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Anthracycline–DNA Interactions Studied with Linear Dichroism and Fluorescence Spectroscopy†

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ABSTRACT: DNA-binding geometry and dynamics of a number of anthracyclines, including adriamycin and 4-demethoxydaunorubicin, interacting with DNA have been studied by means of linear dichroism and fluorescence techniques. The anthracycline chromophore is found to be approximately parallel to the plane of the DNA bases and to have a restricted mobility, as would be expected for an intercalative binding mode, but there are variations between different directions in the chromophore as well as between the drugs. From dichroic spectra of adriamycin in an anisotropic host of poly(vinyl alcohol), absorption components corresponding to transitions with mutually orthogonal polarizations have been resolved. These can be exploited to determine the orientations of the two chromophore axes in the DNA complex relative to the DNA helix axis. In a certain binding regime the long axis of the bound anthracycline chromophores (with the exception of 4-demethoxydaunorubicin) is found to be approximately 10° closer to perpendicular to the helix axis than are the DNA bases. This demonstrates that the average base tilt is at least 10°. By contrast, the short axis of the aglycon moiety is found to be tilted some 20–30° from perpendicular. This may be because it is probing a base direction with a more pronounced, static or dynamic, inclination than the average in DNA. The drug orientation and the DNA orientation (reflecting flexibility) are observed to vary differently and nonmonotonically with binding ratio, suggesting specific binding and varying site geometries. The fluorescence lifetimes of adriamycin and 4-demethoxydaunorubicin in aqueous solution are measured to 1.0 and 1.5 ns, respectively, and are found to change only moderately upon binding to poly(dA-dT) or poly(dG-dC). A high fluorescence polarization anisotropy shows that the anthracyclines in their DNA complexes have a restricted mobility on the time scale of their fluorescence lifetimes. Comparison with ethidium, though, indicates a significantly higher degree of libration of the anthracyclines. This effect is discussed in terms of a selective fluorescence detection of drug molecules that, owing to DNA breathing or (slow) exchange, are in a transient state of semiintercalation.

The anthracycline antibiotics include several hundreds of compounds, isolated from nature or synthesized by man. Many of them are biologically active and are used in cancer chemotherapy; among the various drugs exhibiting efficiencies toward different kinds of solid tumors and leukemia, adriamycin (AM) and daunorubicin (DR) (Figure 1) are the most well-known (Arcamone, 1981).

Nuclear DNA is believed to be the primary target of antitumor activity of the anthracyclines, resulting in inhibition of DNA transcription and replication. In vitro, the anthracycline–DNA interaction mainly occurs by intercalation of the planar ring system between DNA base pairs. The DNA interaction in vivo has been documented by numerous biochemical studies and from structure–activity relationships: compounds that have reached the clinical stage, for example, generally belong to the group with the highest affinity for DNA (Arcamone & Penco, 1987). The planar aromatic aglycon group, which can intercalate, together with the positively charged amino–sugar moiety can be anticipated to provide a high DNA affinity as has been evidenced from binding studies (Valentini, private communication). [It may be noted, though,

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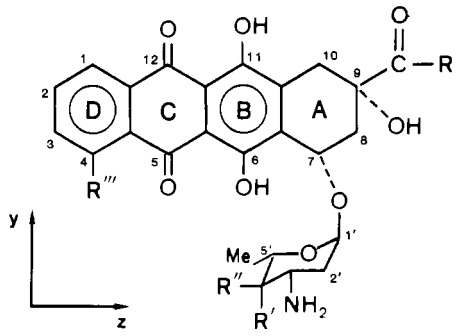

FIGURE 1: Structural formulas of the anthracyclines studied:

	R	R′	R″	R‴
adriamycin (AM)	CH_2OH	OH	H	OCH_3
daunorubicin (DR)	CH_3	OH	H	OCH_3
4′-epidoxorubicin	CH_2OH	H	OH	OCH_3
4′-deoxydoxorubicin	CH_2OH	H	H	OCH_3
4-demethoxydaunorubicin	CH_3	OH	H	H

that adriamycin has been shown to exert cytotoxic activity without entering the cell (Tritton & Yee, 1982) and that DNA intercalation is thus not an absolute requirement for anthracycline activity.]

A number of physicochemical techniques have been exploited in order to verify that AM and DR do intercalate in natural DNAs as well as in synthetic polynucleotides and oligomers: UV/visible absorption, fluorescence, dichroism and viscometry techniques, and X-ray crystallography. The absorption maximum around 480 nm of the anthraquinone chromophore exhibits an ~25-nm red shift upon binding to DNA, and the fluorescence of both AM and DR is substantially quenched compared with the free drugs (Chaires, 1983; Vedaldi et al., 1982). Electric linear dichroism has indicated small angles between the planes of the bound drug chromophores and the DNA base pairs (Fritzsche et al., 1987). Viscometry has shown a DNA lengthening of some 3 Å (Reinert, 1983) and an unwinding by some 10° per bound drug molecule (Fritzsche et al., 1982). All these observations are in qualitative agreement with intercalation. As to the detailed structure of the anthracycline–DNA complexes, however, the only X-ray diffraction study is that of Quigley et al. (1980), recently refined by Wang et al. (1987). According to these studies two daunorubicin molecules were found to intercalate between the GC base pairs in the self-complementary hexanucleotide CGTACG.

The binding of AM and DR is shown to be cooperative at ionic strengths between 0.01 and 1.0 M NaCl (Graves & Krugh, 1983). Upon binding to native DNA, AM and DR have been claimed to exhibit a sequence specificity for two GC base pairs flanked by an AT pair (Jones et al., 1987). According to the crystal structure mentioned, the aglycon moiety intercalates with the ring D (see Figure 1) protruding into the major groove and the puckered saturated ring A residing in the minor groove together with the sugar moiety and the C9 substituents. The O9 of DR was found to form hydrogen bonds to the amino groups N2 and N3 of an adjacent guanine (Wang et al., 1987).

This well-fitting structure seems to strongly favor the B conformation of DNA; DR is observed to completely convert Z-form poly(dG-dC) (2.4 M NaCl) into B-form at binding conditions of one drug molecule per 20 base pairs, indicating a long-range allosteric effect (Chaires, 1985).

Despite both the great interest that anthracyclines have attracted over the years owing to their importance as antitumor pharmaceuticals and the extensive search for structure–activity relationships, the fundamental mechanisms, on a structural molecular level, are still far from clear. Since the structure in solution differs from the crystal-state structure, and there is also considerable variation between the structures that different anthracycline derivatives adopt, spectroscopic studies of the binding geometries of anthracycline–DNA complexes in solution are desirable.

Here we present results from flow linear dichroism (LD) and fluorescence polarization anisotropy (FPA) measurements on AM, DR, and the three derivatives 4′-epidoxorubicin, 4′-deoxydoxorubicin, and 4-demethoxydaunorubicin interacting with DNA (Figure 1). The results confirm intercalative binding and give information about the orientation of the drug chromophore relative to the DNA base pairs as well as about the DNA structure on a larger scale. The substances studied behave very similarly with the exception of 4-demethoxydaunorubicin, which has also been found to exhibit a significantly higher cytotoxicity compared with DR (Arcamone & Penco, 1987).

Materials and Methods

Chemicals. The anthracycline drugs adriamycin (AM), daunorubicin (DR), 4′-epidoxorubicin, 4′-deoxydoxorubicin, and 4′-demethoxydaunorubicin (4-demDR) were kindly supplied by Pharmitalia Carlo Erba, Milano, Italy. Calf thymus DNA (Sigma, type I) was used without further purification. Double-stranded alternating poly(dA-dT) and poly(dG-dC) (P-L Biochemicals) were purchased from Pharmacia, Uppsala, Sweden. The concentration of calf thymus DNA, poly(dA-dT), and poly(dG-dC) was determined from light absorbance at 258 nm by using molar absorptivities of 6600, 6600, and 8400 M^{-1} cm^{-1}, respectively. The samples were prepared in 0.15 M NaCl solution containing 1 mM sodium cacodylate buffer adjusted to pH 7.0. Poly(vinyl alcohol) (PVA) (Elvanol, Du Pont) was used for film preparation as described elsewhere (Matsuoka & Nordén, 1982).

"Binding ratio" r denotes the ratio between total concentration of drug and the DNA phosphate concentration. With a typical DNA phosphate concentration of 10^{-4} M the deviation of r from the ratio bound drug/DNA phosphate is less than 5% for r-values lower than 0.1.

Linear Dichroism. Linear dichroism LD is defined at any wavelength as the differential absorption of linearly polarized light:

$$LD(\lambda) = A_{\parallel}(\lambda) - A_{\perp}(\lambda) \quad (1)$$

where $\parallel$ and $\perp$ denote polarization of the electric field of light parallel and perpendicular to the orientation direction (flow direction). Light is propagated radially through the concentric silica cylinders of a Couette cell of Wada type (Wada & Kozawa, 1964), and the LD is measured differentially by polarization modulation in a modified commercial circular dichrometer (Jasco J-500) as described previously (Davidsson & Nordén, 1976) [for calibration, see Nordén and Seth (1985)]. The LD is normalized to be independent of concentration and path length by dividing it by the isotropic absorbance A_{iso} (measured on a wavelength-matched Cary 219 spectrophotometer) to form the reduced linear dichroism, LD^r:

$$LD^r(\lambda) = LD(\lambda)/A_{iso}(\lambda) \quad (2)$$

LD^r can be related to the orientation of the absorbing transition moments according to (Nordén, 1978)

$$LD^r(\lambda) = \sum F_i \epsilon_i(\lambda)(LD^r)_i / \sum F_i \epsilon_i(\lambda) \quad (3a)$$

$$(LD^r)_i = \tfrac{3}{2} S(3 \langle \cos^2 \alpha_i \rangle - 1) \quad (3b)$$

where $\epsilon_i(\lambda)$ is the decadic extinction coefficient of component

Table I: Fluorescence Lifetimes[a] (ns)

	adria-mycin	χ^2	4-demethoxy-daunorubicin	χ^2
free in solution	0.98	1.7	1.52	1.1
poly(dA-dT), r = 0.02	1.93	1.5	0.61 (57%), 2.4 (43%)	1.0
poly(dA-dT), r = 0.2	1.51	1.1	0.57 (54%), 1.6 (44%)	1.2
poly(dG-dC), r = 0.02	1.03	0.8	1.34	1.2
poly(dG-dC), r = 0.2	1.03	1.2	1.57	1.2

[a] Fluorescence lifetimes measured in buffer solution; concentrations of poly(dA-dT) and poly(dG-dC) were 0.16 and 0.12 mM, respectively. The percentage of the contribution to the fluorescence intensity is given in parentheses when appropriate.

i at wavelength λ, F_i is its fractional concentration, and S is an orientation factor for a local helix axis of DNA. $S = 1$ denotes perfect orientation parallel to the flow direction, and $S = 0$ denotes random orientation (rest). α_i is the angle between the transition moment i and the helix axis.

Component Spectra. Polarized absorption spectra of adriamycin dissolved in a PVA film, stretched approximately five times its original length, were recorded with the film oriented in two orthogonal directions. The spectra thus obtained—$A_\parallel$ and $A_\perp$—were combined according to (Michl & Thulstrup, 1987)

$$A'_y = A_\perp - d_\perp A_\parallel$$
$$A'_z = A_\parallel - d_\parallel A_\perp \quad (4)$$

to yield the shapes of the pure component spectra A'_y and A'_z, which were assumed to correspond respectively to the short and long axis polarized transitions of the aglycon chromophore. A_y and A_z were then obtained by normalizing the contributions from A'_y and A'_z with the isotropic absorbance

$$A_{iso}(\lambda) = \tfrac{1}{3}[A_z(\lambda) + A_y(\lambda)] \quad (5)$$

at wavelengths corresponding to pure polarizations (296 and 500 nm). This procedure relies on an assumption that the aglycon chromophore can be regarded as having effectively C_{2v} symmetry and that only in-plane polarized π–π^* transition contribute, i.e., that any transition moment is polarized along either of the two in-plane symmetry axes. The justification of this assumption will be commented on below.

Fluorescence. Fluorescence was measured on an Aminco SPF-500 spectrofluorometer ("corrected spectra"). Fluorescence polarization anisotropy (FPA) was determined by inserting Polaroid filters in the excitation and emission paths. FPA is defined as FPA = $(I_\parallel - I_\perp)/(I_\parallel + 2I_\perp)$, where $I_\parallel$ and $I_\perp$ denote the intensities of the steady-state fluorescence polarized parallel and perpendicular to the vertically polarized exciting light. For a totally rigid system of immobile chromophores the anisotropy attains a maximum value of +0.4 provided the absorbing and emitting transitions are parallel. In case they are orthogonal a minimum FPA value of −0.2 is expected for immobile chromophores (Cantor & Schimmel, 1980).

Time-Resolved Fluorescence Measurements. To support the interpretation of the fluorescence polarization anisotropy, fluorescence lifetime measurements were made on AM and 4-demDR when free as well as when bound to poly(dA-dT) and poly(dG-dC). The single-photon counting technique was used with a flash lamp filled with hydrogen gas as the excitation source; the excitation light was selected with a 503-nm interference filter and the emission light with a 550-nm long-pass filter. The convolution was done with a scattered response profile at 503 nm, and a least-squares fit was employed to give a minimum in the χ^2 parameter. Results and experimental specifications are summarized in Table I. In order to obtain a reasonable fit to data, an extra lifetime was introduced as a dummy variable to compensate for effects in the lamp and the detection systems. This procedure is justified by the fact that it was needed also for a chromophore known to have a monoexponential decay, rhodamine 101 in ethanol (Kemnitz et al., 1987). The extra lifetime only accounts for a few percent of the total counts within the time window of the experiment, but its value can vary considerably. If this term was excluded, the lifetimes obtained were only slightly affected, but the statistics became much worse. This procedure of data analysis, together with the very long counting times required due to low emission intensities, made it hard to interpret the results in Table I in terms of clearly defined photophysical species. Rather it gives a qualitative picture of the time dependence of the fluorescence emission. Although the fit of data to decay parameters is somewhat loose, a significant improvement in the statistics is obtained for 4-demDR poly(dA-dT) if double-exponential decay is used. The fit is acceptable as judged from the χ^2 parameter, which typically ranged from 0.8 to 1.7.

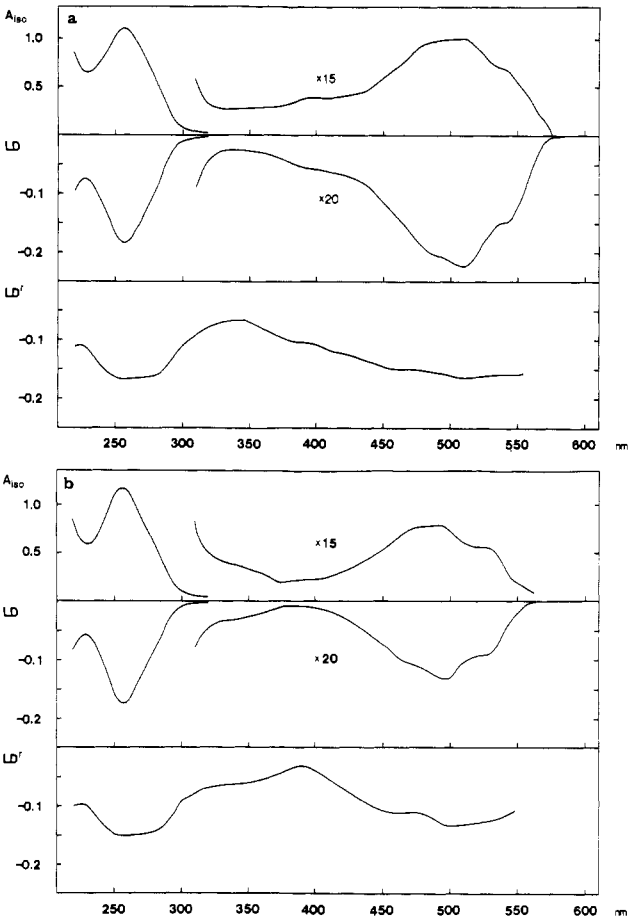

FIGURE 2: From the top: isotropic absorbance (A_{iso}), linear dichroism (LD), and reduced linear dichroism (LD^r) at r = 0.05; DNA concentration = 0.15 mM (a) AM–DNA and (b) 4-demDR–DNA.

RESULTS

Linear Dichroism. The absorption (A), linear dichroism (LD), and reduced linear dichroism [LD^r = LD(λ)/$A_{iso}(\lambda)$] spectra of AM and 4-demDR bound to DNA are shown in Figure 2, panels a and b, respectively, at a drug/DNA phosphate binding ratio of 0.05. The corresponding spectral features of DR, 4′-epidoxorubicin, and 4′-deoxydoxorubicin are very similar to those of AM (results not shown). The negative, almost constant, value of LD^r between 460 and 550

Table II: Reduced Linear Dichroism at Binding Ratio $r = 0.05$

substance	LD^r_{505}	LD^r_{290}	LD^r_{258}	α_z (deg)	α_{DNA} (deg)	α_y (deg)
DNA			−0.151			
AM–DNA	−0.178	−0.09	−0.167	90	82	66
DR–DNA	−0.188		−0.163	90	78	
4′-epidoxorubicin–DNA	−0.177		−0.146	90	76	
4′-deoxydoxorubicin–DNA	−0.162		−0.146	90	79	
4-demDR–DNA	−0.133[a]	−0.06	−0.153	78	90	65

[a] Measured at 495 nm.

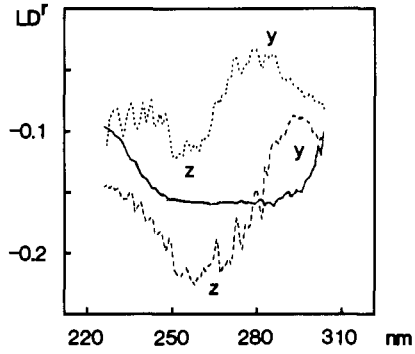

FIGURE 3: Resolved reduced linear dichroism spectra in DNA region. Contributions from AM (−−), 4-demDR (···), and DNA (—). Binding ratio $r = 0.05$.

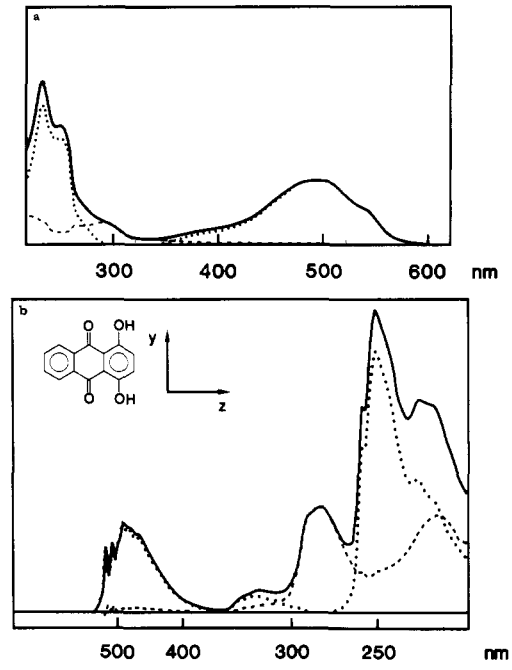

FIGURE 4: (a) Resolved components from polarized absorption spectra of AM oriented in stretched PVA film. A_z (···) and A_y (−−). Isotropic absorbance A_{iso} calculated according to eq 5 (—). (b) Resolved component spectra of quinizarin: A_z (···), A_y (−−), and isotropic absorbance (—) [reproduced with permission from Myrvold et al. (1986)].

nm is in accord with its origin from one single transition moment, which has been assumed to be polarized along the long axis (z) of the aglycon chromophore. This assignment is confirmed in the separate study of polarized absorption spectra of AM oriented in a stretched PVA film (see below). The LD^r amplitude at ~500 nm, by comparison with LD^r of DNA at 260 nm, can be taken as a measure of the orientation of the aglycon long axis relative to the DNA bases. As shown in Table II, LD^r_{505} is close to LD^r_{258} for most of the drugs, indicating approximately parallel orientation, as expected for intercalation.

If the DNA bases were idealized to be perpendicular to the helix axis, the corresponding orientation of the drug can be calculated from eq 3b. [For example, $LD^r_{258} = -0.153$ and LD^r(4-demDR) = −0.133 yield $\alpha_{4\text{-demDR}} = 78°$.] However, most of the drugs exhibit a larger LD^r amplitude than DNA, demonstrating that the DNA bases are not perfectly perpendicular to the helix axis. When α_{drug} is taken to be equal to 90°, $\alpha_{DNA} = 76$–$82°$ is obtained, showing that the average base inclination is at least some 10°.

The polarized absorption spectra of AM oriented in stretched PVA film are shown in Figure 4a. If the chromophore can be treated as having C_{2v} symmetry and only in-plane (π–π^*) transitions giving considerable intensity, the absorption envelope can be decomposed according to eq 4 into two component spectra, A_y and A_z, with polarizations along the two in-plane symmetry axes. As shown in Figure 4a, regions of pure polarization are found at 450–600 nm (z polarization) and 290–330 nm (y polarization). In Figure 4b the corresponding resolved spectra of quinizarin are reproduced [from Myrvold et al. (1986)]; this parent chromophore has C_{2v} symmetry, as is also confirmed by the fact that the A_y and A_z spectra do not have any features in common. At shorter wavelengths another two z-polarized absorption bands (at 240 and 260 nm) and another y-polarized band (at 220 nm) are seen. Table II summarizes the observed transitions. In a recent dichroism study (Samori et al., 1987) of adriamycin and daunorubicin oriented in liquid crystals, it was confirmed that the UV–visible transitions are essentially the same as in the unsubstituted quinizarin.

An attempt to evaluate the short axis orientation of AM and 4-demDR relative to DNA is shown in Figure 3, where the predominant DNA contributions have been subtracted from the A and LD spectra. The features of the drug absorption can be distinguished in the difference spectra. From the LD^r spectra (obtained according to eq 3a) the orientations of the y and z axes can be deduced. Positive contributions at 230 and 290 nm indicate that the y axis has an inclination different from the z axis. The results are presented in Table II in terms of LD^r values and angles relative to the helix axis, calculated on the assumption that $\alpha = 90°$ for the band with the largest LD^r amplitude.

When the drug/nucleotide binding ratios are varied, the orientation of DNA is clearly affected, as seen in Figure 5. At a binding ratio of 0.1 the LD^r amplitudes indicate that the DNA orientation is enhanced by some 10%. The LD^r changes for DNA and AM are not proportional; i.e., the ratio LD^r_{AM}/LD^r_{DNA} is not constant with r. In the case of AM, the curves of LD^r_{AM} and LD^r_{DNA} are even found to cross, and for r-values above 0.02 the amplitude of LD^r is larger for AM than for DNA.

Fluorescence. The fluorescence polarization anisotropy (FPA) was found to be considerable for all compounds studied when they were bound to DNA or to polynucleotides (Figure 6). The values were rather close to the theoretical limit for immobilized chromophores (+0.4) at the lowest binding ratios. In Figure 6a the FPA dependence on the drug/DNA mixing ratio is shown for AM and 4-demDR in DNA. The FPA at high ratios compares well with the FPA of the free drugs. In order to interpret these observations, FPA of AM and 4-demDR was also measured in poly(dA-dT) and poly(dG-dC). As can be seen in Figure 6b, it is clear that for both drugs the FPA decreases more sharply in poly(dG-dC) than in poly(dA-dT) with increasing drug/DNA ratio.

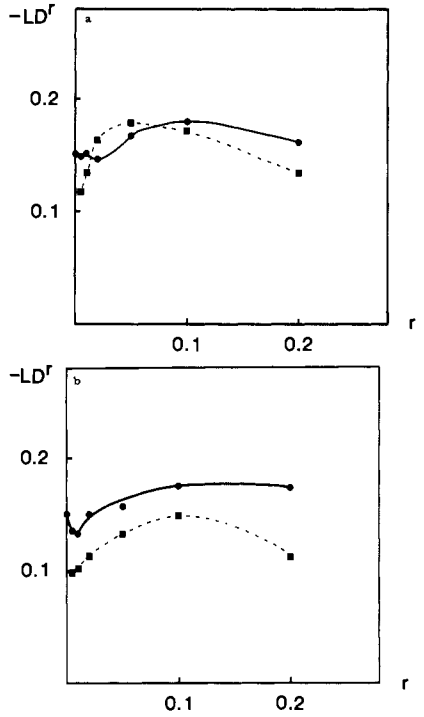

FIGURE 5: Reduced linear dichroism versus binding ratio of (a) AM–DNA LD^r of DNA at 258 nm (●) and of AM at 505 nm (■) and (b) 4-demDR–DNA LD^r of DNA at 258 nm (●) and of 4-demDR at 495 nm (■). LD^r values obtained according to eq 3a with $A_{258}(AM)/A_{480}(AM) = 1.68$ and $A_{258}(4\text{-demDR})/A_{480}(4\text{-demDR}) = 3.0$.

A possible explanation for the high FPA values observed when the drugs are bound to DNA is that the fluorescence lifetime of the chromophore is very short compared to the time scale of the chromophore mobility. Therefore, the fluorescence lifetimes were measured by the single-photon counting technique. The lifetimes of AM and 4-demDR free in solution were found to be approximately 1.0 and 1.5 ns, respectively. Binding to poly(dA-dT) or poly(dG-dC) affects the lifetimes as can be seen in Table I. With AM no lifetime shorter than that of the free drug is found. The proposed explanation can therefore be dismissed. In the case of 4-demDR two lifetimes were found in poly(dA-dT). However, the shortest one is still too long to account for the high FPA. The FPA values of the free drugs, around 0.05 (Figure 6a), are consistent with high chromophore mobility in the absence of DNA.

It is also interesting to know what influence the DNA binding has on the quantum yields. The fluorescence intensity of AM in native DNA shows a nonlinear, sigmoidal dependence on the drug/DNA mixing ratio (Figure 7a). In Figure 7b the effect of adding poly(dA-dT) or poly(dG-dC) to AM in solution is shown. The AM fluorescence intensity in poly(dA-dT) is only reduced to approximately 60% at low binding ratios where all the AM molecules should be bound. In contrast, the AM fluorescence in poly(dG-dC) is very strongly quenched (to more than 99%) at low binding ratios.

Discussion

Linear Dichroism. The orientation of the long axis of all the anthracyclines studied is found to be close to parallel to the DNA base planes, as expected for intercalative binding. Also the orientation of the short axis is more or less parallel to the DNA bases, though a significantly smaller LD^r amplitude indicates a certain tilt or a greater mobility of the chromophore short axis. A qualitatively similar conclusion was made in an electric dichroism study of anthracycline–

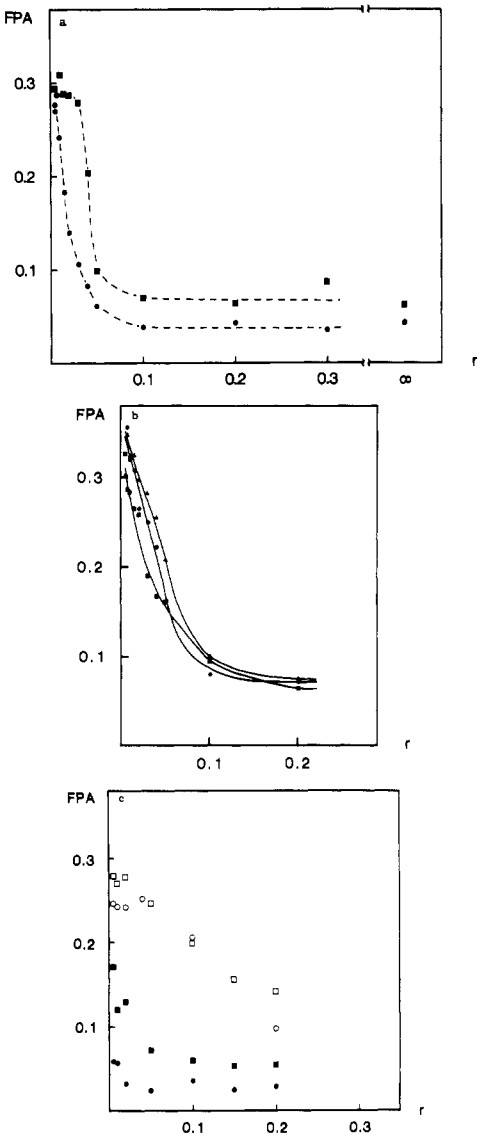

FIGURE 6: Fluorescence polarization anisotropy versus binding ratio for (a) AM–DNA (■) and 4-demDR–DNA (●), λ_{ex} = 510 nm and c_{drug} = 6.5 μM, (b) DR–DNA (■), 4′-epidoxorubicin–DNA (●), and 4′-deoxydoxorubicin–DNA (▲), λ_{ex} = 510 nm and c_{drug} = 10 μM, and (c) AM–poly(dA-dT) (□), AM–poly(dG-dC) (■), 4-demDR–poly(dA-dT) (○), and 4-demDR–poly(dG-dC) (●), λ_{ex}(AM) = 508 nm, λ_{ex}(4-demDR) = 488 nm, and c_{DNA} = 0.15 mM.

DNA interactions (Fritzsche et al., 1987); however, owing to incomplete assignments of transition moments, it was not possible to determine the orientation of the short axis in that study.

An important observation is, at binding ratios larger than 0.02, the larger LD^r amplitude of adriamycin, compared to the LD^r value in the DNA absorption region. This shows that the long axis of the aglycon chromophore at these binding ratios is, on average, closer to perpendicular to the helix axis than are the DNA bases. Such a difference has been observed for a few nonintercalators such as cationic triphenylmethane dyes (Nordén et al., 1978), but not with certainty for any intercalator. If a perpendicular geometry is assumed for the anthracycline long axis relative to the DNA axis, an inclination of the DNA bases of about 10° from perpendicular can be calculated (Table II); the corresponding base inclination in the case of methyl green is ~14° (Nordén & Tjerneld, 1977). The larger LD^r amplitude of the drug chromophore might in principle also arise from a local stiffening of DNA around the binding site. If the intercalated drug reduces the mobility of

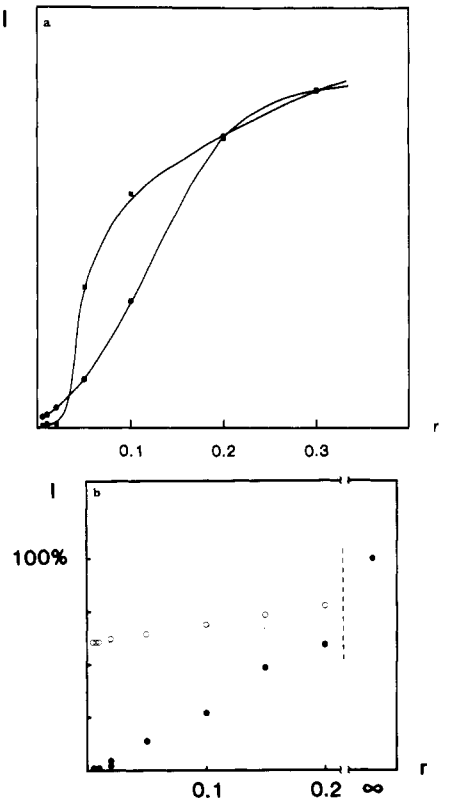

FIGURE 7: Fluorescence intensity versus binding ratio. (a) AM–DNA (■) and 4-demDR–DNA (●) fluorescence intensity in arbitray units, c_{drug} = 6.5 μM. (b) AM–poly(dA-dT) (○) and AM–poly(dG-dC) (●) fluorescence intensity relative to free drug (=100%), c_{AM} = 1.03 μM. λ_{ex} = 480 nm; λ_{em} = 600 nm.

the neighboring bases, the base tilt probed by the drug would appear smaller than the average base tilt at low binding ratios. Alternatively, the transitions in the anthracycline and DNA chromophores are probing directions with different mobility in the DNA bases. In fact, despite the uncertainties in analyzing the AM short axis orientation, all data indicate that this axis is significantly inclined from being perpendicular to the helix axis (Table II). At higher drug/DNA ratios (above 0.1), a decrease in the LD^r_{AM} amplitude is partly due to contributions from free (unoriented) AM.

An interesting observation is the relative change in the LD^r amplitudes for DNA and AM, with $|LD^r_{DNA}| > |LD^r_{AM}|$ at binding ratios lower than r = 0.02 and $|LD^r_{AM}| > |LD^r_{DNA}|$ at higher r-values. It may indicate that, at low r-values, binding to DNA regions where the bases are more inclined or more flexible than on the average is preferred. The same behavior is observed for DM, 4′-epidoxorubicin, and 4′-deoxydoxorubicin (results not shown). In contrast, for 4-demDR the LD^r amplitude for the long-axis transition is lower but approximately proportional to the LD^r value of the DNA bases for all studied r-values. This may indicate a different orientation in the intercalation pocket or alternatively a different binding preference of this drug. In fact, the fluorescence results support a preferential binding of AM to GC in contrast to the binding of 4-demDR (see below). At higher binding ratios ($r > 0.1$) the modest changes in the LD^r of DNA demonstrate that neither the local nor the global (i.e., the flexibility) DNA structure is affected to any large extent by anthracycline interaction.

The resolved polarized absorption spectrum of AM oriented in stretched PVA film (Figure 4a) is in agreement with the component spectra reported for quinizarin in a nematic liquid crystal host (Figure 4b) (Myrvold et al., 1986; Samori et al., 1987). Thus, the long-wavelength absorption band is confirmed to be polarized parallel to the preferential orientation direction, which can be assumed to be the aglycon long axis since the flexibly attached sugar moiety can be anticipated to have only minor influence on the molecular orientation. The resolved spectrum also reproduces a y-polarized component around 290 nm, though with lower intensity than in the quinizarin parent chromophore. Our assignments are also in agreement with relative polarizations obtained in luminescence studies of quinizarin and daunorubicin by Capps and Vala (1980).

The relatively pure polarizations of the absorption bands at 290 and 505 nm, along the y and z axis, respectively, allow study of the inclination of the aglycon chromophore relative to the DNA bases. Wang et al. (1987), in a crystal study on d(CGTACG) containing two intercalated daunorubicin molecules, have found the z axis of the aglycon group to be more or less parallel to the dyad axis of DNA. Thus our observation of a certain tilt of the chromophore y axis is consistent with a corresponding dynamic or static inclination of the long axes of the surrounding base pairs. Such an inclination of the long axis of the intercalation pocket, compared to its short axis, has been evidenced for 9-aminoacridine intercalators (Wirth et al., 1988). Less negative reduced flow linear dichroism than in the 260-nm DNA absorption band has been observed at shorter wavelengths by Edmondson and Johnson (1985), indicating base inclination larger than 15° from perpendicular.

Fluorescence. The time-resolved measurements, indicating lifetimes in the nanosecond regime for AM and 4-demDR when associated to polynucleotides (Table I), show that the fluorescence polarization anisotropy indeed reflects the decreased mobility of the drug chromophores upon DNA binding. All of the studied anthracyclines, when bound to DNA, display a high FPA, around +0.3, in the limit of very low binding ratios (Figure 6a,b). With the exception of adriamycin, for which a narrow plateau in the FPA is seen, the anisotropy decreases sharply toward the value for the free drug when the drug/base ratio is increased. This effect can be ascribed to a dominance in fluorescence from free drug molecules owing to their much higher quantum yield. Not unexpectedly, 4-demDR exhibits a lower FPA when free in solution (higher mobility in the absence of the methoxy group) than AM and the other anthracyclines.

In order to understand the behavior of AM in DNA, it is relevant to compare the fluorescence intensity and FPA measurements in DNA with the corresponding observations in poly(dA-dT) and poly(dG-dC). The fluorescence quantum yield of AM in DNA is very low at small binding ratios (Figure 7a). This excludes any large extent of binding to AT regions where no substantial quenching should have occurred (cf. Figure 7b). The abrupt change at $r \sim 0.035$ of both fluorescence intensity and FPA of AM in DNA is due to increasing amounts of free, strongly fluorescent, drug. The GC specificity in DNA of AM and DR, which has been shown by both CD (Jones et al., 1987) and footprinting techniques (Chaires, 1987), is noteworthy in view of the stronger binding of AM to poly(dA-dT) than to poly(dG-dC) (Chaires, 1983). The plateau shown by AM in Figure 6a and the constant low fluorescence intensity in Figure 7a below r = 0.035 can thus be explained by the high binding constant of AM in combination with the GC specificity.

The high fluorescence polarization of the anthracyclines is in agreement with what can be expected for an intercalator with a lifetime τ_F = 1–2 ns. For example, with ethidium,

which has a radiative lifetime of 23 ns (in DNA), an FPA of 0.23 has been observed in DNA (Härd, 1986). Application of the Perrin equation FPA = $^2/_5(1 + \tau_F 6D_{rot})^{-1}$ (Perrin, 1926), assuming the same effective rotational diffusion constant D_{rot}, gives FPA = 0.39 and 0.38 when taking τ_F to 1 and 2 ns, respectively. This means that the limited mobility of the anthracycline may be explained by the intercalation of the aglycon ring system between the base pairs.

In the limit of low drug/DNA ratios, the FPA approaches values that are high (see, e.g., Figure 6b) but for some compounds still significantly smaller than 0.4. This is particularly clear in the case of AM bound to DNA or poly(dA-dT) (Figure 6a,c) where plateaus are observed at FPA around 0.29 and 0.28, respectively. In poly(dG-dC) the FPA declines very rapidly with increasing drug/nucleotide ratio, and the limiting FPA is difficult to determine. This can be explained by the great difference in quantum yield between free and intercalated dye molecules.

Limiting FPA values which are significantly lower than 0.39 (the value calculated by inference from the mobility of ethidium) could have different origins: wobbling of the anthracycline long axis in the intercalation pocket, mobility of the surrounding DNA base pairs including the intercalator, or enhanced fluorescence intensity from "semiintercalated", more mobile anthracycline molecules.

It is difficult to distinguish intercalator wobbling from motion induced by the surrounding base pairs. Were the depolarization caused by DNA motion, DNA twisting would be the most probable origin, since DNA end-over-end tumbling and bending take place on time scales considerably longer than the anthracycline τ_F (Genest et al., 1985; Shibata et al., 1985). Furthermore, one would then expect the amplitudes of the reduced LD for the anthracycline to follow the corresponding DNA signal, since the orientation of the drug chromophore would depend on the motion of the DNA bases. Such a correlation is not seen (see Figure 5a); depolarization due to DNA twisting can therefore be ruled out.

A semiintercalated anthracycline should be expected to exhibit a lower FPA; it would furthermore experience a less effective DNA quenching. Therefore, small amounts of semiintercalated AM molecules may account for the depolarization observed for AM in DNA as well as in poly(dA-dT) and poly(dG-dC). When discussing semiintercalation as a source of depolarization, the exchange kinetics of the anthracycline–DNA interaction has to be considered. Kinetic measurements on the association/dissociation of daunomycin to DNA have suggested the existence of at least two conformers of the adduct (Chaires et al., 1985). The authors found the rate constants for the reaction steps to be compatible with the existence of an intermediate drug–DNA complex of external nature.

Conclusions

(1) Intercalation of the five studied anthracyclines is supported by the observation of the essentially perpendicular orientation of the aglycon chromophore relative to the DNA helix axis.

(2) Measurements on adriamycin oriented in stretched poly(vinyl alcohol) film indicate pure polarization of the long wavelength band parallel to the long axis of the aglycon chromophore. An absorption band with pure short axis polarization around 290 nm is also seen. This can be used to probe the orientation of the short axis, which is found to be tilted some 20–30° from perpendicular in the adriamycin–DNA complex. The tilt is consistent with the short axis being more mobile than the long axis, or with a preferred tilt of the longest dimension of the base pair.

(3) The anthracyclines studied, except 4-demethoxydaunorubicin, display a more perpendicular orientation relative to the helix axis than the DNA bases (in an intermediate binding regime), indicating heterogeneous DNA flexibility in combination with specific binding.

(4) The fluorescence polarization anisotropy indicates restricted mobility of the anthracyclines within their fluorescence lifetimes (for adriamycin measured to be approximately 1–2 ns, depending on environment) when intercalated in DNA, poly(dA-dT), or poly(dG-dC). Still, the anisotropy is lower than expected for a true intercalator, suggesting the presence of more mobile, strongly fluorescing anthracycline molecules, possibly in a semiintercalative state of DNA binding.

(5) For adriamycin in natural DNA, GC specificity is concluded from fluorescence intensity and polarization anisotropy results.

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Registry No. AM, 23214-92-8; DR, 20830-81-3; 4-demDR, 58957-92-9; PVA, 9002-89-5; poly(dA-dT), 26966-61-0; poly(dG-dC), 36786-90-0; 4′-epidoxorubicin, 56420-45-2; 4′-deoxydoxorubicin, 63521-85-7.

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Atrial Natriuretic Factor R_1 Receptor from Bovine Adrenal Zona Glomerulosa: Purification, Characterization, and Modulation by Amiloride†

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ABSTRACT: The atrial natriuretic factor (ANF) R_1 receptor from bovine adrenal zona glomerulosa was solubilized with Triton X-100 and purified 13 000-fold, to apparent homogeneity, by sequential affinity chromatography on ANF–agarose and steric exclusion high-performance liquid chromatography. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and silver staining of the purified receptor preparation in the absence or presence of dithiothreitol revealed a single protein band of M_r 130 000. Affinity cross-linking of ^{125}I-ANF to the purified receptor resulted in the labeling of the M_r 130 000 band. The purified receptor bound ANF with a specific activity of 6.8 nmol/mg of protein, corresponding to a stoichiometry of 0.9 mol of ANF bound/mol of M_r 130 000 polypeptide. Starting with 500 g of adrenal zona glomerulosa tissue, we obtained more than 500 pmol of purified receptor with an overall yield of 9%. The purified receptor showed a typical ANF-R_1 pharmacological specificity similar to that of the membrane-bound receptor. The homogeneous M_r 130 000 receptor protein displayed high guanylate cyclase activity [1.4 μmol of cyclic GMP formed min^{-1} (mg of protein)$^{-1}$] which was not stimulated by ANF. This finding supports the notion that the ANF binding and the guanylate cyclase activities are intrinsic components of the same polypeptide. Finally, the purified ANF-R_1 receptor retained its sensitivity to modulation by amiloride, suggesting the presence of an allosteric binding site for amiloride on the receptor protein.

Atrial natriuretic factor is a peptide hormone secreted mainly by the atrial cardiocytes to produce a variety of biological effects, including diuresis and natriuresis, relaxation of vascular smooth muscle, and inhibition of aldosterone secretion (Cantin & Genest, 1985; Needleman et al., 1985; Atlas, 1986; Lang et al., 1987). ANF[1] exerts these effects through interaction with specific membrane receptors present in all target tissues (Napier et al., 1984; De Léan et al., 1984; Hirata et al., 1984; Schenk et al., 1985a). Affinity labeling experiments have revealed the presence of two distinct ANF binding proteins with apparent M_r of 60 000–70 000 and 120 000–140 000 (Yip et al., 1985; Misono et al., 1985; Vandlen et al., 1985; Schenk et al., 1985b; Meloche et al., 1986a). Studies in cultured endothelial cells and vascular smooth muscle cells have demonstrated a clear dissociation between ANF binding and ANF-mediated cyclic GMP accumulation (Leitman & Murad, 1986; Leitman et al., 1986; Scarborough et al., 1986). On the basis of these pharmacological and structural criteria, the existence of two subtypes of ANF receptor, designated ANF-R_1 and ANF-R_2 by

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[1] Abbreviations: ANF, atrial natriuretic factor; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; MES, 2-(*N*-morpholino)ethanesulfonic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography.

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