.6 was also found at pH 6.5.

When Hg(II) is added to a solution of poly(A) · poly(U) and the time course of the reaction monitored at various wavelengths, there is an initial rapid phase followed by a slow phase. The relative proportions of these effects are functions of the wavelength. The kinetic curves exhibited in Figure 8 were recorded at 260 and 265 nm. The arrow indicates the time mixing was initiated and the dotted line the estimated absorbance change during mixing. The slow phase was extrapolated to "t = 0" in order to estimate the relative proportions of the effects, which are shown in Figure 9. In Figure 10 the logarithm of the absorbance change as a function of time is shown for the poly(A) · poly(U)–Hg(II) reaction at 275 nm where the slow and fast phases have opposing optical density changes. Curve A exhibits the rate of the slow phase when the poly(U) is of high molecular weight (s_{20} = 5.49 S) and curve B the rate with oligo(U). While the slow phase is not a simple first-order reaction, the rate is clearly a function of the molecular weight of the poly(U). In the linear region of Figure 10, the rate of reaction with poly(A) · oligo(U) is approximately five times the rate with poly(A) · poly(U). Although the slow phase is

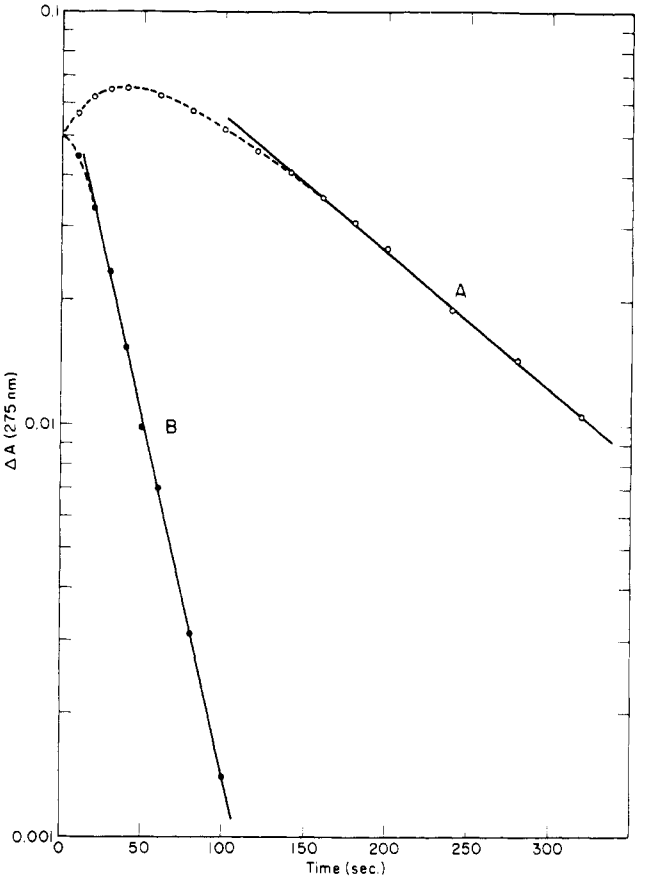

FIGURE 10: Dependence of the slow reaction on the molecular weight of poly(U). Curve A, [poly(A) · poly(U)] = 0.0868 m*M*, [poly(A) · oligo(U)] = 0.0839 m*M*, 250 m*M* $NaClO_4$, 3.3 m*M* phosphate buffer, pH 6.5, temperature, 25°, λ 275 nm, r = 0.25.

dependent on the molecular weight of poly(U), it is independent of the concentration of poly(A) · poly(U) at a constant ratio of Hg(II) to polyribonucleotides which indicates the slow phase is first order. The slow phase is also a function of the ratio of Hg(II) to polyribonucleotide with the rate increasing at low ratios (Figure 11) in a manner analogous to that observed in the rapid phase of the reaction.

Discussion

The experiments reported here reveal several general features of the reaction of Hg(II) with poly(A) · poly(U). In

[2] Mercury(II) binding to poly(A) was performed at slightly higher pH (pH 6.2 as opposed to pH 5.6) so that poly(A) would not be in the form of a double stranded helix (Krakauer and Sturtevant, 1968).

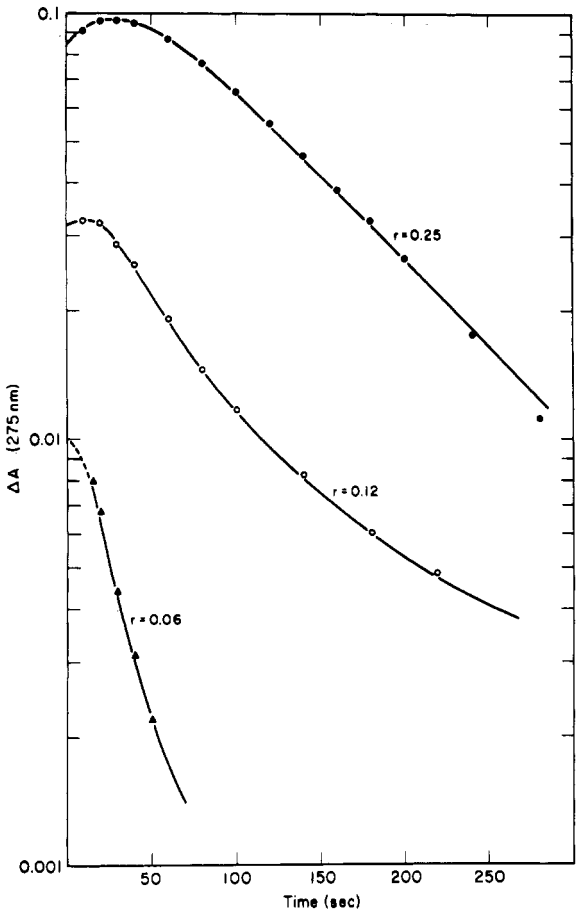

FIGURE 11: Dependence of the slow reaction on the ratio of moles of mercury(II) added/mole of poly(A) · poly(U). [poly(A) · poly(U)] = 0.123 m*M*, conditions same as in Figure 10.

summary, these are the following. (1) The equilibrium binding reaction is cooperative. (2) The kinetic studies show two main reaction phases, differing by about two orders of magnitude in time scale under the conditions used. (3) The fast reaction rate is linearly concentration dependent and hence second order. All proton release occurs in the fast phase, and there is no evidence that this phase is cooperative. (4) The slow reaction phase is concentration independent and hence first order. Since the reaction rate is considerably faster with poly(A) · oligo(U) it must initiate preferentially at oligo(U) chain ends and hence has cooperative features. (5) The double helix poly(A) · poly(U) reacts much more slowly than the constituent polynucleotides, and its reaction rate is more sensitive to ionic strength and temperature than that of the single strands.

These observations imply a reaction mechanism with the following general properties. (1) The initial (fast) phase of the reaction requires a structural fluctuation or "breathing" of the double helix in order to make available the reactive sites blocked by double helix formation. However, since the fast phase is second order, the rate of "breathing" cannot be limiting. (2) Since all proton release occurs in the fast step, both Hg(II) ligand sites must react at that stage. (3) In the rapidly formed complex the presence of Hg(II) at one site does not appreciably increase the reactivity of adjacent nucleotides, since the fast reaction shows no cooperative or autocatalytic features. (4) The slow phase involves a cooperative, first-order structural rearrangement, possibly including substitutions in the ligands bound to Hg(II). (5) Since N_3 of uridine is the preferred site of reaction at both pH 5.5

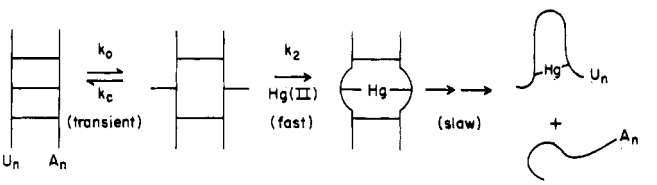

FIGURE 12: Proposed mechanism for reaction of Hg^{2+} with poly(A) · poly(U).

and pH 7 (Simpson, 1964; Nandi et al., 1965; Mansy et al., 1974), the final reaction product (at $r_b < 0.25$) probably involves Hg(II) bound primarily to poly(U). The other possible reaction sites, which may well be occupied transiently, are the adenosine amino group or N_1; there is no evidence for binding to N_7 (Simpson, 1964).

A general mechanism which includes these features is that shown in Figure 12. The first step is transient opening of the double helix. Treating the opened form as a steady-state intermediate gives

$$k_{2\text{nd order}} = k_2 k_0/(k_c + k_2[\text{Hg(II)}])$$

Since $k_{2\text{nd order}}$ was found to be independent of Hg(II) concentration (at constant r_b), k_c must be much greater than k_2[Hg(II)], and therefore

$$k_{2\text{nd order}} = K_{\text{open}} k_2$$

where k_2 is the reaction rate constant with unbonded bases, and K_{open} is the equilibrium constant for the transient opening of a base pair. K_{open} can thus be calculated from the ratio of the second-order rate constant for poly(A) · poly(U) to the rate constant for poly(U). (The poly(U) rate constant is chosen because it is much larger than that for poly(A) and should offer the most rapid path for initial reaction of an opened base pair.)

The mechanism proposed can be considered reliable only if K_{open} calculated in this way has reasonable properties. Our results show $K_{\text{open}} = 2.7 \times 10^3/1.4 \times 10^6 = 2 \times 10^{-3}$ at 15°, 0.5 *M* $NaClO_4$ (Figure 5). The value estimated by Gralla and Crothers (1973) from experiments on model oligonucleotides containing mismatched bases is 1.2×10^{-3} at 25°, 1 *M* Na^+. Correcting the present value for temperature and salt concentration, using the data in Figures 6 and 8, yields 2.4×10^{-3} at 25°, 1 *M* Na^+, which we regard as reasonable agreement considering the errors inherent in both estimates.

The variation of K_{open} with ionic strength and temperature is also consistent with opening a single base pair. The measured heat ΔH_{open} of 9 kcal/mol is reasonable when compared with heats of 5–9 kcal/mol for the average heat of melting all the base pairs in poly(A) · poly(U) (Rawitscher et al., 1963; Krakauer and Sturtevant, 1968).[3] The increase of K_{open} with decreasing sodium ion concentration is also expected by analogy with the known decrease in double helix stability as counterion concentration decreases. A quantitative comparison may be made by the following argument. Let s^{-1} be the equilibrium constant for opening a base pair at the end of a double helix [see, for example, Crothers (1969) for a brief review of the theory of DNA melting]. At T_m the value of s, s_{T_m}, is constant when the Na^+ concentration is varied, so

[3] ΔH_{open} was estimated from the difference of the temperature coefficients of the reaction with poly(U) and poly(A) · poly(U).

$$d \ln s_{T_m} = (\partial \ln s/\partial T)_{Na^+} dT_m + (\partial \ln s/\partial \ln Na^+)_T d \ln Na^+ = 0$$

Setting $(d \ln s/dT)_{Na^+} = (\Delta H/RT^2)$, we obtain

$$\left(\frac{\partial \ln s}{\partial \ln Na^+}\right)_T = \frac{\Delta H}{2.3RT_m^2} \frac{dT_m}{dpNa^+}$$

Using $(dT_m/dpNa^+) = -17°$ (Dove and Davidson, 1962; Schildkraut and Lifson, 1965) and $\Delta H = -8$ kcal at a T_m of 60° (Krakauer and Sturtevant, 1968) we calculate $[d \ln s^{-1}/d \ln (Na^+)]_T = -0.27$. As determined from Figure 6, $[d \ln K_{open}/d \ln (Na^+)] = -0.50$. Thus the quantitative variations of s^{-1} and K_{open} with Na^+ concentration are comparable. The difference (0.23) is probably due to the variation of the loop free energy (Gralla and Crothers, 1973) with salt concentration.

In summary, the properties of K_{open} determined by comparing the rate of reaction of Hg(II) with poly(U) and poly(A) · poly(U) are in agreement with independent estimates of the same quantities. This contrasts with tritium exchange studies of polynucleotide structural fluctuations, which seem to require some novel structural assumptions for interpretation (McConnel and Von Hippel, 1970a,b). The resolution of these differences will require further work. In this context we note that even should the detailed mechanism we propose ultimately require modification, the small ratio of the rate constant for poly(A) · poly(U) to that for poly(U) alone means that the uridine N_3 in poly(A) · poly(U) is available for reaction with Hg(II) *no more* than that same small fraction of the time. In other words, our experiments set an *upper* limit of 2×10^{-3} (15°, 0.5 *M* Na^+) for the fraction of base pairs in poly(A) · poly(U) that are sufficiently open that the U residue can react with Hg(II).

A further conclusion that we can draw concerns the rate of opening the double helix. Since the opening reaction is not rate limiting, the structure must open at a rate that exceeds the fastest observed apparent first-order rate constant, or about 1 sec^{-1} (Figure 5). Hence, $k_0 > 1$ sec^{-1}.

One intriguing question raised by our results is the structure of the product of the fast reaction. Since no further proton release occurs in the slow reaction phase, it is likely that Hg(II) has reacted to displace two protons in the fast phase. Assuming that initial attack occurs at uridine N_3, this would permit structures in which Hg(II) cross-links either a U and an A or two U's. In considering models for such structures, one should keep in mind that the fast reaction does not seem to be cooperative. Hence substitution of Hg(II) at one site should not appreciably increase the reactivity of adjacent residues. It is difficult to see how this could be the case if the double helix were locally disrupted so that two U residues could be bridged. A working hypothesis which we find more appealing is that Hg(II) serves to crossbridge an A and a U, leaving the double helix intact, in the (unstable) product of the fast reaction phase. We built such a model with Hg(II) joining uridine N_3 to the adenine amino group, and found that this structure could be accommodated in the double helix, at least as judged by CPK space filling models.

Finally, this model for the intermediate structure provides a simple picture of the slow reaction phase. The bond joining Hg(II) to A is slowly broken and replaced by a second Hg(II)–U bond, driven by the greater affinity of Hg(II) for U than for A (Simpson, 1964). This process requires disruption of the double helix and should therefore be cooperative. It is reasonable that it should initiate at the ends of oligo(U) chains, peeling the U strand away from its complex with poly(A).

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New Method for Isolation and Sequence Determination of 5′-Terminal Regions of Bacteriophage φX174 in Vitro mRNAs†

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ABSTRACT: We have determined the nucleotide sequences of the 5′-terminal oligonucleotides, produced by RNase T_1 digestion of bacteriophage φX174 mRNAs synthesized in vitro. The major sequences are: pppCpGp(Ap), pppApUpCpGp(Cp), pppAp(Ap)$_2$UpCp(Up)$_2$Gp(Gp), and pppAp(Ap)$_3$UpCp(Up)$_2$Gp(Gp). The sequences of several minor 5′-terminal oligonucleotides were also determined. For this research we have devised a simple isolation procedure, for the 5′-terminal oligonucleotides, based upon hydroxylapatite chromatography and two-dimensional thin-layer separation. This method allows for the rapid and quantitative recovery of all oligonucleotides containing 5′-triphosphate end groups and should be generally useful for sequence on 5′ termini of mRNAs.

Methods allowing the rapid and quantitative recovery of the 5′ terminal oligonucleotides from messenger RNAs are of vital importance to studies on the control of transcription. Present techniques for the isolation of these oligonucleotides involve separation based upon the change in net electric charge after dephosphorylation (Konrad, 1973) or selective digestion of oligonucleotides which are not phosphorylated on the 5′ end (Takeya and Fujisawa, 1974; Sugiura et al., 1969; Smith et al., 1974). These methods tend to be tedious or difficult to reproduce. Based on the observation by Soave et al. (1973), that 5S RNAs can be separated on hydroxylapatite columns according to the degree of phosphorylation at their 5′ ends, we have derived a simple isolation procedure for oligonucleotides from the 5′ ends of in vitro mRNAs transcribed from bacteriophage φX174 RF DNA.

Materials and Methods

Hydroxylapatite (grade HT) was obtained from Bio-Rad Laboratories, DEAE-cellulose (DE-52) was obtained from Whatman. Other materials were the same as described in a previous publication (Smith et al., 1974).

In vitro φX174 mRNAs selectively labeled with α- or γ-^{32}P ribonucleoside triphosphates were prepared as described previously (Smith et al., 1974) from φX174 RFI DNA and *Escherichia coli* K12 RNA polymerase holoenzyme. These α-^{32}P-labeled transcripts range in size from 500 to 1000 nucleotides as judged by sedimentation through dimethyl sulfoxide gradients (Smith et al., 1974). They also hybridize almost exclusively (>97%) to φX174 complementary strand, in agreement with results obtained by Hayashi et al. (1963, 1964) for both in vivo and in vitro φX174 mRNAs.

Digestion with RNase T_1. Aliquots of the ^{32}P-labeled RNA were digested in 0.01 *M* Tris-HCl (pH 7.9)-0.01 *M* EDTA using RNase T_1 to substrate ratio of 1:10 (w/w). Incubation at 37° for 40 min gave complete digestion.

Other enzymatic digestions were performed as described previously (Smith et al., 1974).

Hydroxylapatite Chromatography. Experiments were routinely performed at room temperature, using disposable columns made from Pasteur pipets (0.5 × 5 cm) previously equilibrated with 0.05 *M* potassium phosphate buffer[1] (pH 6.8). Samples (usually 5-10 μg of RNA digest) were applied in digestion buffer (10 m*M* Tris-HCl (pH 7.9)-10 m*M* EDTA) which often contained traces of phenol from the previous extraction step. The presence of phenol or 10 m*M* EDTA did not interfere with absorption and separation on the column. Samples were loaded by gravity and the columns were washed with 1 ml of 50 m*M* KPO_4 buffer. Elution was achieved with 40 ml of a linear gradient of KPO_4 buffer (pH 6.8), between 0.05 and 0.1 *M*, followed by 10 ml of 0.1 *M* KPO_4 buffer (pH 6.8), and a final wash with 10 ml of 0.5 *M* KPO_4 buffer (pH 6.8). A constant effluent flow rate of 20 ml/hr was provided by means of a peristaltic pump (Buchler Instruments). Fractions of approximately 1 ml were collected. The radioactivity content of effluent fractions was measured by applying aliquots of

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[1] Abbreviations used are: KPO_4 buffer, potassium phosphate buffer; $TEAHCO_3$, triethylammonium bicarbonate.