Interaction of Aromatic Imides with Deoxyribonucleic Acid. Spectrophotometric and Viscometric Studies†

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ABSTRACT: A series of naphthyl imides and diimides with varying substituent sizes have been synthesized as probes for major–minor groove interactions in intercalation and for studies of DNA dynamics. The substituents next to the ring, which were found to be most important, vary in size from a methylene (type A compounds) to a methyl group on the carbon next to the ring (type B) to lysine amides, which form the imide through the α-amino group of lysine (type C). The largest type C compound was prepared from the adamantyl amide of lysine. Viscometric titrations with linear and closed circular superhelical DNA, circular dichroism, and UV–visible adsorption spectroscopy indicated that these compounds all interact strongly with DNA through intercalation complexes. All compounds form complexes with significant induced circular dichroism in the presence of DNA. All show slight absorption spectral shifts to longer wavelengths and significant hypochromism when titrated with DNA. The adsorption spectra show isosbestic points and were used to estimate the relative binding affinities for the imides and diimides. The diimides bind at least 10-fold more strongly to DNA than monoimides with corresponding substituents. Type A diimides bind approximately 10 times more strongly than both type B and C diimides. Quantitative measurement of unwinding angles indicated that type A diimides all have unwinding angles near 16°, type B between 13° and 14°, and type C less than 10°. These results, Corey–Pauling–Koltun (CPK) model building experiments, and kinetic experiments (to be reported elsewhere) indicate that the diimides intercalate with one bulky substituent in opposite grooves of DNA at the intercalation site. These diimides are, thus, the first class of intercalators known to have a bulky cationic substituent in the major groove of DNA when intercalated. The fact that the large adamantyl amide easily slides through the double helix to form an intercalation complex indicates that the thermally induced breathing motions of DNA result in large openings in the double helix even at temperatures far below the T_m. The diimide binding affinities and unwinding angle results are interpreted in terms of intercalation complexes with the long axis of the intercalator more parallel to the long axis of the adjacent base pairs with type A compounds while the ring systems are essentially perpendicular to the base pairs in type B and C complexes. The monoimide results are consistent with this interpretation and are used primarily to help understand the more complex diimide–DNA interactions.

Molecules that bind to DNA can enter and complex through either the major or minor groove. These two grooves present different environments to ligands from both a steric and a chemical standpoint (Waring, 1972; Wilson & Jones, 1982). Intercalating molecules without bulky substituents, such as proflavin, can presumably intercalate without having any significant part of the bound molecule in either the major or minor groove. Other important intercalating drugs such as the anthracyclines, daunorubicin and adriamycin (Henry, 1976; Pigram et al., 1972; Quigley et al., 1980), phenanthridine derivatives related to ethidium bromide (Waring, 1972; Sobell et al., 1977), quinacrine (Davidson et al., 1978), and actinomycin (Müller & Crothers, 1968; Sobell, 1973) have bulky substituents that must be in one groove or the other after the planar aromatic ring of the drugs is bound by intercalation. With ethidium, actinomycin, and other intercalating compounds examined to date, most evidence supports location of the bulky groups in the minor groove (Wilson & Jones, 1981, 1982). With daunorubicin and adriamycin the case is more complex. X-ray fiber diffraction studies of a daunorubicin–DNA complex (Pigram et al., 1972) and model building studies (Henry, 1976) suggested that the nonplanar A ring and amino sugar of the anthracyclines were in the major groove. Gabbay et al. (1976), however, conducted solution studies with daunorubicin and several related derivatives and concluded that the amino sugar was in the minor groove. Patel & Canuel (1978) found from NMR and ultraviolet melting experiments with synthetic double-helical polydeoxynucleotides with 5-bromo and 5-iodo substituents in the major groove that anthracyclines still bind quite well to these polymers. A recent X-ray crystallographic determination of a daunorubicin–d-(CpGpTpApCpG) complex showed that it also has the A ring and amino sugar in the minor groove (Quigley et al., 1980). Part of the D ring protrudes into the major groove in this complex as a result of the more or less perpendicular orientation of the long axis of the anthracycline ring to the long axes of the adjacent base pairs of DNA. If this result holds for the solution complex between anthracyclines and DNA, then all intercalators with large bulky substituents, which have been thoroughly studied, have these bulky substituents located in the minor groove (Wilson & Jones, 1981, 1982).

This minor groove specificity raises several interesting questions: (1) Is there a general molecular reason for location of bulky substituents in the smaller groove? (2) Is it possible to have intercalation and bulky substituents in the major groove? (3) Is the protrusion of the D ring of the anthracyclines into the major groove important to their biological properties? (4) What are the size restrictions to groups that can be in either groove of DNA? (5) What are the size restrictions to groups that must slide between base pairs of DNA to allow an intercalation complex to form? These are

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§ Professor Edmond J. Gabbay died during the time the experimental work reported in this paper was being conducted. His influence and ideas are strongly in evidence in this research and in nucleic acid chemistry in general. Those of us who were fortunate enough to know him as a friend and colleague value the experience and miss him deeply.

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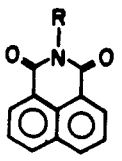
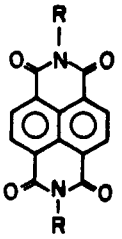


Compound	R
1	$(CH_2)_2N(CH_3)_2 \cdot HBr$
2	$-(CH_2)_4CH(CONH_2)NH_2 \cdot HBr$
3	$-CH(CONH_2)(CH_2)_4NH_2 \cdot HBr$
4	$-CH(CONHAd)(CH_2)_4NH_2 \cdot HBr$ Ad: Adamantyl
5	$-CH(CH_3)(CH_2)_3N(C_2H_5)_2 \cdot HBr$



Compound	R
6	$-(CH_2)_2N(CH_3)_2 \cdot HBr$
7	$-(CH_2)_2\overset{+}{N}(CH_3)_2 \cdot HBr$
8	$-(CH_2)_3N(CH_3)_2 \cdot HBr$
9	$-(CH_2)_4CH(CONH_2)NH_2 \cdot HBr$
10	$-CH(CONH_2)(CH_2)_4NH_2 \cdot HBr$
11	$-CH(CONHAd)(CH_2)_4NH_2 \cdot HBr$
12	$-CH(CH_3)(CH_2)_3N(C_2H_5)_2 \cdot HBr$
13	$-CH(CH_3)(CH_2)_3\overset{+}{N}(C_2H_5)_2(CH_3)I^-$

FIGURE 1: Structures for naphthyl imides **1–5** and diimides **6–13** are shown. The compounds are classified into types A, B, and C based on the size of the substituent on the carbon next to the imide or diimide ring. Type A: **1**, **2**, and **6–9**; the carbon attached to the ring nitrogen is a methylene. Type B: **5**, **12**, and **13**; the carbon attached to the ring nitrogen has a methyl substituent. Type C: **3**, **4**, **10**, and **11**; the carbon attached to the ring nitrogen is the α carbon of a substituent lysine. Note that pairs **2–3** and **9–10** are structural isomers, which fall into either the type A or type C class.

questions that need to be answered in order to have a better understanding of the interaction of intercalating drugs with DNA and to achieve a more rational approach to the design of intercalating drugs.

We have designed and synthesized a new class of intercalating agents (see Figure 1) as potential biologically active drugs and as novel probes for the study of intercalating drug–DNA interactions to answer some of the specific questions listed above. As shown in Figure 1, these compounds can be classified into two major categories, naphthyl imides (**1–5**) and naphthyl diimides (**6–13**). On the basis of the substitution groups attached to the aromatic ring system, they are further classified into three subcategories. Type A compounds, **6–9**, are those with a methylene at the α position to the naphthyl diimide ring. Type B compounds, **12** and **13**, are those with a methyl group on the carbon adjacent to the chromophore. Type C compounds, **10** and **11**, are those with an amide group on the carbon adjacent to the ring. Because of the unique substitution of the naphthyl diimides as shown in Figure 1, if they form an intercalation complex with DNA, one of the side chains must lie in the major groove while the other lies in the minor groove. So that the diimide molecule can adopt this alternate groove intercalation configuration, one of the substituents must slide between base pairs during formation of the complex. The compounds were designed to have a large variation in substituent size with respect to the approximately 3.4-Å thickness of the fused aromatic ring systems. This size variation was accomplished by varying the substituent size (note **6** and **11**) and position of substitution (note **9** and **10**). With **9** it is possible to rotate the lysine side chain into a range of conformations not significantly wider than 3.4 Å. Because of the proximity of the amide group to the ring in **10**, this is not possible and **10** should require wider openings in the double-helix backbone for it to bind by intercalation. The work reported here is designed to determine whether any or all of these types of compounds can bind to DNA by intercalation and, if so, how the substituent variations (Figure 1) affect their complex structures and binding interactions with DNA.

Materials and Methods

Chemicals. The syntheses and medicinal testing results of all the naphthyl imides and diimides, **1–13**, will be reported elsewhere. The structure and purity of these compounds were confirmed by ^1H and ^{13}C NMR,[1] UV–visible spectroscopy, elemental analysis, and thin-layer chromatography.

Buffer. The buffers used in this research unless indicated otherwise contained 1.0×10^{-2} M 2-(*N*-morpholino)ethanesulfonic acid (MES) and 1.0×10^{-4} M ethylenediaminetetraacetic acid (EDTA), pH 6.2, with no (MES 00) or 0.1 M (MES 10) NaCl.

Nucleic Acids. (1) Linear DNA: Calf thymus DNA was purchased from Worthington Biochemical Corp. (lot no. 38N764) and was sonicated under nitrogen with a Heat Systems W-375 sonicator at 0.1-s pulse every 1 s and 90% power for 50 min at 4 °C. The resulting DNA solution was filtered through a 0.45-μm Millipore filter, precipitated with ethanol at 4 °C, and collected by centrifugation. The DNA obtained was redissolved and dialyzed in MES 00 buffer. The molecular weight was determined by a standard electrophoretic method (Jones & Wilson, 1980) and was found to be approximately 300000. (2) Covalently closed superhelical DNA (CCS-DNA): The CCS-DNA was prepared as previously described (Jones et al., 1980).

Circular Dichroism Studies. Circular dichroism (CD) spectra were recorded from 600 to 300 nm on a Jasco J-20 spectropolarimeter. All experiments were carried out in 5-cm quartz cells in MES 00 buffer at 25 °C.

Spectrophotometric Measurements and Relative Binding Affinities. All of the spectrophotometric measurements were performed with either a Cary-17D or a Cary-219 spectrophotometer at 25 °C. The extinction coefficient of each compound in the absence of DNA (E_f) was determined by carefully titrating small aliquots of a concentrated stock solution into a buffer solution and measuring the absorbance. The results were linear over the range of the experiment, 8.0×10^{-5} and 4.0×10^{-5} M for imides and diimides, respectively. The extinction coefficients of the compounds bound to DNA (E_b) were determined by titrating small aliquots of a concentrated drug stock solution into a concentrated DNA solution (typically around 1.0×10^{-3} M). The ratio of DNA phosphate to compound was kept greater than 100 to 1, and

[1] Abbreviations: CCS-DNA, closed circular superhelical DNA; CD, circular dichroism; ν, moles of ligand bound per mole of DNA base pairs; η, relative specific viscosity of a DNA–ligand complex; η_0, relative specific viscosity of DNA alone; NMR, nuclear magnetic resonance.

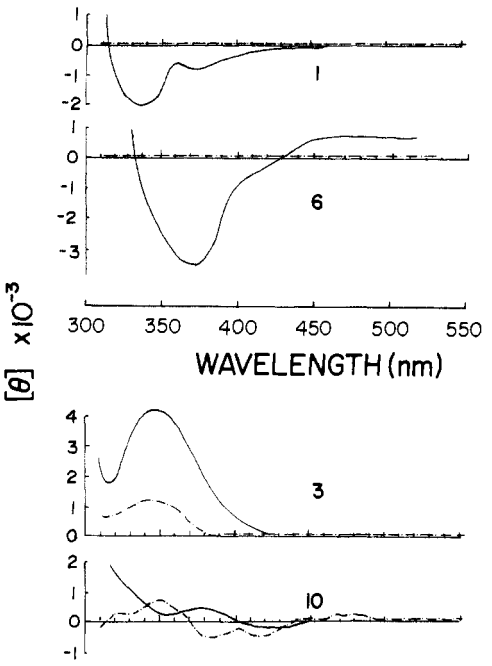


FIGURE 2: Intrinsic and DNA-induced CD spectra for representative imides **1** and **3** and diimides **6** and **10** are shown with molar ellipticity plotted as a function of wavelength. Compounds **1** and **6** contain no chiral center while **3** and **10** have the chiral center on the carbon adjacent to the chromophore. Broken lines in the figure indicate spectra with no DNA present while the solid lines illustrate the CD spectra in the presence of an excess of DNA. For all spectra in the presence of DNA, the concentration of DNA base pairs was between 1 and 1.5 × 10^{-4} M and was always approximately 40 times greater than the imide or diimide molarity. The same concentration of imide or diimide was used in spectra with and without DNA.

in this range linear Beer's law plots were obtained. Using higher DNA concentrations and/or ratios gave plots with the same slope.

The relative binding affinity, which represents the intrinsic equilibrium binding constant (McGhee & von Hipple, 1974; Wilson & Lopp, 1979), was determined either by titrating a concentrated ligand solution (2.5 × 10^{-3} M) into various concentrations of DNA solution or by titrating a concentrated DNA solution (4 × 10^{-2} M) into various concentrations of imide or diimide solution (Wilson & Lopp, 1979). The absorption at 345 nm for imides and 383 nm for diimides was taken directly from the digital reading of the Cary 17D or 219 spectrophotometer. Isosbestic points were obtained for these titrations, and with the previously determined extinction coefficients, the spectrophotometric results were converted into Scatchard plots. Only data for fractions of compound bound to DNA in the range between 0.2 and 0.8 were used for the Scatchard plots (Deranleau, 1969; Wilson & Lopp, 1979).

Viscometric Studies. Both linear and CCS-DNA viscometric titrations were carried out at 25 °C with a Cannon-Ubbelohde semimicro dilution viscometer (series no. 75, Cannon Instrument Co.) as previously described (Jones et al., 1980).

Results

Circular Dichroism Studies. CD spectra for the related imide–diimide pairs **1–6** and **3–10** are shown for illustration in Figure 2. Those compounds without a chiral center (**1**, **6–8**), which have the chiral center five carbons away from the chromphore (**2** and **9**), or which are racemic mixtures (**5**, **12**, and **13**) do not show any significant CD spectra in the range 550–300 nm. For compounds **3** (Figure 2) and **4** a positive CD at 345 nm (molar ellipticity of 4.86 × 10^3 for **3** and 3.91 × 10^3 for **4**) was observed. More complicated CD spectra were observed for **10** (Figure 2) and **11**. CD spectra for **1**, **6**, **3**, and **10** are also shown in Figure 2 in the presence of DNA. When bound to DNA, all of the imides and diimides, even those without intrinsic activity, exhibit induced CD spectra. In the presence of DNA imides **1** and **2** have similar spectra while considerable variation was seen in the spectra for **3–5**. Diimides **6–9** have generally similar induced CD spectra in the presence of DNA, as do **12** and **13**, while **10** and **11** show considerable variation from each other and from the other diimides.

Table I: Spectrophotometric Results for the Naphthyl Imides and Diimides of Figure 1

compd	λ_{free}[a] (nm)	E_f[b]	λ_{DNA}[a] (nm)	E_b[b]	IP[c] (nm)	σ (M^{-1})[d] × 10^{-4}
1	345	12400	348	5910	368	1.7 ± 0.1
2	345	13100	348	6060	368	1.4 ± 0.1
3	345	14000	348	6200	369	0.82 ± 0.1
4	345	12300	348	5850	368	0.44 ± 0.04
5	345	12300	347	6100	367	0.24 ± 0.04
6	382	26600	382	9700	391	46 ± 8
7	382	28800	382	9970	390	36 ± 4
8	383	27300	385	10000	391	30 ± 6
9	384	26000	385	9700	392	44 ± 6
10	384	26600	385	12500	392	5.2 ± 0.6
11	384	26700	385	9090	393	4.8 ± 0.6
12	383	26600	383	10000	391	3.2 ± 0.4
13	384	25900	386	9800	393	2.8 ± 0.2

[a] λ_{free} and λ_{DNA} are the wavelengths of maximum absorbance for the compounds free in solution or bound to DNA, respectively. [b] E_f and E_b are the extinction coefficients of the compounds free and bound, respectively, both determined at the wavelength of maximum absorbance of the free species, λ_{free}. [c] IP is the isosbestic point obtained on titrating the compound with DNA. [d] σ is the relative binding affinity determined as described in the text. The lattice binding unit of DNA for these calculations is viewed as a base pair and the DNA concentrations were calculated in molarity of base pairs. Binding constants calculated relative to DNA nucleotide molarities would be half this value. These experiments were carried out at 25 °C in MES 10 buffer, and it should also be noted that these binding affinities are quite ionic strength dependent.

Spectrophotometric Titrations and Relative Binding Affinities. Representative absorption spectra of imides and diimides are shown in Figure 3 with the related compounds **1** and **6** as examples. In the range from 330 to 410 nm all of the naphthyl imides exhibit a strong absorption peak at about 345 nm whereas two strong absorption peaks are observed for the diimides with maxima near 362 and 383 nm. Wavelengths of maximum absorbance and extinction coefficients at that wavelength are collected in Table I for all compounds. Figure 3 also shows three important effects on the absorption spectra of imides and diimides as a result of titrating these compounds with DNA: first, a large hypochromic effect was observed for all compounds; second, there is a very small shift of the wavelength of maximum absorption to longer wavelengths for all compounds; third, an isosbestic point was observed. Spectral changes induced in all of the imides and in all of the diimides by addition of DNA were quite similar, and the results are also summarized in Table I.

Spectrophotometric titrations such as those shown in Figure 3 were converted to Scatchard plots as previously described (Wilson & Lopp, 1979). Because base pair binding preferences and cooperative effects have not yet been determined for these compounds, no attempt was made to fit these results to a precise binding model. From the relatively linear portions of the Scatchard plot as the amount of bound ligand ap-

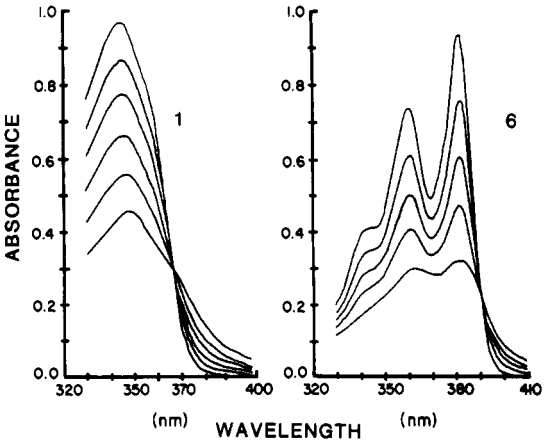


FIGURE 3: Effects of DNA on the absorption spectrum of the related imide **1** and diimide **6** are shown. The spectrum having the largest absorbance at 345 (**1**) and 383 nm (**6**) is that of the free ligand solutions with concentrations of 7.74×10^{-6} and 3.93×10^{-6} M for **1** and **6**, respectively. The absorbances of the compound solutions successively decrease as the concentration of DNA in the solution is increased, finally reaching a point at which no further significant change occurs. The molar ratios of compound to DNA base pairs are 0.00, 0.068, 0.134, 0.24, 0.36, and 0.78 for **1** and 0.00, 0.072, 0.104, 0.192, and 0.38 for **6** from top to bottom, consecutively. The experiments were carried out in MES 00 buffer at 25 °C in quartz cuvettes having a 10-cm light path.

proaches zero, the intercept at $\nu = 0$ was determined. In the lattice binding model, which has been shown to be most appropriate for DNA, this intercept is the intrinsic binding equilibrium constant (McGhee & von Hipple, 1974; Bloomfield et al., 1974; Wilson & Lopp, 1979). Regardless of the binding model, this intercept can be used as a relative binding affinity for comparing a series of ligands that bind to DNA (Müller & Crothers, 1975; Howe-Grant & Lippard, 1979). Intrinsic binding constants, determined in this manner, are collected in Table I for all imides and diimides, and the following can be noted: (1) the relative binding affinities of naphthyl diimides, in general, are about 10-fold larger than those of imides with the corresponding side chain (i.e., 46×10^4 for **6** and 1.7×10^4 for **1**; 3.2×10^4 for **12** and 0.24×10^4 for **5**); (2) all of the type A diimides bind to DNA approximately 10 times stronger than those of type B or type C diimides. It is particularly striking to note the large difference in relative binding affinity for the structural isomers **9** (type A) and **10** (type C). Attempts to measure relative binding affinities in MES 00 buffer were inaccurate due to the considerably larger binding constants for these compounds at the lower ionic strength and the resulting difficulty of obtaining data at low ν values for evaluation of the $\nu = 0$ intercept. This increase in binding affinity for compounds that bind to DNA is expected from the structure and polyanionic charge of DNA (Manning, 1978; Record et al., 1978; Wilson & Lopp, 1979).

Viscometric Studies on Linear DNA. Illustrative results of viscometric titrations of sonicated calf thymus DNA with a naphthyl imide and naphthyl diimides of types A, B, and C are shown in Figure 4. All of the imides and diimides increase the relative specific viscosity of DNA as the molar ratio of the compound to DNA base pairs is increased to a maximum value of approximately 0.6. There are some differences in the maximum η/η_0 ratio reached for the various imides and diimides at high ratios of compound DNA phosphate (Figure 4), but these do not show a regular pattern and, at these high ratios, could be influenced by a small amount of nonintercalative binding (Jones et al., 1980).

Unwinding Angle Studies. Figure 5 shows representative viscometric titrations of CCS-DNA with ethidium bromide

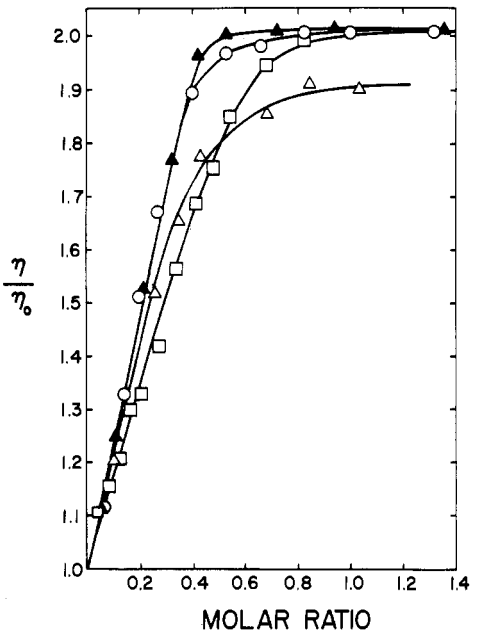


FIGURE 4: Representative viscometric titrations of calf thymus DNA with naphthyl imide **1** (○) and diimides **6** (▲), **10** (□), and **12** (△) are shown with the ratio of the reduced specific viscosity of the DNA–ligand complex, η, divided by the reduced specific viscosity of DNA alone, η_0, plotted as a function of the molar ratio of imide or diimide to DNA base pairs. The DNA concentration in base pairs in all titrations was 1.65×10^{-4} M, and the experiments were conducted in MES 00 buffer at 25 °C.

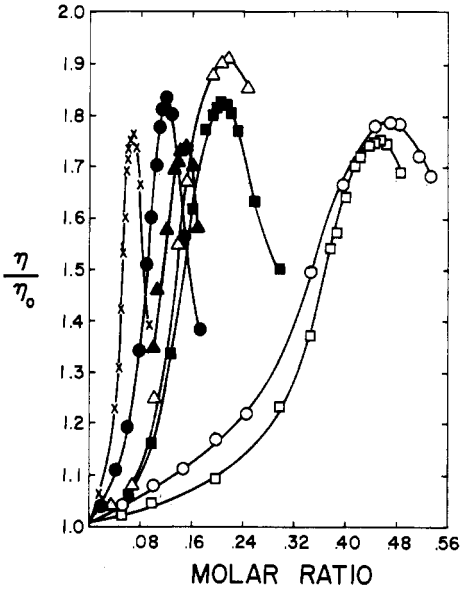


FIGURE 5: Representative viscometric titrations of closed circular superhelical Col E_1 DNA are plotted as described in Figure 4 with ethidium bromide (×), **6** (●), **3** (△), **10** (■), **4** (○), and **11** (□). In this experiment the DNA concentration in base pairs was 4.2×10^{-5} M in MES 00 buffer at 25 °C.

and some naphthyl imides and diimides. The total drug concentration, C_t, and total DNA concentration, N_t, in base pairs were determined at the maximum in these plots. The unwinding of CCS-DNA was quantitatively determined with several viscometric titrations of the type shown in Figure 5 at varying DNA concentrations. The N_t and C_t results from these titrations were analyzed with the following equation as proposed by Vinograd and co-workers (Revet et al., 1971; Jones et al., 1980):

$$C_t = \nu N_t + C_f \quad (1)$$

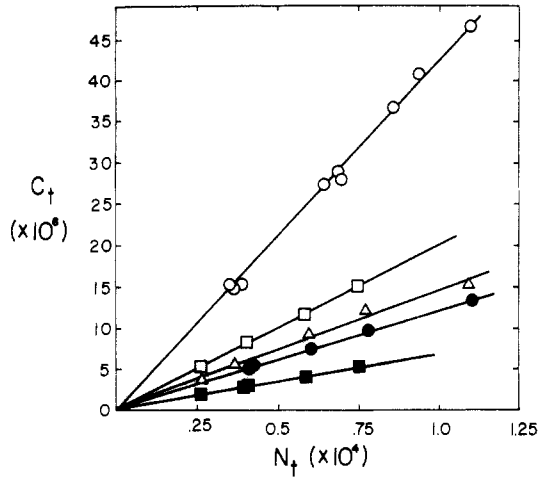


FIGURE 6: Titrations such as those shown in Figure 5 were conducted at several DNA concentrations for the compounds of Figure 1 and the DNA base pair concentrations, N_t, and total imide or diimide concentrations, C_t, determined at the maximum in each titration. Representative plots of C_t vs. N_t are shown for ethidium bromide (■), imide **6** (●), and diimides **10** (□), **11** (○), and **12** (△).

Table II: Apparent Intercalation Unwinding Angles[a]

compd	ν	unwinding angle (deg)
ethidium bromide	0.075	(26.0)[b]
1	0.165	11.8
2	0.170	11.5
3	0.222	8.8
4	0.426	4.6
5	0.220	8.9
6	0.122	16.0
7	0.120	16.3
8	0.120	16.3
9	0.114	17.1
10	0.204	9.6
11	0.426	4.6
12	0.144	13.5
13	0.142	13.7

[a] The experiments were carried out at 25.0 °C in MES 00 buffer. [b] This value is used as a standard.

where C_t and C_f are the molar concentrations of total and free ligand and $\nu = C_B/N_t$, where C_B is the molar concentration of bound drug. Several example plots according to eq 1 are shown in Figure 6. All of the quantities referred to above are those determined at the principal maximum in viscometric titrations of the type shown in Figure 5. Excellent linear plots such as those shown in Figure 6 were obtained for all imides and diimides, and the slopes of those plots, the ν values at the point of exact unwinding of the superhelical DNA, are collected in Table II. The values of ν from Table II are related to the unwinding angles by

$$\nu_s\phi_s = \nu_u\phi_u \qquad (2)$$

where ν_s and ν_u refer to the standard and unknown intercalators, respectively. The 26° unwinding angle for ethidium bromide, resulting from several different determinations (Wang, 1974; Pulleyblank & Morgan, 1975; Bauer, 1978), was used as the standard value in eq 2. The ν_s and ν_u values determined from plots such as those shown in Figure 6 were used in eq 2 to calculate unwinding angles for all of the imides and diimides, and the results are also presented in Table II. It is important to note that all the naphthyl diimides with a methylene group adjacent to the chromophore (type A) have an unwinding angle near 16°. Both type B diimides, **12** and **13**, have slightly lower unwinding angles, between 13° and 14°. There is a dramatic decrease in going to the type C compounds with **10** having an unwinding angle near 10° and **11** near 5°.

From eq 1 it can be seen that the intercept at $N_t = 0$ in Figure 6 is equal to the concentration of free ligand. This value is quite small ($< 5 \times 10^{-7}$ M) for imides **1–3** and all diimides in MES 00 buffer, indicating that these compounds are essentially fully bound to DNA under these concentration conditions. Imides **4** and **5** have greater free ligand concentrations as would be expected from their lower binding constants (Table I). The low unwinding angle and low free ligand concentration of **11** under these conditions suggest that this compound could have some secondary nonintercalative binding occurring (Jones et al., 1980). This compound has low solubility and forms gel-like aggregates in MES 10 buffer, making it impossible to perform reproducible viscometric titrations at the higher ionic strength to test the possibility of outside binding (Jones et al., 1980).

Discussion

The significant induced CD (shown in Figure 2) and the hypochromic effect (shown in Figure 3) of the naphthyl imides and diimides indicate their strong interaction with DNA. As shown in Figure 4, the naphthyl imides and diimides cause a length increase on binding to DNA, and in Figure 5, it is shown that they also remove the superhelical turns of CCS-DNA. The viscometric studies strongly indicate that these molecules interact with DNA through an intercalation mechanism, resulting in base pair unwinding and a DNA length increase (Cohen & Eisenberg, 1969; Bauer, 1978; Jones et al., 1980). Several lines of evidence suggest that the diimides place one cationic substituent in each groove when they intercalate: (1) the strong binding of these compounds to DNA indicates good interaction between the diimide ring and the DNA base pairs; (2) partial insertion of the diimide ring between base pairs would not give the viscosity increases observed for these compounds with DNA and might even lead to a viscosity decrease (Gabbay, 1977); (3) Corey–Pauling–Koltun (CPK) model building studies with diimides **6–13** and DNA indicate that, due to steric restrictions, only a small overlap can be obtained between base pairs adjacent to the intercalation site and the diimide aromatic ring system when the cationic substituents are in the same groove but excellent interaction of the diimide ring system with the base pairs and of the diimide cationic groups with DNA phosphates can be obtained when the substituents are in opposite grooves; (4) stopped-flow kinetic studies indicate that association and dissociation reactions of diimides with DNA are several orders of magnitude slower than for the corresponding monoimides (S.-F. Yen, E. J. Gabbay, and W. D. Wilson, unpublished results). In agreement with this idea, compounds **10** and **11**, which must move the largest groups through the double helix, react the slowest with DNA of any of the compounds of the series. There is also a variation between diimide type A and C association rates, with isomers **9** and **10**, for example, having apparent first-order association rate constants that differ by over an order of magnitude (3.6 and 0.27 s^{-1} for **9** and **10**, respectively, at 2×10^{-6} M DNA base pairs, 25 °C, and MES 10 buffer). Differences of this magnitude for mono- and diimides hold for all temperature, concentration, and ionic strength conditions examined (S.-F. Yen, E. J. Gabbay, and W. D. Wilson, unpublished results). This kinetic criterion for opposite groove binding of diimide substituents is supported by the results of Müller & Crothers (1968) with actinomycin derivatives having bulky substituents at position seven of the chromophore. In their proposed intercalation complex for

actinomycin, substituents at the seven position would have to slide between base pairs and lie in the opposite groove from the cyclic peptide substituents. A derivative with the bulky $-NHCOC(CH_3)_3$ group at the seven position had an apparent second-order association rate constant several orders of magnitude less than for the parent compound (Müller & Crothers, 1968). These differences are similar to those observed for mono- and diimides. Preliminary comparative binding experiments of compound **11** with T4 viral DNA and a similar but nonglucosylated DNA from a mutant T4 bacteriophage have indicated significantly stronger interaction with the nonglucosylated DNA (W. Germon and W. D. Wilson, unpublished results). Since the wild-type and mutant DNA samples are quite similar in every respect except major groove glucosylation, this finding also supports the idea that the diimides have one substituent in the major groove in their intercalation complex. These diimide compounds are, thus, the only known examples, at present, of intercalating compounds that can have bulky cationic substituents in the major groove.

A significant finding from this research is that even far below the melting temperature of DNA (e.g., 25 °C) there are transient openings in the double helix large enough to allow a substituent as large as the adamantyl amide of **11** or the hydrated amide of **10** to slide between base pairs. In our original design of these diimide compounds, we had hoped to synthesize a series of molecular calipers, the larger members of which would be able to intercalate readily into DNA near the melting temperature but would intercalate extremely slowly at low temperature. Even at low temperature, however, the largest diimides readily associated with and dissociate from DNA [e.g., with rates comparable to daunorubicin (Gabbay et al., 1976)]. This finding suggests that a relatively high percentage of the thermally induced openings in DNA are large enough to allow even very large molecules to pass through the double helix. In agreement with this concept Englander et al. (1980) have shown that hydrogen exchange results on nucleic acids (Mandel et al., 1979) are consistent with large sections of open base pairs (10 or more pairs) in the double helix. Recent high-resolution NMR studies of the exchangeable imino protons in homogeneous DNA restriction fragments of varying size (Early et al., 1981), however, have shown that A·T base pairs open for exchange much faster than G·C base pairs and that an A·T base pair can open for exchange while neighboring G·C base pairs remain hydrogen bonded. All of these results illustrate the complexity of thermally induced DNA breathing and the importance of using a variety of techniques in the investigation of DNA dynamics.

Intercalation and major/minor groove positioning of diimide substituents cannot explain the rather large differences in binding affinity and unwinding angle for these compounds. The difference between the structural isomers **9** and **10** is large for both of these physical properties. CPK model building studies with a double-helical section of DNA and diimides suggest that the substituent on the carbon adjacent to the naphthalene ring is particularly important for the final structure of the diimide–DNA complex. The methyl group on type B diimides and the amide group on type C diimides, for example, prohibit the naphthyl diimide ring from having as much overlap with the base pairs as those of type A compounds. This lower degree of overlap decreases the interaction between the intercalator and the two adjacent base pairs and could lead to a decrease in the binding affinity. This proposed structural variation, which is schematically represented in Figure 7, can account for the results of both the binding

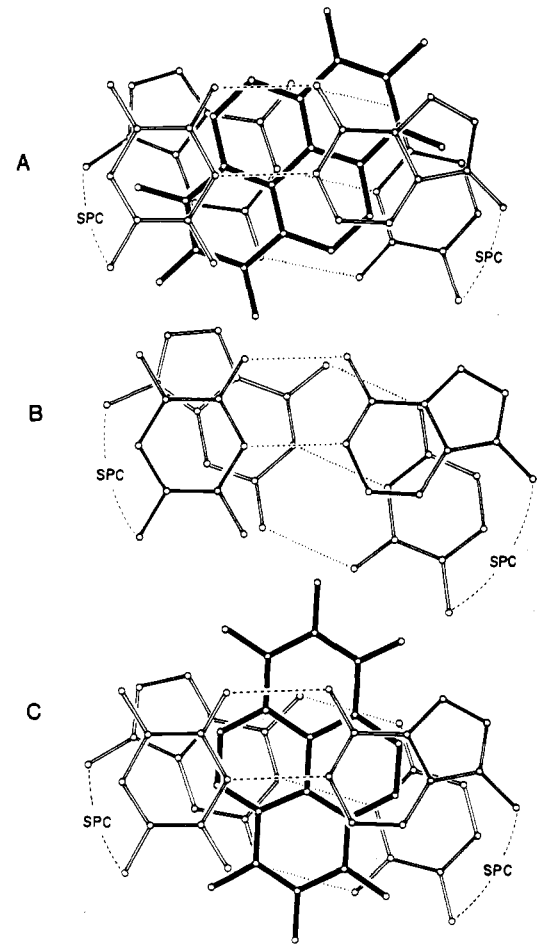


FIGURE 7: Schematic representations of DNA unwinding and base pair stacking interactions for naphthyl diimides are shown. A top view of two adjacent base pairs is shown with (A) a type A diimide intercalated, (B) no intercalator, and (C) a type C diimide intercalated between the base pairs. The drawings are based on binding, hydrodynamic results, and CPK model building studies for the two classes of diimides.

constant and the unwinding angle variations among type A, B, and C diimides. As shown in Table II, all of type A compounds exhibit unwinding angles near 16°. Because of the similar structural features among the four type A compounds, they show a very similar interaction with DNA, including binding affinities and unwinding angles. The small steric hinderance on the side chain of type A compounds (shown in Figure 7A) can allow the long axis of the diimide molecule to become relatively parallel with the long axis of the adjacent DNA base pairs and, thus, achieve a high degree of interaction with these base pairs. On the other hand, the unwinding angle decreases as the size of the α carbon substituent group increases from **6–9** to **12–13** and to **10–11**. This suggests that groups larger than a hydrogen on the α carbon sterically hinder the diimide from having a high degree of interaction with the adjacent base pairs. As proposed in Figure 7C, the long axis of compound **10** is shifted, relative to the nearly parallel arrangement of type A diimides, to close to perpendicular with respect to the long axis of the adjacent base pairs. It is interesting to note that the same trend was observed for the imide system; i.e., the order of the size of the substitution group on the carbon adjacent to the ring is $1 \sim 2 < 5 < 3 < 4$, and the order of unwinding is $1 \sim 2 > 5 \sim 3 > 4$. From all of these results, it seems that, in general, for these intercalators the steric size of the group adjacent to the chromophore is the critical factor for determining the degree of its interaction with

the two adjacent base pairs of DNA and consequently its relative binding affinity and unwinding angle.

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Molecular Characterization of Small Polydisperse Circular Deoxyribonucleic Acid from an African Green Monkey Cell Line†

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ABSTRACT: Several size classes of small polydisperse circular (spc) DNA from the African green monkey cell line BSC1 have been cloned into the bacterial plasmid pBR322. Analysis of the cloned spc DNA fragments as well as total spc DNA reveals that (a) most or all cloned spc DNAs share homologies with chromosomal sequences, (b) both unique and repetitive chromosomal sequences are represented in spc DNA, (c) the repetitive sequences in spc DNA include two known major repeat families (the α and the Alu) as well as a third, as yet unidentified, set of interspersed repetitive sequences, and (d) the α-like sequences are present in an oligomeric series of circular DNA molecules within the spc DNA population. The organizational features of repetitive DNA sequence-carrying circles suggest a mechanism for their generation.

It has been known for about a decade that many eukaryotic cells in culture (as well as some cells isolated from animal tissues) contain small polydisperse circular (spc) DNAs (Radloff et al., 1967; Smith & Vinograd, 1972; Stanfield & Helinski, 1976; Delap et al., 1978; Delap & Rush, 1978). The best characterized of the spc DNAs are those found in three lines of cultured cells: (1) HeLa from human, (2) Schneider line 2 from *Drosophila melanogaster*, and (3) BSC1 from African green monkey kidney. Table I summarizes the known physical characteristics of spc DNA isolated from these three sources. The common features of the spc DNA from these cell lines are the following: (1) average sizes in the range of 0.8–2.5 kilobase pairs (kbp), (2) copy numbers ranging from a few to perhaps a few thousand per cell, (3) the presence of

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