STRUCTURAL AND SEQUENCE-DEPENDENT ASPECTS OF DRUG INTERCALATION INTO NUCLEIC ACIDS

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I. INTRODUCTION

There are three modes whereby foreign molecules interact with nucleic acids. Covalent binding, especially to bases, is shown by reactive metabolites of chemical carcinogens, such as benzo[a]pyrene, acetylaminofluorene, and nitrosoureas.[1] DNA-binding proteins and peptides predominantly employ hydrogen-bonding interactions; when these involve DNA base donors and acceptors, the recognition can be highly specific for particular sequences.[2-4] The third category of interaction involves molecules possessing a planar aromatic chromophore, typically of dimensions similar to those of a standard Watson-Crick base pair (*circa* 10 × 3 Å), corresponding to about three fused six-membered rings. Many (though not all) of these molecules are cationic. It is now generally accepted that this noncovalent binding involves intercalation of the chromophore in-between adjacent stacked base pairs of a double-stranded polynucleotide; the essence of this concept is seen in the model suggested by Lerman[5] (Figure 1). These three classifications are by no means mutually exclusive. Thus, the planar aromatic moiety of many carcinogens may well play a role in early stages of DNA recognition and binding,[6] and intercalation of aromatic amino acid residues into DNA can impart enhanced stability to protein-DNA interactions.[7] Hydrogen bonding involving substituent groups attached to complex intercalating molecules frequently imparts sequence selectivity to the binding process.

Intercalation into double-helical nucleic acids has received widespread attention for over 20 years, not the least because of the well-defined nature of the concept in drug-receptor terms. The medical importance of many intercalative compounds, especially as anticancer drugs, has provided a major impetus to structure activity and mode-of-action studies (see, for example, References 8 to 14). Many of these are ultimately directed to developing compounds clinically superior to those currently available. Anticancer activity and cytotoxicity of intercalative agents are associated with interference in at least some aspects of transcriptional, translational, and replicative processes, as well as with gross DNA damage and consequent misrepair. Breakage of cellular DNA and interference with topoisomerase activity may well be a peculiar property of anticancer intercalating drugs.[15,16]

This review examines the present state of knowledge regarding molecular structural descriptions of drug-nucleic acid intercalation, especially in relation to current concepts of nucleic acid structure. Relationships between this very detailed level of information and the molecular and cell biology of intercalation are, as yet, largely unexplored. It is, however, a central thesis of this review that the interaction process is not a random event in terms of DNA sequence, but that sequence preference and even specificity are important factors which may well have particular consequences in terms of alterations to specific genomic or control sequences.

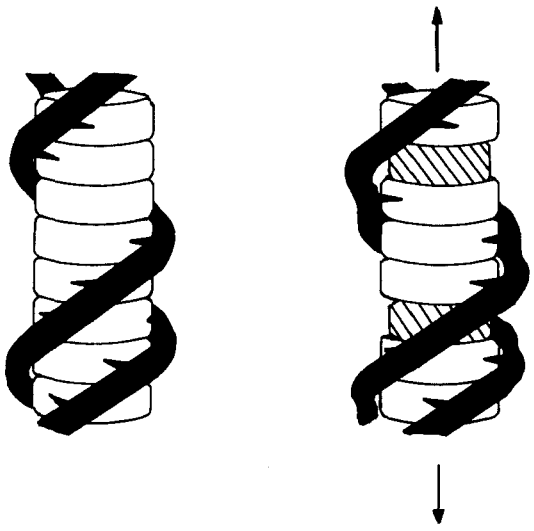


FIGURE 1. The Lerman intercalation model, in schematic form. (A) Illustrates the double-stranded DNA helix; (B) shows DNA with bound-drug molecules as shaded discs intercalated between base pairs, shown as unshaded discs.

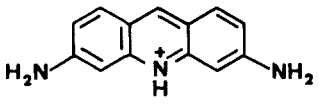


FIGURE 2. Structural formula of proflavine.

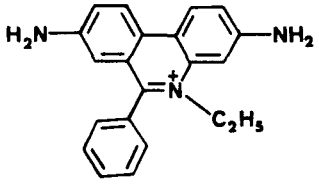


FIGURE 3. Ethidium.

There are, perhaps, several thousand known DNA-intercalating compounds. Only a small number have been studied to the level of kinetics and mechanism of interaction, and even fewer to a point where at least some molecular details of intercalation at defined sites have been elucidated. We shall focus attention in this review on these drugs. Together they span a wide diversity of therapeutic effects as well as showing considerable differences in their DNA-binding behavior.

Proflavine (Figure 2) is structurally one of the simplest of intercalators, and has historical interest in that its frameshift mutagenicity was utilized in deciphering the genetic code. It and many other aminoacridines have bacteriostatic properties that have sometimes been clinically useful. More complex acridines, such as 4′-(9-acridinylamino) methanesulphon-*m*-anisidide (*m*-AMSA),[9,16] can possess antitumor activity. The antitrypanosidal agent ethidium bromide (Figure 3) is well known to molecular biologists as a nucleic acid stain. The anthracyclines daunomycin and adriamycin (Figure 4) are clinically useful anticancer drugs; daunomycin is chiefly effective against acute leukemia, whereas adriamycin has an exceptionally wide spectrum of activity against a variety of neoplasms including solid tumors. It is probably the most potent anticancer drug in current clinical use. Unfortunately, both drugs produce severe cumulative cardiotoxicity. Thus, there has been a continuing search for new equipotent analogs with diminished toxicity, which has prompted numerous studies on their modes of DNA binding. Actinomycin D (Figure 5) contains two pentapeptide units crucial

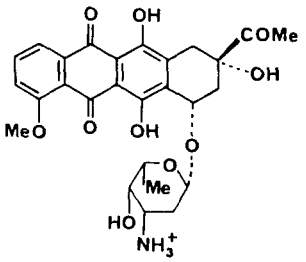


FIGURE 4. Daunomycin.

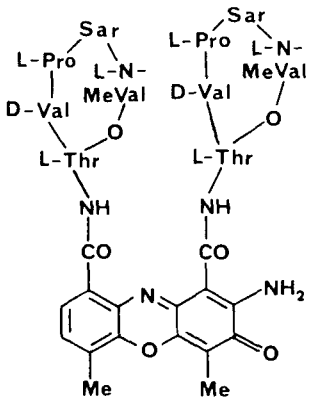


FIGURE 5. Actinomycin D.

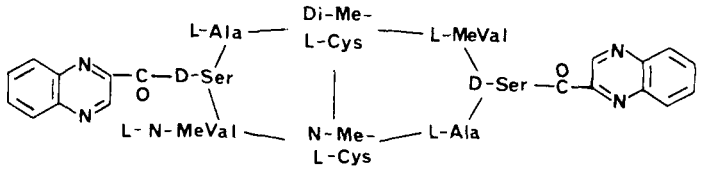


FIGURE 6. Echinomycin.

for its biological activity as a specific inhibitor of RNA polymerase via DNA binding. Its high toxicity precludes its clinical use, except in the treatment of refractory cancers such as Wilm's tumor. Echinomycin (Figure 6) is a naturally occurring quinoxaline antibiotic that has experimental antitumor activity. It is distinguished by having two chromophores linked by a cyclic octapeptide.

II. FUNDAMENTAL FEATURES OF INTERCALATION

The fundamental features of nucleic acid molecular structure have been recently reviewed elsewhere[17-20] and will not be discussed in detail. However, the fact that the classic picture of DNA as a polymeric right-handed double-helical molecule has been modified in several important ways may well have consequences for our understanding of intercalation. The extensive fiber-diffraction studies of Arnott and his school have demonstrated the high propensity of DNA to undergo polymorphic transitions between a wide variety of right-handed structures, dependent upon the environment.[17,18] The major polymorphs are the A and B forms; an important difference between these that is relevant to intercalation concerns the relative dimensions and, hence, accessibility of major vs. minor grooves on the exterior of the double helix. The A form is likely to predominate in natural, average sequences in conditions of relatively greater hydrophobicity, which might well be the situation when surrounded by the planar aromatic groups of intercalating molecules. The relatively sparse X-ray crystallographic data on single crystals of oligonucleotides to date do suggest that certain sequences may be more likely to adopt A-type structures. This is a result of the requirements for particular patterns of first-shell water association being dictated by the sequence of hydrogen-bond acceptors and donors on the base pairs.[22-24] For example, the sequences of TATA[25] and CCGG are on this basis disposed to prefer an A-DNA structure, although neighboring sequences will certainly play important roles in determining the ultimate

Table 1
DNA-BINDING BEHAVIOR OF THE COMMON INTERCALATORS

	Proflavine	Ethidium	Daunomycin	Actinomycin	Echinomycin
Unwinding angle (°)	17	26	11	26	48
Number of base pairs occupied by bound drug	2	2	3	6	5
Dependence on GC content	No	No	?	Yes	Yes
Dependence on B-DNA-type structure	No	No	Yes	Yes	Yes

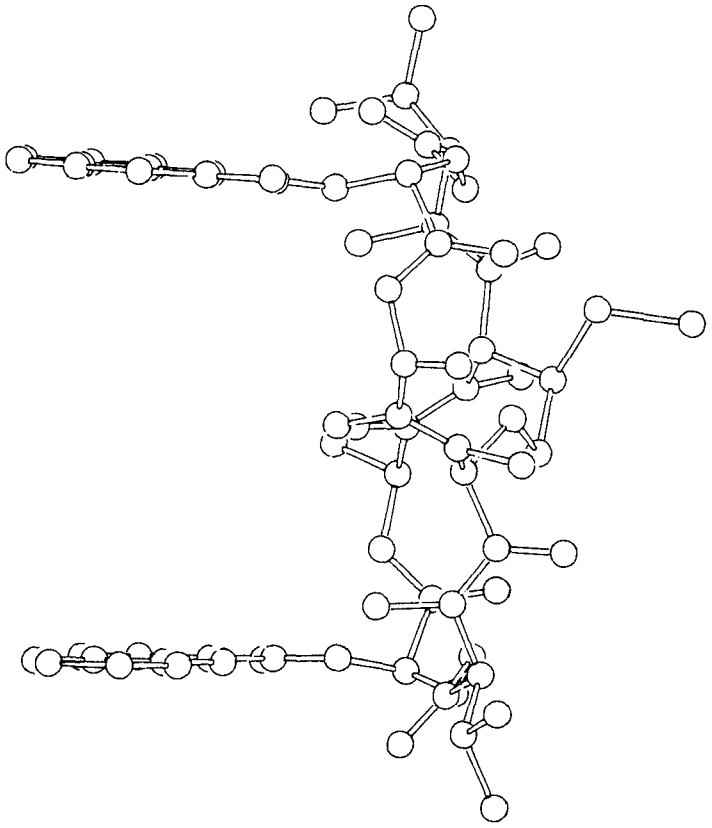


FIGURE 8. The calculated minimum-energy conformation of echinomycin,[37] drawn from coordinates kindly supplied by M. J. Waring.

constants for the common intercalators are all broadly comparable (of the order of 10^5 to 10^6 M^{-1}), indicating that the attractive Van der Waals stacking interaction between drug chromophore and base pairs (which have broadly comparable energies for most of these drugs) is the major stabilizing factor in the interaction of even a molecule with the complexity of actinomycin D. Detailed comparison of affinity constants is not straightforward, not only because of differences in experimental procedures, conditions, and data analysis, but also because of likely mechanistic and kinetic differences in the binding processes themselves.

It is also increasingly clear that the level of drug binding is an important factor in that the very low drug-to-DNA ratios comparable to chemotherapeutic conditions may well result in distinct mechanisms of intercalation.[76,77]

2. Unwinding Angles

Unwinding of base pairs at and around the binding site is a necessary consequence of intercalation. The degree of angular unwinding, measured by the reversal of negative supercoiling in a covalently closed-circular double-stranded DNA,[38] is conventionally calculated relative to the 26° value for ethidium[39] (Table 1); no singly intercalating drug has an unwinding angle larger than this. Angles substantially greater than this, such as the 48° one for echinomycin,[40] are indicative of *bis*-intercalation (Figure 7). The degree of helix unwinding appears to be related to the conformational changes forced in DNA by a drug molecule, and is thus dependent in some manner to the molecular structure of the drug itself. Unwinding angles do not necessarily correlate with DNA-association constants or biological activity; for example, the antitumor acridine *m*-AMSA has a lower affinity for DNA than its inactive positional isomer *o*-AMSA,[41] even though they have virtually identical unwinding angles[42] of 20°. Indeed, relative DNA affinity among members of series of antitumor drugs cannot be taken as a consistently reliable indication of in vivo activity — examination of a series of AMSA derivatives has shown a 6×10^4 range in cytoxicity compared to a 19-fold range of DNA binding constants,[43] with these parameters inconsistently related. It is not surprising that the various measures of DNA binding and intercalative change are not altogether reliable indications of an anticancer effect, since they do not take into account factors such as lipophilicity or metabolism.

The determination of unwinding angles using natural closed-circular DNAs (such as PM2 or SV40) means that the values are, like affinity constants, averaged over all the possible sites. An unwinding angle refers to the total effect produced by an individual drug molecule over all structurally affected nucleotides, and not just those at the intercalation site itself. Since the helix twist angles between adjacent base pairs in both A- and B-DNA are likely to be highly sequence-dependent,[25,44] the extent of changes in them (i.e., unwinding) consequent to intercalation are also probably variable and related in sequence in a yet more subtle manner. As yet, there is no information available on unwinding angles in defined-sequence polynucleotides, which would help to resolve this problem.

3. Other Probes of Intercalation

The technique of transient electric dichroism[45,46] has shown that the simple concepts of DNA length increase and chromophore-base pair coplanarity inherent in simple intercalation models may not be correct in detail. In particular, base pairs around the binding site appear twisted and buckled, and the drug chromophores are invariably not perpendicular to the DNA helix axis. The magnitudes of these changes are dependent on the nature of the drug itself, and presumably on the nucleotide sequence at the site of binding.

The kinetics of intercalation have been studied for several drugs. In general, the reactions are fast and require temperature-jump relaxation techniques. A detailed analysis of proflavine binding[47] has shown that intercalation is preceded by attachment to the exterior of the double helix, largely by electrostatic forces. This initial step, which is very fast, does not involve substantial change in DNA structure. By contrast, ethidium-DNA kinetics, which are in general more complex[48,49] indicate that for proflavine outside-bound forms are unimportant and instead direct ligand transfer mechanisms operate. It is noteworthy that the DNA complexes of both actinomycin[50] and echinomycin[51] show slow dissociation kinetics. It has, accordingly been suggested[50] that this factor is important for the biological activity of actinomycin, in that the long life of the DNA complex very effectively blocks RNA polymerase from moving along the DNA template.

4. Sequence Preference and Specificity

There are 16 distinct dinucleoside sites in DNA for a drug to intercalate in-between. The number of possibilities becomes much larger when account is taken of the adjacent base pairs which may have an effect on binding. Experiments with natural DNAs, including those with high G.C or A.T content, will provide data averaged over all bound sites and perturbed sequences. Even if a single dinucleoside site is absolutely preferred, it is unlikely that there will be an absolute requirement for particular flanking sequences unless they are specifically involved in subsidiary interactions with the drug. Experiments with defined-sequence oligonucleotides are, in principle much more informative, although as recent structural studies have shown, a repetitive polynucleotide may have a structure that is distinct from that of a few repeating units when embedded in another sequence.[21,52,53]

a. Proflavine

There have been numerous studies of proflavine-polynucleotide binding; however, no consensus of base or sequence preference has emerged. Viscometric and kinetic studies[54,55] have found a greater length increase for A.T-rich DNA, and greater external binding with G.C-rich DNA (from *Micrococcus lysodeikticus*). These results appear to conflict with a recent thermodynamic examination[56] of proflavine intercalation, which concluded that the enthalpy of interaction as well as the binding constant depend on DNA base composition. The drug binds to single- and double-stranded RNAs as well as DNAs. A qualitative UV absorbance study of proflavine binding to defined-sequence tetranucleotides[57] has shown greater binding to d(CCGG) and d(CGCG) than to d(GGCC), which has been interpreted in terms of preference for the CG pyrimidine-3′,5′-purine sites in the first two oligomers.

b. Ethidium

Ethidium does not appear to have a marked base-composition dependence. However, a study[58] with several deoxy- and ribo-polynucleotides has revealed a number of subtle differences between their equilibrium binding behavior. Calf thymus DNA and the alternating copolymers poly(dA-dT) and poly(dI-dC) all had high ethidium binding constants, as did some homopolymers such as polyA·polyT. Others, such as polydA·polydT, had much lower affinity for the drug. The behavior was further complicated by differences in a number of neighbor-excluded sites; RNAs had three excluded base-pairs per bound drug molecule, in contrast to two for DNAs. Evidence was found for cooperative binding to the synthetic polynucleotides, though not to DNA itself, possibly because of the averaging of counterbalancing factors. Cooperative binding at low drug levels has been found[59] with *Escherichia coli* DNA and low-salt poly(dG-dC). At high salt concentrations (4.4 *M* NaCl), the binding of ethidium is highly cooperative[31] in promoting the Z→B transition. It has been suggested[32] that this inhibition of Z-DNA is a resultant of kinetic rather than binding-affinity factors; both ethidium and proflavine need to be at a more than 20-fold higher level than the one-drug molecule per 450 base pairs of actinomycin in order to inhibit the transition.

The interactions of ethidium with short-length oligonucleotides have been extensively studied by chirooptic and fluorescence methods. Binding to dinucleoside monophosphates[60,61] has clearly revealed that ethidium binds more strongly to the pyrimidine-3′,5′-purine sequences CG, d(CG), UA, d(TA) than to any others. These sequences form self-complementary base-paired dimers, which can be considered as polynucleotide double-helical fragments. A recent quantification[62] of these bindings has resulted in affinity constants with d(CG), d(TA), and their sequence isomers, the earlier conclusion of the pyrimidine-3′,5′-purine sequence being favored, although, as before, the drug was found to bind to the A.T dinucleosides with a significantly lower affinity. These differences are ascribed to geometric distinctions between the complexes, particularly in respect to drug-phosphate electrostatic interactions. Extension of these analyses to the tetranucleotide level[63,64] has generally upheld

the conclusion of sequence preference, in that d(CGCG), with two C(3′,5′)G sites, binds ethidium more extensively than its sequence isomers. The finding that the difference in binding between this tetramer and d(CCGG), with one such site, is much more than expected has been taken as suggestive of the importance of nearest-neighbor sequences. This, also, implies cooperativity in adjacent-site intercalation.

c. Daunomycin

In contrast to the simpler drugs above, daunomycin appears to intercalatively bind only to polynucleotides displaying a B-type DNA structure. Thus, it does not bind to double-stranded RNA[65] or to poly (I-C),[66] although no serious study of this drug's effects on the B → A transition of DNA in solution have been reported. The literature on daunomycin binding to DNAs of differing G.C content is conflicting (reviewed by Neidle and Sanderson[67]). For example,[68] the apparent affinity constants (from Scatchard plot analysis) for interaction with poly dG·poly dC and poly(dG-dC) are slightly greater than with poly dA·poly dT and poly(dA-dT). However, the last two polynucleotides were found to differ markedly in the number of base pairs occupied and influenced per bound drug molecule, with the alternating copolymer having the greater number. This suggestion of preference for the alternating polynucleotide was borne out by differences in the thermal stabilities and dissociation constants of the complexes.[69] A recent investigation with DNAs of varying G.C content[70] concludes that some G.C preference exists, as a result of both equilibrium dialysis and enhancement of G.C-rich cesium chloride density gradient experiments. It is not unexpected that this preference is greatest at low drug-DNA levels.

d. Actinomycin D

Actinomycin D is unique among intercalating drugs in displaying an apparently almost absolute base specificity. Early studies[71,72] showed that the DNA binding behavior of this antibiotic is strongly dependent on the integrity of the chromophore with respect to the pentapeptide rings, and that the chromophore alone does not show the G.C specificity of the intact molecule. An extensive series of experiments[73] with 17 different DNAs and synthetic polynucleotides has shown that the specificity requirements of this drug are complex. It binds to natural DNAs (except *cancer productus* crab DNA, which contains 3% G.C) and to poly (dG-dC), poly dG·poly dC. The repeating dinucleotide polymers bind actinomycin D only slightly less effectively, whereas neither poly(dT-dG)·poly(dA-dC) and poly(dT-dC)·poly(dG-dA) polynucleotides nor poly (dI-dC) showed detectable interaction. Surprisingly however, strong binding to poly dI was found, which was reflected in the marked inhibition of in vitro poly dI transcription. It was inferred from these results that the previously assumed absolute requirement for a 2-amino purine is not necessary in all cases, especially since poly (dA-dT-dC)·poly (dG-dA-dT) was found not to bind the drug. Nevertheless, as is suggested,[73] the molecular structures of the actinomycin complexes may well be very different in these diverse situations, particularly in the case of the poly dI one, which would not be expected to form an intercalation complex analogous to that with double-stranded B-DNA. It was further inferred that a likely intercalative binding site for most of the polynucleotides examined would have a 2-amino purine-3′,5′-pyrimidine sequence. This has received substantial support from a number of nuclear magnetic resonance studies, detailed in a subsequent section. However, examination of the circular dichroism spectra of actinomycin complexes with several DNAs has suggested,[74] on the basis of calculated first-neighbor frequencies, that C-(3′,5′)-G and G-(3′,5′)-C are the two favored sites for the drug. The former site may be a reflection of adjacent-site influence, as well as of the real possibility that differing polynucleotide polymorphisms can have an unpredictable effect on the spectral analysis. The overall concensus of opinion remains that the GC site is the most favored one for actinomycin. This site in a double helix has twofold symmetry. Since the

drug itself can show almost exact twofold symmetry as well, it has been speculated that the complex of -GC- and actinomycin will have this same symmetry.

It has been known for some while that actinomycin and daunomycin bind to different sites on DNA.[75] Thus, the observation[76] that the latter drug (and its closely related analog Adriamycin) cooperatively enables the binding of actinomycin to double-stranded poly(dA-dT) to take place is a surprising one; as stated above, this suggests that daunomycin produces structural changes on the polynucleotide that result in the accommodation of actinomycin at nearby sites, such that the latter's binding requirement is a steric rather than a 2-amino purine one. This conclusion is supported by the finding[77] of an increase in actinomycin-cooperative DNA interaction with decreasing ionic strength, which indicates that aspects of DNA mobility and flexibility are involved in the binding process.

e. Echinomycin

This *bis*-intercalator does not appear to require specific bases in its interactions with polynucleotides. It is, though, a powerful discriminator of different sequences. Thus, it binds to poly (dG)·poly (dC) about three times more tightly than to poly (dG-dC) which, in turn, binds better to poly (dA-dT), and only very weakly to poly (dA)·poly(dT).[40] In overall terms, there appears to be some preference for G.C base pairs although the significant differences in the number of base pairs per bound drug suggests that nucleic acid structure and the molecular details of the intercalative interaction play a dominant role in the selectivity. This conclusion is reinforced by observations of dramatic changes in polynucleotide selectivity with triostin A (with an additional sulfur atom in the central peptide cross bridge). This drug has a quite different order of interaction (and therefore, sequence preference) with the above polynucleotides, in that poly (dA-dT) binds more strongly than poly (dG-dC). It is suggested[78] from these results that echinomycin has a moderate preference for sites containing three G.C base-pairs, whereas triosin A only requires one G.C per bound drug molecule.

III. NUCLEAR MAGNETIC RESONANCE STUDIES OF INTERCALATION

NMR techniques are undoubtedly the most powerful of all methodologies for investigating molecular structure and dynamics in solution, and thus in principle may complement and even enhance X-ray crystallographic results. The advent of high-field (>400 MHz) instrumentation and associated techniques of analysis such as two-dimensional NMR and nuclear Overhauser effect measurements is now rendering attainable the signal assignment of complex oligonucleotides in terms of conformational and base-pairing properties. We cite as recent examples of the power of the method analyses of the sequence dependence of base stacking in a dodecanucleotide[80] and of the conformation of a partially methylated octanucleotide.[81] NMR studies of drug-nucleic acid complexes have been especially fruitful when monitoring base-pair proton resonances and aromatic protons from drug chromophores.[11,82,83] In principle, analysis of these latter protons, whose chemical shifts are perturbed upon intercalation by the ring-current effect induced by the adjacent base-pairs, can provide information on the geometry of the drug in its binding site.

A. Proflavine and 9-Aminoacridine

A study[57] of proflavine binding to the tetranucleotides d(CCGG) and d(GGCC), monitoring the nonexchangeable proton resonances of base pairs and drug, has been interpreted in terms of a model for proflavine intercalation in which the chromophore is extensively and symmetrically overlapped between base pairs of both strands. This result is in accord with the Lerman model[5] and rules out alternatives which have the drug asymmetrically intercalated, and lying between bases of only one strand.[84] Intercalation into poly (dA-dT)[85] occurs

preferentially at the dT-3′,5′-dA (pyrimidine-3′,5′-purine) sites, with the geometry of the bound drug complex being similar to that found for the tetranucleotide complexes. This study also observed changes in sugar pucker upon intercalation, but was unable to quantify these on account of line-broadening effects.

9-Aminoacridine exhibits significant differences in its binding behavior with oligonucleotides, compared with other simple intercalators. A detailed study with several deoxydinucleoside phosphates[86] has shown only a weak preference for pyrimidine-3′,5′-purine over purine-3′,5′-pyrimidine intercalation, although both are preferred, compared to nonalternating sequences. These latter have the drug molecule stacked asymmetrically over one strand of a dimer duplex, a factor that, it is suggested, could be relevant to the frameshift mutagenic specificity of 9-aminoacridine for the sequence d(GGGG). This drug intercalates into a number of sites in d(AGCT),[87] including GC and AG/CT ones, with the former one being preferred at lower drug levels.

B. Ethidium

The studies at the dinucleoside and tetranucleotide levels on ethidium binding to self-complementary duplexes,[60,61,63,64] discussed in Section II.4.b, employed NMR proton variable-temperature experiments to verify that the drug was, indeed, intercalated into a duplex-type structure. Analysis of overlap geometry suggested[64] that the geometry of the bound ethidium was at least similar to that observed in the crystal (see Section VII.B) and that pyrimidine-3′,5′-purine sequence preference intercalation occurred. More detailed information on the sugar conformations in CG and d(CG) intercalated with propidium (an ethidium analog with a butyl side chain at the protonated ring nitrogen atom) suggests[88] that mixed C3′*endo*-3′,5′-C2′*endo* puckers are the major populations for each strand at low temperatures. Similar conclusions have been reached for ethidium and CG. However, an alternative examination[89] of this data indicates that the situation is complicated by the lack of complete drug complexation, but that it does indicate a significant population of complexes with nonmixed sugar puckers, and, indeed, some with a 50:50 mixture of C2′*endo* and C3′*endo* at the 3′ end of each strand. Thus, a simple view of the puckering in dinucleoside complexes seems inappropriate and, in any case should not be directly extrapolated to larger oligomers. The trimer CUG forms ethidium intercalation complexes that probably involve the U residues being displaced from the site of drug binding;[90] the C.G residues surrounding the ethidium molecule have sugar puckers indistinguishable from the unbound trimer.

C. Daunomycin

In view of the fact that this drug excludes three base pairs of DNA from further binding, the relevance of studies with dinucleosides is not clear. The duplex of the tetranucleotide d(GCGC) was found to be stabilized by daunomycin,[91] and the differences in proton chemical shifts observed for various groups on the drug molecule upon intercalation were interpretable in terms of some aspects of the geometry. This has ring D and the sugar group of the drug almost unperturbed by base pairs, a conclusion also found in the daunomycin-poly (dA-dT) complex.[91-93] This system has been analyzed in terms of chromophore-base-pair overlap and geometry, and a molecular model obtained, which is in excellent agreement with X-ray crystallographic data (Section VII.C). However, the question of site (TA vs. AT) selectivity in this complex could not be answered, even though two resolved ^{31}P resonances were observed. These indicate that differential binding to these two sites was indeed taking place. This polynucleotide-daunomycin complex did not show any changes in the proton resonances of the sugar moiety of the drug upon binding, indicating little, if any, interaction between this group and the nucleic acid, although an NMR study of the daunomycin-d$(TA)_3$ system[94] does indicate that this may not be generally true.

D. Actinomycin D

The guanine preference shown by this drug has prompted a number of NMR studies with relatively simple systems modeling nucleic acids. These have enabled the interactions involved to be analyzed in very considerable detail.[89]

Purine mononucleotides form 2:1 complexes with actinomycin.[95] It is, however, at the dinucleoside level and above that specificity is observable. A study with several different deoxynucleotides[96] concluded that only d(GC) cooperatively formed a 2:1 intercalation complex with the drug, whereas others such as d(CG), d(TA), and d(AT) did not. This conclusion has been confirmed and extended by both proton and ^{31}P NMR studies on the actinomycin-d(GC) complex,[97-99] which have been interpreted in terms of possible geometries for the chromophore-base-pair part of the complex. These appear to support the X-ray crystallographic-derived model (see Section VII.D), which has been extrapolated from the crystal structure of an actinomycin-deoxyguanosine complex. These studies could not define the structure of the complex as a whole, which would represent the conformation averaged over the NMR time scale. Nonetheless, they suggest the presence of hydrogen-bond interactions between N2 of guanine and the peptides of the drug, since the amino protons are shifted downfield.

The binding at a G-(3′,5′)-C site has been verified in several studies with oligonucleotides. A 2:1 oligomer drug complex is formed with d(ATGCAT),[100] which does not have the exact twofold symmetry of the hexamer duplex alone. This was found by observing the ^{31}P resonances of the central G-(3′,5′)-C phosphates which increased in number upon drug binding, indicating different environments for these phosphates on the two strands. This effect was also observed with d(CGCG) and d(CGCGCG),[101] which have been interpreted in terms of changes in phosphodiester torsion angle, possibly following hydrogen bonding between one phosphate group and the amino substituent on the phenxazone chromophore of the drug. Furthermore, there was some evidence of phosphate groups distant from the intercalation site being affected by the drug binding. Binding of actinomycin to the 12-mer d(CGCGAATTCGCG) has shown that intercalation at the G.C sites results in slight kinetic destabilization of the central A.T base-pairs,[102] thus altering the opening/closing properties of these relatively weaker base-pairs. This provides further evidence for the general hypothesis of drug intercalation resulting in structural and dynamic changes that are relatively remote from the binding site. This dodecanucleotide can also bind actinomycin after interaction in the minor groove with the peptide antibiotic netropsin,[102,103] which binds to the central AATT residues. The ternary complex has significantly stabilized A.T and G.C base-pairs compared to either the oligomer alone or its actinomycin complex.

Extensive use has been made of nuclear Overhauser effects in order to obtain distance and, hence, geometric information on the actinomycin-d(AGCT) complex.[104] To date, this is the most detailed NMR study of a drug-oligonucleotide intercalation complex. As with many complexes, line-broadening effects made a number of the chemical shifts difficult to assign. The absence of effects between adjacent G.C base-pairs indicates that intercalation takes place between them. Confirmation of the crystallographic results was obtained which show the peptide groups of the drug fitting into the oligomer minor groove (see Section VII.D). A more surprising finding is that the terminal A.T base-pairs of the complex are twisted with respect to the G.C ones.

IV. BIOCHEMICAL APPROACHES TO INTERCALATING DRUG SITE PREFERENCE

A number of techniques derived from DNA biochemistry have been used as a means of accurately probing the DNA sequence preference of intercalating drugs. This is a fairly new area of investigation and most of the work to date has concentrated on actinomycin D site preference. There have been only limited studies on other intercalating drugs.

Table 2
THE PROTECTION OF RECOGNITION SITES OF VARIOUS RESTRICTION ENZYMES OF LAMBDA DNA BY ACTINOMYCIN

Restriction enzyme	Recognition sequence (↓ = cleavage site)	Actinomycin D(M) Partial protection	Actinomycin D(M) Complete protection
Eco RI	5'G↓AATTC 3'CTTAA↑G	5×10^{-4}	None
Eco RII	5'↓CCTGG 3'GGACC↑	2×10^{-5}	0.6×10^{-4}
Hpa I	5'GTT↓AAC 3'CAA↑TTG	3×10^{-5}	
Hpa II	5'↓CCGG 3'GGCC↑	1×10^{-5}	4×10^{-5}
Hind II	5'GTPyPuA↓C 3'CAPuPyT↑G	1×10^{-5}	5×10^{-4}
Hind III	5'A↓AGCTT 3'TTCGA↑A	3×10^{-5}	None

A. Restriction Enzyme Inhibition

Restriction endonucleases are a group of DNA-cleaving enzymes, each recognizing a small number of unique sites of particular nucleotide sequence. The class II restriction endonucleases have been particularly useful for the analysis of DNA structure and function, since they both recognize and cut at a specific nucleotide sequence.

For any enzyme, the restriction sites themselves are identical, but each site has a different surrounding base sequence because of its unique location on the DNA studied. The principle behind the restriction enzyme inhibition method[105,106] is that the unique restriction endonuclease pattern for a particular type of DNA, for example, the plasmid pBR322, may be altered when the DNA is preincubated with a drug which may bind preferentially to the endonuclease cleavage site.

This change is seen because endonuclease activity is inhibited as the drug now occupies the enzyme's binding site. The cleavage rates may differ from the plasmid without drug and the o ler of reactivity can be determined by monitoring the appearance and disappearance of DNA fragments on a gel electrophoresis system. Nonselective inhibition would only alter the rate of digestion, whereas selective inhibition also affects the order of cleavage.

1. Actinomycin D

The G.C. requirements of actinomycin D intercalation have been used[107] to select restriction endonuclease recognition sequences in lambda (λ) DNA, which may be protected by actinomycin D. Six sites were examined and the results are summarized in Table 2. The restriction endonuclease Hpa II, EcoR II, and Hind III all contain GC or CG in their recognition sequences, which are protected from cleavage by actinomycin D. The results obtained for Hpa I and EcoR I were unusual. Hpa I was partially sensitive, while EcoR I was insensitive to the drug. In both bases the G.C pairs are at the ends. It was suggested

Table 3
PROTECTION OF HIND III SITES WITH DIFFERENT CONCENTRATIONS OF ACTINOMYCIN D

Actinomycin D concentration (*M*)	Fragments produced
0	A | F | E | B | G | C | D
3×10^{-5}	AF | EB | GC | D
5×10^{-5}	AFEB | GC | D

that the difference in sensitivity could be due to differences in the sequence environment of these recognition sites.

It is known that all EcoR I sites in DNA are bordered by dA or dT,[108] and either TGA or TGT are the G-containing sequences in the region of λ DNAs EcoR I sites. These sequences are not satisfactory for actinomycin D binding.[109]

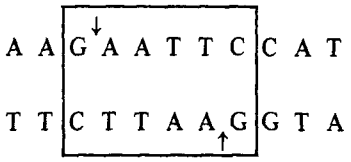
The partial sensitivity of Hpa I could mean that some of the 14 recognition sites are sensitive, having GC pairs near them, but others are insensitive since they have AT pairs near them. The sequence of λ DNA has since been published;[110] examining the sequences on either side of the Hpa I sites, it appears that the explanation for the partial sensitivity to actinomycin D is probably more complex than the one initially proposed,[107] since there are no clear-cut GC- or AT-rich regions. Another explanation for Hpa I's partial sensitivity could be that unlike the sites for Hpa II, EcoR II, and Hind III, actinomycin intercalation occurs quite far from the actual cleavage site (see Table 2). So, intercalation may have an effect at a distance, although this is not so protective as intercalation occurring at the cleavage site. It has also been pointed out[111] that site protection was dependent on the actinomycin D concentration. With increasing concentrations, new and larger DNA restriction fragments appeared and some of the original fragments disappeared (Table 3). The different affinities of actinomycin D for different sites of the same endonuclease could be due to differences in the surrounding sequences of the individual sites.

A similar system[112] was used to look at the effect of actinomycin D, ethidium bromide, and also proflavine on the cleavage of the oligomer d(GGAATTCC) as well as pBR322 DNA by the restriction endonuclease EcoR I (recognition sequence GAATTC). The C_{50}'s values (concentrations at which 50% cleavage occurred) were compared for the oligomer. It was found that actinomycin D had a C_{50} of 300 m*M* for the oligonucleotide. This C_{50} was higher than that for proflavine and ethidium bromide, which had C_{50}'s values of 60 and 150 *M*, respectively. This indicated that actinomycin had the lowest affinity for this site. Preincubation of pBR322 DNA with actinomycin D resulted in a C_{50} of greater than 500 m*M*. The C_{50}'s values for proflavine (150 m*M*) and ethidium bromide (200 m*M*) were also higher than those observed for the oligonucleotide. Thus, actinomycin D has the lowest affinity of the three drugs for the EcoR I site.

The differences in affinity of these intercalating drugs for the EcoR I site on the oligomer, compared to the same site on the plasmid DNA, were interpreted in terms of a scavenging effect: the large excess of DNA bound outside the recognition sequence helped direct actinomycin to particular sites, and kept others free. It may also be viewed as indicating that these drugs prefer other sites on the DNA more than the EcoR I site. For actinomycin D, this agrees with previous results,[107] which indicated that the EcoR I site in λ DNA was insensitive to this drug. This result is unsurprising since the surrounding sequence of EcoR I in pBR322, like that of λ, is also AT rich.

Table 4
INHIBITION OF THE HPA I SITES ON ϕX174 DNA BY DAUNOMYCIN AND ACTINOMYCIN D

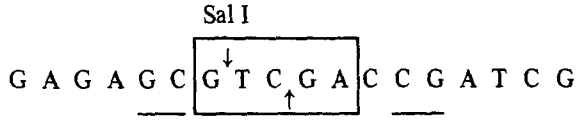
Drug	I_{50} (μ*M*)	
	Site 1292 GACTGTTAACCAAAC	Site 5022 ATACGTTAACAAAAG
Daunomycin	6.7	12.4
Actinomycin	25	40



A quantitative means of measuring the different rates of digestion by restriction enzymes at their recognition sites has been developed.[106] This involved arithmetical treatment of the integrated areas from a densitometer scan of an ethidium bromide stained gel. With this method the effect of actinomycin D and daunomycin on the cleavage of ϕX174 by the restriction enzymes Hpa I (recognition sequence as in Table 2) and on pBR322 by Hinc II and Sal I was investigated. Hpa I has three recognition sites on ϕX174, two of which, at positions 1292 and 5022, were studied.

The effect of each drug on the sites was compared by measuring the I_{50} level, which is the concentration of drug at which 50% inhibition occurs (Table 4). From these results it appears that daunomycin has a greater affinity for Hpa I sites than actinomycin. This probably indicates that daunomycin has a greater preference for Hpa I's AT-rich recognition sequence. Actinomycin D is the less effective inhibitor, probably because neither Hpa I site has a G.C sequence, even within one helical turn of the cleavage site. Also, for both drugs, the I_{50} at the 5022 site is almost twice that at the 1292 site. This implies a preference for the 1292 site, which is less AT rich than 5022. However, with an error of 50%, because of the difficulty in determining the point of maximum density for each band on the gel, these results for the site comparisons are marginally significant.

The digestion of linearized pBR322 by Hinc II (recognition sequence GTPyPuAC, where Py Pu is GC) and Sal I (GTCGAC), after preincubation with actinomycin D, has been studied. Preliminary results showed that both these enzymes were strongly inhibited at one site with an



I_{50} of less than 3 m*M*. The environments of these sites are GC rich, with the dinucleotide sequence GC situated close to both sides of the site.

2. Echinomycin

The same method[113] has been used to examine the site preference of the quinozaline antibiotics echinomycin, triostin A, and several of their analogs. Echinomycin (6.5 m*M*) was found to preferentially bind to the Hpa I site at position 5022, compared to the site of

the 1292 position. This is surprising since echinomycin is known to have a broad GC preference, and the 5022 site is the more AT rich of the two Hpa I sites investigated.

It was suggested that this was because the 5022 site possesses a tight binding sequence not present in the 1292 site. A search for dinucleotide sequences unique to each of these sites showed that the 5022 site has AT and GC in its vicinity and it was suggested that echinomycin might bind more tightly to one or both of these sequences than to all others. Quinomycin C, an analog of echinomycin, in which the *L-N*-methylvalines are replaced by *N*-γ-dimethyl-allo-isoleucines, shows a preference. The biquinoline analog of echinomycin, in which the quinoxalines have been substituted with quinolines, shows some inhibition at the 5022 site. At the 1292 site, cleavage by Hpa I is substantially increased.

Triostin A inhibits both sites at substantially lower concentrations (0.66 m*M*) than echinomycin (6.5 m*M*). However, at double the concentration (1.3 m*M*) it was found that triostin A inhibited both the Hpa I sites equally. Triostin C is an analog of triostin A, in which the *L-N*-methylvalines have been replaced by *N*-γ-dimethyl-allo-isoleucines. At low concentrations (3 m*M*) it seems to slightly activate cleavage at the 1292 site. At higher concentrations, like triostin A, it inhibits both Hpa I sites equally. The *des-N*-methyl analog of triostin A, TANDEM, is a weaker inhibitor of cleavage than triostin A, possibly since it has a lower binding affinity for natural DNAs. TANDEM, however, preferentially inhibits the cleavage of the 5022 site more than the 1292 site, like echinomycin and its analogs. The *bis* quinoline analog of triostin A is unusual, in that it inhibits the cleavage of the 1292 site more than the 5022 one. This could mean that this analog has a GC preference since the 1292 has a relatively higher GC base pair content in its environment compared to the 5022 site. A more detailed examination of the base sequence around the 1292 sites shows that C.C and G.A dinucleotides are found in the vicinity of 1292 but not near 5022. Perhaps this analog binds more tightly to one or both of these sequences than to other dinucleotides present.

The restriction enzyme inhibitor method for explaining base sequence preference does seem to be a useful technique. Although only a few drug-DNA interactions have been extensively investigated, from the results to date, the effects of base sequence on drug binding preference appear to be complex. The observed preferences extend beyond simply the presence or absence of GC or AT base-pairs in the recognition site. However, the effect of the nucleotide sequence neighboring the recognition site is still obscure. The complexity of some results may also be a resultant of the restriction enzymes used and of the sites studied. If the restriction enzyme cleaves DNA to give "sticky ends" (i.e., EcoR I), interpretation of the inhibition by intercalating drugs may be difficult, since intercalation extends over both strands at the binding site. The enzyme EcoR I being dimeric will be inhibited even if only one site is occupied, so whether the drug occupies one or both sites will not be known. This is important since the neighboring sequences in either side of the recognition site are likely to differ.

Also, it has been shown, at least for EcoR I, that there is a low amount of nonspecific binding of the restriction enzyme to DNA. At high concentrations nonspecific binding may have a significant effect on the DNA cleavage pattern. It is important to be sure that the concentration of enzyme used is such that nonspecific binding is insignificant. Otherwise, it may prove difficult to interpret the cleavage inhibition patterns observed after preincubation with the drug. Nevertheless, the restriction enzyme inhibition techniques will probably yield increasingly valuable information on drug binding preferences.

B. "Footprinting" Techniques

Two "footprinting" techniques have recently been developed in the search for a means of mapping drug binding sites.

One method uses[114] a simple bifunctional molecule, methidiumpropyl EDTA (MPE), to probe each base-pair in a given sequence. MPE, in the presence of ferrous iron and oxygen,

produces relatively nonsequence-specific single-strand breaks in DNA, effectively mimicking the action of DNAase I. The treatment of drug-protected DNA fragments with MPE.Fe II is followed by Maxam-Gilbert sequence analysis,[115] to determine the preferred binding site of intercalating drugs.

The other method uses DNAase I[116] in an analogous manner. The technique, originally designed to look at protein-specific binding,[117] involves the partial degradation by DNAase of a 5′ end-labeled double-stranded DNA segment in the presence and absence of the binding drug. The fragments produced are visualized by electrophoresis and autoradiography, next to the base-specific reaction products of the Maxam-Gilbert sequencing method.[115]

In addition, microdensitometric scanning of the gel autoradiograms is applied to aid visualization of band intensity changes and initiate the determination of thermodynamic parameters of drug binding as a function of base sequence.

"Footprinting" with MPE.Fe II[114] has been used to compare the binding sites of actinomycin D and daunomycin on a 516-base-pair fragment of pBR322 DNA. Actinomycin D showed a series of alternating high- and low-density bands on the autoradiogram. The sharpest inhibition patterns were seen at high concentrations. The low-intensity bands, indicating reduced MPE.Fe II cleavage, appear to be 4 to 16 nucleotides in length and centered around at least one or more GC base-pairs. By contrast, daunomycin showed a uniform cleavage pattern, which was not very different from that of the MPE.Fe II control (MPE.Fe II cleavage in the absence of drug).

This apparent inability of daunomycin to generate a nonrandom DNA cleavage inhibition pattern could indicate a lack of base composition specificity. This result conflicts with evidence obtained by enzyme inhibition experiments,[106] which indicated that daunomycin had a preference for the AT-rich Hpa I recognition site (GTTAAC). An alternative explanation is that the binding site size and/or the dissociation rate from DNA for daunomycin may not be in a suitable range to give site-specific protection from MPE.Fe II cleavage. High-affinity sites for this drug might not be present in the relatively small DNA studied. Other investigations on the site specificity of daunomycin[65-70, 91-93] are also conflicting. So whether or not daunomycin has any site specificity is still not known.

The sequence specificity of actinomycin D binding to a fragment of pBR322 has also been investigated by the DNAase I "footprinting" technique.[116] By varying the actinomycin D concentration at constant DNA levels, it was found that the drug is capable of binding to many sequences at high drug concentrations, with an r_t of greater than 0.1.

$$r_t = \frac{\text{input drug molecules}}{\text{total base pairs of DNA molecules}}$$

At an r_t of 0.09, only three sites showed a higher affinity than the others, with over 75% protection from cleavage. The sites (ACGCAGTCA, GCGCTCA, and GTGGATGCT) occurred at positions 38—46, 72—78, and 102—110, respectively, along the 11-base-pair Hind III/Hae III restriction fragment. Each of these sites is 7 to 9 base-pairs long and about 32 base-pairs apart. All three sites were GC rich and contained the dinucleotide G(3′,5′)C. Other GC sites are present between these three high affinity sites, but are not occupied by actinomycin. The explanation of why actinomycin D binds at certain GC-rich sites but not at others and the significance of the 32-base-pair periodicity are still obscure. The effect of lowering the actinomycin D-to-DNA ratio was observed by densitometric scans of a DNAase partial digest for a 25-base-pair segment including the 72 to 78 binding sites. Enhancement of cleavage was observed at base 82, which was interpreted as being caused by a conformational change in the DNA induced by actinomycin binding. This result is consistent with NMR[82,102] and block copolymer studies.[118]

The footprinting techniques examine base sequence preference from a completely opposite viewpoint to that of the restriction enzyme inhibition techniques. The former involve taking a relatively small fragment of plasmid DNA instead of looking at the whole plasmid, and determining by sequencing techniques the sites of inhibition of a relatively nonspecific DNA cleavage agent. Restriction enzyme inhibition looks at inhibition at the specific recognition sites of endonucleases. Further studies of sites that footprinting indicates have high affinity for intercalating drugs promise to be a powerful combination of the two techniques.

C. Nick-Translation Inhibition

It is not possible to look at site selectivity of intercalating drugs by directly using one of the two major DNA sequencing techniques.[115,119] These rely on chemical modification of DNA bases, whereas intercalation as an equilibrium binding process will not change the unbound sequencing patterns. Sanger's dideoxynucleoside technique[119] has been modified into an assay which makes use of nick translation to look for sites of DNA polymerase inhibition.[120] Nick translation is a process whereby DNA polymerase binds to a nick in the DNA template and in the presence of the required deoxynucleoside triphosphates, fills in the gap with missing nucleotides. This action is coupled to 5′ → 3′ exonuclease action which hydrolyzes the DNA so that the nick is gradually moved along the sequence, followed by the DNA polymerase inserting new deoxynucleotides.

In this technique a nick is specifically induced in a double-stranded DNA plasmid. The nicked plasmid is incubated with deoxynucleoside triphosphates and drug and the reaction is then initiated by DNA polymerase I. After a given time, the reaction is stopped and the samples are run on a sequencing gel.

Actinomycin D has been found to inhibit nick translation[120] in a highly sequence-specific manner at just four sites in a 200 nucleotide sequence of pBR313. The two major sites of inhibition, represented by stops in nick translation of the template, were at nucleotides 109 to 116 (GTCAGGTG) and 135 to 141 (TGCGCGG). Although there were other GC-rich regions in the sequence, where binding would be expected, only two other sites showed long-term inhibition, nucleotides 65 to 72 (CGCCATTT) and nucleotides 186 to 189 (GGCT). However, binding at these last two sites was less pronounced. During a time-course study, DNA was exposed to 100 mg/mℓ of actinomycin D. Inhibition was initially observed at many GC-rich sites. Twenty minutes after the addition of the DNA polymerase, the only major stop observed was at nucleotide 116. After 35 min, stops occurred at nucleotide 135, and even after 2 hr this remained the major stop.

With ten times less actinomycin D, the only major stop was at the 135 to 141 sequence (TGCGCGG). These preliminary experiments using nick translation clearly indicate that short dinucleotide sequences alone do not determine strong drug binding sites. As shown with the restriction enzyme work, longer sequences enriched in GC or AT are required.

Several observations suggest that perhaps more than the primary sequence of DNA is involved in determining actinomycin D site preference. Firstly, as seen in the DNAase "footprinting" experiments, not all GC-rich sequences have the same affinity for actinomycin D. Secondly, the DNA fragment used is capable of forming an energetically stable hairpin loop between positions 116 and 139. One of the strong actinomycin inhibition sites coincides with the start of this hairpin. This region contains GC sequences, so the hairpin could be stabilized by bound drug. A hairpin structure could only occur if a stretch of DNA was rendered single stranded. This is possible if the 5′ → 3′ exonuclease activity were uncoupled from polymerization.

Also, caffeine at a concentration of 25 m*M* was shown to completely remove the stop induced by actinomycin at nucleotide 116. This would be expected, since caffeine has a much higher affinity for denatured coiled DNA than double-stranded helical DNA, and would be expected to prevent hairpin loops forming.

However, caffeine has such a wide range of effects, including modification of a number of DNA repair functions and partial suppression of DNA polymerase I exonuclease activity, that these interpretations of the effects remain tenuous without more extensive experimentation. Further examination of sequences for inverted repeats which could form hairpins is also called for.

At present the "rules" for total site preference, even for a well-characterized DNA-binding drug such as actinomycin D, are still vague and cannot be clearly stated. The techniques mentioned in this section, as pointed out in another recent review,[121] will probably be most useful in leading to a greater understanding of them.

Although much of the earlier work in this field has been on small linear segments of DNA, more recent studies have been extended to supercoiled DNA, and now methods of looking at DNA in chromatin-drug interactions are being currently developed.[122,123]

V. OVERVIEW OF X-RAY STRUCTURAL STUDIES

The remarkable success of fiber diffraction, combined with model building techniques in determining the molecular structures of semicrystalline natural and synthetic polymeric nucleic acids, has prompted parallel studies on drug-nucleic acid complexes. These have, in general, been unable to define these structures to any detailed level that even approaches those obtained for the unbound nucleic acids. In order to understand the reasons for this lack of success, it is necessary to appreciate the scope and limitations of the fiber diffraction method.[17,18] The diffraction patterns obtained from polynucleotide fibers, which have their molecular helix axes aligned with the fiber axis, are, in general, relatively rich in detail. The spacial positions of the diffraction maxima define the geometric parameters of the helix, such as the rotational relationship between adjacent base-pairs and the axial translation per residue. The information contained in the diffraction intensities corresponds to structure averaged over all residues and, hence, averaged over stereochemical differences between them. It thus assumes that all repeating units are equivalent in order for a meaningful, yet averaged, structure to be derived. The resolution limit of the intensity data is typically about 3 Å; this factor, combined with the inherent difficulty of using conventional structure-solving methods, has meant that sophisticated methods of postulating and refining trial structures have had to be devised.[124] These methods are currently being used to extend the level of complexity of several polynucleotide structures by invoking the repeating unit to be larger than "averaged" mononucleotide.[52,53]

The great power of the fiber diffraction method lies in its unique ability to provide structural information at the polynucleotide level. It has also defined aspects of the extraordinary polymorphism of DNA; doubtless yet more forms of DNA remain to be discovered. However, the method has not, as yet, yielded unaveraged structural information on particular oligonucleotide sequences other than purely repetitive or alternating ones. It is here that single-crystal methods have recently been very powerful in elucidating *ab initio* the structures of small-length oligonucleotides having A-, B-, and Z-DNA features, with special relevance to subtle features of sequence dependence, base stacking, sugar pucker, and patterns of hydration. Nevertheless, it is clear that such X-ray crystallographic studies have several distinct limitations and problems that are not always sufficiently appreciated: (1) the relative lack of resolution (typically 2.5 to 3.0 Å at best) of several short-length oligonucleotide crystals, combined with as yet incompletely understood problems of sugar flexibility disorder, has meant that some of the structures have within them a marked gradation of structural motion and, hence, reliability; (2) it is not, at present, possible to fully assess the significance and role of end effects in these structures. Since most of them are of short sequences, one cannot be confident that many structural features, especially on residues near the ends of these short helical fragments, are particular to the structure concerned, rather than being

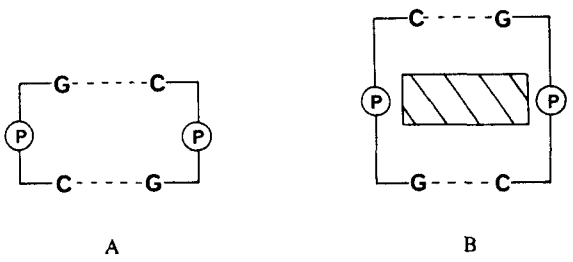


FIGURE 9. Schematic outline of a base-paired self-complementary dinucleoside monophosphate dimer (A) without drug, having the sequence GC; (B) with drug, having the sequence CG. Dashed lines indicate base pairing.

more general properties. Crystal packing forces are also capable of introducing aspects such as overall helix bend. Thus, assessment of these structures will be meaningful only when considerably more single-crystal analyses have been performed.

The fiber and single-crystal data on poly- and oligonucleotides, respectively, should then be viewed, not in contrast with each other, but as complementary. Each provides a differing perspective of nucleic acid structure, and the most fruitful interpretations arise from their synthesis together.

The situation with respect to drug-nucleic acid complex structural data is, unfortunately, less encouraging in several ways. Firstly, fiber diffraction studies have generally yielded X-ray patterns of exceptionally poor quality, resolution, and amount of data, so that accurate model building has only rarely been possible. Secondly, to date, the single-crystal work has, with one exception, been confined to dinucleosides which, perhaps surprisingly, have been beset with problems of crystal quality, lack of atomic resolution data, and consequent difficulties in structural refinement. Although dinucleosides can be considered as the simplest possible models for nucleic acids themselves (Figure 9), end effects in such systems are inherently serious and, as yet, in the absence of further structural data cannot be fully assessed. In spite of these relatively discouraging factors, a coherent picture of dinucleoside intercalation in structural terms is beginning to emerge. It cannot be overemphasized then that our structural knowledge of intercalation is almost entirely confined to such model systems and that current generalizations to polynucleotide complexes still remain speculative.

VI. FIBER DIFFRACTION ANALYSES

The poor quality of diffraction patterns reported for fibers of natural DNA complexed with intercalative drugs may be ascribed to several causes: (1) nonrandom binding due to sequence preference, (2) nonequivalent structural changes to backbone and/or base-pairs on binding, and (3) disordered multiple orientations of intercalated drug molecules. As yet, no successful studies have been reported with synthetic, defined-sequence polynucleotides, which could overcome such problems.

The investigation of fibers of the proflavine-DNA complex by Lerman[5] showed that the 3.4-Å spacing of B-DNA itself was retained. This indicates that the planar drug is coplanar with base-pairs and is crucial evidence for the intercalation hypothesis. The X-ray pattern did not have any layer-line detail at all, so it was not possible to fit or postulate any model more detailed than that in Figure 1. Somewhat improved diffraction patterns for proflavine and acridine orange-DNA complexes have subsequently been reported,[125] which, although still indicative of disorder, had some interpretable layer-line detail. Although no detailed models were produced, optical transform fitting with differing rotation per residue models

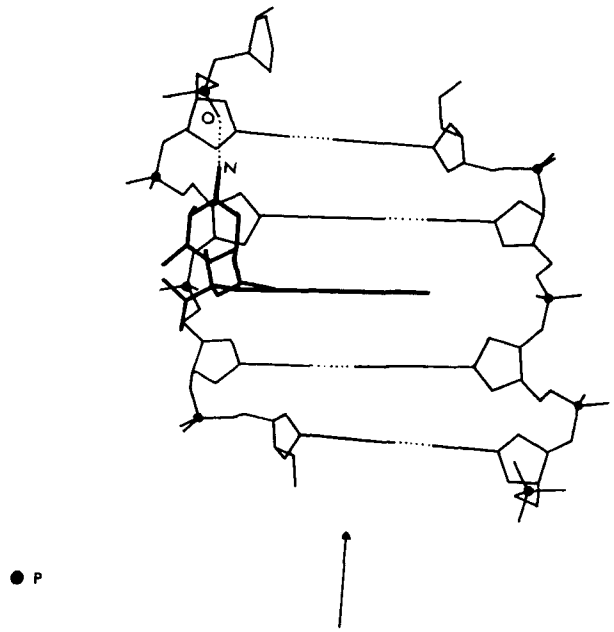


FIGURE 10. The major-groove model for the daunomycin-DNA complex, derived from model building and X-ray fiber diffraction studies. (Reproduced from Neidle, S. and Sanderson, M. R., in *Molecular Aspects of Anticancer Drug Action*, Neidle, S. and Waring, M. J., Eds., Macmillan, London. With permission.)

for DNA suggested that base-pair unwindings per drug molecule of between 12 and 45° were acceptable. On the other hand, molecular modeling of the ethidium-DNA complex[126] suggested that only a 12° unwinding was required in order to produce a 6.8 Å base-pair separation at the intercalation site. The diffraction pattern produced in this study did not of itself provide such information. It showed the characteristic 3.4 Å spacing and indicated that the drug complex forms a thinner helix than B-DNA itself.

The ethidium-DNA model[125] was the first detailed molecular description of an intercalation complex. It has the drug intercalated from the DNA major groove, with its phenyl and ethyl groups protruding into it. The 12° unwinding angle is of historical importance in being the reference standard for intercalative drugs[38] until the definitive 26° determination with closed-circular DNA.[39]

The variability of diffraction from daunomycin-DNA fibers with respect to varying drug-to-DNA ratios and relative humidity has been examined,[127] with particular reference to observed changes in helix pitch correlating with postulated structural models, one of which was an intercalative type. The detailed model produced (Figure 10) has the amino sugar of daunomycin in the DNA major groove, such that there is a strong hydrogen bond between the protonated nitrogen atom and a phosphate group, two base-pairs per drug molecule participating in the binding. The model incorporates a 12° unwinding angle and extensive chromophore-base-pair overlap. It was noted that the alternative minor groove intercalation would have poor drug-base-pair overlap, and so such a model was rejected.

Fibers of an actinomycin-DNA complex[128] had visible dichroism that was interpreted as indicating a perpendicular orientation of the actinomycin chromophore to the fiber axis. Diffraction patterns showed essentially the same, albeit more diffuse and less ordered patterns, as B-DNA. Moreover, unlike other intercalating drugs, no apparent change in helix

pitch was observed with progressively increasing amounts of bound drug. Accordingly, the structural model proposed has the chromophore nonintercalatively situated in the DNA minor groove, forming hydrogen bonds from its amino and carbonyl groups to N3 and N2 atoms of a guanine residue. The peptide rings pack tightly, again in the minor groove.

The most extensive fiber diffraction patterns of any drug-DNA complex have been obtained with a platinum-containing terpyridine compound.[129] This planar molecule had been previously found, by solution studies, to bind intercalatively to DNA. The strong 10.2-Å diffraction maximum was interpreted as the repeating separation of individually bound drug molecules. This requires the drug-drug separation to be two base-pairs, providing powerful support for the neighbor exclusion hypothesis (Figure 7). The excluded-site DNA double helix considered most acceptable in principle had 22 ± 6° unwinding per drug molecule, although they were unable to fully reconcile otherwise stereochemically plausible models (for example, having "mixed" sugar puckers at the intercalation site), with the details of the observed diffraction patterns. A subsequent reinterpretation[130] of these patterns has resulted in a strikingly different molecular structure. The unambiguously smaller unit cell now apparent, in fact, implies that the DNA in this structure cannot be double helical. It is completely unwound so that each intercalated dinucleoside repeat relates to the next solely by translation. This unpresented structure has an alternating *syn-anti* pattern of glycosidic angles along the backbone, pattern of glycosidic angles along the backbone, and resembles part of the left-handed Z-DNA repeat. It has been pointed out[130] that this zero-wound DNA represents a high-energy state intermediate between left- and right-handed forms.

VII. CRYSTALLOGRAPHIC ANALYSES OF DRUG-NUCLEIC ACID COMPLEXES

There are two distinct structural classes of drug-oligonucleotide complexes: those that are truly intercalative and double stranded, and (a minority) that have nonduplex yet base-paired structures. The latter category invariably has opened-out dinucleoside conformations (Figure 11), while still retaining base-drug stacking in some manner[131,132] (Figure 12). Only in the case of the actinomycin-d(GC) complex[133] is there clear relevance to intercalation, so that with the exception of this one structure, the "single-stranded" complexes will not be discussed in detail here.

It is remarkable that all of the true intercalation complexes crystallized appear to follow the sequence-dependence described in earlier sections. Thus, the pyrimidine-3′,5′-purines (Table 5) are the only ones that form an intercalation site. It appears that at the dinucleoside level, others such as (CC + GG) or GC have a greatly diminished, if not a total lack of, ability to form such duplex dimer structures (erroneously termed miniature double helices). As at the time of writing, crystal structures have been reported for complexes involving a number of the structurally simple intercalators. It appears that the practical difficulties involved in obtaining cocrystals with the more complex molecules, daunomycin and echinomycin, have only been successfully overcome in one instance with the former drug. Other drug-oligomer crystals of useable quality remain elusive, in spite of very considerable effort in a number of laboratories.

A. Proflavine and Related Acridines

The 3.2 proflavine:CG complex[134,135] is one of the more accurately determined in the series, with observed diffraction data extending to 0.85 Å and reliably refined atomic parameters. The structure (Figure 13) has an exact twofold symmetry passing through the central ring atoms of the intercalated proflavine molecule, and so the two antiparallel ribodinucleoside phosphate strands are equivalent. The intercalated drug is an equidistant average of 3.35 Å from each Watson-Crick base-pair. These are bent and twisted (by up to

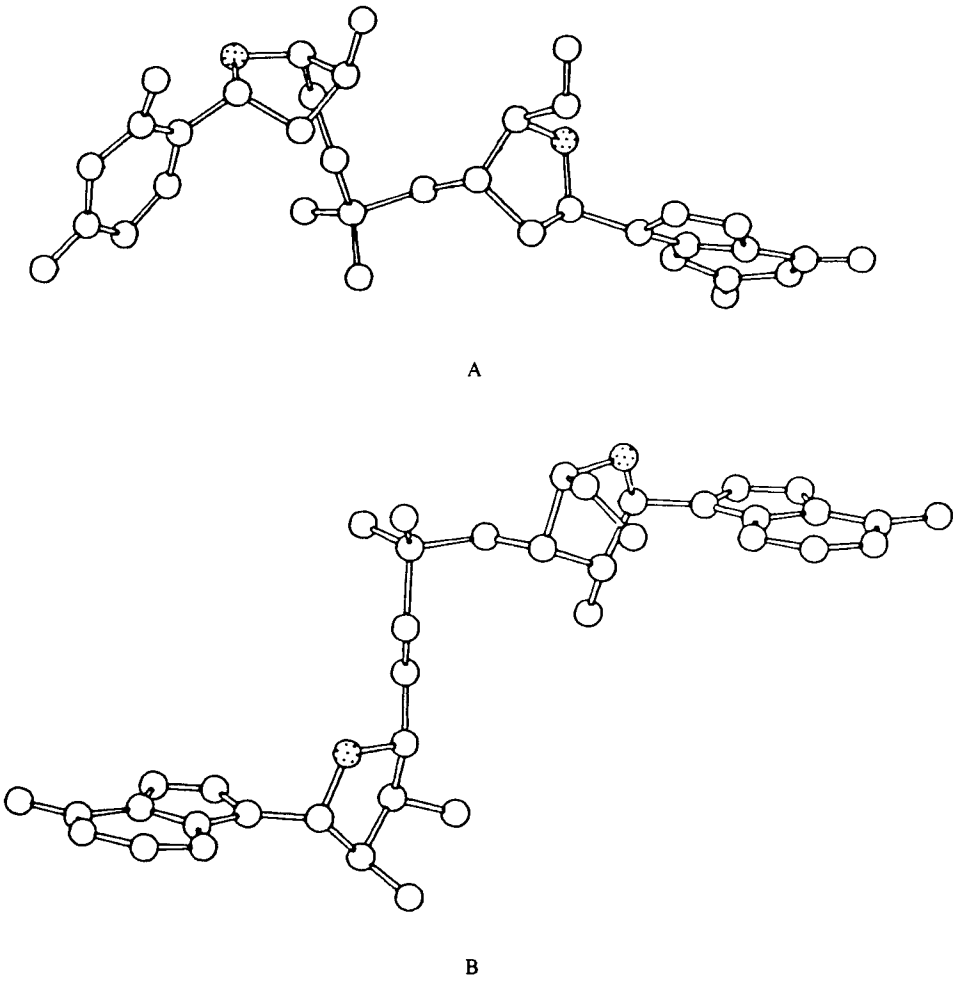


FIGURE 11. The dinucleoside phosphate conformations in the crystal structures of (A) actinomycin D-d(GC)[133] and (B) proflavine-AA.[132]

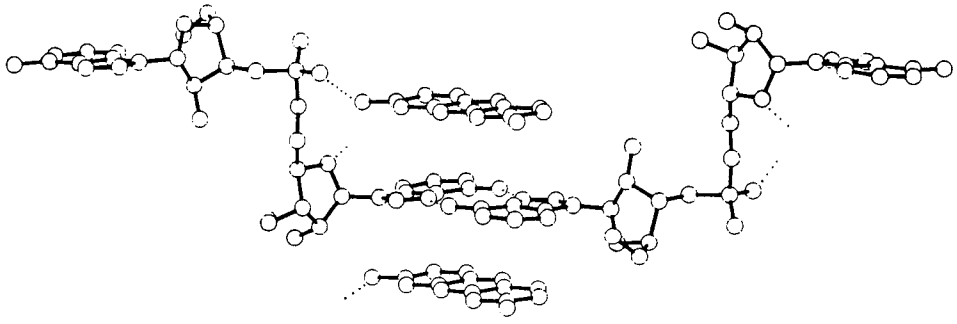


FIGURE 12. The proflavine-AA complex, showing A.....A base pairing between N6 and N7, and the stacking of proflavine molecules onto this base pair.

Table 5
OLIGONUCLEOTIDE-DRUG COMPLEX CRYSTAL STRUCTURES

Drug	Oligomer
Intercalative structures	
Proflavine	CG
	5-Iodo-CG
	d(CG)
	CA
	CA + UG
Acridine Orange	CG
	5-Iodo-CG
9-Aminoacridine	5-Iodo-CG
Ethidium	5-Iodo-CG
	5-Iodo-UA
Daunomycin	d(CGTACG)
Ellipticine	5-Iodo-CG
3,5,68-Tetramethyl-*N*-methyl phenanthrolinium	5-Iodo-CG
Terpyridine platinum	d(CG)
Nonintercalative and pseudointercalative structures	
Proflavine	AA
9-Aminoacridine	AU
Actinomycin	d(GC)
Ethidium	tRNA

14°). The intercalated proflavine has its exocyclic amino groups hydrogen bonded to oxygen atoms of the phosphate groups. These amino groups are pointed into the major groove of the complex. The positive charge of the proflavine cation is carried on the central-ring nitrogen atom. It is at first sight unexpected that the intercalated drug is stacked symmetrically between both strands (as in the Lerman model), rather than stacking on only one strand with negatively charged phosphate group and positively charged proflavine ring nitrogen in close proximity. This suggests that the energetics of the experimentally observed chromophore-base-pair stacking interactions more than compensate for this putative electrostatic interaction (see Section IX). At the present time, there is no experimental or theoretical data available on charge localization in this drug when intercalated. One might expect considerable charge delocalization in view of the polarizing effects of the bases. The unique stoichiometry of this complex dictates that there are four nonintercalated proflavine molecules associated with the exterior of each duplex. These are hydrogen bonded to the phosphate group and a ribose 02′ hydroxyl group, thus providing a model for the external proflavine binding to double-stranded RNA and DNA. Since the same phosphate oxygen atom hydrogen-bonds to both external and internal proflavines, it is tempting to speculate that this structure illustrates a frozen-out early intercalation state, which solution studies have shown to involve external binding prior to insertion.

The 2:2 proflavine-to-d(CG) structure[136] does not have exact twofold symmetry (Figure 14), although it retains the basic features of C.G base-pairs flanking the drug. The base pairs are much flatter than in the ribo complex. The two dinucleoside strands are not precisely conformationally equivalent, and the proflavine is asymmetrically intercalated. There is no amino-phosphate hydrogen bonding, since the phosphate groups are further apart (16.7 Å) than in the ribo complex (15.9 Å). The ternary proflavine-CA-UG complex[137] (Figure 15)

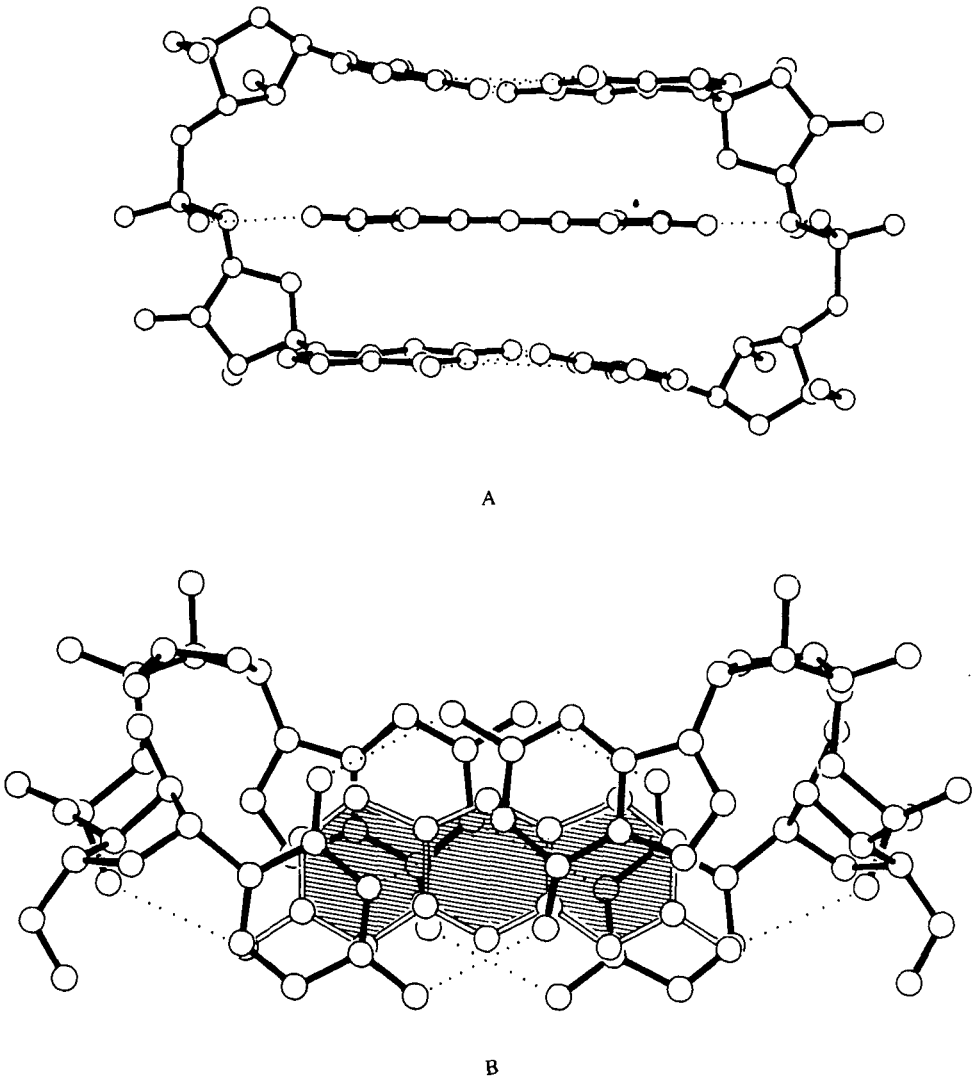


FIGURE 13. The structure of the proflavine-CG complex. (A) Shows a computer-drawn view along the drug plane, looking into the major groove. Dashed lines indicate hydrogen bonds; (B) viewed onto the base-pair plane. The drug molecule is shaded for clarity.

is the only complex reported to date with nonself-complementary strands and thus has the intercalated drug stacked between a C.G and a U.A base-pair. It has several features intermediate between the above two structures. The intercalated proflavine is oriented pseudo-symmetrically and resembles the situation in the CG complex structure. There is a weak hydrogen bond (3.15 Å long) between the phosphate group of the UG strand and a drug exocyclic nitrogen atom, but no corresponding one to the CA strand — the nitrogen-oxygen distance here is over 3.8 Å. The two base-pairs are significantly (0.1 Å) different in C1′-C1′ interstrand dimensions, which may be a factor contributing to its dissimilarities to the ribo CG complex structure. These and other differences, discussed in Section VII.E.3, are suggestive of subtle sequence-dependent distinctions between the proflavine complexes, even though they are all of pyrimidine-3′,5′-purine type. One particular consequence of the different sequences is an asymmetry in residual charge on the base substituent atoms in the

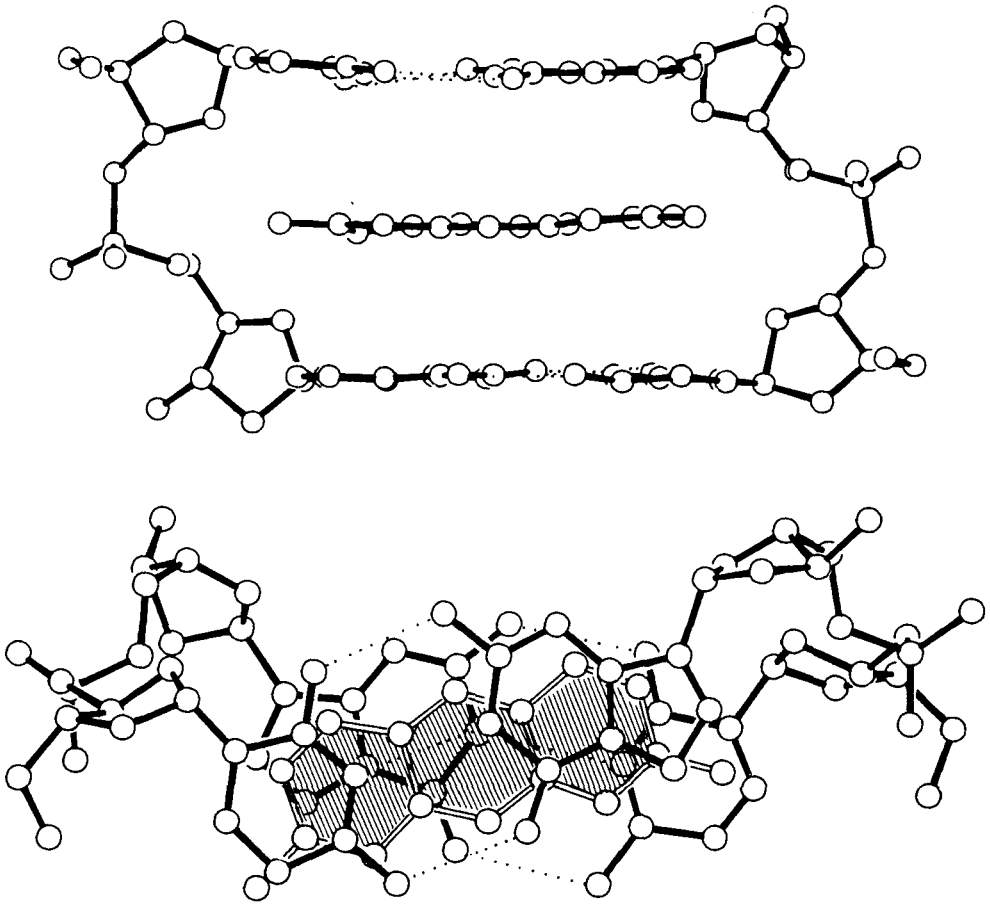


FIGURE 14. The molecular structure of the proflavine-d(CG) complex.

major groove. 06G carries a −, N6A a +, O4U a −, and N4C +. So, in the CG and d(CG) complexes, each side of the concave curve bounded by these four atoms has an alternation of these potentials (which have a total zero net charge). In the CA-UG complex, on the other hand, the CA side has two + charges in this area, and the UG side has two − charges. It is unsurprising, then, that the proflavine molecule is asymmetrically oriented in order for its central positively charged ring nitrogen atom to be somewhat closer to the UG negative side of the major groove, and thus enable the phosphate-amino hydrogen bond to be formed.

The structure of the 2:2 proflavine-5-iodo-CG complex[138] is similar to that of the 3:2 noniodinated one, although the former does not have the exact twofold symmetry of the latter, and some backbone angles in this relatively low-resolution structure appear to differ by as much as 70° comparing one strand with the other. The proflavine complex with CA[139,140] is of considerable interest. This dinucleoside cannot form Watson-Crick base-pairs, and so it might be expected that the crystal structure would be analogous to, say, the AA complex, with an extended conformation for the CA. Instead, two CA molecules self-associate through A.A and C.C base-pairs to form a parallel duplex with an intercalated proflavine molecule in the standard position 3.4 Å midway between them. There is weak, partial hydrogen bonding between the proflavine's exocyclic nitrogen atoms and phosphate backbone. The backbone conformation is essentially the same as that found for the antiparallel

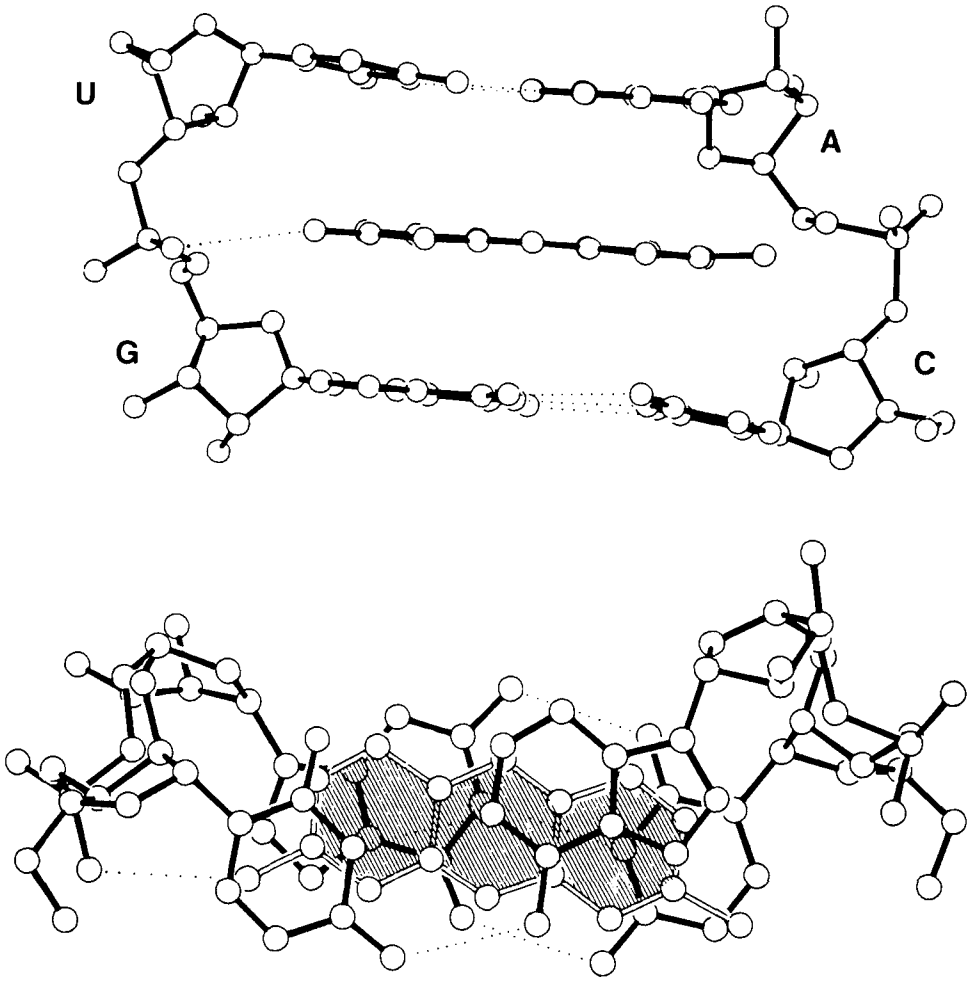


FIGURE 15. The molecular structure of the proflavine-CA-UG complex.

duplex intercalated structures (see Section VII.E.1), even though there is a marked difference in size between the A.....A and C.....C base-pairs (the C1′.....C1′ interstrand separations are 10.97 and 9.59 Å, respectively). The fact that, in spite of these factors, the CA complex is an intercalative one argues strongly that, at least at the dinucleoside level, there is a pronounced preference for the pyrimidine-3′,5′-purine sequence to be the one that forms intercalated structures with drugs.

The crystal structures of several other acridine complexes have been reported. Acridine orange, which has methyl groups replacing the hydrogen atoms on the exocyclic amino groups of proflavine, cannot form analogous intermolecular hydrogen bonds, and, thus, the intercalation geometry is asymmetric[138,141] and closely resembles proflavine's disposition in the d(CG) complex. Again, intercalation is from the major groove side. The 5-iodo-CG complex with 9-aminoacridine[142,143] has two distinct intercalated duplex complexes in the crystal asymmetric unit. One has the 9-amino group pointing into the minor groove and the drug stacked between G.C base-pairs very much in the manner of the proflavine-d(CG) complex. The other has the drug molecule rotated 180° and intercalated asymmetrically between the bases of one strand only. The two duplexes do have similar backbone confor-

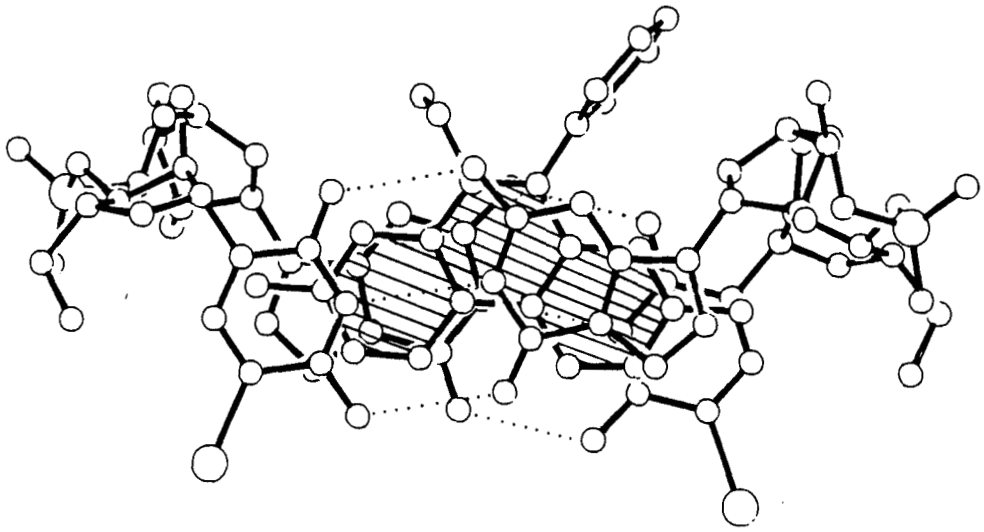


FIGURE 16. The molecular structure of the ethidium-5-iodo CG complex.

mations. The interstrand phosphorus..... phosphorus distances in these two units are the same (17.3 Å), inferring that the width of the intercalation pocket is of sufficient size to accommodate a proflavine molecule. 9-Aminoacridine, not having terminal amino groups, does not then fit tightly into this pocket, and several approximately equi-energetic stacking positions are possible, as are observed. It will be of interest to ascertain whether other 9-aminoacridine intercalation structures have a similarly oversized drug binding site, especially since it has been suggested[142,143] that the single-strand intercalative stacking of 9-aminoacridine is responsible for ultimate looping-out and stabilization of such regions in DNA. Such a feature has been proposed as an integral part of a mechanism for acridine frameshift mutagenesis.

B. Ethidium

The 5-iodo-CG and UA complexes[144,145] of this drug were the first intercalated crystal structures to be reported (Figure 16). These very similar structures have ethidium bound from the minor groove direction, so that the drug's ethyl and phenyl substituents are pointing into this groove. There is no hydrogen bonding between the drug's exocyclic amino groups and backbone phosphates; the minimum separations are 3.6 Å. This contrasts with suggestions from the earlier model building,[126] although there are weak hydrogen bonds (varying from 3.0 to 3.3 Å in length) between these amino groups and the purine 05′ atoms on each chain.

Yeast phenylalanine transfer RNA has also been used as a model for ethidium binding.[146] There is evidence that intercalation occurs, presumably to the helical stem regions. However, soaking crystals of the tRNA in drug followed by examination of a different Fourier electron density map showed an alternative binding mode. A single-drug molecule was found in a fold of the tertiary structure, near (though not stacked with) a Hoogsteen U.A base pair and a nonstandard G.C in general agreement with NMR solution data and assignments. It is not known whether cocrystallization of an intercalating drug and tRNA would produce the same externally attached structure.

C. Daunomycin

The only drug complex for which structural information at greater than the dinucleoside

level is available at present is that between daunomycin and d(CGTACG).[147] This drug is structurally more complex than the simple intercalators discussed above, since it potentially has two functional groups, the protonated amino sugar and the anthracycline chromophore. The link between these two groups has been shown by crystallographic analysis[148] and energy calculations on the drug itself to be relatively inflexible, so that daunomycin has a single low-energy conformation,[149,150] apart from some flexibility in the cyclohexene ring of the anthracycline. In view of these two groups in the drug-molecule separating charge and intercalative potential, it is unsurprising that it requires more than two base-pairs in its total DNA binding site. Thus, a dinucleoside is in principle an inadequate model for the daunomycin-DNA complex.

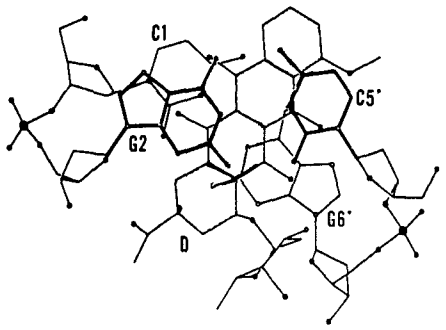
The structure consists of two antiparallel Watson-Crick base-paired hexamer duplexes, into which two drug molecules are intercalated. The intercalation sites are the C(3′,5′)G sequences at the terminii of the helical fragment. Thus, the drug has bound in a sequence-specific manner, not only to pyrimidine-(3′,5′)-purine sites, but has selected out T.A in favor of the C.G. However, this seeming C.G preference may be more apparent than real, since thermodynamic grounds would favor two drug molecules binding rather than one, assuming the site affinities are comparable. This is somewhat unfortunate, since intercalation at the terminal site does not eliminate the possibility of asymmetric end effects perturbing nucleotide conformational features from what would occur at the central intercalation site. Discussion of the conformation of this structure is deferred to Section VII.E.

The complex (Figure 17) has the drug molecules intercalated from the minor groove side, with its bulky sugar group resting in the groove. The severe steric requirements of the narrow minor groove in this B-DNA-like structure force the anthracycline chromophore to lie almost at right angles to the base-pair long axis. The base pair-chromophore overlap is notably slight, with the methoxy-substituted terminal aromatic ring of the drug directed freely into the major groove. NMR data showing small chemical shifts associated with the protons of this ring are fully interpretable in terms of this model.[92,93] A recent study with T4 phage DNA (which has a major groove sterically blocked by glycosylation) has also confined minor-groove intercalation.[151]

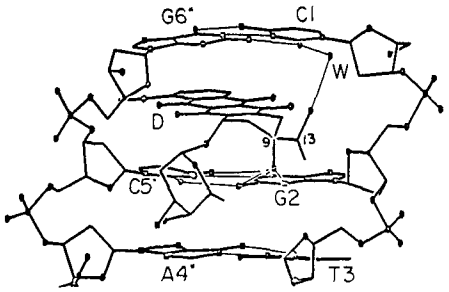
The daunomycin sugar group does not interact with the backbone by hydrogen bonding, in contrast with a central feature in the model derived from fiber-diffraction data.[127] This model, which also incorporates major-groove insertion, is thus at total variance with the single-crystal structure, even though the lack of specific amino group-phosphate interaction does not explain the extensive data showing that this group is an important factor in daunomycin binding to DNA, as well as its biological activity.[67] However, the crystal structure does show the existence of several previously unsuspected hydrogen bonds involving the substituents at the C9 position on the cyclohexene ring. These are to the C(3′,5′)G bases on one strand of the intercalation site. The interaction between the hydroxyl group at C9, and N2 and N3 atoms of the guanine suggests a weak preference for this residue compared to adenine.

D. Actinomycin D

The deoxyguanosine complex with this drug was the first to be reported between an intercalative drug and a nucleic acid component.[152,153] In retrospect, the relevance of this structure to nucleic acids is necessarily limited, since a nucleoside does not have the conformational constraints of base-pairing or adjacent residues. The complex has approximate twofold symmetry with a deoxyguanosine molecule stacked on each side of the phenoxazone chromophore. Hydrogen bonds connect the N2 amino groups of the guanines to the carbonyl oxygen atoms of the *L*-threonine residues, providing the specificity observed in solution. These features have been used as the basis for a model of the actinomycin-DNA complex[154,155] using the sequence d(ATGCAT) with drug bound at the center. This self-complementary



A



B

FIGURE 17. Two views of the daunomycin d(CGTACG) complex. (Reproduced from Quigley, G. J., Wang, A.H.-J., Ughetto, G., van der Marel, G., van Boom, J. H., and Rich, A., *Proc. Natl. Acad. Sci. U.S.A.*, 77, 7204, 1980. With permission.)

G(3′,5′)C intercalation site is in correspondence with the pseudo twofold symmetry of the crystal structure. It has been pointed out[156] that a number of aspects of the backbone stereochemistry in this model is unsatisfactory, with, for example, several eclipsed torsion angles. Such steric strain can to some extent be relieved by relaxing the overall twofold symmetry of the complex; extensive energy minimization studies along these and related lines remain to be performed.

The actinomycin-d(GC) complex[133] has standard Watson-Crick G.C base-pairs stacked on either side of the drug chromophore (Figure 18). However, instead of the cytosine and guanine bases on each side being linked together to be part of a single d(GC) molecule, they are linked to adjacent pseudo-intercalated units. Thus, the dinucleoside backbone adopts an extended conformation, in apparent accord with the overriding requirement of a pyrimidine-3′,5′-purine intercalation sequence in order for a dinucleoside to form a duplex-type structure. This sequence preference conflicts with the guanine 5′ end requirement of actinomycin — the structure incorporates a guanine N2 to threonine-carbonyl hydrogen bond which clearly can only be formed with the G(3′,5′)C sequence. The conformations of the peptides in this structure are similar to that found in the deoxyguanosine complex;[153] the role of the peptides in stabilizing the structure by hydrophobic shielding is discussed elsewhere.[157] Model-building studies on possible true-intercalated actinomycin-dinucleoside structures have shown[157] that the restrictions of base-chromophore overlap and hydrogen-

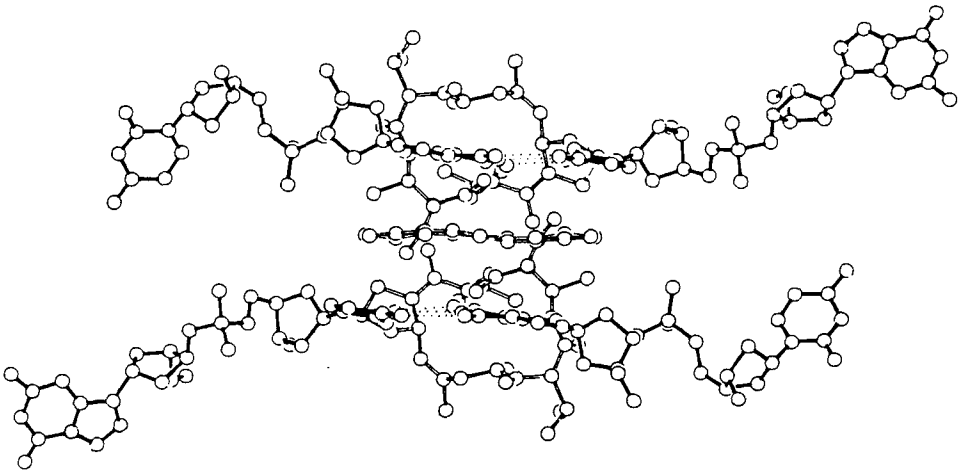


FIGURE 18. The actinomycin-d(GC) complex, showing several dinucleoside units (with solid bonds). The drug molecule has open bonds.

Table 6
CONFORMATIONAL CHARACTERISTICS OF NUCLEIC ACIDS AND DRUG-DINUCLEOSIDE COMPLEX

	χ(5′)	C3′-03′	03′-P	P-05′	05′-C5′	C5′-C4′	χ(3′)
A-DNA	*anti*	*t*	g^-	g^-	*t*	g^+	*anti*
B-DNA							
A-RNA							
A′-DNA							
Z-DNA	*anti*	g^-	g^+	g^+	*t*	*t*	*syn*
	syn	*t*	g^- g^+	*t*	*t*	g^+	*anti*
Intercalated dinucleosides	*anti*	*t*	g^-	g^-	*t*	g^+	High *anti*
Actinomycin d(GC)	*anti*	*t*	g^+	g^+	*t*	g^+	*anti*
Proflavine-AA	*syn*	g^-	g^-	g^-	*t*	*t*	High *anti*

Note: *t* = *trans*, 180° ± 60°; g^- = *gauche*$^-$, 300° ± 60°; g^+ = *gauche*$^+$, 60° ± 60°.

bond specificity place stringent requirements on possible backbone conformations. It has also been noted[157] that a plausible model of drug binding to bases flipped out from a Z-DNA helix can be built, since the conformation of the d(GC) dinucleoside in the crystal structure bears some resemblances to a unit of Z-DNA. This novel noncovalent cross-linking may be relevant to some of the observed actinomycin-DNA behavior in solutions, both in vivo and in vitro.

E. Conformational Correlations

1. The Backbone Conformations

The intercalated ribo- and deoxynucleosides all have a common backbone conformation,[158-161] with all being in the same conformational class (Table 6) as the standard right-handed DNA and RNA double helices. Detailed examination of the dinucleoside conformations has shown that, taking account of experimental error in some of the structures (up to ten degrees in torsion angles), the close equivalence of conformation is independent of

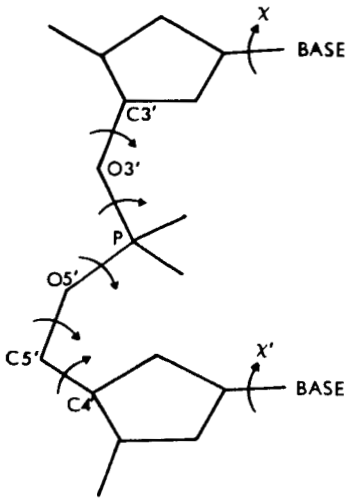


FIGURE 19. The backbone torsion angles in a dinucleoside monophosphate. The glycosidic angles are defined as the O1′-C1′-N1-C6 for torsion angle for pyrimidines and O1′-C1′-N9-C8 for purines.

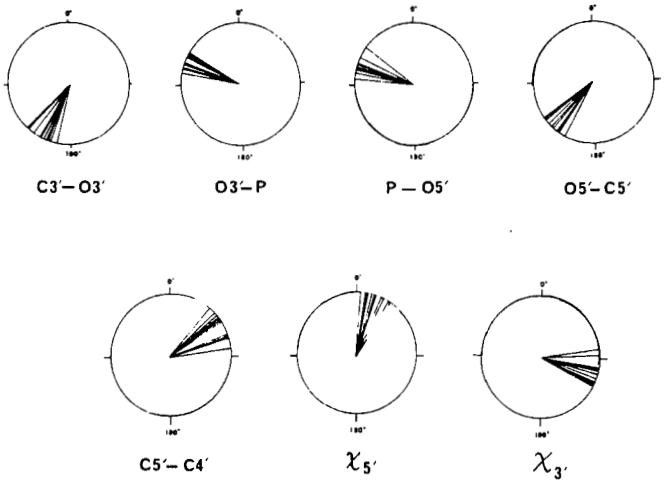


FIGURE 20. Histograms showing the distribution of backbone torsion angles, found in the crystal structures of intercalated dinucleosides.

the nature of the intercalated drug (Figures 19 and 20). In general, both strands of the duplex retain their twofold relationship. The individual torsion angles in the backbones mostly neighbor those of the A-RNA or A′-DNA polymorphs. The only significant changes from these are in the O5′-C5′ torsion angle and the glycosidic angle at the purine 3′ end. These differ in being 30 to 40° and 60 to 80°, respectively, higher than in A-RNA or A′-DNA dinucleosides (Figure 21). The increase in the O5′-C5′ torsion angle serves to increase base-base separation on each strand, and the change in glycosidic angle preserves the base parallelism. Thus, the base-pair separation is altered from 3.4 to 6.8 Å. Increasing these angles still further opens up the intercalation site to a maximum of ~8 Å, at which point the O5′-C5′ angle has a pure *trans* value and the purine glycosidic angle tends to be in the *syn* domain. The limited data available to date suggest that sequence changes do not substantially modify the observed conformations.

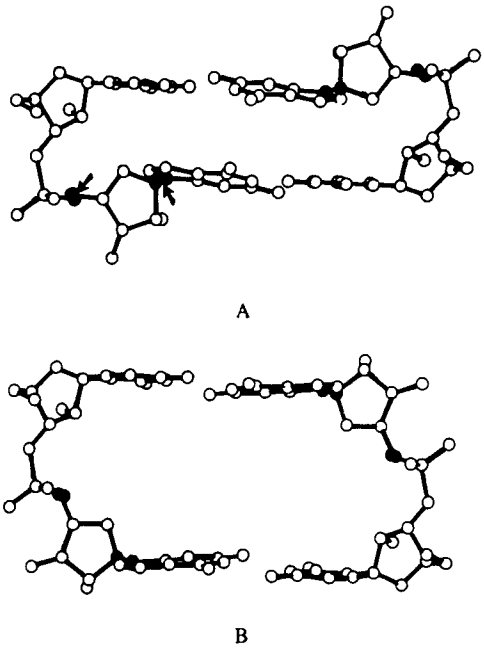


FIGURE 21. (A) A dinucleoside with an A-RNA conformation, with the 05′-C5′ and 5′ end glycosidic angles on one strand indicated by arrows. Structure (B) has these angles on both strands increased relative to (A) so that the base pair separation is 6.8 Å.

It is remarkable that this intercalation conformation is adopted both by the known ribo- and deoxydinucleoside complexes. The consistent adoption of A-type nucleic acid geometry may be related to the relatively hydrophobic minor groove surface found in several of the complexes, especially those with proflavine. This major groove entry drug has its protonated nitrogen atom in the major groove. A study of the water structure in the proflavine-d(CG) complex[162] has shown extensive ordered water clusters in the major groove cavity, and a sparsely hydrated minor groove. This is analogous to the hydration pattern observed in the crystal structure of an A-DNA oligmer.[163] There are no structural data available as yet on ethidium deoxydinucleoside complexes, which probably have the same minor groove mixture of protonated nitrogen and hydrophobic phenyl group as the 5-iodo-CG and -UA complexes.

The detailed conformations of the daunomycin-hexamer complex has yet to be published. Preliminary details suggest that the glycosidic angles at the 3′ ends of the two intercalation sites have high *anti* values similar to those in the dinucleoside complexes. However, other features appear to be at variance with the consistent pattern in these structures. The torsion angles around the C3′-03′ and 03′-P bonds are reported to be the ones that alter to accomodate the intercalator. More detailed discussion of this important structure must await the results of a high-resolution, low-temperature crystallographic analysis that is currently in progress at M.I.T. Even then, since intercalation is at the end sites of the hexamer duplex, relevance to the conformational properties of generalized intercalation sites remains to be demonstrated.

2. Sugar Pucker

The nature of the sugar-ring pucker in a polynucleotide is an important determinant of overall structure. Particular puckers are correlated with ranges of backbone torsion angles. The belief has accordingly arisen that ring pucker in intercalated structures, especially dinucleoside duplexes, is a critical factor. The ethidium complexes have a consistent pattern of C3′*endo*-3′,5′-C2′*endo* pucker on each strand. This alternation has also been observed in several other complexes. It has been suggested[164,165] that a change in pucker from ho-

mogeneous to "mixed" is the major factor in producing intercalation sites in both dinucleosides and B-DNA itself. This change has been termed "kinking", and has been related to structural changes in DNA required to wrap it around the histone core in the nucleosome.

The sugar puckers in the proflavine-CG complex[134,135] were all found to be C3′*endo*, which suggested that the nature of the pucker at the 3′ side of a dinucleoside was not necessarily important for intercalation. This was confirmed by a detailed modeling study[158] which examined ribodinucleosides in the A-RNA conformation, having either C3′*endo*-3′,5′-C3′-*endo* or C3′*endo*-3′,5′-C2′*endo* pucker for each strand in an antiparallel duplex. In both cases, increases of the 05′-C5′ and 3′ end glycosidic angles were sufficient to result in intercalative (6.8 Å base-pair separation) geometry. No sugar pucker changes were necessary in order to produce this effect. The suggestion has been advanced[166] that the sugar pucker features found in the proflavine-CG structure are peculiar to it, and relate to the drug-backbone intramolecular hydrogen bonding. The determination of the proflavine-d(CG) structure[136] finally settled this issue. This complex, which does not have such drug-backbone interaction, has one strand (1) with both sugars in the C3′*endo* pucker state, and the other strand (2) with mixed C3′*endo*-3′,5′-C2′*endo* pucker. A second, less-ordered crystal had strand (1) with the pucker at the 3′ end disordered between 50:50 populations of C3′*endo* and C2′*endo* on account of a lack of constraining water structure around the sugar. Differing 3′ end puckers have also been found in the proflavine-CA-UG complex.[137] The overall conclusion is that sugar pucker at the 3′ ends of intercalated dinucleosides is a relatively unimportant variable and is largely dependent on environmental factors. This is in accord with NMR studies in solution, which are interpretable in terms of multistate populations.[88] The flexibility of pucker can be correlated with the 3′ end glycosidic angle (Figure 22), which for the known complexes shows two clusters of values corresponding to the two main puckering modes.

At the oligomer level, the nature of the sugar pucker on the 3′ side of an intercalation site is more critical in determining conformation at adjacent residues. The daunomycin-hexanucleotide complex[147] has mostly C2′ *endo* sugar puckering, in line with the general B-DNA character of the structure. However, apparent exceptions to this occur around at least one of the drug binding sites. As with other conformational features, clarification and examination of sequence-dependence will require further oligomer crystal structures to be determined.

3. Unwinding Angles

The helical twist angles in exact A-RNA and B-DNA dinucleoside duplexes are 32.7 and 36°, respectively, taking no account of possible sequence dependence. The ethidium-5-iodo-CG[145] and -5-iodo UA complexes[144] have reported angles of about 10°, so it was initially suggested that these structures have been unwound by 26° relative to B-DNA, in apparent close agreement with solution data. It now seems that the "unwinding" relationship between dinucleosides and polynucleotides is not straightforward, not least because of the lack of adjacent-site residues in the former, which would be expected to be involved in the total unwinding process.

The point is made in the following section that intercalated dinucleosides do not retain the helical symmetry of their unintercalated precursors. It is thus inappropriate to use the terms "helical twist" or "helical unwinding". Instead, a base-turn angle may be defined as the angle subtended by interstrand C1′.....C1′ vectors. This is independent of helical symmetry. Values for various drug complexes are given in Table 7. The base-turn angle for the proflavine-CG complex is almost identical to that for A-RNA itself, and clearly in this case the angle does not relate to that found in solution with closed-circular duplex DNA. The very different base-turn angle in the CA-UG complex suggests that sequence plays an important role in determining its value, although at present it is not possible to formulate

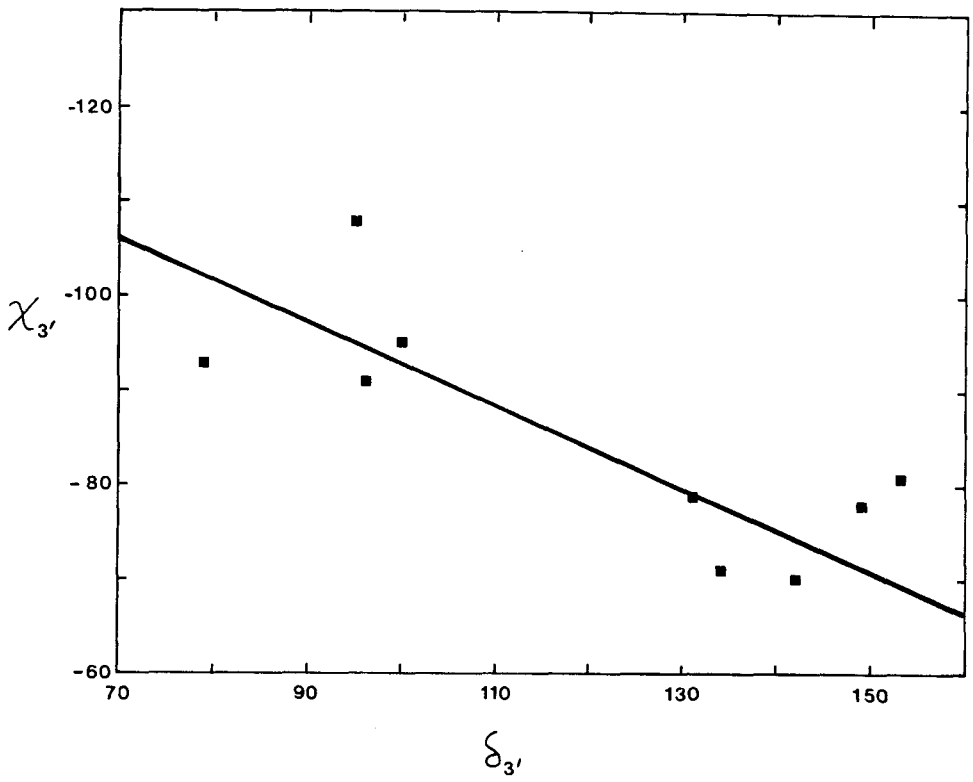


FIGURE 22. Plot of 3′ end glycosidic angle against the C4′-C3′ torsion angle (δ) in the sugar ring, for a number of intercalated dinucleosides. δ is a measure of pucker type, with lower values corresponding to C2′ *endo* pucker. Glycosidic angles are here defined in terms of the O1′-C1′-N1-C6 torsion angle for pyrimidines and O1′-C1′-N9-C8 for purines.

Table 7
BASE-TURN ANGLES (IN °) IN DRUG INTERCALATION COMPLEXES

Complex	Base-turn angle
Ethidium-5-iodo-CG	5
Ethidium-5-iodo-UA	6
Proflavine-CG	32
Proflavine-d(CG)	17
Proflavine-CA-UG	16
Proflavine-CA	40
Daunomycin-d(CGTACG)*	36

* Angle is at the CG sites.

rules governing this. Since there is now good evidence that -CG- and -CA- steps in DNA have different helical twist angles, their drug-unwound values are unlikely to be the same. There is also good evidence from the daunomycin-hexamer complex that helical unwinding may not be confined to the base-pairs immediately surrounding the drug. Table 7 shows that for this structure, the base-turn angle at this position is the same as the helical twist angle in B-DNA itself (36°), whereas the adjacent site base-turn angle is 8° less.

FIGURE 23. Schematic views of (A) major (M) and (B) minor (m) groove intercalation. The drug molecule in (B) is shown with an attached nonplanar group.

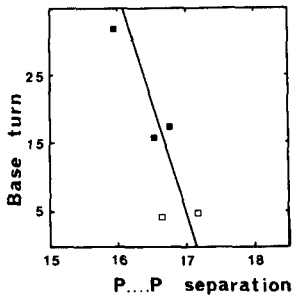


FIGURE 24. Plot of base-turn angle (in °) vs. P.....P separation (in Å) for dinucleoside complexes with proflavine (■) and ethidium (□).

Base-turn angles in intercalated dinucleosides relate in some manner to the extent of base-pair bend and propellor twist.[136,137] Thus, the proflavine-CG complex does not have planar, parallel base-pairs, in contrast to the d(CG) complex. It is not possible at present to quantify relationships between base-turn angle and the various parameters of base and base-pair noncoplanarity. We suggest that when larger deviations from planarity correlate with larger base-turn angles, such structures are, in doing so, attempting to maximize drug-base-pair overlap. Similar factors have been proposed for oligonucleotides themselves, so as to take account of observed sequence-dependent helical twist angles.[166,167] The details of these factors for drug-dinucleoside complexes are probably rather less easily understood than for the unbound oligomers, as shown by the fact that ethidium (whose known complexes have small base-turn angles), nonetheless, induces marked single-strand twist in them. Direct comparisons are not straightforward, since in these complexes ethidium intercalates from the minor groove direction (Figure 23), whereas proflavine is a major groove intercalator. This itself produces distinct tiltings of the two base-pairs at the sites in the direction of entry, which are accentuated in the case of ethidium by the appendage of a bulky phenyl group.

There is a correlation between base-turn angle and interstrand phosphorus-phosphorus distance (Figure 24). It has been pointed out that this correlation has a simple physical rationalization. A small base-turn angle implies that groups on the same dinucleoside strand would be closer together than with a larger value. Potential steric strain would then be relieved (Figure 25) by backbone, and hence phosphate group movement, away from the bases.

4. Relationship to Intercalation into Oligonucleotides

Dinucleosides directly derived from helical polynucleotides retain the helical symmetry

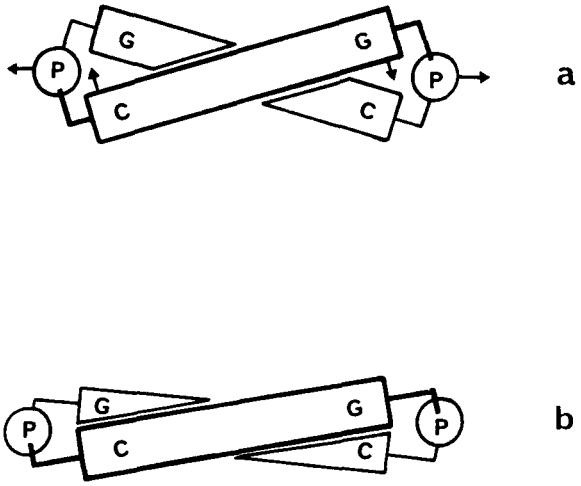


FIGURE 25. Representation of drug-dinucleoside complexes (a) with a large base-turn angle and (b) with a small base turn angle.

of their parent in that the two nucleoside units on each strand are helically equivalent, and the two strands are identical (Figure 21A). It is unsurprising that the latter feature is not always precisely the case for the intercalated dinucleosides. However, the profound loss of helical symmetry, as seen in Figure 21B, is an unexpected finding.[158,159] It is seen as a marked imbalance in the spacial disposition of each monomer unit on a strand with respect to each other, which is caused by the single backbone torsion angle change (around the 05′-C5′ bond) compared to A-RNA or A′-DNA. The consequences of this are (1) that the intercalated structure cannot be simply inserted back into a standard polynucleotide, and (2) that adjacent residues with "standard" conformations cannot be added.

This problem has been examined in two distinct ways. In one,[164] the crystallographically observed dinucleoside intercalation geometry has been relaxed so that immediate incorporation into a B-DNA double helix appeared to be straightforward. Relaxation involved substantial (up to 40°) changes to a number of the backbone torsion angles, as well as some alterations of conformation up to two nucleotides distant from the drug binding site. This category of model has not been subjected to a detailed steric or energetic analysis, so it is not known whether they are low-energy structures with no unacceptable close contacts. The fact that plausible space-filling molecular models have been built to represent them is not a rigorous test, since it is not possible to accurately represent conformation of this complexity without unwitting physical changes in these space-filling models.

The second category of extrapolation from the crystallographic results has rigidly constrained the geometry of the central intercalated duplex dinucleosides to that observed in the crystal structure of proflavine-CG.[159] The principal difficulties in attaching base-paired residues with standard nucleic acid conformations to this structure reside in its asymmetry of sugar residues and glycosidic angles. This makes it impossible to have a normal A-RNA geometry adjacent to the experimentally determined intercalation site and thus a drug molecule cannot be intercalated at the adjacent site. Neighbor exclusion is then a natural consequence of the intercalation geometry, and concepts such as mixed-sugar pucker do not necessarily have to be invoked in order to explain this phenomenon. Model-building studies[159] with the tetranucleotide sequence GCGC resulted in two classes of models, distinguished by conformational differences at the end residues. Both were constrained to retain the twofold

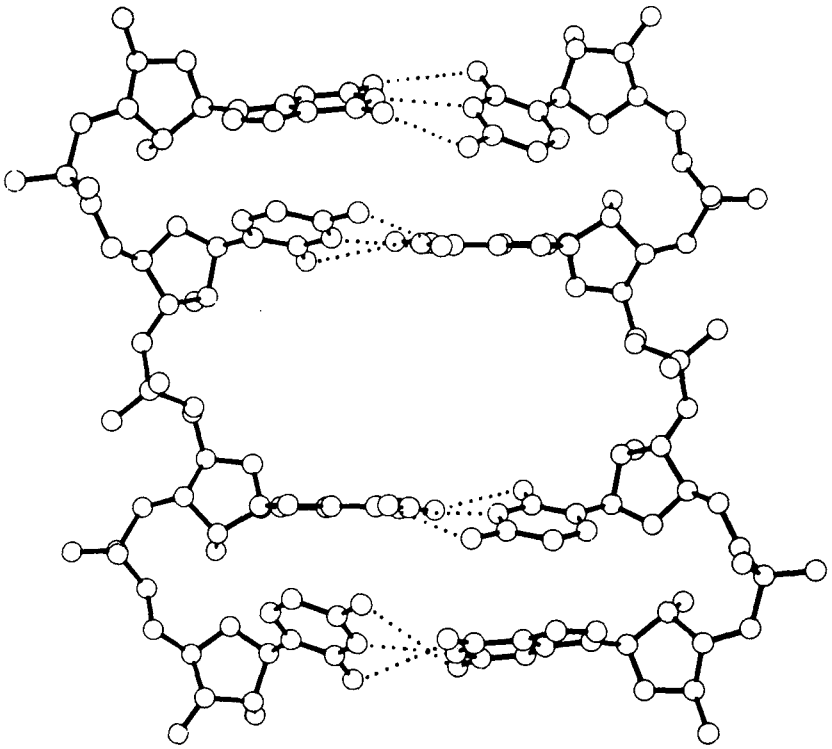


FIGURE 26. Model for an intercalated GCGC tetranucleotide, having neighbor-exclusion geometry at residues 1 and 4.

symmetry relationship between strands, as well as approximate Watson-Crick base-pairing, and were checked for absence of close contacts and energetic feasibility. Both types of models have "nonstandard" adjacent-site conformations, with base-pairs at the ends of the tetramer being twisted and bent. The neighbor-exclusion model (Figure 26) allows normal A-RNA residues to be appended to it, which could then open up for drug intercalation. The alternative model has more acceptable base-pairing geometry at residues 1 and 4, and so is slightly more energetically favored. However, this feature renders standard A-RNA geometry unavailable immediately beyond the 5′ and 3′ ends. The consequence of this extended-site exclusion is that the next pyrimidine-3′,5′-purine intercalation site would be four residues distant from the central site, assuming an alternating CG sequence. It is notable that extended-site neighbor exclusion has been recently found[58] for ethidium binding to double-stranded ribopolynucleotides.

The unusual geometric features in these two models could be relaxed in a deoxy tetramer analog, which would not have the somewhat severe steric constraints of the sugar 02′ hydroxyl group. Also to be explored are possibilities such as additional relaxations of base-pairing at residues 1 and 4 in the tetranucleotide model by, for example, *anti* → *syn* glycosidic angle changes followed by mispairing so as to have a two-hydrogen bond G.C base-pair. No study has, as yet, been reported on the effects of varying the nature of the terminal residues in these tetramer models.

However, until structural data on intercalated ribo- and deoxyoligonucleotides with non-terminal binding sites are available, all such models must remain conjectural. It must be stressed that it is not known whether, or how far, the dinucleoside intercalation geometry relates to oligomers or polymers. Extrapolations in terms of, for example, theories of frame-shift mutagenesis are thus premature at the present time.

VIII. THEORETICAL STUDIES OF INTERCALATION

In general, current theoretical methods are not sufficiently powerful to predict or explain many of the structural and other effects of drug-nucleic acid intercalation. Instead, the majority of studies have sensibly been limited to particular defined questions that have arisen out of the experimental work on model intercalation systems, especially dinucleoside ones. Current methodology of both quantum-mechanical and force-field molecular mechanics approaches has only relatively recently begun to be applicable to nucleic acids themselves — drug complexes are by common consent more difficult systems to tackle, not least because of the lack of structural and solvation data. Reliable force-field and solvent models are still not generally available. It is, however, apparent that the near future will see many developments relevant to drug design in the interface between computer graphics, molecular modeling, and semiempirical energy calculations.

A. Sequence Selectivity and Specificity

The findings that the simple intercalators ethidium and proflavine prefer to bind to pyrimidine-3′,5′-purine sequences have led to a number of theoretical studies. Empirical potential-function calculations on CG and GC with these drugs[168-170] used coordinates from the ethidium bromide-5-iodo-CG structure[145] as a starting point. The GC isomer was constructed from this by reversal of bases, thus assuming that a purine-3′,5′-pyrimidine intercalation structure will have the same backbone conformation. Total energy minimization of trial structures including B-DNA dinucleosides lead to the conclusion that the observed sequence specificity is due to the greater ease of unstacking a CG rather than a GC base-pair, as well as to differences in base-backbone interaction energy. Molecular orbital studies on ethidium dinucleoside[171] as well as proflavine intercalation,[172] on the other hand, suggest that differences arise from the relative ease of unwinding pyrimidine-3′,5′-purine sequences compared to others, and not to differences in stacking energies. Again, the assumption of identical backbone torsion angle changes in different sequences has been made. It is for example, clearly energetically less favorable to have a high *anti* 3′ end glycosidic angle with a pyrimidine rather than a purine base. This has been revealed by analyses of the 05′-C5′ torsion angle and 3′ end glycosidic angle flexibility in ribodonucleosides[173] and deoxydinucleosides,[174] which also indicated that the significant differences in stacking for dinucleoside sequence isomers are important factors in the 3.4 Å → 6.8 Å structural change.

There have been few calculations on the specificity of actinomycin intercalation. A molecular-orbital study of this drug's interaction with guanine has unsurprisingly found that charge-transfer interactions between base and chromophore are important.[175] Electrostatic potential maps for actinomycin with ten possible base-paired dinucleosides[176] have shown that the chromophore interacts best with the CG duplex, although hydrogen bonding between peptides and bases was not taken into account. This work also suggests that differences in backbone conformations (between structures derived from X-ray analyses[145] and those from model building[156]) may not significantly affect base specificities.

B. Structure Predictions for Drug-Oligonucleotide Complexes

1. Dinucleoside Complexes

Many of the studies reported in this category have been directly related to the crystal-structure results and indeed often use them as starting points in energy-minimization procedures. As implied in Section VII.E.4, it is only for these structures that such comparisons of theory vs. experiment can be made.

Molecular-mechanics studies on proflavine and acridine orange intercalation in d(CG) and CG[177] duplexes have shown relatively little structural change after energy minimization, compared to the crystal structures of the complexes. The mixed-sugar pucker C3′*endo*-3′,5′-

C2-*endo* structures were found to be more stable with acridine orange, and perhaps with intercalators in general. The proflavine situation was complicated by intermolecular hydrogen bonding between phosphate oxygen atoms and this drug's exocyclic amino groups; the energy of these interactions was found to be sufficient to stabilize the 3′ end sugar puckers in their C3′*endo* forms. The conclusion that the 3′ end pucker in dinucleoside complexes is a soft parameter, largely determined by the hydrogen-bonding factors of drug and solvent, is entirely in accord with the range of puckers found experimentally in the proflavine complexes. A comparable range of structural data on complexes of other drugs with differing dinucleosides is not as yet available; it will be of interest to ascertain whether this conclusion is a general one.

It has been shown[178] that the (observed) proflavine major groove intercalation model is energetically preferred over minor groove binding into a CG complex. The energy-refined very distorted TA duplex has a small minor groove site, so that in this case the preference is reversed. Examination[179] of proflavine intercalation into CG and d(CG) from all possible directions and orientations has concluded that the crystallographically observed drug positions are, by far, the most stable and that alternatives such as the perpendicular mode found for daunomycin in its hexanucleotide complex[147] are of much higher energy because of diminished drug-base-pair dispersion interactions. Barriers to minor groove intercalation arise from close contacts between the exocyclic amino groups and the dinucleoside backbone.[180]

A detailed study[181] of ethidium intercalation into ribo-dinucleoside duplexes by molecular mechanics procedures, employing explicit estimation of hydration, has shown the importance of the cationic charge on this drug. The energy-minimized structures (which kept the backbone fixed) had the drug moved only 0.5 to 1.0 Å away from its crystallographically observed position[144,145] in the intercalation site. A distinct approach to these problems has been adopted by a molecular orbital-derived electrostatic potential investigation of ethidium intercalation into a d(CG) duplex.[182] Since at the time of this work no atomic coordinates were available for a deoxydinucleoside complex, the geometry used was constructed by a series of torsion angle changes (around both glycosidic angles, and around the C4′-C5′ and 03′-C3′ bonds), that do not relate to those specified in Section VII.E.1. Considerations of three-dimensional electrostatic potential charge indicated that minor-groove intercalation would be favored, and that the fine details of binding geometry could be examined by this "lock and key" approach. By contrast, conventional molecular mechanics calculations on intermolecular fit have steric van der Waals terms as the dominent factors, and explicit electronic charge contributions are of substantially lesser importance for drug-oligonucleotide complexes. A major disadvantage of the electrostatic potential method lies in the inability to conveniently alter backbone conformations during or after simulated interaction, in realistic computer time-scales.

A molecular mechanics study of Adriamycin (the 14-hydroxyl derivative of daunomycin) intercalating into CG and TC-AG duplexes[183] has suggested that the minor groove perpendicular intercalation mode observed in the crystal structure of the daunomycin-hexanucleotide complex[147] is sometimes, though not invariably, the lowest energy complex. It is noteworthy that in this perpendicular mode, additional stabilization is given by a hydrogen bond between a backbone phosphate group and the charged amine of the drug's sugar group; this controversial interaction was not seen in the crystal structure. There appears to be some dependence of minor vs. major groove binding on the dinucleotide sequence; it was pointed out that this conclusion requires elaboration with longer oligonucleotide duplex models.

2. *Oligonucleotide and Polynucleotide Complexes*

An extensive series of model-building studies has been performed on intercalation into the central site of a duplex tetranucleotide[184-186] between base-pairs 2 and 3. In all, three distinct structural types have been classified in this approach, which uses classical potential-

energy functions to determine stable states. All the intercalated tetramers produced have the capability of insertion back into polynucleotide double helices, in contrast with the experimental X-ray drug-dinucleoside structures. This property has been achieved by retaining base-pair parallelism, whether they are 3.4 or 6.8 Å apart, and then altering backbone conformations. The three classes of intercalation site are distinguished by distinct unwinding and glycosidic angles at base-pairs 2 and 3. Accordingly, the intercalating drugs and their unwinding angles listed in Table 1 have been classified into the three classes of low, medium, and high unwinding angles. This approach thus highlights in importance the unwinding angle parameter, at the site of intercalation, and implies that the backbone conformation both at, and adjacent to, the site is flexible in terms of accommodating the requirements of a particular drug. It remains to be seen whether structural data at the tetranucleotide or larger level will be in accord with these models; data at the dinucleotide level do not, with the strong implication that unwinding angles, though specific for a particular drug, may be distributed within the sequence affected by the binding.

Model-building studies of proflavine intercalation into A- and B-DNA[156,187] have highlighted the possible differences between these two categories of site. Constraints of cohelicity and twofold symmetry between the residues immediately at the binding site, the drug molecule, and the nucleic acid helix as a whole were imposed, particularly with respect to glycosidic angles. For B-DNA, two closely related models were produced, both of which had the backbone torsion angles around the P-05′ and 5l′ C5′-C4′ bonds of both strands at the intercalation site, altered by 120° from their B-DNA values and small changes around various bonds at adjacent sites. Total unwinding over affected residues was 18°, with 13° arising at the site itself. No intermolecular hydrogen bonding between drug and backbone was found, in contrast with the A-DNA model.[187] This had P-O5′ and C5′-C4′ torsion angles in the same *trans* range as in the B-DNA intercalated structure, although other backbone angles here adopted their A-form values. It was noted that the intermolecular hydrogen bonding and all C3′*endo* sugar puckering, together with the remarkable absence of helical unwinding, have resemblance to the proflavine-CG crystalline complex[134,135] (though not in terms of individual backbone conformational angles).

IX. CONCLUDING REMARKS

The evidence of the biophysical, structural, and biochemical studies described in this review is that the processes and results of intercalation are much more complex than was until now realized. In particular, biochemical probes of DNA structure and dynamics have begun to show that, for the small number of drugs studied to date, intercalation can be remarkably site-selective, and that there may be until now unsuspected ramifications of drug binding on nucleotides quite remote from the site itself. Much more information from fine-probing techniques is needed on these phenomena, and the ever-increasing power of NMR methods will undoubtedly play a major role. X-ray crystallographic studies, although providing the greatest level of structural detail, are inherently slower and future results must depend critically on the overcoming of current difficulties in producing suitable crystals. It is unrealistic to expect that this method will, in the near future, provide data on systematic variations in oligomer sequence for a variety of intercalating drugs. The results of X-ray analyses also suffer from the disadvantage of being quasistatic pictures of structure in the solid state — although oligonucleotide and oligonucleotide crystals are invariably heavily hydrated, so intermolecular interaction effects are largely negated. Information on molecular dynamics and motion can, in principle, be obtained from high-resolution crystallographic data, and may complement dynamic NMR studies in solution. Empirical energy calculations are rapidly approaching the point of providing realistic predictions of low-energy forms of oligomer structures, sequence-dependent properties, and consequent effects on electrostatic potentials.

Increases in our knowledge of intercalation will be directly useful in drug design. These will be aimed at (1) optimizing the affinity of a compound for a DNA site, using the not unreasonable supposition that strength of interaction can be correlated with at least some measures of biological, especially antitumor, effectiveness,[188,189] provided the compound can pass through the cell membrane; (2) developing more complicated drugs tailored for specific sequences which may have particular functional roles (although at present it is not clear what sequences should be selected).

It is now apparent that random binding to genomic sequences is implausible, although the roles of sequence-dependent binding in the DNA-related biological effects of intercalation remain to be elucidated.

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