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X-Ray Crystallographic Visualization of Drug–Nucleic Acid Intercalative Binding: Structure of an Ethidium–Dinucleoside Monophosphate Crystalline Complex, Ethidium: 5-Iodouridylyl(3′-5′)Adenosine

(drug–nucleic acid interactions/intercalation/double helix unwinding)

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ABSTRACT **We have cocrystallized the drug ethidium bromide with the dinucleoside monophosphate 5-iodouridylyl(3′–5′)adenosine and have solved the three-dimensional structure to atomic resolution by x-ray crystallography. This has allowed the direct visualization of intercalative binding by this drug to a fragment of a nucleic acid double helix.**

Ethidium bromide is one in a class of phenanthridinium drugs that has been found to be useful in the treatment of certain trypanosome infections (1). See Fig. 1. Its medicinal action stems from the ability of the drug to bind to DNA and RNA and to inhibit nucleic acid function (2, 3). The precise nature of ethidium binding to nucleic acids has been the object of study by many workers over the years (3–9). It is generally thought that ethidium binds to DNA (and perhaps to RNA) through intercalation in much the same manner as that postulated for the aminoacridines (10, 11). Evidence for this has been advanced by means of a variety of physical techniques. Viscometric and hydrodynamic measurements with linear DNA molecules indicate a lengthening and stiffening of the helix in the presence of increasing concentrations of the drug (3), an interpretation directly supported by electron microscopic measurements (4). The ability of this drug to unwind circular supercoiled DNA is also considered to be another sensitive criteria for intercalative binding (5, 6). This is thought to reflect unwinding of the DNA helix by the drug at the immediate site of intercalation. The enhancement of fluorescence and a red shift in the ethidium absorption spectrum accompany the binding reaction (7, 8). These spectral perturbations are most easily explained by the stacking of the phenanthridinium ring system on the nucleic acid base pairs. Fiber x-ray diffraction studies have been carried out on the complex, and a tentative molecular model for ethidium–DNA interaction has been proposed (9).

We have previously reported the successful cocrystallization of ethidium with several self-complementary dinucleoside monophosphates (12). We have now solved the three-dimensional structure of one of these [ethidium: 5-iodouridylyl(3′-5′)adenosine] to atomic resolution by x-ray crystallography. This has allowed us to directly visualize intercalative binding by this drug to a fragment of a nucleic acid double helix.

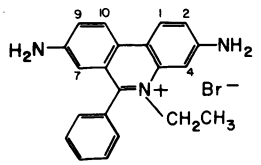

FIG. 1. Chemical structure of ethidium bromide.

MATERIALS AND METHODS

Ethidium bromide was purchased from Sigma Chemical Co. and used without further purification. The dinucleoside monophosphates uridylyl(3′-5′)adenosine (U-A) and cytidylyl(3′-5′)guanosine (C-G), were obtained as the ammonium salts from Sigma Chemical Co. and used directly. Iodination of both dinucleoside monophosphates was accomplished through use of a synthesis devised by Prof. David C. Ward and his colleagues at Yale University. The dinucleoside monophosphates were first converted to a stable mercury intermediate by incubating with mercuric acetate for several hours at 50°C, pH 5.5, in 0.1 M sodium acetate buffer (13). This intermediate was then reacted with elemental iodine to give the iodinated dinucleoside monophosphate. In both reactions, the overall yield was estimated to be over 80%. Excess iodine was removed by repeated chloroform extractions. The aqueous layer was then diluted 10-fold with distilled water, and the material was applied to a DEAE-cellulose DE-52 ion exchange resin, equilibrated with 0.01 M sodium acetate, pH 5.5. The iodinated dinucleoside monophosphate was purified through use of a triethylammonium bicarbonate salt gradient. Triethylammonium bicarbonate is a volatile salt, and rotary evaporation of the purified column material yielded the free acid form of the iodinated dinucleoside monophosphate. We estimate the final product to be between 95 and 98% pure by spectral criteria.

Plate-like crystals were obtained by slow evaporation over several days of equimolar mixtures of ethidium and the dinucleoside monophosphates dissolved in a 50% water–methanol solvent system. Preliminary characterizations of these crystals were done by comparing the ultraviolet absorption spectra of solutions obtained from washed single crystals with solutions containing known stoichiometric mixtures of these compounds. These spectral studies indicated complexes containing equimolar quantities of both compounds. Space groups and unit cell dimensions were initially obtained from precession photographs with nickel-filtered CuKα radiation, and then refined by least squares from 12 independent reflections measured on a Picker FACS-1 automatic diffractometer. This information is tabulated in Table 1.

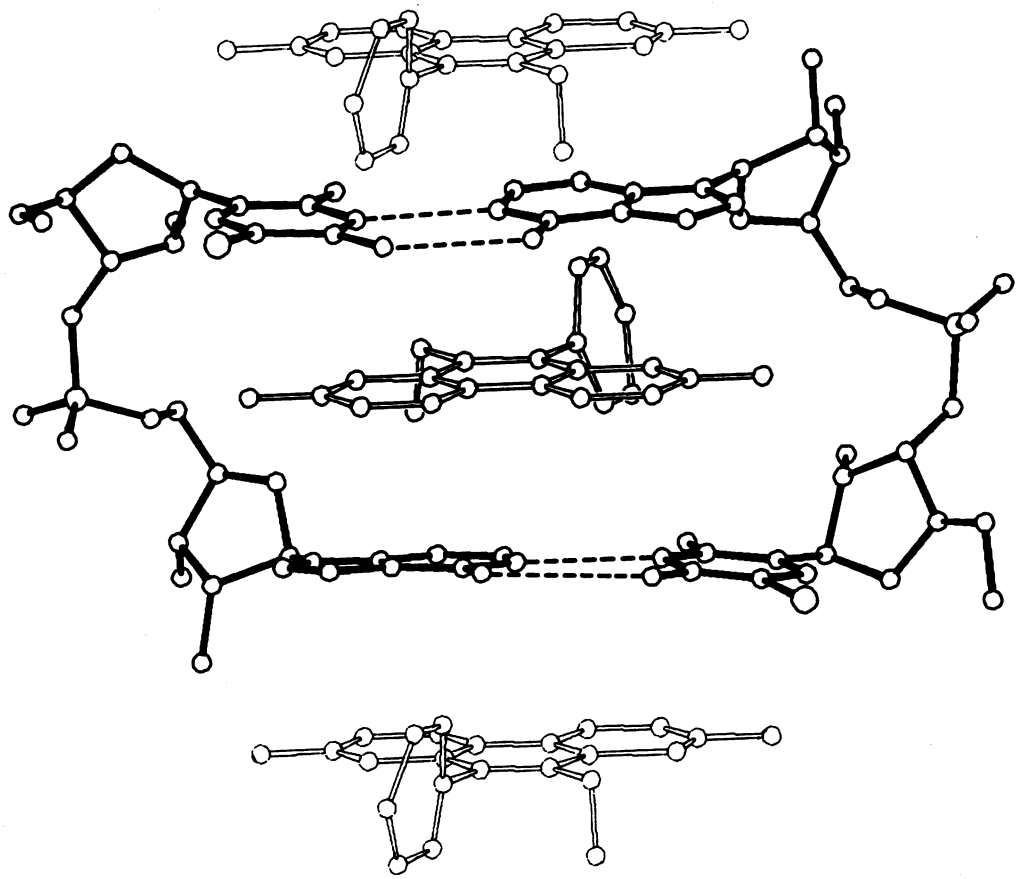

FIG. 2. A computer-drawn illustration of a portion of the ethidium: ioU-A crystal structure viewed approximately parallel to the planes of the adenine·uracil base pairs and ethidium molecules. ioU-A molecules are drawn with dark solid bonds; the intercalative ethidium molecule is shown with dark open bonds, while stacked ethidium molecule(s) are drawn with light open bonds. Hydrogen bonding between adenine·uracil base pairs has been indicated by broken lines.

A single crystal of the ethidium: 5-iodouridylyl(3′-5′)-adenosine complex measuring approximately 0.8 mm × 0.5 mm × 1.0 mm was mounted in a 1.0-mm glass capillary with some mother liquor. Data were collected at room temperature with nickel-filtered CuKα radiation using the theta–two theta scan method out to a maximum two-theta angle of 70°; 3038 reflections were measured, of which 2017 were significantly above background. The intensities were corrected for the Lorentz and polarization factors; no absorption corrections were used. The overall isotropic temperature factor and scale factor were derived by Wilson statistics (14), and normalized structure factors were then computed using the K-curve method (15). The positions of the iodine atoms were determined by examining the ($E^2 - 1$) Patterson map, and, independently, through use of direct methods using a computerized phase-determining program (16). With this information, the structure could then be developed by Patterson superposition using a minimum function. This revealed the positions of *two* ethidium molecules and *two* iodouridylyl-(3′-5′)adenosine molecules in the asymmetric unit. Further structural detail was added by using a sum-function Fourier synthesis (where amplitudes are $\{|2F_o| - |F_c|\}$ and phases are the calculated phases) (17), to include an additional 20 water molecules and four methanol molecules, a total of 156 atoms (excluding hydrogens) in the asymmetric unit. The structure has been refined by successive sum-function and difference Fourier syntheses, and the current residual based on observed reflections is 20.3%. A complete report of the structure analysis will be reported elsewhere.

RESULTS

Figs. 2 and 3 show a portion of the ethidium: 5-iodouridylyl-(3′-5′)adenosine crystal structure (hereafter, denoted ethidium: ioU-A) as determined by this crystallographic study. The structure consists of *two* ioU-A molecules (dark solid bonds) held together by adenine·uracil Watson–Crick base pairing. Adjacent base pairs within this paired ioU-A structure and between neighboring ioU-A molecules in ad-

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

Ethidium: U-A (or C-G)	*Ethidium: ioU-A*
a = 13.66 Å	a = 28.45 Å
b = 30.45 Å	b = 13.54 Å
c = 14.03 Å	c = 34.13 Å
β = 100.8°	β = 98.6°
$P2_1$	C2
Ethidium: C-G	*Ethidium: ioC-G*
a = 13.79 Å	a = 14.06 Å
b = 31.94 Å	b = 32.34 Å
c = 15.66 Å	c = 16.53 Å
β = 117.6°	β = 117.8°
$P2_1$	$P2_1$

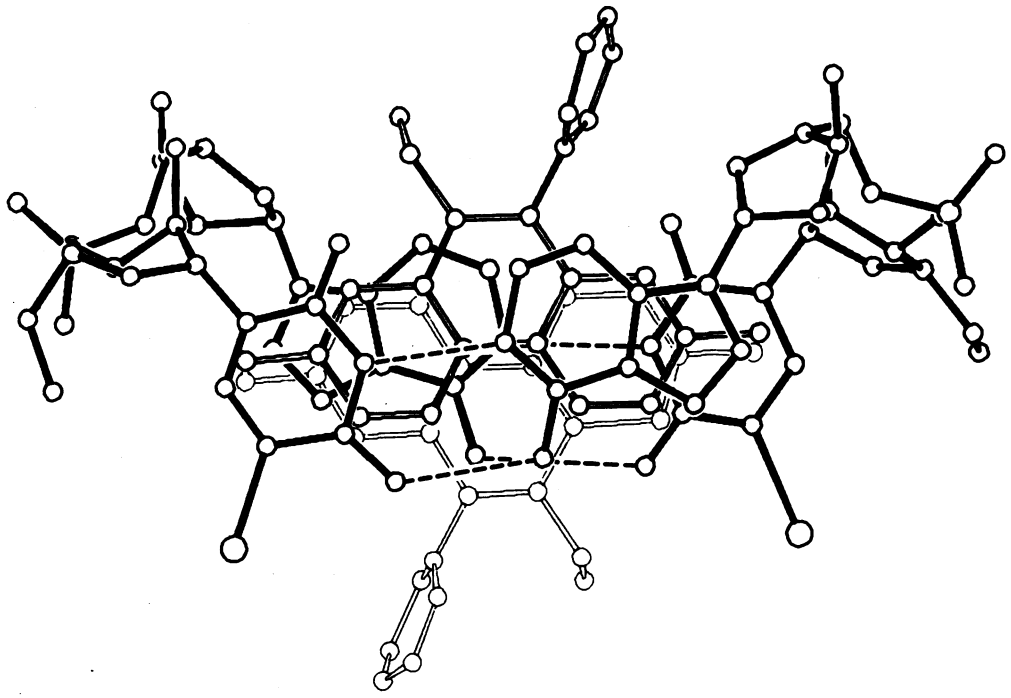

FIG. 3. A computer-drawn illustration of the ethidium: ioU-A complex viewed perpendicular to the planes of the adenine·uracil base pairs and ethidium molecules. This figure illustrates the noncrystallographic 2-fold symmetry that is used in this model drug–nucleic acid interaction.

joining unit cells are separated by 6.8 Å. This separation results from 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 (see Fig. 2). Noncrystallographic 2-fold symmetry is utilized in this model drug–nucleic acid interaction. This reflects the pseudo-2-fold symmetry of the phenanthridinium ring system in ethidium coinciding with the (approximate) 2-fold symmetry that relates sugar–phosphate chains and adenine and iodouracil base pairs both within and between neighboring ioU-A molecules (see Fig. 3). The intercalative ethidium is oriented such that its phenyl and ethylsubstituents lie in the narrow groove of the miniature ioU-A double helix. Both amino groups on this ethidium molecule are juxtaposed to the adenosine O-5′ phosphodiester oxygen and their contacts (3.3 Å; 3.5 Å) suggest possible weak electrostatic and hydrogen bonding interactions. The stacked ethidium lies in the opposite direction; its phenyl and ethyl groups neighbor iodine atoms on uracil residues. The amino groups on this ethidium molecule are not in immediate apposition to the charged phosphate groups, and instead form hydrogen bonds to neighboring water molecules. Of particular interest is the distance that separates glycosidic carbon atoms between base pairs; this distance corresponds to the interchain separation in DNA and RNA. This value is 10.4 Å for both base pairs and may be significantly shorter than that found in DNA and RNA and in single crystal studies of self-complementary dinucleoside monophosphates (18–20); however, because of the limitations in the accuracy of this analysis, we hesitate to be overconfident on this point. Another interesting number describes the relative angular orientation of base pairs within the paired dinucleotide; this is estimated from the relative twist between vectors connecting glycosidic carbon atoms within each base pair. This value is about 7°, significantly smaller than the corresponding twist angle in DNA (36°) and double helical RNA (32.7°), and in the single crystal dinucleoside monophosphate studies (about 32°). The small angular twist between base pairs in this study directly reflects the presence of the intercalative ethidium molecule, and this corresponds to an unwinding of double-helical nucleic acid polymers at the immediate site of drug intercalation. Conformational changes in the sugar–phosphate chains accompany this unwinding. These conformational changes partly reflect the differences in ribose sugar ring puckering that are observed (both iodouridine residues have C3′ *endo* sugar conformations, whereas both adenosine residues have C2′ *endo* sugar conformations), as well as small but systematic changes in the torsional angles that describe the phosphodiester linkage and the C4′–C5′ bond. The detailed nature of these conformational changes will be discussed elsewhere.

A considerable amount of water structure surrounds the columns of ethidium: ioU-A complexes stacked in the *y* direction of 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. The complex is heavily solvated in the crystal lattice, and this leads us to believe that the association of these compounds in the solid state is not significantly different from their solution associations (35). Further study of other ethidium: dinucleoside monophosphate complexes in different lattices will provide useful information on this point. A detailed description of the water structure will be discussed elsewhere.

DISCUSSION

It is now over a decade since Lerman proposed his intercalation hypothesis to explain the strong binding mode of the aminoacridines to DNA (10). A large body of evidence now

supports the intercalation concept and it has become increasingly apparent that (in addition to the aminoacridines) a large number of other synthetic drugs and antibiotics utilize intercalation in their binding to DNA. These include anticancer drugs such as actinomycin and daunomycin (21–23), antimalarial drugs such as chloroquine and quinacrine (24), the antischistosomiasis drug hycanthone (a hydroxylated metabolite of Miracil D) (25), and the antitrypanosomal compounds ethidium bromide and propidium iodide (26). In addition to their usefulness as clinical chemotherapeutic agents, several of these drugs (in particular, actinomycin, ethidium bromide, and acridine orange) have been widely used in the study of molecular and cellular biology. Partly as a result of the drugs' enhanced affinity in binding to superhelical DNA, one observes such diverse phenomena as the selective inhibition of eukaryotic mitochondrial RNA transcription in the presence of ethidium bromide and the "curing" of episomes in bacteria by acridine orange (27, 28). This enhanced affinity for supercoiled DNA reflects the structural ability of these drugs to unwind the DNA helix at the immediate site of intercalation; this acts to relieve the strain in a right-handed supercoiled DNA structure, a reaction that is thermodynamically favorable (29). Ethidium bromide has also been used to create covalently closed circular DNA molecules that contain various numbers of supercoils in their structure (30). These molecules have helped to serve as useful probes in understanding the nature of protein–DNA interactions and control mechanisms that regulate RNA transcription (31, 32).

The current work is of interest for several reasons. In the first place, it provides an x-ray crystallographic structural determination of a model drug–nucleic acid intercalative crystal structure and this has provided a wealth of stereochemical information that can be correlated with the physical and chemical data for ethidium–nucleic acid binding in model systems and in polymer studies. Second, the binding of ethidium to this dinucleoside monophosphate demonstrates what may be a more general principle in the binding of small symmetric (or pseudo-symmetric) ligands to DNA—the use of symmetry in drug–DNA interactions. Although the binding of ethidium to DNA and RNA shows no detectable base specificity, spectral studies with ethidium and a series of ribodinucleoside monophosphates and deoxyribodinucleotides carried out recently have indicated a marked preference for ethidium binding to the C-G and dC-dG sequences (35). This finding is of particular interest in view of the ethidium: dinucleoside monophosphate crystalline complexes we have isolated (see Table 1). Complexes can readily be obtained between ethidium: C-G and ethidium: U-A (as well as with ioC-G and ioU-A); however, repeated attempts to obtain complexes with the reverse sequences (i.e., G-C and A-U) have been unsuccessful. This may indicate a sequence specificity in these dinucleotide studies of the type: pyrimidine-purine, and it is possible that a similar sequence specificity (although, clearly, not an absolute one) exists in ethidium: DNA binding. Studies analogous to those of Wells and Larson (33) (who studied actinomycin binding to a series of synthetic DNA-like polynucleotides) would provide valuable information in this regard.

In their model for ethidium: DNA binding, Fuller and Waring (9) make two interesting predictions concerning the stereochemistry of ethidium intercalation, and it is of interest to compare these with the results of this crystallographic study. The first concerns the magnitude of the unwinding angle that accompanies ethidium intercalation; this is estimated in their model to be about $-12°$. The second concerns the relative orientation of the ethidium molecule with respect to the base pairs; the phenyl and ethyl groups of ethidium are postulated to lie in the wide groove of the DNA helix. As discussed previously, the relative angular orientation between adenine·iodouracil base pairs in the intercalated ethidium: ioU-A structure is about 7°; this means (assuming that the results of this model study can be carried over to the RNA and DNA polymer studies) the unwinding angle is $-26°$ for RNA intercalation and $-29°$ for DNA intercalation, a value considerably larger than the Fuller–Waring figure. [It may be of interest in this regard that recent alkaline titration studies in cesium chloride density gradients of superhelical DNA molecules containing various degrees of superhelicity have suggested the unwinding angle to be about $-26°$ for ethidium intercalation (34).] An important variable in understanding the polynucleotide conformation in intercalation is the ribose sugar ring puckering; this was assumed to be C2′ *endo* for all nucleotide residues in the Fuller–Waring model. The ethidium: ioU-A structure, however, demonstrates a mixture of C2′ and C3′ *endo* sugar conformations and this (in part) can explain the angular unwinding difference that is observed. The other feature of the Fuller–Waring model concerns the orientation of the intercalative ethidium molecule with respect to the base pairs and can be compared with the results of this study. The intercalative ethidium is oriented in a manner exactly *opposite* to the Fuller–Waring model; the phenyl and ethyl groups of ethidium are found to lie in the narrow groove of the miniature double helix. This difference could conceivably reflect the presence of the iodine atoms on uracil residues, which, because of their bulk, interfere with intercalation from the wide groove. This possibility can be tested by solving the light atom ethidium: U-A and ethidium: C-G crystal structures. However, it is also possible that the ethidium associations with these dinucleotides may not be a completely accurate model for the ethidium–nucleic acid polymer associations, and further data are necessary to clarify this point.

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1. Newton, B. A. (1964) "Mechanisms of action of phenanthridine and aminoquinaldine trypanocides," in *Advances in Chemotherapy*, eds. Goldin, A. & Hawking, F. (Academic Press, Inc., New York), Vol. 1, pp. 35–83.
2. Elliot, W. H. (1963) "The effect of antimicrobial agents on deoxyribonucleic acid polymerase," *Biochem. J.* **86**, 562–567.
3. Waring, M. J. (1964) "Complex formation with DNA and inhibition of *Escherichia coli* RNA polymerase by ethidium bromide," *Biochim. Biophys. Acta* **87**, 358–361.
4. Freifelder, D. (1971) "Electron microscopic study of the ethidium-DNA complex," *J. Mol. Biol.* **60**, 401–403.
5. Crawford, L. V. & Waring, M. J. (1967) "Supercoiling of polyoma virus DNA measured by its interaction with ethidium bromide," *J. Mol. Biol.* **25**, 23–30.
6. Bauer, W. & Vinograd, J. (1968) "The interaction of closed

circular DNA with intercalative dyes. I. The superhelix density of SV40 DNA in the presence and absence of dye," *J. Mol. Biol.* **33**, 141–172.

7. LePecq, J. B. & Paoletti, C. J. (1967) "A fluorescent complex between ethidium bromide and nucleic acids," *J. Mol. Biol.* **27**, 87–106.
8. Waring, M. J. (1965) "Complex formation between ethidium bromide and nucleic acids," *J. Mol. Biol.* **13**, 269–282.
9. Fuller, W. & Waring, M. J. (1964) "A molecular model for the interaction of ethidium bromide with deoxyribonucleic acids," *Ber. Bunsenges. Physik. Chem.* **68**, 805–808.
10. Lerman, L. S. (1961) "Structural considerations in the interaction of DNA and acridines," *J. Mol. Biol.* **3**, 18–30.
11. Lerman, L. S. (1963) "The structure of the DNA-acridine complex," *Proc. Nat. Acad. Sci. USA* **49**, 94–102.
12. Tsai, C., Jain, S. C., Lam, C. H. & Sobell, H. M. (1974) *Concerning the Stereochemistry of Ethidium Bromide Binding to DNA and RNA: Structural Studies of Ethidium-Dinucleotide Crystalline Complexes*, American Crystallographic Association Abstracts, March 24–28, University of California, Berkeley, M1.
13. Dale, R. M. K., Livingston, D. C. & Ward, D. C. (1973) "The synthesis and enzymatic polymerization of nucleotides containing mercury: potential tools for nucleic acid sequencing and structural analysis." *Proc. Nat. Acad. Sci. USA* **70**, 2238–2242.
14. Wilson, A. J. C. (1942) "Determination of absolute from relative x-ray intensity data," *Nature* **150**, 151–152.
15. Karle, J. & Hauptman, H. (1953) "Application of statistical methods to the naphthalene structure," *Acta Crystallogr.* **6**, 473–476.
16. Tsai, C. (1968) *A Program for Phase Determination for Noncentrosymmetric Crystals*, Ph.D. Thesis, Part III, Indiana University, Bloomington, Ind.
17. Sussman, J. L. & Kim, S. H. (1974) *Group Scatterers in Macromolecules. A Method to Bootstrap a Macromolecular Structure from a Partial Structure*, American Crystallographic Association Abstracts, March 24–28, University of California, Berkeley, J1.
18. Fuller, W., Wilkins, M. H. F., Wilson, H. R. & Hamilton, L. D. (1965) "The molecular configuration of deoxyribonucleic acid. IV. X-ray diffraction study of the A form," *J. Mol. Biol.* **12**, 60–75.
19. Rosenberg, J. M., Seeman, N. C., Kim, J. J. P., Suddath, F. L., Nicholas, H. B. & Rich, A. (1973) "Double helix at atomic resolution," *Nature* **243**, 150–154.
20. Day, R. O., Seeman, N. C., Rosenberg, J. M. & Rich, A. (1973) "A crystalline fragment of the double helix: the structure of the dinucleoside phosphate guanylyl-3′,5′-cytidine," *Proc. Nat. Acad. Sci. USA* **70**, 849–853.
21. Muller, W. & Crothers, D. M. (1968) "Studies of the binding of actinomycin and related compounds to DNA," *J. Mol. Biol.* **35**, 251–290.
22. Sobell, H. M. & Jain, S. C. (1972) "Stereochemistry of actinomycin binding to DNA. II. Detailed molecular model of actinomycin-DNA complex and its implications," *J. Mol. Biol.* **68**, 21–34.
23. Pigram, W. J., Fuller, W. & Hamilton, L. D. (1972) "Stereochemistry of intercalation: interaction of daunomycin with DNA," *Nature New Biol.* **235**, 17–19.
24. O'Brien, R. L., Allison, J. L. & Hahn, F. E. (1966) "Evidence for intercalation of chloroquine into DNA," *Biochim. Biophys. Acta* **129**, 622–624.
25. Weinstein, I. B. & Hirschberg, E. (1971) "Mode of action of Miracil D," in *Progress in Molecular and Subcellular Biology*, ed. Hahn, F. E. (Springer-Verlag, New York), Vol. 2, pp. 232–246.
26. Waring, M. J. (1970) "Variation of the supercoils in closed circular DNA by binding of antibiotics and drugs: evidence for molecular models involving intercalation," *J. Mol. Biol.* **54**, 247–279.
27. Zylber, E., Vesco, C. & Penman, S. (1969) "Selective inhibition of the synthesis of mitochondria-associated RNA by ethidium bromide," *J. Mol. Biol.* **44**, 195–204.
28. Hirota, Y. (1960) "The effect of acridine dyes on mating type factors in *Escherichia coli*," *Proc. Nat. Acad. Sci. USA* **46**, 57–64.
29. Bauer, W. & Vinograd, J. (1970) "Interaction of closed circular DNA with intercalative dyes. II. The free energy of superhelix formation in SV40 DNA," *J. Mol. Biol.* **47**, 419–435.
30. Wang, J. C. (1969) "Variation of the average rotation angle of the DNA helix and the superhelical turns of covalently closed cyclic λ DNA," *J. Mol. Biol.* **43**, 25–39.
31. Maniatis, T. & Ptashne, M. (1973) "Multiple repressor binding at the operators in bacteriophage λ," *Proc. Nat. Acad. Sci. USA* **70**, 1531–1535.
32. Saucier, J. M. & Wang, J. C. (1972) "Angular alteration of the DNA helix by *E. coli* RNA polymerase," *Nature New Biol.* **239**, 167–170.
33. Wells, R. D. & Larson, J. E. (1970) "Studies on the binding of actinomycin D to DNA and DNA model polymers," *J. Mol. Biol.* **49**, 319–342.
34. Wang, J. C. (1974) "The degree of unwinding of the DNA helix by ethidium. I. Titration of twisted PM2 DNA molecules in alkaline cesium chloride density gradients," *J. Mol. Biol.* **89**, 783–801.
35. Krugh, T. R., Wittlin, F. N & Cramer, S. P. (1975) "Ethidium bromide dinucleotide complexes: evidence for intercalation and sequence preferences in binding to double stranded nucleic acids," *Biopolymers*, in press.