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Binding of Mercury(II) to Poly(dA-dT) Studied by Proton Nuclear Magnetic Resonance†

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ABSTRACT: The binding of Hg(II) to poly(dA-dT) has been examined with proton NMR spectroscopy. Addition of $HgCl_2$ between r (Hg^{2+}/nucleotide) = 0 and 0.25 results in loss of the exchangeable imino N3H resonance of thymine, indicating preferential binding at this site. The nonexchangeable base resonances AH8, AH2, and TH6 shift their intensity downfield in a cooperative manner, indicating complexation which is slow on the NMR time scale and changes in the polymer conformation upon binding. At r = 0.25, the polymer is cross-linked, and an increase in temperature does not result in denaturation of the polymer, as evidenced by the thymine proton resonance chemical shifts. The chemical shifts of the AH2 and T(CH_3)5 base resonances allow some general conclusions to be made about the stereochemistry of this complex.

Metal ions exert a wide range of effects on the structure and biological properties of nucleic acids. These effects are seen to be closely tied to the manner in which the particular ion concerned binds to nucleic acids. [For reviews, see Barton & Lippard (1980) and Marzilli et al. (1980) inter alia.] Of particular interest has been Hg(II) because of its specific binding to the DNA bases as opposed to the more common phosphate backbone binding (Thomas, 1954). This has led to its use as a probe of DNA structure both kinetically (Williams & Crothers, 1975) and statically, as in viral (Katz & Santilli, 1962; Dorne & Hirth, 1970) and chromatin complexes (Simpsom & Sober, 1970; Bryan et al., 1976; Ding & Allen, 1980a). Furthermore, Hg^{2+} has been used to separate DNAs of differing base composition (Nandi et al., 1965; Wang et al., 1965; Davidson et al., 1965), while the related CH_3Hg^+ ion is used as a standard method for denaturing both RNA and DNA (Gruenwedel & Davidson, 1966).

The binding of Hg(II) to DNA is poorly understood at a detailed molecular level. It has been determined that the preferred mononucleoside binding sites are N3 of thymidine and N1 of guanosine, with several weaker sites available in adenosine and cytosine (Simpson, 1964; Eichorn & Clark, 1963). However, these preferences may not pertain in the polymeric situation where stereochemical factors may also be important. Furthermore, there has been little progress in understanding the effects of Hg(II) binding on the local conformation of DNA though it is known to result in a large change in its viscosity (Katz, 1952).

A suitable method for monitoring the effects of metal binding in nucleic acids would appear to be proton NMR spectroscopy, particularly when the sequence is repetitive and individual resonances can be clearly resolved and assigned. This is the case for poly(dA-dT) (Patel & Canuel, 1976), which has also been examined for its Hg(II) binding spectrophotometrically (Yamane & Davidson, 1961; Katz, 1963; Nandi et al., 1965) and so is a good candidate for critical study. This paper reports the analysis of Hg(II) binding to poly(dA-dT) as monitored by proton NMR. Binding at the N3 site of thymidine is confirmed, and we are led to suggest a variant of the model of Katz (1963) to explain the data.

Materials and Methods

Poly(dA-dT) was purchased from Sigma and dialyzed extensively against 10 mM Tris-HCl (pH 7) and water and then concentrated by lyophilization. Thermal denaturation of this polymer monitored by proton NMR gave results essentially identical with those obtained previously (Patel & Canuel, 1976), except that the T_m was 57 °C in the present conditions. Poly(dA)·poly(dT) was obtained from Collaborative Research, Waltham, MA, and was similarly dialyzed and lyophilized.

Proton NMR titrations were performed in 0.01 M NaCl-O_4-0.1 M sodium phosphate buffer (pH 8.0) and spectrophotometric titrations were performed in this and in 0.01 M $NaClO_4$-0.01 M sodium cacodylate buffer (pH 7.1). The nonexchangeable proton spectra were taken in D_2O solutions. The D_2O was purchased from Wilmad (99.8%) and vacuum distilled to remove divalent ions before use.

Proton NMR spectra were taken on a Bruker WH-360 spectrometer operating at 360 MHz in the Fourier-transform mode (nonexchangeable resonances) or in the correlation mode (exchangeable resonances). Temperature control was effected by the Bruker regulator operating on a continuous flow of air. Areas under resonances were determined gravimetrically by using a linearly extrapolated base line. Chemical shifts were measured relative to internal DSS (sodium 4,4-dimethyl-4-silapentanesulfonate).

Spectrophotometric changes were monitored on a Perkin Elmer Model 552 recording spectrophotometer at room temperature.

Results

Spectrophotometry of the Binding of $HgCl_2$ to A-T Containing Polymers. In Figure 1 is shown the binding of Hg^{2+}

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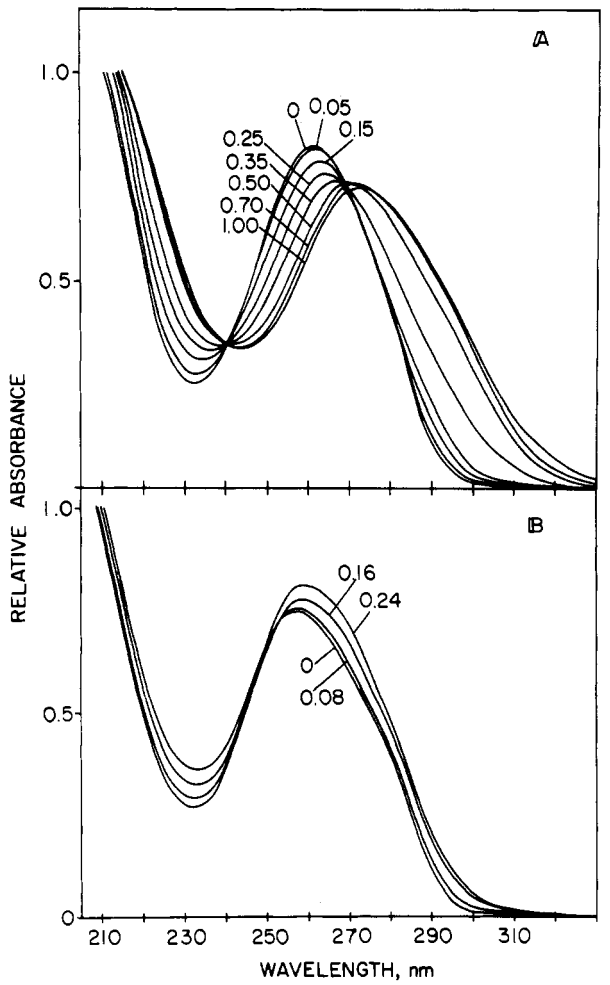

FIGURE 1: Effect of addition of $HgCl_2$ on the absorption spectra of (A) poly(dA-dT) and (B) poly(dA)·poly(dT) in 10 mM $NaClO_4$–10 mM cacodylate, pH 7.1, at ambient temperature. The numbers assigned to each spectral line refer to r, the Hg(II)/nucleotide ratio.

to poly(dA-dT) and poly(dA)·poly(dT) at pH 7.0. These can be seen to differ substantially in appearance. The spectrophotometric changes for poly(dA-dT) on addition of Hg^{2+} are identical with those reported previously (Nandi et al., 1965; Yamane & Davidson, 1961) with the binding mode halting at a ratio r of Hg^{2+}/nucleotide = 0.25. The decrease in absorbance and shift to a slightly longer wavelength in this region are highly reminiscent of the binding of the Hg^{2+} to uridine and, given that the N3 of uridine is the preferred site among nucleosides (Simpson, 1964), suggest that the Hg^{2+} ion is binding between the N3 atoms of thymines on opposite strands (Katz, 1963; Nandi et al., 1965). This conclusion is further supported by the limiting ratio of 0.25 (Hg^{2+} nucleotide) observed for poly(dA-dT) which contrasts with the limiting ratio of 0.5 (Hg^{2+}/nucleotide) observed for DNA (Nandi et al., 1965; Yamane & Davidson, 1961). Further binding results in a shift to even higher wavelengths with little change in absorbance, presumably corresponding to binding at the adenine residues (Simpson, 1964). Smaller spectra are obtained for the binding at pH 8.0 in the conditions used in the NMR experiments below.

In contrast, the binding of Hg^{2+} to poly(dA)·poly(dT) is accompanied by hyperchromism, and the lack of any isosbestic points is consistent with a multiplicity of sites or resulting states. The hyperchromism itself suggests some loss of secondary structure, and indeed at a ratio of r = 0.24 (Hg^{2+}/nucleotide), the absorbance continues to increase with time. This behavior is comparable to that seen for Hg(II) binding

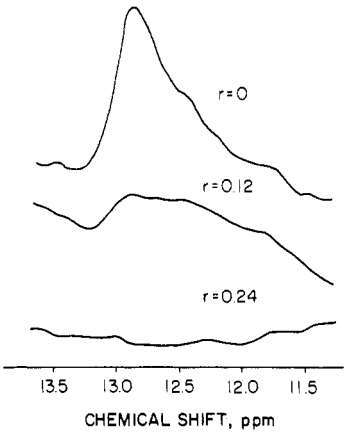

FIGURE 2: Spectra of the low-field region of poly(dA-dT) showing the loss of the Watson–Crick hydrogen-bonded imino resonance upon addition of $HgCl_2$. Conditions were the following: 11 mM in 0.01 M $NaClO_4$–0.1 M sodium phosphate, pH 8.0, 50 °C, 454 transients in correlation mode, 60-Hz line broadening. r is the Hg(II)/nucleotide ratio.

to poly(A)·poly(U) (Yamane & Davidson, 1962; Kawade, 1963; Williams & Crothers, 1975).

Proton NMR Spectroscopy of Hg(II) Binding to Poly(dA-dT). (*a*) *Exchangeable Resonances.* At temperatures below 57 °C, the T_m of poly(dA-dT) determined in this study [see also Patel & Canuel (1976)], the exchangeable thymine imino hydrogen can be observed at a downfield location due to its involvement in base pair hydrogen bonding (Shulman et al., 1973; Patel & Tonelli, 1974), which limits its rate of exchange with water. Because binding of one Hg^{2+} ion results in a loss of two protons at pH 9.0 (Nandi et al., 1965), then binding at the thymine N3 atom should cause the imino resonance to disappear. That this is indeed the case is indicated by the spectra in Figure 2. The nonuniform envelope of the resonances in this figure is probably due to noise in the base line and not to dispersion in the resonance frequency.

(*b*) *Nonexchangeable Resonances.* The effect of addition of Hg^{2+} to poly(dT-dT) at 40 °C on the nonexchangeable proton resonances is illustrated in Figure 3. The most obvious features between r = 0 and r = 0.25 are the transfer of intensity of AH2 and TH6 from 7.06 to ca. 8.1 ppm and of both of the ribose 1′ resonances from 6.14 to 6.67 ppm and from 5.6 to 5.97 ppm, respectively. These transfers of intensity are maintained upon raising the temperature of the r = 0.24 complex, whereupon extensive narrowing occurs, especially in the broad envelope at 8.1 ppm which loses its chemical shift heterogeneity (Figure 4). Such an intensity transfer is characteristic of a process which is slow on the NMR time scale as established by the frequency shift between the initial and final resonance positions (i.e., less than ca. 400 s^{-1}). This is what would be expected for a strong, covalent interaction with the nucleobases (Simpson, 1964). The transfer of intensity is indicated more quantitatively in Figure 5 and indicates a cooperative process.

The binding of Hg^{2+} to poly(dA-dT) is also accompanied by a small change in the chemical shift of the T(CH_3)5 proton resonance between r (Hg^{2+}/nucleotide) = 0 and 0.25 (Figure 6a), in parallel with the shifts in resonance intensity for the other nonexchangeable base resonances mentioned above. In addition, the T(CH_3)5 resonance is a good monitor of the state of the secondary structure after addition of Hg^{2+} to r = 0.24. In Figure 6b are plotted the melting curves for this resonance in the presence and absence of Hg^{2+}. Despite a conformational transition at 45 °C, the resonance remains upfield from its denatured position in "free" poly(dA-dT) when Hg^{2+} is bound

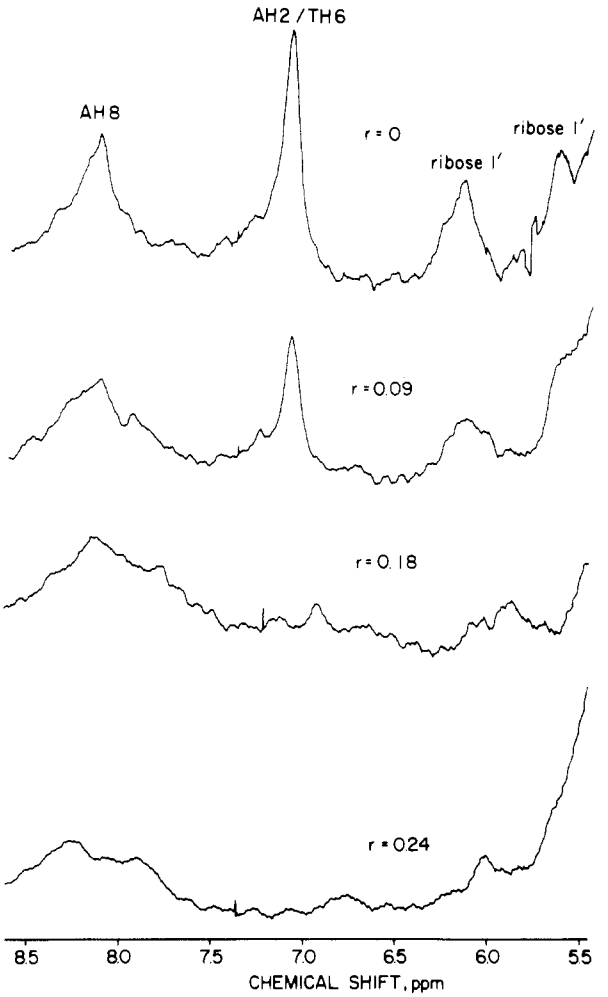

FIGURE 3: Spectra of nonexchangeable base and sugar proton resonances of poly(dA-dT) upon addition of $HgCl_2$. Conditions were the following: 11 mM in 0.01 M $NaClO_4$–0.1 M sodium phosphate, pH 8.0, 40 °C, 200 transients in the Fourier-transform M mode, 12–20-Hz line broadening. r is the Hg(II)/nucleotide ratio.

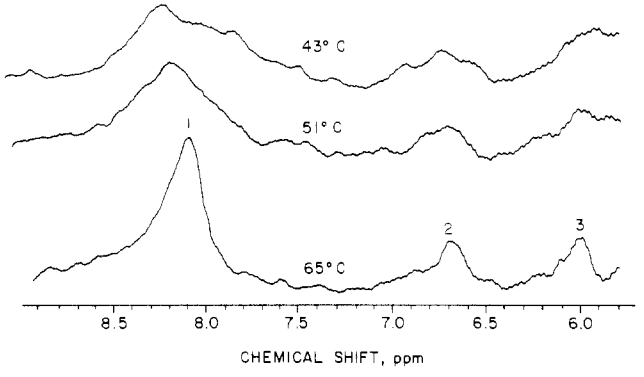

FIGURE 4: Effect of thermal denaturation on the nonexchangeable proton NMR spectrum of the 0.24 Hg(II)/nucleotide complex of poly(dA-dT). Resonance 1 is 3 times the intensity of either resonances 2 or 3 and represents AH8 + AH2 + TH6. Resonances 2 and 3 are ribose 1′ protons.

(Table I) and, along with the minimal changes in the other resonance chemical shifts (Figure 4), indicates the persistence of significant secondary structure.

The changes in chemical shift observed for poly(dA-dT) could reflect either intrinsic shift changes due to the covalent binding of Hg^{2+} to the nucleobase ring system or changes in secondary structure induced as consequence of binding Hg^{2+}. The dichotomy can be resolved directly by studying the effect of Hg^{2+} binding on the spectrum of thymidine, which, like

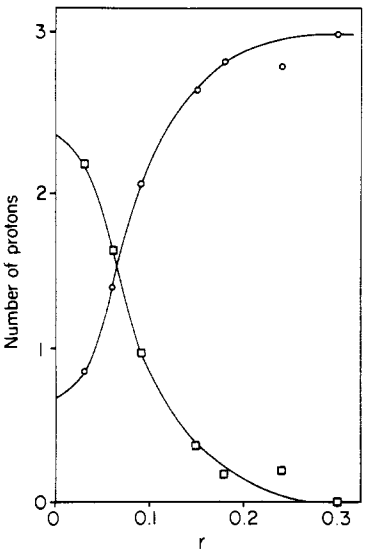

FIGURE 5: Changes in the intensity of AH8 (open circles) and AH2/TH6 (open squares) resonances with addition of $HgCl_2$, as determined from peak areas. The broad resonance at ~8 ppm was considered as one resonance when selecting the base line for the area determination. With this assumption in mind, the errors in area determination in most cases are less than 10%.

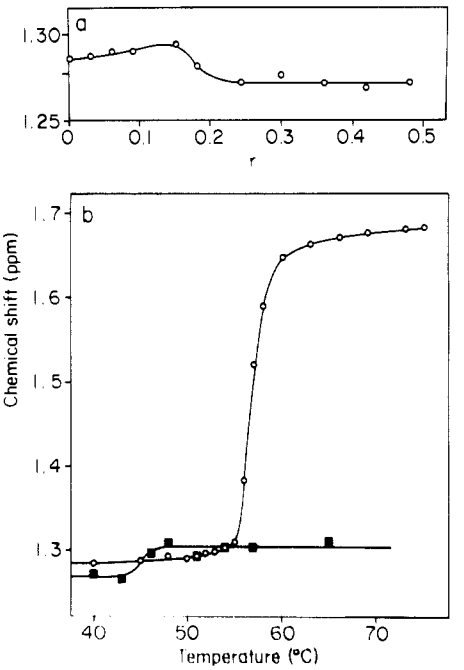

FIGURE 6: Chemical shift changes of the thymine 5 methyl proton resonance of poly(dA-dT) (a) upon addition of $HgCl_2$ at 40 °C and (b) upon temperature increase in the absence of Hg(II) (circles) and the presence of 0.24 Hg(II) per nucleotide (squares).

Table I: Chemical Shifts of Poly(dA-dT) Resonances

proton resonance	chemical shift (ppm) uncomplexed native	uncomplexed denatured	complexed with Hg(II) at $r = 0.24$
AH8	8.11	8.28	
AH2	7.06	8.08	8.11 (±0.25)
TH6	7.06	7.26	
$T(CH_3)5$	1.29	1.68	1.27
ribose 1′(1)	6.14	6.29	6.67
ribose 1′(2)	5.61	5.96	5.97

thymine in poly(dA-dT), has just N3 as the major binding site at pH 8 (Simpson, 1964). The changes in the chemical shift

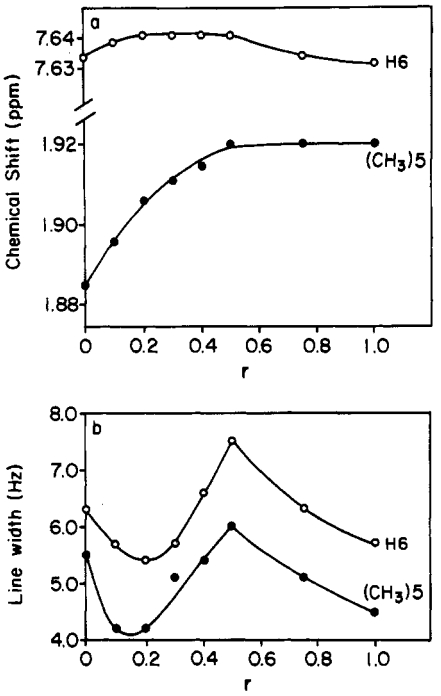

FIGURE 7: Effect of $HgCl_2$ addition to thymidine on (a) the chemical shifts and (b) the line widths of its nonexchangeable base proton resonances.

and line widths of the base proton resonances upon $HgCl_2$ addition are plotted in Figure 7. These show a discontinuity at 0.5, which suggests that a 1:2 (Hg^{2+}/thymidine) complex is formed. Such a complex has in fact been observed in a crystal structure (Kosturko et al., 1974) in which the Hg cross-links two (methyl) thymines through their N3 atoms. This is probably the complex being formed in solution, since N3 is known to be the preferred thymidine binding site (Simpson, 1964) and the small downfield shift of the $T(CH_3)5$ resonance is consistent with a coplanar rather than a stacked configuration. Hence, the binding of Hg^{2+} does not result in significant changes in the intrinsic thymidine proton resonance chemical shifts. This is similar to previous observations on Hg(II) binding to uracil in dimethyl sulfoxide (Kan & Li, 1970) which had led these authors to conclude that there was no binding in this solvent. Since the spectroscopic and exchangeable proton NMR evidence points to the Hg^{2+} binding primarily to thymine residues up to Hg^{2+} nucleotide ratio of 0.25, it can be assumed that for adenine resonances, as for thymine resonances, the chemical shift changes in this region are due to changes in secondary structure.

The effect of binding of Hg^{2+} between $r = 0.25$ and $r = 0.5$ was also examined via proton NMR, but the broadening and apparent changes in the base and ribose 1′ resonances are not simple to interpret and are not considered further.

Discussion

The present studies confirm and extend our understanding of the nature of the Hg(II) reaction with poly(dA-dT). (1) Reaction is shown to occur initially at thymidine N3, resulting in loss of the imino proton as evidenced by its disappearance from the NMR spectrum in H_2O. Previous studies have not been unequivocally able to confirm such a reaction for polynucleotides. It has in fact been suggested that binding of $HgCl_2$ to the keto oxygen of thymine in the A–T base pair might occur, followed by hydrolysis of the $HgCl_2$ complex (Carrabine & Sundaralingam, 1971). (2) Cross-linking of thymines between strands occurs in poly(dA-dT). This is evidenced not only by the end point of this first phase of the binding at 0.25 Hg^{2+} DNA phosphate but also by the lack of denaturation in the NMR melting curves of Figure 6b when compared to the polymer before binding; i.e., the thymine methyl group clearly remains shielded by adjacent nucleobases. Furthermore, poly(dA)·poly(dT), which has the thymidines all on one strand rather than dispersed alternately on each strand as in poly(dA-dT), exhibits a completely different change in spectrophotometric absorbance profiles with no clear binding end points. The hyperchromism observed suggests a loss of secondary structure, which might be a result of the binding of Hg^{2+} between thymines on the same strand, as suggested for uridines in poly(A)·poly(U) (Kawade, 1963; Williams & Crothers, 1975). These results therefore support the Katz model of Hg cross-linking via thymine N3 atoms for poly(dA-dT).

What does this metalated structure look like? The crystal structure of two methyl thymines linked by Hg(II) has been previously determined (Kosturko et al., 1974) and provides us with a detailed model of the kind of geometry to be expected for the thymine–Hg–thymine bridge. Circular dichroism studies of Hg(II) binding to synthetic polynucleotides have further shown that the thymines which cross-link are disposed in the sequence (5′–3′) TpA rather than (5′–3′) ApT (Ding & Allen, 1980b). Neither of these results gives us insight into the disposition of the adenine and thymine residues and the backbone conformational changes. The present NMR results address these questions since it has already been shown above that all chemical shift changes are due to changes in secondary structure.

In Table I are compared the chemical shifts for the base and ribose 1′ nonexchangeable proton resonances in poly(dA-dT) for the uncomplexed native and denatured polymer and Hg(II) ($r = 0.25$) complexed polymer. Two resonances [TH6 and ribose 1′(1)] have chemical shifts substantially different from those observed for these resonances in the uncomplexed polymer native or denatured, due most likely to change in the glycosidic (χ) angle (Giessner-Prettre & Pullman, 1977a,b; Prado et al., 1978), as evidenced for a ribose 1′ proton chemical shift in the alternating oligomer $(dG\text{-}dC)_n$, which is affected by the B-DNA to Z-DNA transition (Patel et al., 1979). The AH2 and $T(CH_3)5$ resonances adopt chemical shifts similar to those of the denatured and native polymer, respectively. Since these two resonances are expected to be sensitive mainly to the ring current effects of adjacent stacked nucleobases (Prado et al., 1978; Patel, 1980), their shifts suggest that the stacking arrangement is quite different to that of the native polymer in which the A/T bases form coplanar stacks. Furthermore, an alternative complex in which the adenines are pushed out of the helix so that the cross-linked thymine pairs can stack on each other, while explaining the denatured-like chemical shift of AH2, cannot account for the maintenance of a large upfield shift by $T(CH_3)5$, since the stacked thymine residues would have too low a ring current effect (Giessner-Prettre & Pullman, 1976).

A model consistent with the observed shifts is illustrated in Figure 8. Here the cross-linked thymines alternate with coplanar stacks of adenine residues. Given that thymine has a much lower ring current than adenine (Giessner-Prettre & Pullman, 1976), it can be easily seen that the downfield shift of AH2 in the complexed polymer would reflect the fact that adenine is now surrounded by four nearest-neighbour thymines as opposed to two thymines on the same strand and two adenines on the opposite strand in the native polymer. Similarly, the $T(CH_3)5$ resonance would shift slightly upfield because it is surrounded by four adenines with which it can stack as

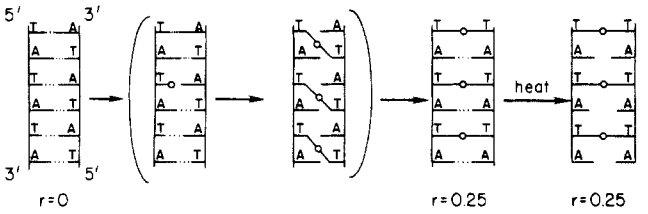

FIGURE 8: Scheme of the complexes formed upon addition of Hg(II) to poly(dA-dT). Unequivocal evidence for or against the bracketed intermediate is not available from the present data. See text for full explanation.

opposed to two adenines on the same strand and two thymines on the opposite strand. A further enticing feature of this model is the possible hydrogen bonding between the coplanar adenine residues, which would be expected to be thermolabile and hence explain the observed thermal transition of the complex at 46 °C (see Figure 6b). Attempts to observe these hydrogen-bonded adenine amino resonances have so far met with little success due to the proximity of this region of the spectrum (~6 ppm) to the H_2O peak and the attendant effects on base-line reproducibility. The model also has a good disposition of the adenines for the subsequent cross-linking by Hg between $r = 0.25$ and 0.5 (Yamane & Davidson, 1961; Nandi et al., 1965; Ding & Allen, 1980b; this paper). Indeed the $T(CH_3)5$ resonance experiences little change in chemical shift and hence stacking in this latter region of the binding (Figure 6a).

Figure 8 illustrates one other important aspect of the binding process, namely, its cooperativity. There are two possibilities consistent with the present data: (1) Hg^{2+} binds to separate thymine residues, as has been observed for the monovalent CH_3Hg^+ (Anderson et al., 1980), and only when enough Hg is bound does cross-linking and a cooperative conformational change occur; (2) every Hg^{2+} cross-links two thymines and results in a conformational change, as suggested by Kosturko et al. (1974), but the binding itself is cooperative. Although this version of the model is easier to reconcile with the observation that two protons are released uniformly during this binding stage (Nandi et al., 1965), discrimination between the two versions rests on whether or not $r = r_b$, where r_b is the ratio of Hg^{2+} *bound* per nucleotide. If the total concentration of Hg^{2+} is identical with r_b, only the first model is tenable, since no simple cooperative binding process can account for the sigmoidal form of the data in Figure 5.

In conclusion, this study has shown that proton NMR is a very useful probe of ion-induced conformational changes in nucleic acids, as well as being able to define the site of interaction of the ion in the circumstance of proton release. Hence, it offers a way to extend measurements on the interaction of metal ions with mononucleotides to the polymeric situation.

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