Drug-Nucleic Acid Interaction: X-ray Crystallographic Determination of an Ethidium-dinucleoside Monophosphate Crystalline Complex, Ethidium: 5-Iodouridylyl (3'-5') Adenosine

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Drug–nucleic acid interaction: X-ray crystallographic determination of an ethidium–dinucleoside monophosphate crystalline complex, ethidium: 5-iodouridylyl(3′–5′)adenosine

By C.-C. Tsai, S. C. Jain and H. M. Sobell

Department of Chemistry, The University of Rochester, River Campus Station,
Rochester, New York 14627 *and*
Department of Radiation Biology and Biophysics, The University of Rochester,
School of Medicine and Dentistry, Rochester, New York 14620, *U.S.A.*

The intercalative trypanosomal drug, ethidium bromide, forms a crystalline complex with the dinucleoside monophosphate, 5-iodouridylyl(3′-5′)adenosine (iodoUpA). These crystals are monoclinic, space group C2, with unit cell dimensions, $a = 2.845$ nm, $b = 1.354$ nm, $c = 3.413$ nm, $\beta = 98.6°$. The structure has been solved to atomic resolution by Patterson and Fourier methods, and refined by full matrix least squares to a residual of 0.20 on 2017 observed reflexions. The asymmetric unit contains *two* ethidium molecules, *two* iodoUpA molecules, twenty water molecules and four methanol molecules, a total of 156 atoms excluding hydrogens. The two iodoUpA molecules are held together by adenine–uracil Watson–Crick base-pairing. Adjacent base-pairs within this paired iodoUpA structure and between neighbouring iodoUpA molecules in adjoining unit cells are separated by 0.68 nm. This separation results from intercalative binding by one ethidium molecule and stacking by the other ethidium molecule above and below the base-pairs. Non-crystallographic twofold symmetry is utilized in this model drug–nucleic acid interaction, the intercalative ethidium molecule being oriented such that its phenyl and ethyl groups lie in the narrow groove of the miniature nucleic acid double helix. Solution studies have indicated a marked sequence specificity for ethidium–dinucleotide interactions and a probable structural explanation for this has been provided by this study.

The antitrypanosomal drug, ethidium bromide, is one in a class of phenanthridinium compounds that bind to DNA and RNA and inhibit nucleic acid function (Newton 1964; Elliot 1963; Waring 1964) (see figure 1). The precise nature of ethidium binding has been the object of study by many workers over the years, and it is generally believed that this drug intercalates into DNA (and perhaps into RNA) in much the same manner as the aminoacridines (Lerman 1961; Lerman 1963). A variety of physical techniques have been used to provide evidence concerning this. For example, viscometric and hydrodynamic measurements with linear DNA molecules in the presence of ethidium have suggested that lengthening and stiffening of the

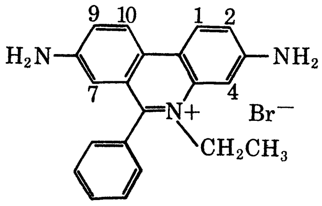

Figure 1. Chemical structure of ethidium bromide.

helix accompanies ethidium binding (Waring 1964), an interpretation that has been supported by electron microscopic DNA length measurements (Freifelder 1971). The ability of a drug to unwind circular supercoiled DNA is also considered to be another criterion for intercalative binding (Crawford & Waring 1967; Bauer & Vinograd 1968). This reflects unwinding of the DNA helix at the immediate site of intercalation by a drug or dye, and is observed with ethidium–DNA binding. An enhancement in fluorescence and a red shift in the ethidium absorption spectrum accompanies the binding reaction (LePecq & Paoletti 1967; Waring 1965); these spectral changes suggest stacking of the phenanthridinium ring system on the nucleic acid base-pairs. Fibre X-ray diffraction studies have been carried out on this complex, and a tentative molecular model for ethidium–DNA interaction has been advanced (Fuller & Waring 1964).

We have previously reported the successful co-crystallization of ethidium with several self-complementary dinucleoside monophosphates and the determination of the three-dimensional structure of one of these (ethidium: 5-iodouridylyl(3′-5′)adenosine) to atomic resolution by X-ray crystallography. This structure is of particular interest in that it demonstrates intercalative binding by this drug to a fragment of a nucleic acid double helix (Tsai, Jain & Sobell 1975). This paper will discuss further details of the structure and its biological implications. Particular reference will be made to solution studies that have indicated a marked sequence specificity in ethidium–dinucleoside monophosphate binding. These studies, along with the solid state studies, have suggested a sequence specificity of the type: pyrimidine–purine for ethdium–dinucleotide interactions and it is possible that a similar sequence specificity (although, clearly, not an absolute one) may carry over to ethidium–DNA binding. In this paper, we discuss a probable structural explanation for this sequence specificity.

Solution studies of ethidium–dinucleotide interactions

The interaction between ethidium and a large number of deoxyribodinucleotides and ribodinucleoside monophosphates has been studied by Krugh and his colleagues using a variety of spectroscopic techniques (Krugh, Wittlin & Cramer 1975). Figure 2 shows the effect of adding the self-complementary ribodinucleoside monophosphates, cytidylyl(3′-5′)guanosine (CpG), guanylyl(3′-5′)cytidine (GpC), uridylyl(3′-5′)adenosine (UpA) and adenylyl(3′-5′)uridine (ApU) to ethidium bromide solutions as followed by absorbance changes at 460 nm. Although all four dinucleoside monophosphates interact with ethidium, the sequence isomer CpG clearly binds ethidium tightest and in a cooperative fashion (as evidence by the sigmoidal nature of the binding curve). The difference curves for the four self-complementary ribodinucleoside monophosphates are shown in figure 3 in the visible spectral region. These curves all have approximately the same overall shape except that the magnitude of the induced spectral change for complex formation of ethidium with CpG is much larger than with the other nucleotides. A consideration of the nucleotide concentrations that produce a half-maximal change in the absorbance indicates that ethidium binds more strongly to CpG than to GpC. A more detailed analysis to determine the binding constants requires a number of assumptions and will not be attempted here.

Although ethidium by itself is optically inactive, a characteristic circular dichroism (c.d.) spectra can be induced in the 300–600 nm region when the molecule binds to double helical nucleic acids (see, for example, Aktipis & Martz 1974; Douthart, Burnett, Beasley & Frank

1973). Figures 4 and 5 show the induced c.d. spectra of ethidium in the presence of the self-complementary dinucleotides, dC-dG, CpG and UpA. These spectra are very similar to the published c.d. spectra for ethidium–DNA and ethidium–RNA complexes; this suggests that the geometry of these complexes are all very similar (i.e. involve intercalation). Under the same conditions, however, there is only a weak c.d. spectrum in the presence of the GpC sequence and no detectable c.d. spectrum with the ApU sequence. This suggests a pyrimidine–purine sequence specificity for ethidium binding to these dinucleotides, a conclusion reinforced by

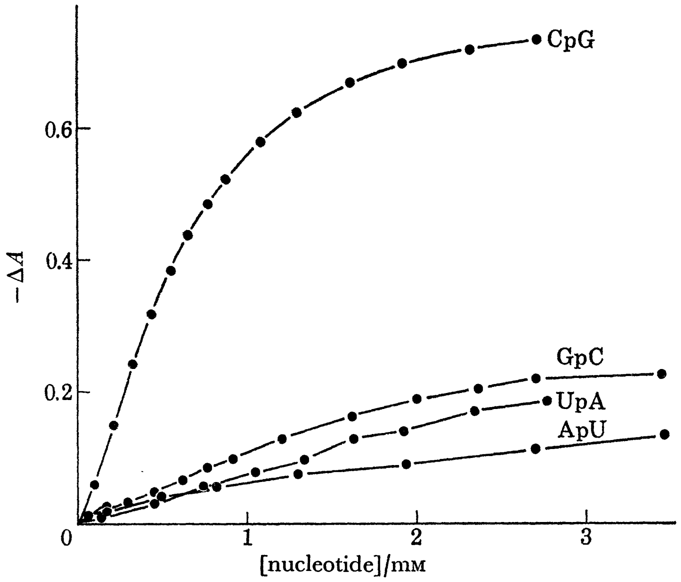

FIGURE 2. Effect of adding the self-complementary ribodinucleoside monophosphates on the absorbance of ethidium bromide at 460 nm. The ethidium bromide concentration at the start of each titration was 2.7×10^{-4} M. (Redrawn from Krugh *et al.* 1975.)

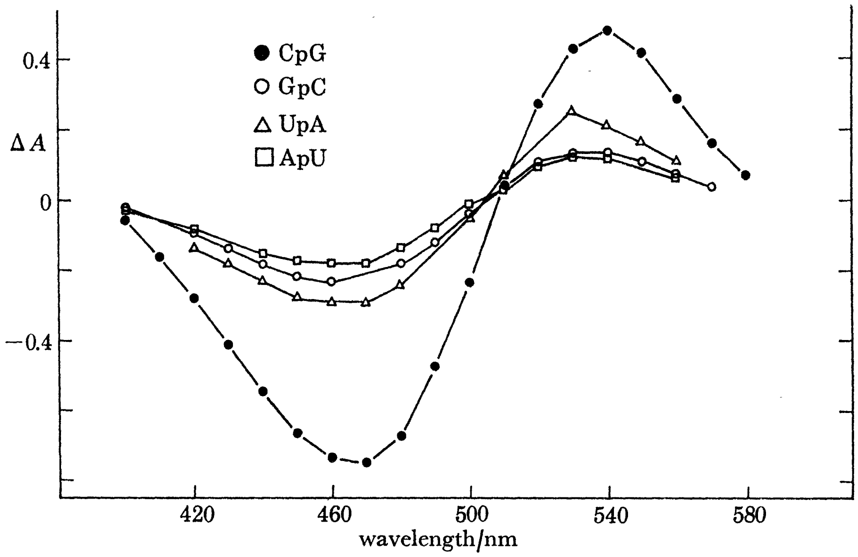

FIGURE 3. Difference curves for the ethidium bromide complexes with the ribodinucleoside monophosphates. The curves were calculated point by point from a spectrum obtained at the end of each titration. The final nucleotide concentrations were CpG (2.7 mM); GpC (2.7 mM); UpA (4.7 mM); and ApU (6.3 mM). (Redrawn from Krugh *et al.* 1975.)

further c.d. studies with mixtures of complementary and non-complementary dinucleotides (shown in figure 6). When ethidium is mixed with equimolar amounts of the complementary dinucleotides, pdT-dG and pdC-dA, one observes the characteristic c.d. spectrum for ethidium binding. If, however, ethidium is mixed with the *non-complementary* dinucleotides, pdT-dG and pdA-dC, a very weak c.d. spectrum is observed. Complementary dinucleotides that contain the *reverse* sequence, pdG-dT and pdA-dC, interact weakly with ethidium as evidenced by their c.d. spectrum. Results such as these are consistent with the general concept of an intercalative complex between ethidium and base-paired dinucleotides and dinucleoside monophosphates possessing the general sequence: *pyrimidine–purine*.

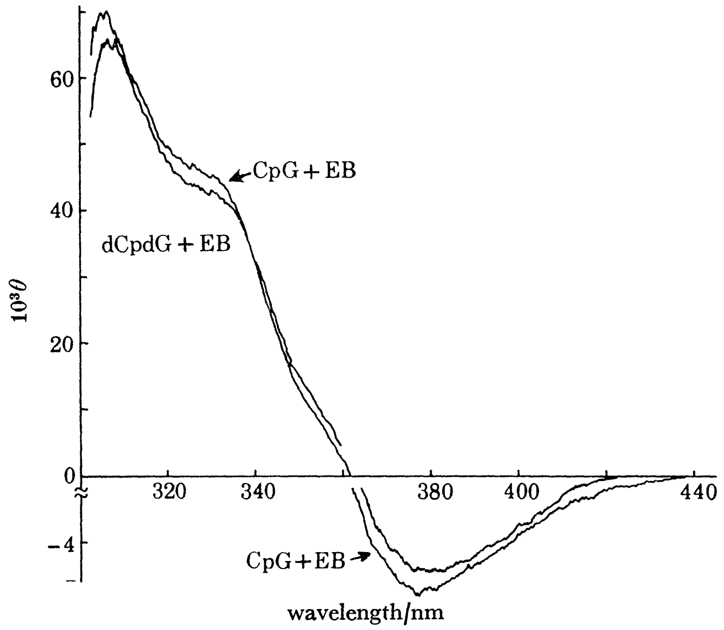

FIGURE 4. Induced circular dichroism spectra of ethidium bromide upon addition of CpG and dC-dG in a 1 mm thermostatted cell at 3 °C. The samples were (1) 0.75 mM ethidium bromide and 2.3 mM CpG and (2) 0.75 mM ethidium bromide and 3.2 mM dC-dG. Both samples were dissolved in a D_2O potassium phosphate buffer (5 mM) pD = 7.4. The theta values are direct readings from the recorder. Note that the scale of the ordinate has been expanded below the zero level. (Redrawn from Krugh *et al.* 1975.)

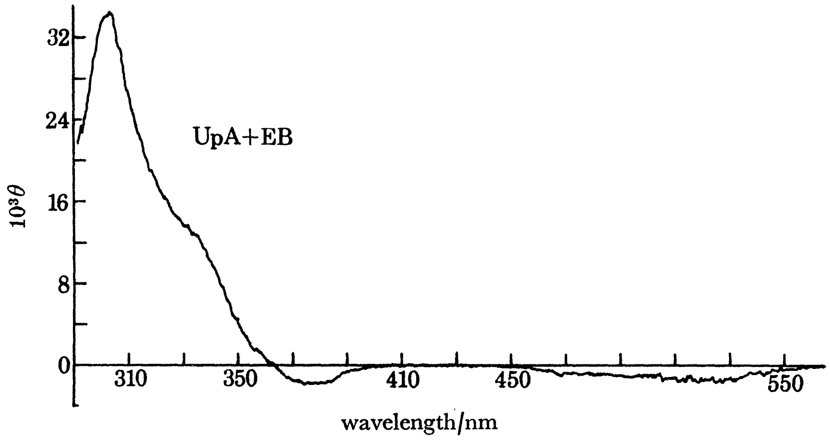

FIGURE 5. Induced circular dichroism spectra of ethidium bromide (0.41 mM) upon addition of UpA (2.0 mM) in a 1 mm cell at 0 °C. A similar sample containing ethidium bromide (0.41 mM) and ApU (2.4 mM) did not exhibit an induced circular dichroism spectrum in the 300–600 nm region. (Redrawn from Krugh *et al.* 1975.)

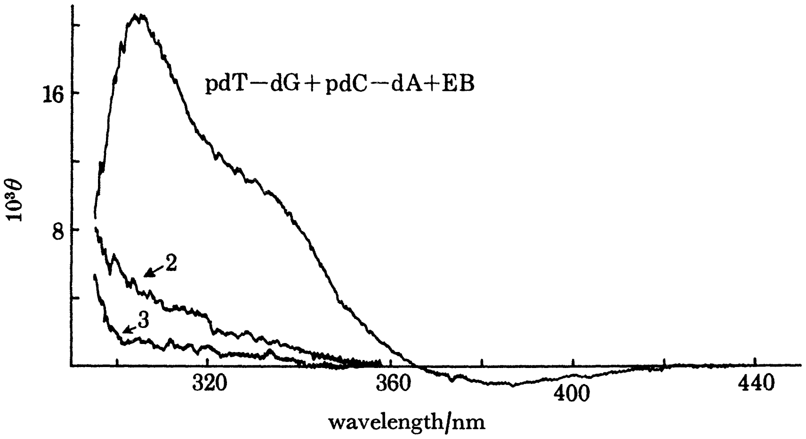

Figure 6. Induced circular dichroism spectrum of ethidium bromide (0.41 mM) upon addition of the complementary nucleotides pdT-dG (1.2 mM)+pdC-dA (1.2 mM) in a 1 cm cell at 0 °C. A mixture of the non-complementary nucleotides pdT-dG+pdA-dC (both 1.2 mM) and ethidium bromide (0.41 mM) gave rise to curve 3 under identical conditions used above. Curve 2 is the induced circular dichroism spectrum for a solution of ethidium bromide (0.41 mM) and the complementary nucleotides pdG-dT (2.7 mM)+pdA-dC (2.6 mM) containing the purine–pyrimidine sequence. (From Krugh *et al.* 1975.)

Isolation of ethidium–dinucleoside monophosphate crystalline complexes

It is against this background information that our isolation of ethidium–dinucleoside monophosphate crystalline complexes take on particular meaning. Plate-like crystals of these complexes can be obtained after several days by slow evaporation of ethidium: UpA and ethidium: CpG mixtures from water–methanol solutions. (In addition, we have synthesized 5-iodouridylyl(3′-5′)adenosine (iodoUpA) and 5-iodocytidylyl(3′-5′)guanosine (iodoCpG) and have obtained crystalline complexes with these as well.) These crystals are 2:2 stoichiometric complexes, and their space groups and unit cell dimensions are tabulated in table 1. Repeated attempts to obtain crystalline complexes with dinucleoside monophosphates possessing the reverse sequences (i.e. GpC and ApU, or their iodinated derivatives) have been unsuccessful.

Three different crystalline modifications exist for these complexes. One of these modifications contains isomorphous structures of ethidium: UpA and ethidium: CpG. This indicates that (in this crystal form) both complexes must be extremely similar. Ethidium: CpG and ethidium:

Table 1. Space groups and unit cell dimensions for 2:2 ethidium: dinucleoside monophosphate crystalline complexes

ethidium: UpA (or CpG)	ethidium: iodoUpA
a = 1.366 nm	a = 2.845 nm
b = 3.045 nm	b = 1.354 nm
c = 1.403 nm	c = 3.413 nm
β = 100.8°	β = 98.6°
$P2_1$	C2
ethidium: CpG	ethidium: iodoCpG
a = 1.379 nm	a = 1.406 nm
b = 3.194 nm	b = 3.234 nm
c = 1.566 nm	c = 1.653 nm
β = 117.6°	β = 117.8°
$P2_1$	$P2_1$

iodoCpG form a second crystalline modification; however, these structures are not exactly isomorphous with each other and this limits the effectiveness of heavy atom isomorphous replacement techniques in assisting their structure analysis. Ethidium: iodoUpA crystallizes in a third crystalline modification. This structure possesses different unit cell dimensions and space group symmetry compared with the other two. We have now solved this structure to atomic resolution (Tsai *et al.* 1975), and report further details of this structure in this paper.

VISUALIZATION OF DRUG–NUCLEIC ACID INTERCALATION

Figure 7 shows a portion of the ethidium: iodoUpA crystal structure as determined by our X-ray crystallographic study. The structure consists of *two* iodoUpA molecules (dark solid bonds) held together by adenine–uracil Watson–Crick base-pairing. Adjacent base-pairs within this paired iodoUpA structure and between neighbouring iodoUpA molecules in adjoining unit cells are separated by 0.68 nm. This separation reflects intercalative binding by one ethidium molecule (dark open bonds) and stacking by the other ethidium molecule (light open bonds) above and below the base-pairs. It is seen that non-crystallographic twofold symmetry is utilized in this model drug–nucleic acid interaction (see figure 8). This reflects the pseudo twofold symmetry of the phenanthridinium ring system in ethidium coinciding with the (approximate) twofold symmetry that relates sugar-phosphate chains and adenine and iodo-uracil base-pairs both within and between neighbouring iodoUpA molecules. The phenyl and

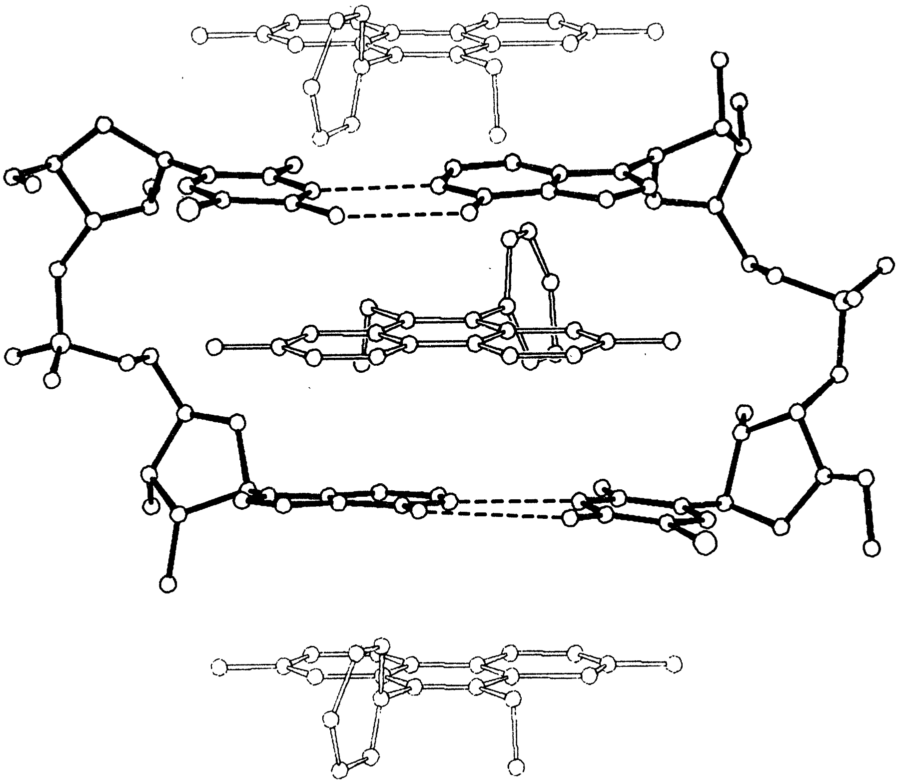

FIGURE 7. A computer-drawn illustration of a portion of the ethidium: iodoUpA crystal structure viewed approximately parallel to the planes of the adenine–uracil base-pairs and ethidium molecules. IodoUpA molecules are drawn with dark solid bonds; intercalative ethidium molecule is shown with dark open bonds, while stacked ethidium molecule(s) are drawn with light open bonds. Hydrogen-bonding between adenine–uracil base-pairs has been indicated by dashed lines. (Redrawn from Tsai *et al.* 1975.)

ethyl groups of the intercalative ethidium lie in the narrow groove of the miniature iodoUpA double helix. Both amino groups on this ethidium molecule neighbour the adenosine O5′ phosphodiester oxygen and the contacts (0.33; 0.35 nm) suggest possible weak electrostatic and hydrogen-bonding interactions. The other (stacked) ethidium molecule is oriented in the opposite direction; its phenyl and ethyl groups lie next to iodine atoms on uracil residues. The amino groups on this ethidium molecule are not immediately adjacent to any charged phosphate group, but instead form hydrogen bonds to neighbouring water molecules.

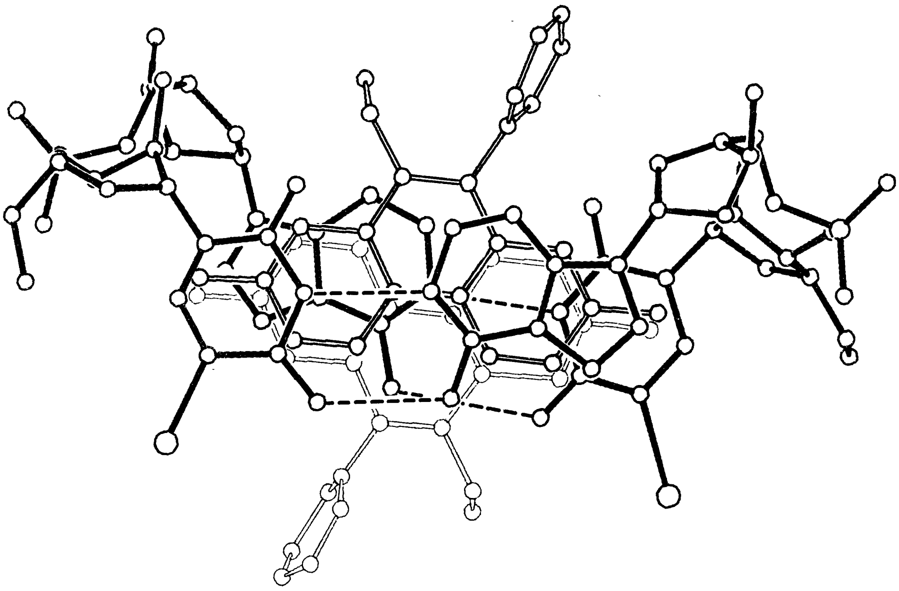

Figure 8. A computer-drawn illustration of the ethidium: iodoUpA complex viewed perpendicular to the planes of the adenine–uracil base-pairs and ethidium molecules. This figure illustrates the non-crystallographic twofold symmetry that is found in this model drug–nucleic acid interaction.

The distance that separates glycosidic carbon atoms between base-pairs is of particular interest; this distance corresponds to the interchain separation in DNA and RNA. The value is 1.04 nm for both base-pairs and may be significantly shorter than in DNA and RNA, as well as in single crystal studies of self-complementary dinucleoside monophosphates (Fuller *et al.* 1965; Rosenberg *et al.* 1973; Day *et al.* 1973). Another interesting number describes the relative angular orientation of base-pairs within the paired dinucleotide; this is estimated from the relative angular twist between vectors connecting glycosidic carbon atoms within each base-pair. This is about 8°, significantly smaller than the corresponding twist angle in DNA and double helical RNA (i.e. 36° and 32.7°, respectively), and in the single crystal dinucleoside monophosphate studies (about 32°). This directly reflects the presence of the intercalative ethidium molecule which acts to unwind double-helical nucleic acid polymers at the immediate site of drug intercalation. Conformational changes in the sugar-phosphate chains accompany this unwinding. These reflect (in part) the differences in ribose ring puckering that are observed (both iodouridine residues have C3′ *endo* sugar conformations, whereas both adenosine residues have C2′ *endo* sugar conformations). In addition, small but systematic changes in the torsional angles describing the phosphodiester linkage and the C4′–C5′ bond occur, and these, as well as other details will be described elsewhere.

The complex is heavily hydrated in the crystal lattice. Twenty water molecules have been found in the asymmetric unit, many of these forming hydrogen-bonded water–water tetrahedral-like structures and water-hydroxyl linkages to the sugar-phosphate chains. Differences in water structure could account for the different ethidium–dinucleoside monophosphate crystalline modifications described earlier, and we are currently continuing these X-ray analyses with this in mind. The water structure in this complex will be described in detail elsewhere.

STRUCTURAL EXPLANATION FOR SEQUENCE SPECIFICITY

As discussed earlier, the solution studies of Krugh *et al.* have demonstrated that ethidium exhibits a marked sequence specificity in binding pyrimidine–purine dinucleotide sequences. Our crystallographic study has provided structural information relevant to understanding the basis for this specificity.

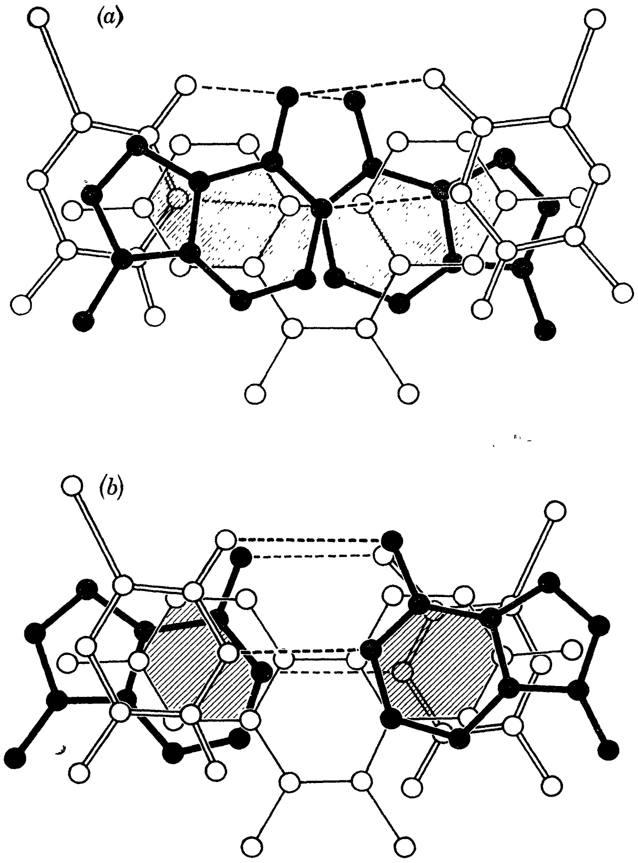

FIGURE 9. The stacking of adenine–uracil base-pairs on ethidium molecule intercalating in (*a*) UpA, (*b*) ApU. Shaded regions show that portion of the ethidium molecule directly involved in base-stacking.

Figure 9*a* shows the stacking of adenine–uracil base-pairs on the intercalative ethidium molecule, as determined by this study. We have shaded those regions of the ethidium molecule directly involved in base stacking. If the positions of the adenine–uracil base-pairs are now interchanged so as to generate a hypothetical ApU sequence, one can compare the relative stacking of base-pairs in this structure with ethidium. As can be seen in figure 9*b*, the ApU sequence stacks strikingly less with ethidium than does UpA. This difference in stacking follows from structural differences of pyrimidine–purine and purine–pyrimidine sequences in double helical nucleic acid structures, and can explain the specificity ethidium exhibits in binding

UpA and CpG. We will discuss this feature of ethidium binding more completely in a subsequent communication.

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Discussion

D. B. WETLAUFER (*Department of Biochemistry, University of Minnesota, Minneapolis MN* 55455, *U.S.A.*)

It seems that a different set of constraints may operate in the dinucleotide complex in the crystal, from those operating in the solution complex of the polymer. How reliable do you think the ‘unwinding angle’ you obtained is for solution complexes?

C. C. TSAI

Of course, the study described here is only a model study and it is possible that the ethidium–DNA interaction may differ significantly.

However, Wang (1974) has recently been able to measure directly the unwinding angle associated with ethidium–DNA binding and his value ($-26°$) agrees very well with our own observations (i.e. the twist angle of 8° in this structure implies an unwinding angle of $-28°$ if one assumes the B form of DNA). We think that the magnitude of the unwinding angle directly reflects the altered geometry of the sugar-phosphate backbone, in particular, the mixed sugar-puckering pattern of the type we describe.

It is of particular interest that an obligatory pattern of mixed sugar-puckering associated with drug intercalation immediately suggests a nearest neighbour exclusion binding model (i.e. intercalative binding occurring between every *other* base-pair in the double helix at maximal drug–nucleic acid binding ratios) for ethidium binding. This is consistent with the data that show that one ethidium molecule binds every four nucleotide bases at saturating conditions (Bauer & Vinograd 1970).

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C. C. B. Southgate (*Christ's College, Cambridge*)

If I am correct in thinking that in ethidium–DNA complexes two ethidiums cannot be bound between adjacent base-pairs, could not an explanation for the high unwinding angle found in your model complex be electrostatic repulsion between the two bound drug molecules producing an extended conformation not found in ethidium complexes with polynucleotides?

C. C. Tsai

I think this is unlikely on structural grounds. I prefer the explanation for nearest neighbour exclusion in ethidium–DNA binding that I have just described.

A. D. B. Malcolm (*Department of Biochemistry, Glasgow University*)

In your data illustrating the binding of various dinucleoside monophosphates to actinomycin it was pointed out that all except GpC bind with a stoichiometry of 1:1 and with a simple hyperbolic binding curve. However, 2 mol of GpC are bound per mole of actinomycin and the binding curve is sigmoid suggesting cooperativity. Are you able to say whether the cooperativity arises entirely from the fact that when the second GpC binds it can hydrogen bond to the first GpC in the usual way or do you think that the actinomycin itself has a role in the cooperativity? The free energy of interaction, derived from a Hill plot, should help to answer this question. Have you measured it?

C. C. Tsai

Krugh (1972) has recently compared the dimerization of dG–dC in the absence and in the presence of actinomycin. The dimerization constant for dG–dC in the absence of actinomycin is about 10 mol^{-1}, whereas, in the presence of actinomycin it is about 2000 mol^{-1}. The enhancement for dimerization in the presence of actinomycin undoubtedly reflects strong interactions between actinomycin and the dinucleotides, probably arising from stacking with the phenoxazone ring as well as from interactions with the peptide chains. It is of interest that the Hill coefficient is almost 2.0, indicating almost perfect cooperativity in the interaction.

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