Anthracycline Antibiotics. Interaction with DNA and Nucleosomes and Inhibition of DNA Synthesis†

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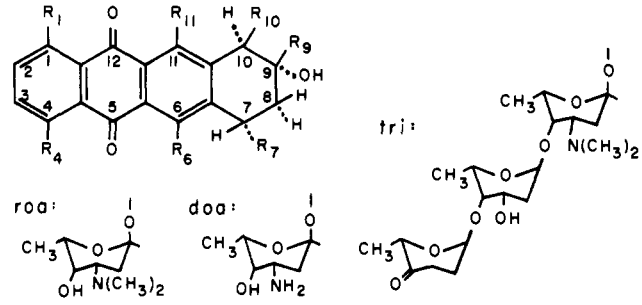
ABSTRACT: We report studies of the interaction of four anthracycline antibiotics, iremycin (IM), daunomycin (DM), aclacinomycin A (AM), and violamycin B1 (VM), with naked DNA, nucleosomal core particles, and 175 base pair (bp) nucleosomes lacking histone H1. In all cases the binding strength increases in the order IM < DM ≈ AM < VM. The binding substrates increased in affinity for the drugs in the following order: core particles < 175-bp nucleosomes < DNA. The apparent DNA length increment per drug bound decreases in the progression IM > DM > AM > VM, the same serial order as is characterized by increasing binding affinity. Dichroism amplitude measurements show that for all drugs the long-wavelength absorbance transition moment is tilted by 26–29° relative to the plane perpendicular to the helix axis; this angle probably corresponds to the long axis tilt of the intercalated chromophore. Finally, it was found that the ability of the drugs to inhibit DNA synthesis by *Escherichia coli* DNA polymerase I increases in the same order as their binding affinity.

Anthracycline antibiotics are among the most potent known chemotherapeutic drugs with antitumor activity (Crooke & Reich, 1980). Their mechanism of action remains uncertain, although antitumor action via intercalation of the anthracycline chromophore between DNA base pairs has been widely acknowledged to be a reasonable starting hypothesis (Arcamone, 1978). However, there are indications suggesting interaction of these drugs with other cell components (Tritton & Lee, 1982) as well as with metabolic processes. At present, the most persuasive proposal is that the drugs act by binding to the DNA–topoisomerase II complex and inducing DNA strand breakage (Tewey et al., 1984; Pommier et al., 1985). Despite the lack of knowledge about the detailed mechanism of action, the potential of DNA both as drug target and competitor for drug binding justifies continued intensive study of the interaction of the anthracycline antibiotics with DNA and chromatin subunits.

We selected four anthracycline antibiotics for the present study, iremycin (IM), daunomycin (DM), aclacinomycin A (AM), and violamycine B1 (VM); their structures are given in Chart I. Two are clinically used antitumor drugs (DM and AM), while the other two were isolated at ZIMET, Jena (Ihn et al., 1980; Fleck et al., 1974). Violamycin B1 (VM) exists as a mixture of three main components (Chart I). Most of the known anthracycline antibiotics are sugar-substituted at position 7, whereas IM is substituted with the amino sugar at position 10, and the main components of VM have two identical amino sugars, in both position 7 and position 10 (Koch, private communication).

In eukaryotic cells, most of the DNA is highly organized in chromatin or chromosomes. Hence the drugs interact in vivo with packaged DNA, which may behave quite differently from free DNA. We have chosen 145 base pair (bp) core particles, H1-depleted, as a suitable subunit of chromatin in which the whole DNA is tightly bound to the histone core. We compared the binding affinity of the four drugs to free DNA and to core particles and present also some comparison experiments on larger (175-bp) nucleosomes and even larger dinucleosomes. We found that the apparent binding constant of the anthracycline–DNA interaction is decreased appreciably in the drug–nucleosome complexes but the sequence of increasing binding affinity, with IM < DM ≈ AM < VM, is identical for both DNA and nucleosome interactions. The binding affinity is reflected in the DNA synthesis inhibitory capacity of these four antibiotics, which also increases in the order IM < DM < AM < VM. On the other hand, we found that the drug-induced apparent length increase of DNA by intercalation decreases in this order, possibly due to changes of the overall shape of the ~150-bp DNA rods. The intercalation geometry of the drug–DNA complexes is rather

Chart I: Structures of Drugs Studied



Drug	R_1	R_4	R_6	R_{11}	R_7	R_9	R_{10}
Iremycin (IM)	H	OH	OH	OH	H	C_2H_5	roa
Daunomycin (DM)	H	OCH_3	OH	OH	doa	$COCH_3$	H
Aclacinomycin A (AM)	H	OH	OH	H	tri	C_2H_5	$COOCH_3$
Violamycin BI/1[a]	H	OH	OH	OH	roa	C_2H_5	roa
" BI/2[a]	OH	OH	OH	OH	roa	C_2H_5	roa
" BI/3[a]	OH	OH	H	OH	roa	C_2H_5	roa

[a] These are the three main components of violamycin BI (VM).

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uniform, with the possible exception of the daunomycin–DNA complex (Fritzsche et al., 1982a).

Materials and Methods

Materials. DNA fragments of ~150-bp length were used for both the electric dichroism and the fluorescence titration experiments. One of the preparations was a sonicated, phenol-extracted and Sepharose-fractionated calf thymus DNA, and the other was a purified restriction fragment of *Escherichia coli* λ phage.

The H1-depleted 145-bp nucleosomes were prepared from calf thymus glands as described previously (Wu et al., 1980). The 175-bp nucleosomes were obtained by a procedure previously described (Crothers et al., 1978) and purified on two successive 5–20% sucrose gradients in TE buffer: 10 mM tris(hydroxymethyl)aminomethane (Tris) and 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.5.

Iremycin and violamycin B1 (both as hydrochlorides) were isolated in ZIMET, Jena (Ihn et al., 1980; Fleck et al., 1974). Daunomycin hydrochloride was obtained from Sigma Chemical Corp., and aclacinomycin A was generously supplied by Prof. H. Umezawa, Institute of Microbiological Chemistry, Tokyo, Japan.

DNA polymerase I from *E. coli* and the deoxyribonucleotides were purchased from Boehringer (Mannheim, FRG), and [^{3}H]dTTP was from Amersham (Amersham, England).

Methods. The electric dichroism studies were done with an instrument described elsewhere (Hogan et al., 1978; Fritzsche et al., 1982a) interfaced to a PDP 11/40 computer. Both the rise time of the dichroism amplitude and the dichroism spectra of the drug chromophores were measured at 12 °C. All solutions were in 2.5 mM sodium cacodylate buffer, pH 6.8.

The fluorescence titrations were carried out at ambient temperature (23 ± 1 °C) on a microprocessor-equipped instrument (Perkin Elmer Model 650-40). Increments of 5 μL of a stock solution of the drug were added to 3 mL of 10^{-5} M (bp) DNA or 9×10^{-6} M (bp) nucleosomes, dissolved in 2.5 mM sodium cacodylate (DNA) or TE buffer (nucleosomes and DNA).

The spectrophotometric titration of IM–DNA complexes was done with high molecular weight calf thymus DNA dissolved in 2.5 mM sodium cacodylate at pH 6.9 and 23 °C on a Specord M 40 spectrophotometer (VEB Carl Zeiss, Jena).

The general assay for DNA polymerase was performed by using an incubation mixture containing in 125 μL: 50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 6 nmol each of dATP, dGTP, dCTP, and [^{3}H]dTTP, 0.065 mM (bp) calf thymus DNA activated with pancreatic DNase according to the method of Richardson (1966), and 0.05 μg of DNA polymerase I from *E. coli*. The antibiotic was added 5 min before starting the reaction by addition of the enzyme. After incubating the reaction mixture for 30 min at 37 °C, aliquots were treated on filter paper disks according to the method of Bollum (1966). The newly synthesized DNA was measured by liquid scintillation counting (Wähnert et al., 1975). The control (100% of synthesis) ran without antibiotic.

Results

Apparent Length Increase of DNA. Ideally, intercalation of a drug between two DNA base pairs is expected to create a 0.34-nm length increase of the DNA molecule for each intercalated drug (Lerman et al., 1961). Several physical and physicochemical methods have been used to measure this effect, including transient electric dichroism, in which an electric field of extremely short rise time (<50 nsec) is applied to the DNA solution at low ionic strength (Hogan et al., 1978). The rise time of the UV linear dichroism reflects the orientation of the DNA rod under the action of the electric field and is approximately proportional to the third power of the DNA length (Hogan et al., 1978). Typical intercalators have been investigated by this method (Hogan et al., 1979); the apparent length increase ΔL per intercalated drug deviates in several cases markedly from the classical figures of 0.34 nm and has been found in the range of 0.20–0.37 nm (Fritzsche et al., 1982b). In our previous paper (Fritzsche et al., 1982a), we showed that the anthracycline antibiotics DM and IM differ significantly in the apparent DNA length increase ΔL induced by their intercalation, with ΔL of the IM–DNA complex greater than that of the DM–DNA complex. In this study, we have extended our investigations to the corresponding AM–DNA and VM–DNA complexes under the same solution conditions (2.5 mM cacodylate, pH 6.8, 12 °C). Surprisingly, we find extremely low ΔL values for those complexes, with 0.1 nm for VM and 0.16 nm for AM (Table I); ΔL decreases in the sequence IM > DM > AM > VM. Besides these differences in the absolute value of ΔL, we observe saturation of the drug-induced apparent length increase of DNA at a relatively low r_t value (r_t = drug added per base pair) of about 0.2 for AM and VM (Figure 1); the corresponding curve for DM does not level out even at $r = 0.25$ drug molecule bound per base pair (Fritzsche et al., 1982a).

Table I: Apparent Relative DNA Length Increase ΔL and Orientation of the Chromophore of Anthracycline Antibiotics in Their DNA Complexes[a]

antibiotic	ΔL (nm/bound drug)	transition moment orientation (deg)[b] long wavelength[c]	short wavelength[d]
IM	0.394	25 ± 1	29 ± 4
DM	0.265	25 ± 1	3 ± 4
AM	0.160	23 ± 2	28 ± 3
VM	0.099	20 ± 2	26 ± 3

[a] At 12 °C, 2.5 mM Na^+, pH 6.8. The results for IM and DM are taken from Fritzsche et al. (1982a), with additional measurements taken into account for calculation of ΔL. ΔL values refer to $r = 0.1$ (drug per DNA base pair) and are accurate to about 10%. [b] Relative to the plane perpendicular to the DNA helix axis. [c] ~490 nm; corresponds to the chromophore long axis (Capps & Vala, 1981). [d] ~390 nm; orientation is uncertain, possibly off-axis, and may be variable from one drug to another.

Geometry of Intercalation. We measured the electric dichroism of the VM- and AM–DNA complexes in the long-wavelength (~490-nm) and short-wavelength (~390-nm) transition regions of the spectrum and extrapolated the field dependence of the dichroism ρ to infinite field, as described earlier for IM and DM (Fritzsche et al., 1982a). The results, collected in Table I, show that the long-wavelength transition moment is inclined within a narrow angular range (20–25°) relative to the plane perpendicular to the DNA helix axis, for all the drugs studied. In contrast, the tilt of the short-wavelength transition is very similar for IM, AM, and VM (26–29°) but much smaller for DM (3°).

Binding Affinity to DNA and to Nucleosomes. Comparative fluorescence titrations of DNA and nucleosomes with the four anthracycline drugs result in the same qualitative sequence of increasing binding affinity: IM < DM ≈ AM < VM (Figure 2). [Note that the Scatchard plots of the two titration experiments for DNA (Figure 2a) and nucleosomes (Figure 2b) differ in their ordinate scales]. In the case of IM–DNA, a spectrophotometric titration in 2.5 mM cacodylate buffer

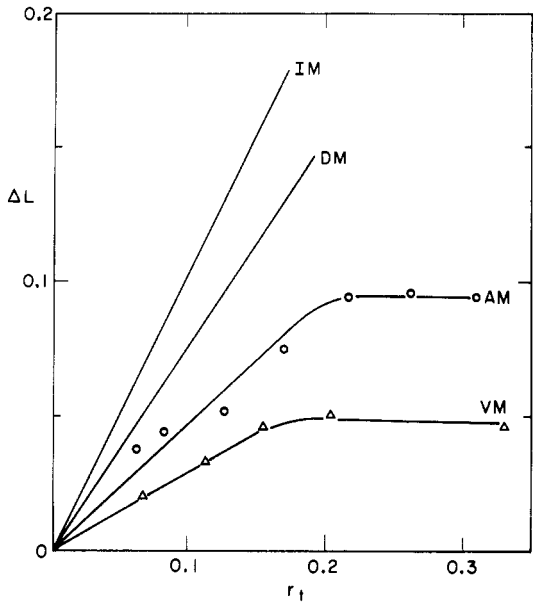

FIGURE 1: Length increase ΔL of DNA induced by interaction with anthracycline antibiotics, showing the dependence on r_t, the number of added drug molecules per DNA base pair. DNA, length ~150 bp, was dissolved in 25 mM sodium cacodylate buffer, pH 6.8 at 12 °C. The results for IM and DM are taken from Fritzsche et al. (1982a), but for DM additional measurements have also been included.

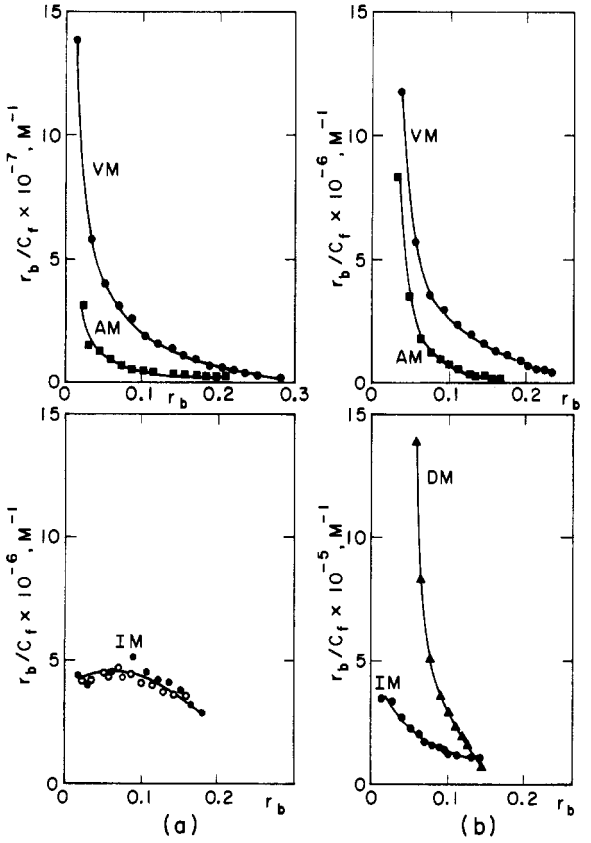

FIGURE 2: Scatchard plots of fluorescence titration experiments on (a) DNA (~150 bp) and (b) 145-bp nucleosomes (from calf thymus) with anthracycline antibiotics. All experiments were done at ambient temperature (~23 °C) in (a) 2.5 mM sodium cacodylate buffer, pH 7.3, and (b) TE buffer containing 10 mM Tris-HCl and 1 mM Na_2EDTA at pH 7. The DM data are taken from Chaires et al. (1983). The open symbols are the results of a spectrophotometric titration of DNA with IM at 23 °C in 2.5 mM sodium cacodylate buffer at pH 6.9.

agrees fairly well with the fluorescence titration (Figure 2a). This agreement confirms the proper setting of the relative

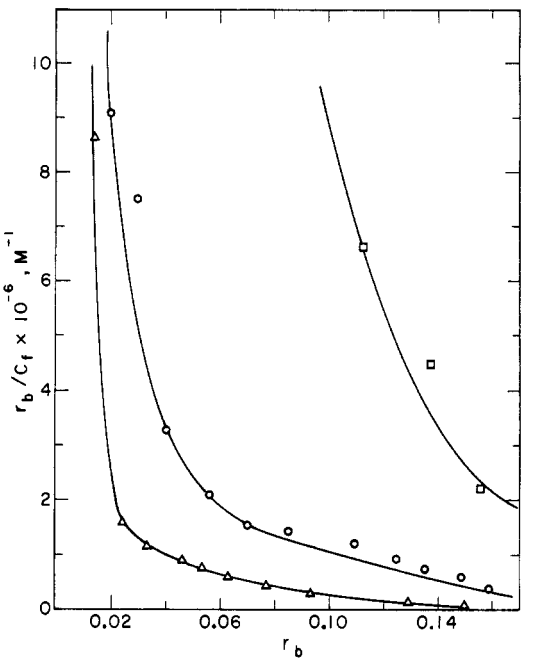

FIGURE 3: Scatchard plots for binding of daunomycin to DNA (□), 175-bp nucleosomes (○), and core particles (△) in TE buffer, 23 ± 1 °C. DNA data are taken from Chaires et al. (1983).

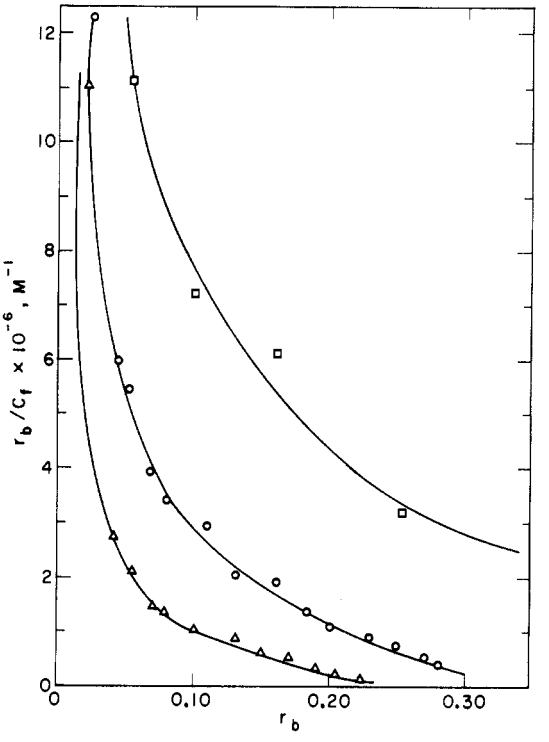

FIGURE 4: Scatchard plots for binding of violamycin to DNA (□), 175-bp nucleosomes (○), and core particles (△) in TE buffer, 23 ± 1 °C.

fluorescence intensity of the bound drug, which is known to be a crucial parameter (Peters & Pingoud, 1979).

Figures 3 and 4 present comparative Scatchard isotherms for binding DM (Figure 3) and VM (Figure 4) to DNA, core particles, and 175-bp nucleosomes, all measured in TE buffer. For both drugs, the binding strength increases in the following order: core particles < 175-bp nucleosomes < DNA; and we find for all three species that the binding of VM is stronger than that of DM.

Inhibition of DNA Synthesis. Inhibition of DNA synthesis by the four anthracycline antibiotics was investigated in order

Table II: Amount of Anthracycline Antibiotic That Inhibits [^{3}H]dTMP Incorporation by 50% Relative to Control

antibiotic	concn at 50% inhibition (μM)[a]	antibiotic	concn at 50% inhibition (μM)[a]
iremycin	29	aclacinomycin A	17
daunomycin	23	violamycin B1	14

[a] ± 1 μM.

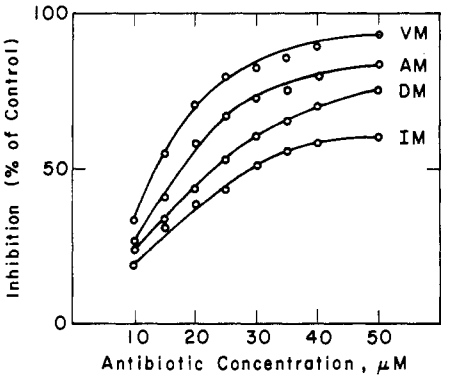

FIGURE 5: Inhibition of DNA synthesis monitored by [^{3}H]dTMP incorporation rate (in percent of the control) as a function of the concentration of anthracycline antibiotics.

to explore one possible molecular mechanism of their biological action. In our assay, the amount of drug that inhibits the synthesis rate by 50% decreases in the sequence IM > DM > AM > VM (Table II and Figure 5). In summary, the binding affinity of the anthracycline antibiotics to DNA as well as to nucleosomes parallels their inhibition capacity.

Several controls were done to exclude alternative interpretations. In the case of DM, the inhibition estimated by the incorporation rate of either [^{14}C]dGMP or [^{3}H]dTMP was the same. Furthermore, inhibition was unaffected by varying the incubation times of the antibiotics (0, 5, or 10 min at 37 °C) before addition of enzyme to initiate synthesis. In contrast to the behavior of DNA, increase of the enzyme concentration did not relieve the inhibition. Addition of bovine serum albumin (66–200 μg of albumin per 125 μL of reaction mixture) did not interfere with the inhibitory action of the antibiotics. The [^{3}H]dTMP incorporation as a function of incubation time was checked by addition of water as a control and of VM (3×10^{-5} M), at both 5- and 15-min incubation times. The incorporation rate was not affected in the two control experiments but was enormously slowed down by addition of the antibiotic VM (Figure 6).

Discussion

Apparent Changes of DNA Length by Anthracycline Intercalation. The dichroism rise time reflects the apparent length increase ΔL of DNA under the assumption that the DNA behaves as a stiff rod, a reasonably good approximation for DNA of ~150-bp length (Hogan et al., 1978). Intercalation according to the classical picture would result in a ΔL of approximately 0.34 nm per bound drug molecule. We find that the drug-induced length increase is the smaller the higher is the binding affinity to DNA. Thus ΔL for the strong binding drugs AM and VM is much less than 0.34 nm (Table I). At least three alternative explanations can be given for this fact: (i) the lower ionic strength may favor an outside-binding mechanism so that a substantial fraction of the bound drug may not be intercalated; (ii) compensating structural changes may shorten the double helix adjacent to the binding sites; (iii) the overall shape of DNA may be changed as a consequence of drug binding, particularly by bending or kinking. Reinert

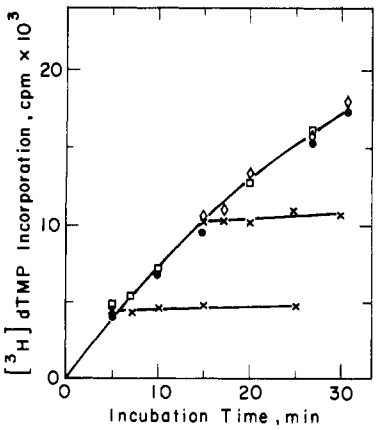

FIGURE 6: Dependence of DNA synthesis rate, monitored by [^{3}H]-dTMP incorporation (in cpm), on the incubation time. (×) 3×10^{-5} M VM was added after 5- and 15-min incubation times. (●, □, and ◊) An equivalent volume of water was added as a control after 0, 5, and 15 min, respectively.

(1983) has shown that ligands with two or more attachment sites produce bending of the DNA section involved in binding. Bending or any similar tertiary structural change reduces the DNA end-to-end separation and thus would lower the apparent length increase upon intercalation. At present, we favor the third explanation, that complexation of DNA with the four anthracyclines may produce bending of DNA around the binding site. If this hypothesis is correct, and if the length increment ΔL is effectively independent of the nature of the drug, then bending must increase in the same order as the binding strength, namely, IM < DM < AM < VM. This suggestion would at least partially remove apparent discrepancies between our appparent ΔL values and corresponding data found by viscometry (Reinert, 1983).

Geometry of the Intercalated Chromophore. In our earlier paper (Fritzsche et al., 1982a), we used the conclusion of Gabbay et al. (1976) that the long-wavelength and short-wavelength transition moments are directed respectively across the short and long axes of the drug. The experiments of Capps and Vala (1981) on DM show that this interpretation is incorrect: the long-wavelength transition is clearly polarized along the long axis of the drug molecule. They found in addition that the ~390-nm transition has a smaller positive fluorescence polarization than the ~490-nm transition, but the polarization is not sufficiently negative to be oriented along the short axis of the drug. Our results for DM bound to DNA also show that the 390-nm transition is not oriented parallel to the 490-nm transition, since the two have different angular orientations relative to the DNA helix axis. It seems likely that the 390-nm transition is off-axis, lying between the long and short axes of the drug chromophore, but the orientation cannot be specified more precisely at this time.

With this revised view of the transition moment orientation, the results in Table I show that all four drugs have similar tilt values of the long axis of the chromophore, as indicated by similar angular orientations for the ~490-nm transition. DM differs in the orientation of the ~390-nm transition, but it is also the only drug with an $-OCH_3$ residue at position 4 (Chart I). Hence the alteration of the short-wavelength transition could be due to a different structure of the drug–DNA complex, or it could be due to an influence of the $-OCH_3$ group on the transition moment direction within the chromophore. Further work will be required to decide this issue.

Binding of Anthracycline Antibiotics to Nucleosomes Is Weaker Than to DNA. The fluorescence titration data suggest a weaker binding of all four anthracycline antibiotics to nu-

cleosomes than to DNA over the whole investigated drug/base pair region (Figures 2–4). This is in contrast to the results found for ethidium binding (Wu et al., 1980): the binding curve of ethidium to nucleosomes crosses that of the ethidium–DNA complex, implying a stronger ethidium–nucleosome than ethidium–DNA interaction at low r values.

Binding of the anthracycline drugs was weakest to 145-bp core particles lacking any linker DNA, with all sections of the DNA in contact with the core histones. Accordingly, the data reflect directly the difference in the binding affinity of the drugs between free DNA and nucleosomal DNA. Obviously, the DNA–anthracycline interaction energy is reduced by the DNA–histone interaction in the nucleosomal particles. Nevertheless, the qualitative order of increasing binding affinity of the four investigated drugs is the same for DNA and nucleosomes. The stronger binding found for 175-bp nucleosomes presumably reflects the increased binding affinity of the linker DNA present in those particles. This conclusion is supported by the observation (Schlessinger, 1982) that VM binds more weakly to dinucleosomes when H1 is present than when it is absent.

Inhibition of DNA Synthesis by Anthracycline Antibiotics Parallels Their Binding Tendency to DNA as well as to Nucleosomes. Knowledge of the inhibitory effect of anthracycline antibiotics on DNA synthesis is of importance for an insight into the mechanism of action of these drugs. Our experiments clearly demonstrate an increasing inhibition of DNA synthesis by the four drugs in the order IM < DM < AM < VM (Figure 5 and Table II). The action of DNA polymerase is the more inhibited the stronger the corresponding drugs bind to DNA or to nucleosomes.

Preliminary results have shown (Förster & Stutter, 1984) that the association rate constants of DNA complexes with the three anthracyclines adriamycin, DM, and IM are almost constant but the corresponding dissociation rate constants differ significantly in the ratio of 1:3:10. These results suggest a correlation between the lifetime of the anthracycline antibiotic–DNA complexes and the biological effectiveness of the drugs on the inhibition of DNA synthesis. Thus a simple competition for the binding sites between enzyme and the drug could explain our findings.

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Registry No. IM, 75634-51-4; DM, 20830-81-3; AM, 57576-44-0; VM, 51938-70-6; DNA polymerase, 9012-90-2.

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