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Interaction of Netropsin and Distamycin with Deoxyribonucleic Acid: Electric Dichroism Study†

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ABSTRACT: We report dichroism and equilibrium binding studies of netropsin (Net) and distamycin A_3 (Dist) binding to deoxyribonucleic acid (DNA). We show that at low degrees of binding (r) to calf thymus DNA, Net induces a considerable increase in the apparent DNA length (14 Å/drug molecule bound), closely analogous to the results reported earlier for Dist. In addition, we show that chicken erythrocyte DNA shows length changes similar to those of calf thymus DNA upon distamycin binding. DNA length reaches a maximum at 1 bound drug/20–30 base pairs and then decreases to its initial value by $r = 0.1$. This effect is not seen for two other DNAs with nearly identical A + T base pair content and may therefore arise from the details of base sequence or base modification in eukaryotic DNA. We also show that Dist binding to calf thymus DNA at low r values is positively cooperative and shows a DNA affinity which is primarily nonionic. We demonstrate that independent of the DNA to which they are bound, the Net and Dist transition moments are inclined by 43 ± 3° from the helix axis, consistent with the idea that both drugs bind inside and parallel to the DNA small groove. From dichroism measurements, we show that the conformational change induced in calf thymus DNA by Dist does not kink or bend the helix and does not substantially alter the average inclination of the bases. Finally, we outline a statistical mechanical theory for calculation of binding isotherms when binding is coupled to a DNA structural change.

Distamycin A (Dist) and netropsin (Net) are basic oligopeptides which are potent antibacterial (Finlay et al., 1951; Thrum, 1959; Thrum et al., 1972; Sanfilippo et al., 1966), antiviral (deRatuld & Werner, 1970), and antineoplastic (DiMarco & Arcamone, 1963) agents whose pharmacological activity is based upon binding to DNA (Hahn, 1975). The binding of the two to DNA[1] shows similar properties and is by any standard quite remarkable. Both Dist and Net show a DNA binding affinity which is in some cases 100–1000 times larger than that of typical intercalating drugs, yet binding is only weakly ionic (Luck et al., 1977) and does not involve intercalation (Wartell et al., 1974); drug attachment most likely occurs outside the helix in the small groove (Wartell et al., 1974; Krey & Hahn, 1970; Kolchinskii et al., 1975). When bound, both Dist and Net induce changes in the DNA CD spectrum which have been interpreted as arising from a substantial DNA conformational alteration (Luck et al., 1974, 1977). Binding is accompanied by viscosity changes which indicate a length increase and an increase in persistence length (Luck et al., 1977; Reinert, 1972; Reinert et al., 1979), but the nature of the conformational change which occurs is unclear. In an earlier study (Hogan et al., 1979) we used the transient electric dichroism method to demonstrate a large length increase in calf thymus DNA upon Dist binding, interpreting the accompanying cooperative binding as an allosteric transformation of DNA structure. The present paper is an extension of that work.

The binding mechanisms of both Dist and Net are not understood in detail, but it is clear that they show a preference for DNA base sequence which is more complicated than any known for smaller molecules. Net and Dist are capable of preferentially inhibiting, at very low concentration, the action of endonucleases on A·T base pair rich sequences (Nosikov & Sain, 1977). CD and viscosity measurements all indicate that Net and Dist bind most tightly to A·T base pair rich regions of DNA.

Here, we describe electric dichroism and equilibrium binding studies whose goal is to define in more detail the structure of the Net and Dist complexes with DNA and to determine the kind of structural distortion they produce. Measurements were

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[1] Abbreviations used: DNA, deoxyribonucleic acid; EDTA, ethylenediaminetetraacetic acid; Me_2SO, dimethyl sulfoxide; bp, base pair; CD, circular dichroism; CPK, Corey–Pauling–Koltun.

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made on monodisperse DNA fragments isolated from DNAs with varied base content to determine the effect of that variable on binding.

Experimental Procedures

Materials

Distamycin A_3 hydrochloride was purchased commercially (Boehringer and Sigma Chemical Co.) and was then purified by LH20 chromatography (Pharmacia) using as solvent MeOH–$CHCl_3$ (9:1 v/v). Dist was then dried in vacuo and stored at −20 °C. Aqueous solutions of Dist decompose after several days; therefore, stock solutions were prepared immediately before use.

Netropsin hydrochloride was a gift from Dr. E. L. Patterson (Lederle Laboratories) and was used without further purification. The concentration of Net in dichroism buffer was determined by absorbance using ϵ_{295} = 21 000 M^{-1} cm^{-1} (Zimmer, 1975).

1-Pentanol (Aldrich Chemical Co.) and Me_2SO (Matheson Coleman and Bell) were spectral grade and used without purification. The 140-bp DNA fragment from the micrococcal nuclease digestion of calf thymus chromatin (Klevan & Crothers, 1977) was a generous gift from Dr. L. Klevan and was used without further purification. Other DNAs were purchased commercially, deproteinized, and fractionated as described elsewhere (Hogan et al., 1978). The DNA produced by this procedure was 140–150 base pairs in length as determined by electric dichroism and gels calibrated against a *Hae*III digest of ϕX174 (Maniatis et al., 1975).

DNA was prepared for dichroism by extensive dialysis at 4 °C against dichroism buffer (0.5 mM Na_2HPO_4, 1 mM NaH_2PO_4, and 0.25 mM Na_2EDTA, pH 7.0). Drugs were added directly from concentrated stock solutions.

Methods

Electric Dichroism. The bases in DNA form an extremely regular lattice. Therefore, when DNA is oriented in an electric field, chromphores bound to DNA will, like the bases, display linear dichroism. At any wavelength where the ligand absorbs light, the reduced dichroism ρ is defined by

$$\rho = (A_{\parallel} - A_{\perp})/A \quad (1)$$

in which $A_{\parallel}$ and $A_{\perp}$ are the absorbances measured with light polarized, respectively, parallel and perpendicular to the applied electric field and A is the absorbance in absence of the field. The dichroism due to a particular transition moment can be expressed by (Okonski & Yoshioka, 1959)

$$\rho = \tfrac{3}{2}(3\cos^2\alpha - 1)\Phi \quad (2)$$

where α is the angle between the transition moment and the axis of orientation and Φ is the fractional orientation (Okonski & Yoshioka, 1959), which is equal to the ratio (ρ/ρ_∞) of the measured dichroism to the dichroism at perfect orientation, achieved in the limit of infinite field.

As seen previously (Hogan et al., 1978), DNA dichroism values can be determined precisely. Nearly 80% orientation is achieved at the highest field strengths. The extrapolation of the measured ρ values to perfect orientation is consistent with a field-independent dipole moment or the ion-flow model for DNA orientation (Hogan et al., 1978). The angles α for Dist and Net were calculated from the extrapolated dichroism values by using eq 2.

Length Changes. As we have shown (Hogan et al., 1978), rodlike DNA molecules orient in an electric field as predicted for an induced-type moment (Tinoco, 1955), with the dichroism $\rho(t)$ rising to the steady-state value ρ_{ss} according to

$$\rho(t) = \rho_{ss}(1 - e^{-t/\tau}) \quad (3)$$

where $\tau = {}^1/_6 D_r$. For DNA, the rotational diffusion coefficient D_r is as derived by Broersma (1960) for long rods

$$D_r = \frac{3kT}{\pi\eta L^3}(\ln L/b - 1.57) \quad (4)$$

where L and b are respectively the DNA length and half-width.

This expression for D_r applies to any cylindrical particle with a large axial ratio and therefore can be used to measure the length of DNA–ligand complexes. D_r is dominated by its L^3 term and is relatively insensitive to thickness changes. Therefore, dichroism rise times can be used to measure drug-induced length changes with an accuracy equal to 1–2% of the length.

Phase Partitioning. The binding of Dist to calf thymus and *Escherichia coli* DNAs was measured by using the phase partitioning technique (Waring et al., 1975) rather than by dialysis because Dist is moderately unstable in aqueous buffers and would degrade during dialysis. Also, the affinity of Dist for DNA is so high that binding parameters cannot be determined by dialysis at low and moderate degrees of saturation.

The phase partitioning technique is performed with a mixture of an aqueous buffer and an immiscible organic phase. Drug bound to DNA is unavailable for partitioning. Consequently, at partition equilibrium the concentration of drug in the organic phase is a direct measure of free ligand concentration c_f in the aqueous phase, i.e.

$$c_f = c_{org}/K$$

where K is the partition coefficient.

Partition equilibrium can be achieved in a few minutes, which allows binding measurements on ligands that would be unstable in aqueous buffers during a 2–3-day dialysis experiment. Also, if the organic solvent is chosen such that K is large, the free concentration of ligand in the aqueous phase can be amplified substantially in the organic phase, allowing measurement of c_f at very low degrees of saturation.

1-Pentanol was chosen as the organic phase in these experiments. The aqueous phase was 1 M Pes (1 M NaCl, 6 mM Na_2HPO_4, 2 mM NaH_2PO_4, and 1 mM EDTA, pH 6.5). Binding was measured at 11 °C in a thermostated bath. Pentanol (2 mL) was added to 2 mL of 1 M Pes buffer which contained DNA and Dist at known total concentrations. The mixture was then shaken vigorously for 2 h at 11 °C; further shaking induced no change in the distribution of Dist. The two phases were than allowed to separate at 11 °C. The top (pentanol) phase was removed, and the concentration of Dist, c_{org}, was determined from its absorbance at 306 nm, ϵ_{306} = 42 400 M^{-1} cm^{-1}.

Since the concentration of free Dist in the aqueous phase $c_f = c_{org}/K$, the concentration of bound Dist $c_b = c_a - c_f$. The concentration c_a was determined by dissociating the Dist–DNA complex by addition of an equal volume of Me_2SO. The total concentration of Dist c_a was then determined from its absorbance at 306 nm in 1:1 Pes/Me_2SO, ϵ_{306} = 32 800 M^{-1} cm^{-1}. The Dist partition coefficient K was measured in the same way and averaged over five experiments.

Results and Discussion

The effect of Net and Dist on DNA length is shown in Figures 1–4. As can be seen in Figure 1, the drugs induce remarkable changes in calf thymus and chicken erythrocyte DNA. When binding is below 1 distamycin/30 base pairs (r_{Dist} = 0.033), the DNA length is increased by ~14 Å/bound drug (Hogan et al., 1979). A similar effect is seen for Net, which

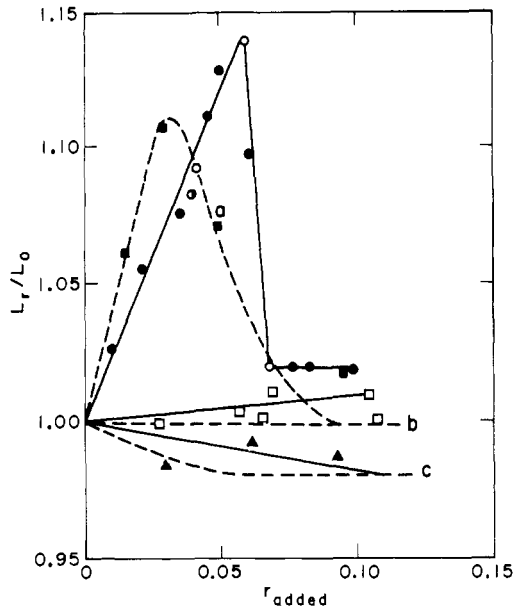

FIGURE 1: Netropsin and distamycin-induced DNA length changes. The length L_r of the drug–DNA complex was measured as a function of r (the ratio of bound drug/total DNA base pairs). Lengths were normalized to DNA length L_0 in the absence of drug, L_r/L_0. DNA samples were monodisperse, 140 ± 10 bp in length (see Materials). DNA concentrations were 1×10^{-5}–3×10^{-5} M base pairs in 2.5 mM dichroism buffer at 11 °C. Lengths were calculated from dichroism rise times measured at 265 nm. Experimental points refer to netropsin–DNA complexes, for different sources of DNA: (●) calf thymus, sample 1; (◑) calf thymus, sample 2; (○) calf thymus, 140-bp fragment from nucleosome; (□) *E. coli*; (▲) human placenta. Dashed lines represent distamycin-induced length changes (Hogan et al., 1979): (a), (b), and (c) are for DNAs from calf thymus, *E. coli*, and human placenta, respectively. Also shown are experimental points (■) for distamycin with chicken erythrocyte DNA.

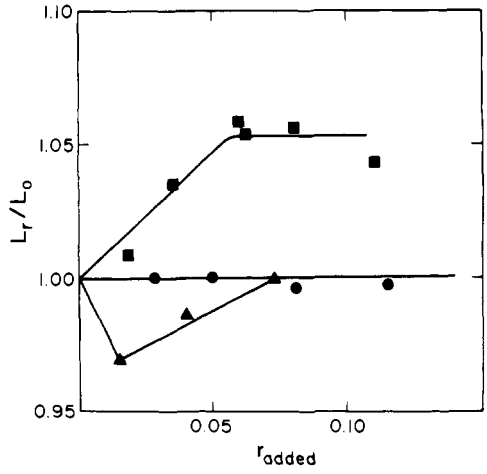

FIGURE 2: Distamycin-induced length changes. Conditions were identical with those in Figure 1. DNA: (■) *C. perfingens*; (●) *E. coli*; (▲) *M. luteus*.

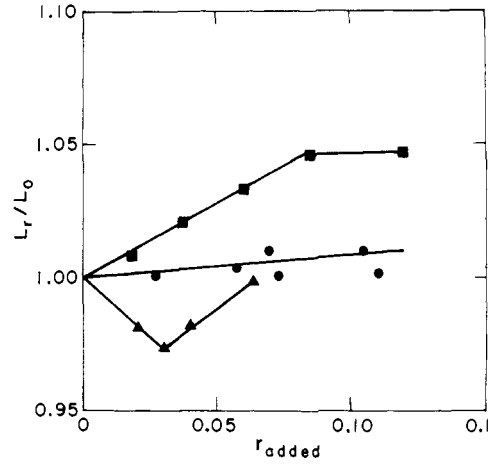

FIGURE 3: Netropsin-induced length changes. Conditions were as described in Figure 1. DNA: (■) *C. perfringens*; (○) *E. coli*; (▲) *M. luteus*.

shows a length maximum at $r_{Net} = 0.05$, and also exhibits an ~14-Å DNA length increase/bound molecule in the low r value region. This unusual effect suggests that with only 1 drug bound/20–30 base pairs (5–7 molecules/140-bp DNA fragment) the length of the helix has been increased by ~13%. We have elsewhere shown additional evidence that the effect of Dist on calf thymus DNA results from an allosteric conversion of DNA from one form to another (Hogan et al., 1979).

As shown in Figure 1 the effect is seen both for the 150-bp fragments fractionated from whole calf thymus DNA and for the 140-bp fragments from chromatin, suggesting that the capacity to undergo lengthening is a property of most of the DNA in calf thymus. A similar length increase is also seen (Figure 1) for chicken erythrocyte DNA, and we have observed the same lengthening effect in rat liver nucleosome DNA prepared in another laboratory (R. Sarma and N. Dattagupta, unpublished results). Among eukaryotic DNAs tested, only human placenta DNA has failed to show the dramatic length increase at $r \simeq 0.03$.

Lengthening is less dramatic for the prokaryotic DNAs tested and seems to be dependent on A·T base content. *Clostridium perfringens* DNA (72%) shows the greatest lengthening (4–5 Å/bound drug molecule) (Figure 2 and 3). However, this increase is monotonic up to $r = 0.1$ implying that it differs qualitatively from that seen for calf thymus DNA.

The length of *Micrococcus luteus* DNA (28% A·T) decreases in the presence of the two drugs (Figures 2 and 3) implying that the effect on G·C-rich regions is fundamentally different. However, the ability of Dist to lengthen DNA is not dependent on gross A·T content in a simple way, as evidenced in Figure 1, in which are presented the measured length changes for *E. coli* (50% A·T), calf thymus (56% A·T) chicken erythrocyte (57% A·T), and human placenta DNA (52% A·T). Neither Dist nor Net has an effect on the length of *E. coli* DNA, and both reduce slightly the length of human placenta DNA. Since these three DNAs are nearly identical in base composition and in their other physical properties, the enormous difference in the effect of Net and Dist binding implies that the ability to undergo conformational change in response to these two drugs is determined by details of base sequence or base modification.

Interestingly, neither Net nor Dist induces a length change in poly(dA)·poly(dT) (Figure 4a), reinforcing the idea that although the effect of Net and Dist is related to A·T base content, the effect is complex. Sequences $A_n \cdot T_n$ may not be binding sites capable of producing length change, or, as has been noted elsewhere (Arnott & Selsing, 1975), the structure of the poly(dA)·poly(dT) helix differs from B-type helices and this may alter drug binding. It should be made clear that under all the conditions cited, Dist and Net bind to these DNAs with high affinity (Luck et al., 1974, 1977); only the effects of binding differ among the DNAs.

The salt condition used for our experiments, 2.5 mM, is considerably below the 200 mM concentration used by Reinert et al. (1979) for their determination of DNA length and

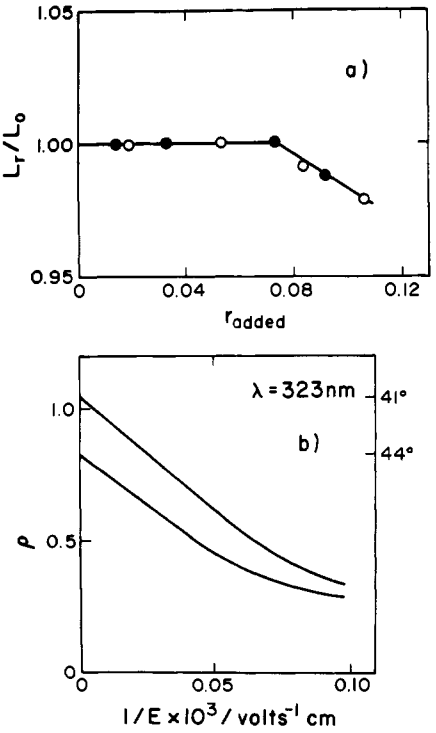

FIGURE 4: Netropsin and distamycin effects on poly(dA)·poly(dT). (a) The length of the drug–DNA complexes at different r values: (○) netropsin; (●) distamycin. Up to $r = 0.08$, DNA length is unchanged. (b) Drug dichroism amplitudes at 323 nm of the Net and Dist complexes with poly(dA)·poly(dT): (upper curve) Net; (lower curve) Dist. Dichroism amplitudes measured with poly(dA)·poly(dT) are representative of those with other DNAs. The dichroism for Net and Dist complexes is very similar, positive, and indicative of a 43° angle (41–45°) between the drug backbone and the helix axis. The 43° angle is nearly independent of r and the DNA used (see Table I).

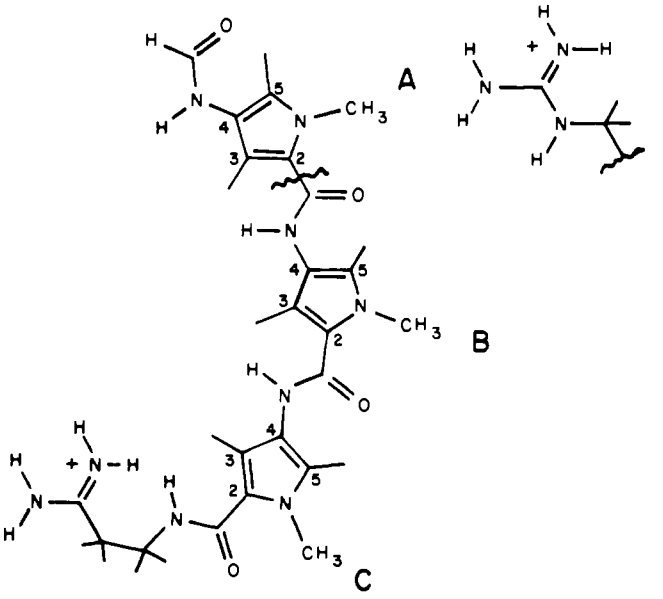

FIGURE 5: The structure of netropsin and distamycin. The lower structure is distamycin. As indicated, the terminal pyrrole group is replaced by methylguanidine to form netropsin. Both molecules are aromatic polypeptides and therefore expected to be rigid in solution. As drawn, the molecules are curved with six to seven hydrogen bond donors facing the inner radius (see text).

stiffness changes. Possibly this explains their smaller observed length increase, with no dramatic difference between prokaryotic and eukaryotic DNA samples. Unfortunately, 200 mM salt concentration is not accessible to electric dichroism measurements, so no direct comparison is possible. However, we show below that cooperative distamycin binding to calf thymus DNA is retained even in high salt concentration. Hence, it is possible that allosteric alteration of DNA by distamycin binding occurs under physiological conditions but that the length of the altered form is not so different from the standard form in higher salt concentration.

Finally, our calculation of length changes has assumed that there is no contribution of stiffening of DNA to the measured change in the rotational correlation time. Using the Hearst (1963) theory for a weakly bending rod and assuming a persistence length of 600 Å, we estimate that τ would be ~10% smaller for 140-bp DNA than if the DNA were perfectly rigid. Therefore, a maximum of 3–4% apparent length increase (τ varies with L^3) could actually be due to stiffening, compared to the observed maximum length increase of ~13%.

Orientation of Net and Dist on DNA. When Net and Dist are bound to DNA, the absorption maxima of both shift to 325 nm. Hence, we can measure the dichroism of the two when bound to DNA, without any contribution from DNA dichroism. As can be seen for the poly(dA)·poly(dT) complex in Figure 4b, Net and Dist show similar positive dichroism values at 323 nm, which indicates that the polypyrrole transition moment for each drug is oriented <54.5° from the helix axis.

The direction in the drug molecule of the transition moments responsible for absorption by the pyrrole–peptide system has not been measured experimentally. Recent work suggests that the pyrrole subunits themselves display degenerate lowest energy transitions directed along the pyrrole C_2 axis (z) and parallel to the C_2–C_5 vector (x) (Sutherland et al., 1978). Aminopyrrole-2-carboxamido substitution, as occurs in both Net and Dist, should greatly favor the x-directed transition.

The Dist derivative with only one pyrrole ring ($Dist_1$) absorbs at 280 nm (Arcamone et al., 1969); with two pyrrole rings, $Dist_2$ absorbs at 300 nm, which suggests a substantial delocaliation of π electron density between adjacent pyrroles, mediated by the intervening amide (Arcamone et al., 1969).

It has been argued that the delocalizaton of pyrrole π electron density is strong only for adjacent pyrroles, and for that reason, UV absorption in Net and Dist is determined by individual pyrrole rather than molecular transition moments (Sutherland et al., 1978). However, if pyrrole groups are in conjugation with the intervening peptide group, as indicated by both UV spectroscopy and the NMR properties of the intervening amides (Arcamone et al., 1967; M. Hogan, unpublished results), then the pyrrole system will as a whole be rigid and planar. With the exception of cyclic peptides such as proline, peptides and oligopeptides show a strong preference for a trans orientation of the amide group. All of the possible rigid planar conformers of Dist and Net with trans amides have a long molecular axis which is roughly parallel to the C_2–C_5 axis in individual pyrroles (Figure 5). Consequently, the direction of the lowest energy transition moment measured for either Net or Dist should be nearly coincident with the long molecular axis whether or not the pyrrole rings are in conjugation beyond nearest neighbors; in the limit of strong mixing of pyrrole transitions, the resulting molecular transition moment is necessarily coincident with the long axis of the linear polypyrrole system; in the limit of weak mixing, absorption arises as an average of independent transitions, each of which is nearly parallell to the long molecular axis.

Dichroism spectra of distamycin bound to calf thymus and *E. coli* DNA are presented in Figure 6. The spectra clearly indicate that more than one transition moment contributes to the absorption in the wavelength range between 310 and 350

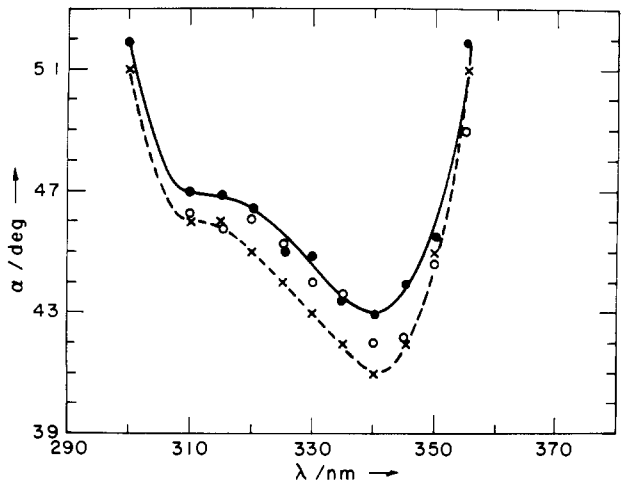

FIGURE 6: Electric dichroism spectra of distamycin DNA complexes. (●) *E. coli* (r = 0.075); (×) calf thymus (r = 0.085); (○) calf thymus (r = 0.032). Other conditions were as described in Figure 1.

Table I: Chromophore Orientation Angles for Distamycin and Netropsin

ligand	DNA	r_{added}	α_{323} (deg)
distamycin A	calf thymus	0.033	46
		0.058	45
		0.065	44
	C. perfringens	0.058	45
	E. coli	0.075	46
	human placenta	0.092	44
	M. luteus	0.075	39
	poly(dA)·poly(dT)	0.072	44
	poly(dG)·poly(dC)	0.080	*a*
netropsin	calf thymus	0.036	41
		0.085	42
	C. perfringens	0.071	45
	human placenta	0.095	44
	M. luteus	0.033	45
	poly(dA)·poly(dT)	0.082	41
	poly(dG)·poly(dC)	0.080	*a*

[a] No dichroism signal at 323 nm.

nm, but all have similar (within ±3°) orientation angles relative to the DNA helix. This supports the idea that all the transition moments are approximately parallel to the molecular axis of the drug. This result is important for the interpretation of dichroism measurements for it assures that the average orientation angle α measured by dichroism is an accurate measure of the orientation on DNA of the pyrrole drug backbone.

We have calculated the orientation angle α from the extrapolated dichroism data and eq 2 for several DNAs at several r values. The results of these calculations are presented in Table I. The similarity among DNAs is striking. Regardless of the effect of Net or Dist on DNA length, the angle between the drug backbone and the helix axis is 43 ± 3°. The Net and Dist complexes with calf thymus DNA show angles which are independent of r. This means that when DNA is lengthened by the drugs, the complex remains a linear rodlike helix and the orientation of the drugs on the helix is unchanged. If the drugs kinked or bent the helix in an irregular way, the measured dichroism values would decrease significantly with increasing r (Dattagupta et al., 1978). It should also be noted in Table I that when bound to poly(dG)·poly(dC), neither Net nor Dist shows dichroism at 323 nm. As shown by CD (Luck et al., 1977) and absorbance titrations (Wartell et al., 1974), Net and Dist bind strongly to poly(dG)·poly(dC) in low ionic strength buffers, but the complex appears to be a nonspecific ionic interaction, perhaps with the backbone phosphates (Zimmer, 1975). The failure of the bound drug to show dichroism implies either that this complex occurs without a fixed geometry or (less likely) that the transition moment happens to be α = 54°, for which ρ = 0.

There is considerable evidence which suggests that Net binds to the DNA small groove. Net binds tightly to glucosylated T_2 DNA (Kolchinskii et al., 1975) (the glucose residue is in the large groove), and the small groove is selectively protected from methylation by Net binding (Kolchinskii et al., 1975). If Net (and by analogy Dist) binds to the small groove in a regular fashion, its orientation should reflect the orientation of the groove in the helix.

The DNA pitch angle β is a direct measure of the inclination of the small groove and is given by (Bloomfield et al., 1974)

$$\beta = \tan^{-1}(2\pi r/P)$$

where r is the radius from the center of the helix and P is the helical pitch.

For B-type helices, P = 34.6 Å; the center of the small groove has a radius r of ~6 Å. Using r = 5.5Å and P = 34.6 Å, we calculate β to be 45°. The 43 ± 3° angle measured for Net and Dist therefore argues that both drugs are bound inside and parallel to the DNA small groove on all DNAs except poly(dG)·poly(dC).

This kind of binding has an interesting geometrical implication. If Net and Dist bind inside the small groove and interact directly with 3–5 base pairs, as implied from binding (Luck et al., 1974, 1977; Wartell et al., 1974) and methylation protection studies, then, since the drugs are rigid linear molecules, they must themselves be helical or at least curved; (the DNA helix advances through 36 × 4 or 144° over 4 base pairs). For interaction with both the first and fourth member of a base pair sequence, the drugs would need to wrap around 144° of helix. If, as is indicated, Net and Dist are rigid, then the drugs must already be curved. The structure of the drugs as drawn in Figure 5 is curved so that both drugs fit snugly into a CPK model of the small groove and can span as many as five consecutive bases (180°). NMR work indicates that this curved planar structure is the preferred conformation of Dist in solution (M. Hogan, unpublished results). Recent crystallographic evidence (Berman et al., 1979) shows that the netropsin molecule in a crystal is also bowed, with the amide groups on the concave side and the carbonyl and methyl groups on the convex side.

Characterizing the Conformational Change Induced by Net and Dist: Equilibrium Binding of Distamycin. At r = 0.03–0.05, Dist and Net have increased the length of DNA by 14 Å/bound drug molecule. This much lengthening must be spread over several base pairs beyond the actual site of binding. If conformational changes are propagated beyond the binding site, bound drugs could interact with each other at some distance. This interaction could be evidenced as binding cooperatively (positive or negative).

Figure 7 shows a Scatchard (1949) plot of the equilibrium binding of Dist to calf thymus DNA at high ionic strengths. As we reported previously for binding at low ionic strength (Hogan et al., 1979), the steep convex slope of the plot is indicative of high positive cooperativity at low r values (type I binding). Above r = 0.03, a second kind of binding is apparent (type II) which shows a concave slope that would be expected for the noncooperative binding of Dist to several base pairs [the "neighbor exclusion" effect (Crothers, 1968; McGhee & von Hippel, 1974)]. The solid curve in the figure was calculated from a statistical mechanical theory (see Appendix) of cooperative alloseric transition of DNA from its

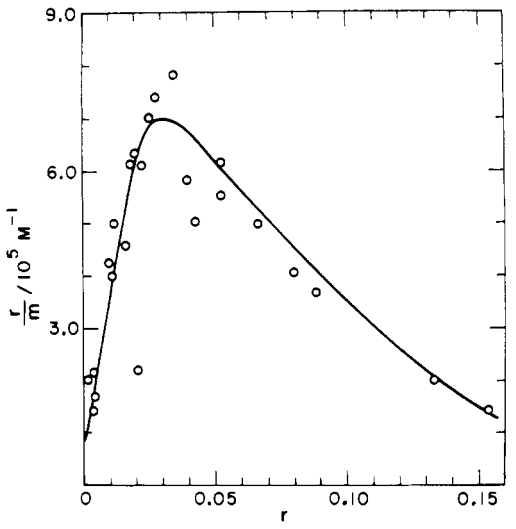

FIGURE 7: Distamycin binding to calf thymus DNA. Equilibrium binding was measured by the solvent partition method (see text) and is plotted in the Scatchard formalism; r is the ratio of bound Dist to total base pair concentration. m is the concentration of free Dist. Binding was measured in 1 M Pes, partitioning against 1-pentanol at 11 °C. The solid curve was calculated from a statistical mechanical theory (see Appendix), with parameters (see Appendix) $s = 0.977$, $\sigma = 0.003$, $K_1 = 0.95 \times 10^5$ M^{-1}, $K_2 = 0.95 \times 10^6$ M^{-1}, $n_1 = 15$, and $n_2 = 4$.

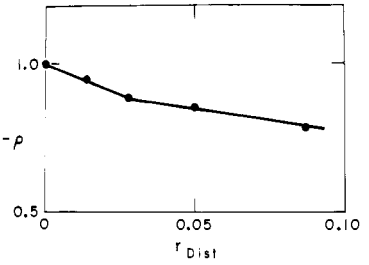

FIGURE 8: Variation of the dichroism of the Dist–calf thymus DNA complex. Dichroism was measured at 265 nm, increasing the concentration of bound Dist. The measured dichroism at 265 nm, ρ, is plotted vs. the ratio of bound Dist/DNA base pairs, r. Conditions were as described in Figure 1.

initial form to anther structure that has high affinity for distamycin.

The Structure of the Distorted DNA Complex. We have measured DNA dichroism in the Dist–calf thymus DNA complex in order to characterize the structure of this altered form of DNA. Figure 8 shows the average dichroism at 265 nm for the Dist–calf thymus DNA complex as a function r_{Dist}. Dist absorbs strongly at 265 nm, so the measured dichroism is a weighted average of DNA dichroism and some unknown contribution from Dist. As has been shown, Dist dichroism is positive at 323 nm; if the dichroism contribution from Dist absorption at 265 nm is also positive, then the average dichroism at 265 nm should decrease monotonically with increasing r_{Dist}, so long as the negative DNA contribution remains constant. If the DNA base tilt changed significantly due to Dist binding, this would be evidenced as a large deviation from the monotonic positive change induced by Dist.

A small change in slope is seen at $r = 0.03$ in Figure 8, implying a minor effect occurs over the range where DNA length is changing. However, the small magnitude of the decrease in $-\rho_{265}$ implies that there is no major change in DNA base tilt up to $r_{Dist} = 0.1$ (a decrease in $-\rho$ from 1.0 to 0.9 corresponds to a total average change in angle of 2°). This means that although DNA conformation is altered, the average base tilt angle remains nearly unchanged. Further, the small magnitude of the total change in dichroism at 265 nm reinforces the conclusion that no kinking or bending is associated with the DNA conformational change.

Earlier we showed that Dist was capable of inducing a cooperative change in DNA structure which extends over large DNA domains (hundreds of base pairs) (Hogan et al., 1979). We also showed that the ability of Dist to induce that conformational change was not simply related to DNA base composition; the length of *E. coli* DNA, with base composition nearly identical with calf thymus and chicken erythrocyte DNA, is unaffected by Dist binding (Hogan et al., 1979).

Here, we have shown that the functionally similar antibiotic netropsin is also able to induce massive conformational changes in calf thymus DNA and that this effect is not simply related to DNA base composition.

We have proposed that this length increase results from a Dist- or Net-induced conversion of DNA between conformations which are very nearly isoenergetic at room temperature (Hogan et al., 1979). On the basis of that previous work and the results of the present study, we can now list a few of the properties of this altered DNA cmplex. (1) The helix is 12–15% longer than unaltered DNA. (2) The helix remains linear (unbent and unkinked). (3) The DNA base tilt angle is not greatly altered. (4) Dist (and presumably Net) binds to this DNA with higher affinity. (5) The ability of Dist to induce the altered conformation is only weakly salt dependent. (6) Ethidium bromide intercalates into the altered DNA forming a complex tilted by 20° with respect to its complex with unaltered DNA. (7) Ethidium shows a lower affinity for the altered DNA but binds to it with substantial positive cooperativity.

Appendix

Theory of Allosteric Transitions in DNA. An allosteric transition requires that a macromolecule have (at least) two structural forms. A simple model for such transitions in DNA allows each base pair to exist in one of the two double-helical forms 1 and 2, with equilibrium constant s for the conversion of a base pair at the interface between the two forms:

$$...(1)(1)(2)(2)... \overset{s}{\rightleftharpoons} ...(1)(2)(2)(2)... \qquad (A1)$$

The difficulty of nucleating one structural form within a helix of the other kind is accommodated by including in the equilibrium constant a factor σ for each new interface between the forms which is created by the reaction. Thus the equilibrium constant for the nucleation process

$$...(1)(1)(1)... \overset{\sigma^2 s}{\rightleftharpoons} ...(1)(2)(1)...$$

is $\sigma^2 s$.

The second requirement for an allosteric conversion in DNA is that the two helical forms have different affinities for a ligand, which in our case is the drug distamycin. We let K_1 be the intrinsic equilibrium constant for binding a ligand to form 1 in isolation from interaction with other ligands; K_2 is the analogous quantity for DNA form 2. In principle, each form may also have different neighbor exclusion (Crothers, 1968) or base sequence preference properties.

Statistical mechanical description of the allosteric transition for this model is straightforward if one ignores possible base specificity of the ligand affinity. With the assumption also that the DNA molecules are very long, the problem is readily solved by a modification of the method of sequence-generating functions (Lifson, 1964). The partition function includes a factor y for each base pair; y is subsequently adjusted so that

the partition function summed over all chain lengths is on its circle of convergence, ensuring that the average chain length is infinite. Let, in addition, a be the concentration (activity) of the free drug molecule and n_1 (n_2) be the number of base pairs occluded by a bound drug molecule in form 1 (2). At saturation the extent of binding is 1 drug/n_1 (or n_2) base pairs; n for standard neighbor exclusion binding of an intercalator is 2 (Bauer & Vinograd, 1970).

We next define the series B and F which respectively represent sequences of successive occupied sites at the closest allowed spacing and sequences of free or unoccupied base pairs. These series are defined for both forms 1 and 2:

$$B_1 = \sum_{j=1}^{\infty} (K_1 a y^{n_1})^j$$

$$B_2 = \sum_{j=1}^{\infty} [K_2 a (sy)^{n_2}]^j$$

$$F_1 = \sum_{j=1}^{\infty} y^j$$

$$F_2 = \sum_{j=1}^{\infty} (sy)^j$$

The statistical weight of all chain configurations can be generated by repeated multiplication of the matrix **M** by itself.

$$\mathbf{M} = \begin{pmatrix} 0 & F_1 & \sigma B_2 & \sigma F_2 \\ B_1 & 0 & \sigma B_2 & \sigma F_2 \\ \sigma B_1 & \sigma F_1 & 0 & F_2 \\ \sigma B_1 & \sigma F_1 & B_2 & 0 \end{pmatrix}$$

The criterion for placing the partition function on its circle of convergence is that the largest eigenvalue of **M**, λ_{max}, be unity. This is accomplished by adjusting y.

Averages are calculated by differentating λ. For example, the average number of bases in form 2, Θ_2, is

$$\Theta_2 = \frac{\partial \ln \lambda_{max} / \partial \ln s}{\partial \ln \lambda_{max} / \partial \ln y}$$

We have written a Fortran computer program which calculates the revelant quantities by an iterative procedure. Given an initial estimate of y, the program calculates λ_{max} and adjusts y until $\lambda_{max} = 1$. It then calculates the appropriate derivatives numerically by making small individual changes in s, y, etc. and recalculating λ_{max}. The program is available by inquiry to the authors.

The theory contains six adjustable parameters; data such at those shown in Figure 7 are sufficient to specify five of these parameters within a narrow range. K_2 and n_2 reflect the binding affinity of form 2 and are adjusted to fit the magnitude and slope of the binding isotherm for r values greater than the maximum in r/m. K_1 is determined from the intercept on the r/m axis or from the binding isotherm for a DNA such as *E. coli* which does not undergo the allosteric transition. The neighbor exclusion range n_1 is not accessible from the data in Figure 7, but it can be estimated from the binding isotherm for *E. coli* DNA (Hogan et al., 1979). The parameters s and K_2/K_1 determine the r value at the midpoint of the rising portion of the isotherm (at small r values), and σ determines the steepness with which the conversion from form 1 to form 2 binding is accomplished. We fitted the theory to the data by trial and error adjustment of the parameters, recalculating the whole binding isotherm for each new set of values. The calculation can be accomplished much more rapidly than the experiment. Table II lists the parameters determined from the binding data at two different salt concentrations.

Table II: Parameters Describing the Allosteric Binding of Distamycin to Calf Thymus DNA

parameter	1 M salt[a]	66 mM Na^+ [b]
s	0.977	0.962
σ	0.003	0.003
K_1	0.1×10^6 M^{-1}	0.3×10^6 M^{-1}
K_2	1.0×10^6 M^{-1}	4.8×10^6 M^{-1}
n_1	15	15
n_2	4	6

[a] This work. [b] Hogan et al. (1979).

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Hypoxanthine in Deoxyribonucleic Acid: Generation by Heat-Induced Hydrolysis of Adenine Residues and Release in Free Form by a Deoxyribonucleic Acid Glycosylase from Calf Thymus†

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ABSTRACT: A slow conversion of adenine residues to hypoxanthine occurs in single-stranded DNA when heated in neutral aqueous buffers. The rate of this reaction at pH 7.6 and 110 °C is $k = 4 \times 10^{-8}$ s^{-1}, as determined by base analysis of heat-treated DNA that contains radioactively labeled adenine residues. It is proposed that adenine deamination is one of several forms of hydrolytic damage that may occur as spontaneous premutagenic lesions in DNA in vivo. Cell extracts from calf thymus and human fibroblasts contain a DNA glycosylase activity which specifically catalyzes the release of free hypoxanthine from DNA or polydeoxyribonucleotides that contain dIMP residues. Several properties of the purified enzyme from calf thymus are described: It has an approximate molecular weight of 31 000. No cofactors are required for activity. The enzymatic release of hypoxanthine occurs readily from double-stranded polydeoxyribonucleotides that have either thymine or cytosine residues in the complementary strand. Single-stranded polymers are 10–20-fold more slowly attacked, and there is no detectable cleavage of monomeric dIMP. Hypoxanthine is liberated from DNA directly as a free base. Thus, when poly(dI)·poly(dC) containing both [^{3}H]-dIMP and [^{32}P]dIMP residues was employed as the substrate, ^{3}H-labeled hypoxanthine but no ^{32}P-labeled material was released in ethanol-soluble form. The hypoxanthine-DNA glycosylase presumably acts in DNA repair by preventing deaminated adenine residues from being expressed as mu

DNA molecules undergo slow decay at neutral pH as a result of a number of hydrolytic reactions. One of the most important of these may be the deamination of the constituent bases. Conversion of cytosine to uracil has been shown to occur for dCMP residues in single-stranded DNA (Lindahl & Nyberg, 1974) after incubation in solution at elevated temperatures and neutral pH. A DNA repair enzyme which specifically removes uracil from DNA, uracil-DNA glycosylase, is present in extracts from both bacterial and mammalian cells (Lindahl, 1974; Friedberg et al., 1975; Sekiguchi et al., 1976). *Escherichia coli* mutants, *ung*, deficient in this enzyme have been isolated and found to exhibit an increased spontaneous mutation frequency (Duncan et al., 1978). Most of the spontaneous mutations in *ung* strains are G·C→A·T transitions (Duncan & Weiss, 1978). This is consistent with the notion that deamination of cytosine residues in DNA occurs at a significant rate in vivo and that such lesions remain unrepaired in *ung* strains. Further, the discovery of Coulondre et al. (1978) that spontaneous base substitution hot spots in *E. coli* occur at 5-methylcytosine residues further implicates cytosine deamination as relevant to mutagenesis, because the methylated derivative is converted to nonrepairable thymine residues by deamination.

Hydrolytic deamination of purines is a slower reaction than cytosine deamination (Shapiro & Klein, 1966). Nevertheless, even a very slow conversion of purine residues in DNA to deaminated forms may be of physiological significance in view of the very large amount of unique information present in a DNA molecule. In particular, heat-induced degradation at 37 °C of adenine to hypoxanthine would result in A·T→G·C transition mutations after DNA replication unless a repair mechanism exists to remove hypoxanthine from DNA. We have previously shown that *E. coli* cell extracts contain low levels of a DNA glycosylase which excises hypoxanthine from deaminated DNA and polydeoxyribonucleotides (Karran & Lindahl, 1978). Here we report that slow hydrolytic deamination of dAMP residues in DNA can be observed at neutral pH and, further, that mammalian cells contain a hypoxanthine–DNA glycosylase with properties similar to, but not identical with, those of the bacterial enzyme.

Experimental Procedures

Purines, Nucleotides, Polydeoxyribonucleotides, and DNA. Uniformly labeled [^{14}C]adenine (309 mCi/mmol), [2-^{3}H]-adenine (15 Ci/mmol), [8-^{3}H]dATP (29 Ci/mmol), and [α-^{32}P]dATP (30 Ci/mmol) were obtained from the Radiochemical Centre, Amersham, England. Radioactively labeled dITP was made by deamination of labeled dATP with 3 M

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