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Viscometric and Fluorometric Studies of Deoxyribonucleic Acid Interactions of Several New Anthracyclines†

Jonathan A. Pachter,*,‡ Cheng-Hsiung Huang, Virgil H. DuVernay, Jr., Archie W. Prestayko,§ and Stanley T. Crooke§

ABSTRACT: Interactions involved in the binding of the anthracycline analogues adriamycin, carminomycin, pyrromycin, musettamycin, marcellomycin, and aclacinomycin to calf thymus DNA and covalently closed circular PM-2 DNA have been studied. Fluorescence quenching experiments revealed that denaturation of calf thymus DNA and increasing ionic strength each resulted in a marked decrease in the DNA binding affinities of all of the anthracyclines studied. These results suggest that intercalative and electrostatic interactions are both important in the DNA binding of these analogues. Viscometric studies indicated that under high ionic strength conditions which negated electrostatic effects, all of the anthracyclines induced an unwinding–rewinding process of the closed superhelical PM-2 DNA typically observed for DNA intercalators. Relative to the 26° unwinding angle of ethidium bromide, anthracyclines with a daunomycinone-like aglycon induced an unwinding angle of approximately 13°. This differed slightly from the unwinding angles of 10.3°–11.1° which were induced by anthracyclines with a pyrromycinone-like aglycon. Increases in the length of the glycosidic side chain did not elicit significant differences in PM-2 DNA unwinding ability, implying a lack of effect of glycosidic side chain length on the anthracycline intercalation process. The unwinding angles also showed little sensitivity to decreases in ionic strength, suggesting that the fraction of bound anthracycline molecules which are in a nonintercalated state is similar to the fraction of bound ethidium bromide molecules which are in a nonintercalated state.

The interaction of anthracycline antitumor antibiotics with DNA has been the subject of many studies using a wide variety of techniques including thermal denaturation (Kersten & Kersten, 1965; Zunino et al., 1972), X-ray diffraction (Pigram et al., 1972), spectrophotometric titration (Gabbay et al., 1976), fluorometric titration (Tsou & Yip, 1976; Zunino et al., 1977; DuVernay et al., 1979a), equilibrium dialysis (Zunino et al., 1972; Gabbay et al., 1976), and viscometry (Calendi et al., 1965; Kersten et al., 1966; DiMarco et al., 1971). This interaction is believed to inhibit DNA-dependent nucleic acid synthesis (Calendi et al., 1965; Zunino et al., 1972; Neidle, 1978; Crooke et al., 1978). It is now generally accepted that the biological activity of many anthracyclines may result, to a large degree, from their DNA interactions (Zunino et al., 1972).

At least two modes of interaction between anthracyclines and DNA have been distinguished (Zunino et al., 1972). Intercalation (Lerman, 1961), which consists of insertion of the hydrophobic chromophore between adjacent DNA base pairs, is thought to be the predominant mode of anthracycline–DNA interaction (Pigram et al., 1972) and has been most rigorously demonstrated (Kersten et al., 1966; Waring, 1970; Pigram et al., 1972; Gabbay et al., 1976). Electrostatic binding, which is thought to be a weaker mode of binding than intercalation, consists of an ionic interaction between the amino group on the anthracycline amino sugar and a phosphate group on the DNA backbone (Pigram et al., 1972; Zunino et al., 1972; Gabbay et al., 1976; Patel & Canuel, 1978).

Recently, carminomycin (CMM),[1] pyrromycin (PYM), musettamycin (MSM), marcellomycin (MCM), and aclacinomycin (ACM) have been isolated (Gause et al., 1974; Oki et al., 1975; Nettleton et al., 1977) and their molecular pharmacology has been investigated (Crooke et al., 1978; DuVernay et al., 1979a,b, 1980) in an attempt to develop less toxic anthracyclines for clinical use. Their structures, along with those of the prototypic anthracyclines adriamycin (ADM) and daunomycin (DNM), are shown in Figure 1. Previous studies in this laboratory have attempted to characterize the nature of anthracycline–DNA interactions. Structure–activity relationships regarding DNA-binding affinities and base composition preferences for DNA binding have been demonstrated (DuVernay et al., 1979a, 1980). The lack of DNA breakage activity of several of these analogues under moderate experimental conditions has also been reported (Mong et al., 1980). Using spectrofluorometric and viscometric techniques with linear DNA and covalently closed circular DNA, the present study investigates relationships between the structures of ADM, CMM, PYM, MSM, MCM, and ACM and the

† From the Bristol-Baylor Laboratory, Department of Pharmacology, Baylor College of Medicine, Houston, Texas 77030 (J.A.P., C.H.H., V.H.D., A.W.P., and S.T.C.), and Bristol Laboratories, Syracuse, New York 13201 (A.W.P.). *Received April 6, 1981.* This work was supported in part by a grant from Bristol Laboratories and by a grant (CA-10893-P12) from the National Cancer Institute.

‡ Predoctoral trainee supported by Baylor College of Medicine Institutional Funds. Present address: Program of Neuroscience, Cullen Eye Institute, Baylor College of Medicine, Houston, TX 77030.

§ Present address: Smith Kline & French Laboratories, Philadelphia, PA 19101, and Department of Pharmacology, Baylor College of Medicine, Houston, TX 77030.

[1] Abbreviations: ADM, adriamycin; DNM, daunomycin; CMM, carminomycin; PYM, pyrromycin; MSM, musettamycin; MCM, marcellomycin; ACM, aclacinomycin; EtBr, ethidium bromide; NaP_i, sodium phosphate buffer; ccc DNA, covalently closed circular DNA.

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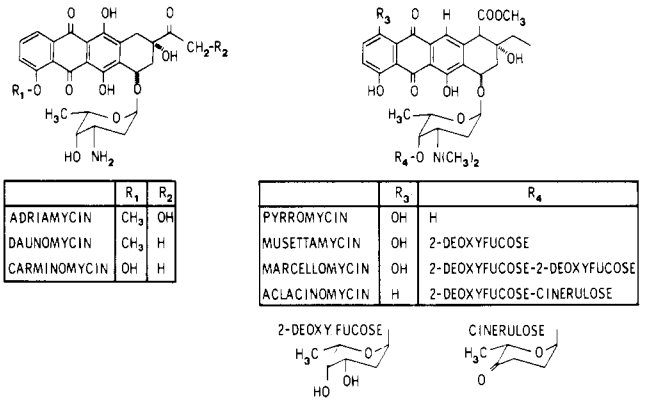

	R_1	R_2
ADRIAMYCIN	CH_3	OH
DAUNOMYCIN	CH_3	H
CARMINOMYCIN	OH	H

	R_3	R_4
PYRROMYCIN	OH	H
MUSETTAMYCIN	OH	2-DEOXYFUCOSE
MARCELLOMYCIN	OH	2-DEOXYFUCOSE-2-DEOXYFUCOSE
ACLACINOMYCIN	H	2-DEOXYFUCOSE-CINERULOSE

FIGURE 1: Structures of anthracycline analogues.

relative contributions of intercalative and electrostatic binding to their DNA interactions.

Materials and Methods

Chemicals. Covalently closed circular PM-2 bacteriophage DNA (ccc PM-2) was isolated as previously described (Salditt et al., 1972; Strong & Hewitt, 1975). All preparations employed in this study were shown to contain greater than 85% ccc DNA by agarose gel electrophoresis. Calf thymus DNA and ethidium bromide (EtBr) were obtained from Sigma Chemical Co., St. Louis, MO, and the DNA was sheared to approximately 10^6 daltons by sonication. DNA denaturation was accomplished by heating the DNA in a boiling water bath for 20 min, quick cooling to 0 °C, and subsequently adding distilled water to replace the evaporated weight. The anthracycline antibiotics, ADM, CMM, PYM, MSM, MCM and ACM, were all supplied by Bristol Laboratories, Syracuse, NY. Working drug solutions were prepared by wetting the drug crystals with dimethyl sulfoxide and 500 mM sodium acetate, pH 4.5, followed by dilution in the appropriate buffer. The concentrations of EtBr, anthracycline, and DNA solutions were determined on a Zeiss PMQ3 spectrophotometer. The molar extinction coefficients were determined in methanol for the anthracyclines and in water for EtBr. The values employed in this study were 14400 M^{-1} cm^{-1} at 477 nm for ADM, 14250 M^{-1} cm^{-1} at 492 nm for CMM, 13200 M^{-1} cm^{-1} at 490 nm for PYM, 15600 M^{-1} cm^{-1} at 490 nm for MSM, 14400 M^{-1} cm^{-1} at 490 nm for MCM, 13400 M^{-1} cm^{-1} at 434 nm for ACM, and 4600 M^{-1} cm^{-1} at 460 nm for EtBr. A molar extinction coefficient of 6600 M^{-1} cm^{-1} was used for both calf thymus and PM-2 DNAs in aqueous solutions.

Fluorescence Measurements. Fluorescence assays were performed in a 1-cm quartz cuvette with an Aminco-Bowman 4-8106 spectrofluorometer. The procedure was as previously described (DuVernay et al., 1979a) with the following modifications. Each binding determination consisted of a minimum of seven different DNA concentrations in duplicate. These determinations were repeated at least once. Each cuvette contained 2 μM drug, buffer, and DNA such that the DNA/drug concentration ratio varied from 0 to 100. The DNA/drug ratio of 100 was taken as the end point in this titration assay with the drug considered to be maximally bound, based on the observation that the ratio of bound drug/input drug reached a plateau at this point.

To vary ionic strength, we used three different buffers. The low-salt buffer contained 5.0 mM NaP_i, pH 6.2, and 1.0 mM EDTA. The medium-salt buffer contained 50 mM NaCl, 50 mM NaP_i, pH 6.2, and 1.0 mM EDTA. The high-salt buffer contained 200 mM NaCl, 50 mM NaP_i, pH 6.2, and 1.0 mM EDTA. Experiments with native and heat-denatured calf thymus DNA were performed in the medium-salt buffer at room temperature.

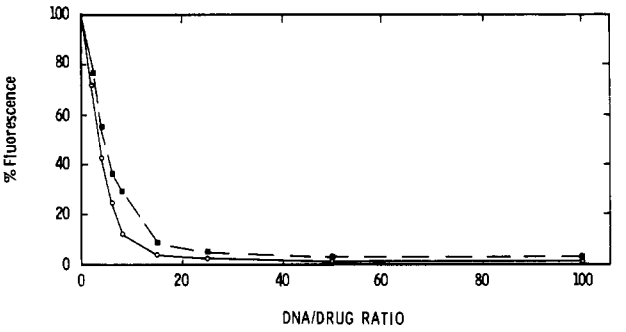

FIGURE 2: Quenching of fluorescence of MCM by increasing concentrations of native (○) and heat-denatured (■) calf thymus DNA. Increasing concentrations of DNA were titrated against 2 μM MCM in a buffer containing 50 mM NaCl, 50 mM NaP_i, pH 6.2, and 1.0 mM EDTA. The reaction mixture was incubated for 1 h at room temperature, and fluorescence measurements were taken by using the excitation and emission wavelengths described previously (DuVernay et al., 1979a).

The binding data were analyzed by the Scatchard (1949) method. The Scatchard variables r_b (moles of ligand bound per nucleotide) and C (the molar concentration of free ligand) were calculated from the fluorescence data according to the method of Peacocke & Skerrett (1956). Binding parameters were determined from plots of r_b vs. r_b/C, in which K_{app} (apparent association constant) is the negative slope and n_{app} (the apparent number of binding sites per nucleotide) is the x intercept.

Viscometric Studies. Viscometric titrations were performed with a type 75 Cannon-Ubbelohde semi-micro dilution viscometer. A Cannon constant-temperature bath (Cannon Instrument Co., College Station, PA) was used in combination with a recirculating water cooler (Savant Instruments Inc., Hicksville, NY) to maintain a temperature of 25 °C. A Model 221 Wescan automatic viscosity timer, which was calibrated with a Model 229-600 viscosity test set (Wescan Instruments Inc., Santa Clara, CA), was used to measure the flow time. Drug stock solutions for viscometry were prepared in 50% ethanol to prevent immediate precipitation of DNA–drug complexes upon drug addition. Appropriate control experiments were performed to show that the effect of the ethanol on the viscosity of DNA was negligible (data not shown). Microliter volumes of drug stock solutions were sequentially added with glass micropipets to an initial 1.0 mL of buffer solution containing 0.5 mM ccc PM-2 DNA. Mixing was accomplished by bubbling air through the solution. The final volume was always less than 1.05 mL. Flow times were measured in triplicate with standard deviations less than 0.5%.

Results

Anthracycline Binding to Native and Heat-Denatured DNA. Interactions of anthracyclines with native and heat-denatured calf thymus DNA were compared to assess the degree to which drug binding was dependent on the double strandedness of the DNA. Figure 2 illustrates quenching of the fluorescence of MCM by increasing concentrations of native and heat-denatured calf thymus DNA. Greater than 98% of the fluorescence was quenched by both DNA types at a DNA/drug concentration ratio of 50. Similar extents of quenching were observed with ADM, CMM, PYM, MSM and ACM (data not shown). Scatchard plots, such as those shown in Figure 3 for MCM, allowed resolution and quantitation of differences in the fluorescence quenching abilities of native and heat-denatured DNAs. The parameters which were derived from these

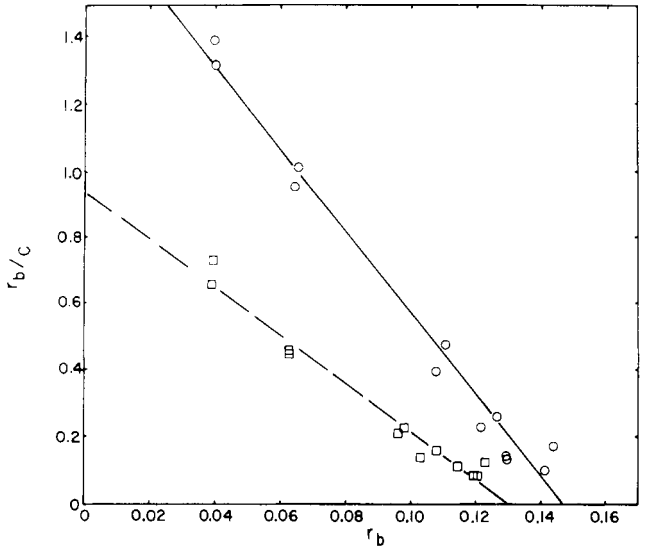

FIGURE 3: Scatchard analysis of the quenching effects of native (○) and heat-denatured (□) calf thymus DNA on the fluorescence of MCM. The parameters r_b (moles of ligand bound per nucleotide), C (moles per liter of free ligand), and r_b/C were calculated by the method of Scatchard (1949).

Table I: Fluorescence Quenching Results for Native and Heat-Denatured Calf Thymus DNA[a]

drug	native DNA K_{app} ($\times 10^6$ M^{-1})[b]	native DNA n_{app}[c]	heat-denatured DNA K_{app} ($\times 10^6$ M^{-1})	heat-denatured DNA n_{app}	heated K_{app}/native K_{app}
adriamycin	11.8 ± 1.8[d]	0.12	6.9 ± 0.9	0.12	0.58
carminomycin	5.3 ± 1.1	0.12	2.2 ± 0.4	0.19	0.42
pyrromycin	5.1 ± 0.6	0.13	1.8 ± 0.3	0.16	0.35
musettamycin	7.4 ± 1.2	0.13	4.6 ± 0.7	0.14	0.62
marcellomycin	12.3 ± 0.6	0.15	7.3 ± 0.4	0.13	0.59
aclacinomycin	3.4 ± 0.3	0.16	1.2 ± 0.3	0.20	0.35

[a] Ten or more points were used to construct composite Scatchard curves. p values, as determined from tables of significance limits for correlation coefficients (Diem & Lentner, 1970; DuVernay et al., 1979a), were all less than 0.001. [b] K_{app} values, apparent association constants, were obtained by linear regression analyses of composite Scatchard curves obtained from two or more separate experiments, each of which contained duplicate values at each DNA concentration. [c] n_{app}, the apparent number of binding sites per nucleotide, was also determined by linear regression. [d] Standard deviations of slopes (K_{app}) were calculated from linear regression analyses (Diem & Lentner, 1970; DuVernay et al., 1979a).

Scatchard plots are shown in Table I. The K_{app} value of 11.8 $\times 10^6$ M^{-1} and the n_{app} value of 0.12, which were obtained for the interaction of ADM with native calf thymus DNA, are in approximate agreement with previously published values (Zunino et al., 1972; Gabbay et al., 1976; Tsou & Yip, 1976; DiMarco et al., 1977; DuVernay et al., 1979a). Although only slight differences were observed in the n_{app} values upon DNA denaturation, decreases in the K_{app} values were consistently apparent. The ratios of heat-denatured to native K_{app}'s demonstrate that the binding affinities of all of the anthracyclines studied for heat-denatured DNA were only 35–62% of the corresponding binding affinities for native DNA.

Effects of Ionic Strength on DNA Binding. To define the contribution of electrostatic interactions to the overall binding of anthracyclines to DNA, we measured quenching of fluorescence of anthracyclines by DNA in buffers of differing ionic strength. Superhelical PM-2 DNA was used in this study to facilitate the interpretation of viscometric studies which will be discussed subsequently. A comparison of binding parameters as affected by three different salt conditions is presented in Table II. A significant increase in affinity for DNA was observed for ADM, CMM, and MCM as the ionic strength of the reaction buffers was decreased. The n_{app} values for the three drugs were also higher in low ionic strength buffer. It may also be noted that the binding parameters for ADM, CMM, and MCM with ccc PM-2 DNA in medium ionic strength conditions are similar to those obtained with native calf thymus DNA under the same conditions (see Table I).

Table II: Fluorescence Quenching Results with ccc PM-2 DNA in Buffers of Various Ionic Strengths[a]

drug	high salt[b] K_{app} ($\times 10^6$ M^{-1})	high salt[b] n_{app}	medium salt[c] K_{app} ($\times 10^6$ M^{-1})	medium salt[c] n_{app}	low salt[d] K_{app} ($\times 10^6$ M^{-1})	low salt[d] n_{app}
adriamycin	7.43 ± 0.88	0.11	13.23 ± 1.79	0.11	57.7 ± 7.1	0.18
carminomycin	3.26 ± 0.53	0.13	6.95 ± 0.41	0.12	29.8 ± 4.0	0.21
marcellomycin	5.06 ± 1.00	0.18	14.38 ± 0.52	0.14	64.4 ± 5.2	0.19

[a] All p values were less than 0.001. K_{app} is the apparent association constant. n_{app} is the apparent number of binding sites per nucleotide. See Table I footnotes for details. [b] High salt: 200 mM NaCl, 50 mM NaP_i, pH 6.2, and 1.0 mM EDTA at 25 °C. [c] Medium salt: 50 mM NaCl, 50 mM NaP_i, pH 6.2, and 1.0 mM EDTA at 25 °C. [d] Low salt: 5.0 mM NaP_i, pH 6.2, and 1.0 mM EDTA at 25 °C.

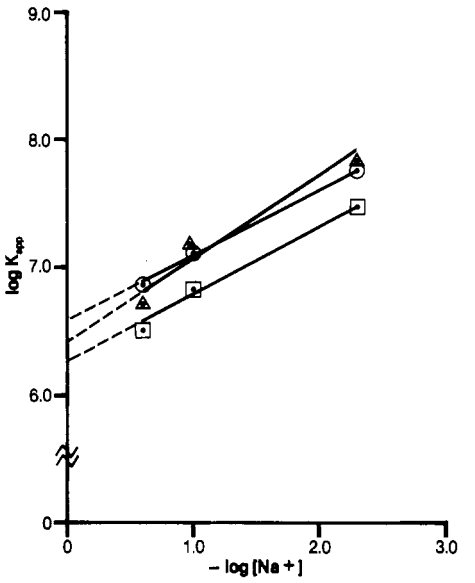

FIGURE 4: Dependence of the apparent binding constant (K_{app}) for the interactions of ADM (○), CMM (□), and MCM (△) with PM-2 DNA on the ionic strength ([Na^+]) of the buffer. (---) Extrapolated portion of the curves.

For determination of the number of ion pairs which are formed between a given anthracycline and PM-2 DNA (m'), the following equation was used in which [M^+] is the monovalent cation concentration and ψ is the fraction of a counterion which is thermodynamically bound per phosphate (Record et al., 1976):

$$-\frac{\partial \log K_{app}}{\partial \log [M^+]} = m'\psi$$

Plots of log K_{app} vs. -log [Na^+] for the interactions of ADM, CMM and MCM with ccc PM-2 DNA are shown in Figure 4. By use of the above equation with [M^+] equal to [Na^+], values for m' were determined. A value of $\psi = 0.82$ was used to represent the fraction of a counterion which is thermody-

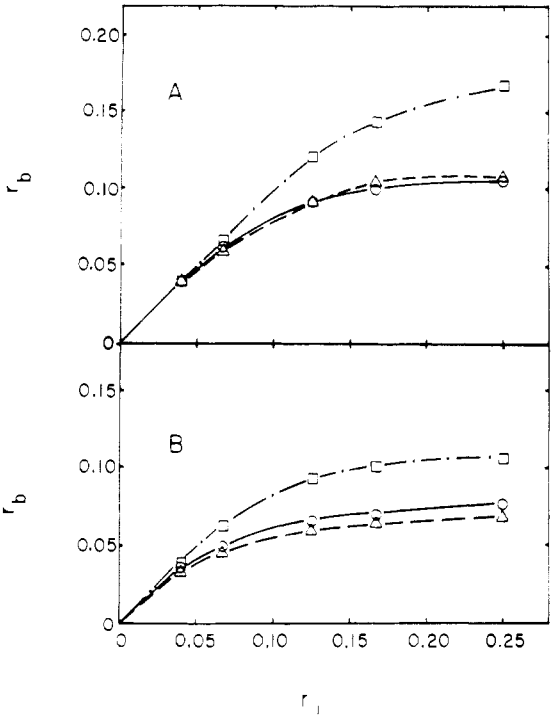

FIGURE 5: Relationship of the input drug/DNA ratio (r_i) to the bound drug/DNA ratio (r_b) as calculated from fluorescence quenching by ccc PM-2 DNA. Results are shown with high salt [(△) 200 mM NaCl, 50 mM NaP_i, pH 6.2, and 1.0 mM EDTA], medium salt [(○) 50 mM NaCl, 50 mM NaP_i, pH 6.2, and 1.0 mM EDTA], and low salt [(□) 5.0 mM NaP_i, pH 6.2, and 1.0 mM EDTA] for CMM (A) and EtBr (B).

namically bound per phosphate in a native DNA–intercalator complex (Wilson & Lopp, 1979). The values of m' for ADM, CMM, and MCM were 0.71, 0.61, and 0.65, respectively. The intercept of a linear extrapolation to a 1 M Na^+ standard state was taken to indicate the nonelectrostatic component of the binding free energy (Record et al., 1976). The nonelectrostatic binding components for ADM, CMM, and MCM were 3.8 × 10^6 M^{-1}, 1.9 × 10^6 M^{-1}, and 2.8 × 10^6 M^{-1}, respectively.

Figure 5 relates the ratio of input drug/DNA (r_i) to the ratio of bound drug/DNA (r_b) as derived from Scatchard calculations. In this type of plot (Huang et al., 1980), a 45° line would indicate that all of the drug that was present was bound to DNA. The curves obtained for CMM in low, medium, and high ionic strength buffers (Figure 5A) show that while CMM was totally bound at r_i ratios up to 0.12 under low ionic strength conditions, unbound CMM began to appear at r_i ratios as low as 0.05 under the higher ionic strength conditions. Similar curves were obtained with ADM and MCM (data not shown). Figure 5B shows that, like the anthracyclines, a larger proportion of EtBr molecules was bound to DNA in the lower ionic strength conditions.

Viscometric Measurements. Viscometric titrations were done to further investigate the intercalative interaction of the anthracyclines with DNA. Figure 6 illustrates the changes in the viscosity of ccc PM-2 DNA (measured as flow time in seconds) which resulted from the addition of increasing amounts of EtBr (Figure 6A), ADM (Figure 6B), and MCM (Figure 6C) in a medium ionic strength buffer solution. The initial increase in viscosity is an indication of removal of the negative superhelicity of the ccc DNA by drug intercalation (Waring, 1970; Revet et al., 1971; Festy & Duane, 1973; LePecq et al., 1975; Jones et al., 1979). The peak in viscosity represents the point at which the negative superhelicity was completely removed, yielding relaxed (form I^0) DNA. This was achieved at an input EtBr/DNA ratio of 0.062, an input

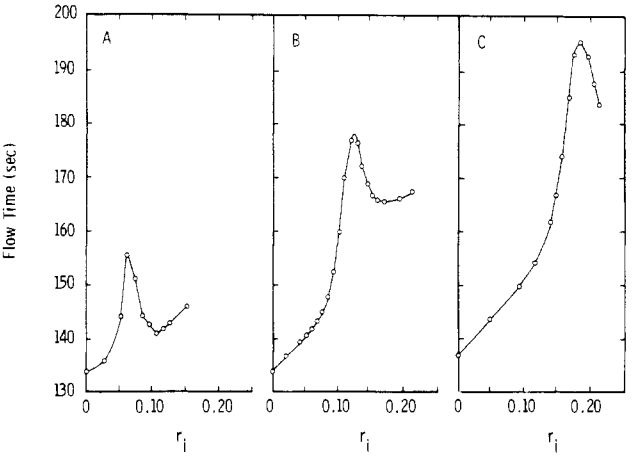

FIGURE 6: Viscometric titrations of the effects of EtBr (A), ADM (B), and MCM (C) on ccc PM-2 DNA in 50 mM NaCl, 50 mM NaP_i, pH 6.2, and 1.0 mM EDTA at 25 °C. Viscosity expressed by flow time was plotted with increasing input drug/DNA ratios (r_i) for each drug. The concentration of the DNA was 0.5 mM.

ADM/DNA ratio of 0.122, and an input MCM/DNA ratio of 0.186. The subsequent decrease in viscosity corresponds to a rewinding process to form positively supercoiled turns in the DNA. The failure of the viscosity to return to a level close to that of the untreated DNA, when titrations were carried out to high r_i values, was typical of all of the anthracyclines in this study (data not shown). This partial return to base line has been observed with a number of intercalative drugs, including DNM and BBM-928A (Saucier et al., 1971; Huang et al., 1980). Viscometric titrations which were performed under other ionic strength conditions yielded curves which differed quantitatively from those shown in Figure 6, but were qualitatively similar (data not shown).

Unwinding Angles. The ratio of input drug to DNA which was sufficient to remove the negative superhelicity of the DNA (r_i') may be used to calculate an unwinding angle. An unwinding angle is a measure of the average degree of uncoiling of the DNA helix which results from the binding of one drug molecule. To derive an unwinding angle, r_i' must first be converted into the *bound* drug to DNA ratio at the viscosity peak (r_b'). Plots of r_i vs. r_b (see Figure 5), which were obtained from fluorescence quenching studies, were used for this conversion. From the medium ionic strength curve in Figure 5B, for example, it was found that the r_i' of 0.062 for EtBr corresponds to an r_b' value of 0.047.

Since all intercalating drugs must remove the same number of superhelical turns to yield completely relaxed PM-2 DNA, the following equation may be used to calculate the unwinding angle (ϕ) for a given anthracycline based on an ϕ of 26° for EtBr (Wang, 1974; Liu & Wang, 1975; Pulleybank & Morgan, 1975; Baase & Johnson, 1979).

$$(\phi r_b')_{\text{anthracycline}} = (\phi r_b')_{\text{EtBr}}$$

The unwinding angles which were determined for ADM, CMM, PYM, MSM, MCM, and ACM in buffers of three different ionic strengths are shown in Table III. Under all conditions studied, the anthracyclines exhibited unwinding angles that were approximately half that of EtBr. This is consistent with previous findings reported for ADM and DNM (Waring, 1970, 1975; Saucier et al., 1971).

A comparison among anthracyclines within high ionic strength conditions shows that the anthracyclines may be divided into groups based on their unwinding angles. ADM and CMM induced the greatest amount of uncoiling with unwinding angles of 13.1°, while PYM, MSM, and MCM

Table III: Unwinding Angles with ccc PM-2 DNA in Buffers of Various Ionic Strengths

drug	high salt[a] ϕ[d] ± SD	medium salt[b] ϕ ± SD	low salt[c] ϕ ± SD
ethidium bromide	(26°)[e]	(26°)	(26°)
adriamycin	13.1° ± 0.4	13.0° ± 0.7	12.4° ± 0.6
carminomycin	13.1° ± 0.3	12.9° ± 0.3	12.0° ± 0.5
pyrromycin	10.5° ± 0.8	ND[f]	ND
musettamycin	10.3° ± 0.3	ND	ND
marcellomycin	10.3° ± 0.7	10.0° ± 0.1	11.6° ± 0.4
aclacinomycin	11.1° ± 0.2	ND	ND

[a] High salt: 200 mM NaCl, 50 mM NaP_i, pH 6.2, and 1.0 mM EDTA at 25 °C. [b] Medium salt: 50 mM NaCl, 50 mM NaP_i, pH 6.2, and 1.0 mM EDTA at 25 °C. [c] Low salt: 5.0 mM NaP_i, pH 6.2, and 1.0 mM EDTA at 25 °C. [d] Each anthracycline unwinding angle represents the average of four determinations derived from two viscometric titrations and two r_i vs. r_b curves. [e] Wang et al. (1974). [f] Not determined.

exhibited unwinding angles between 10.3° and 10.5°. Two-sample t tests showed that the differences between the two groups were statistically significant ($p < 0.001$). Within the groups, the differences between the unwinding angles of ADM and CMM and between the unwinding angles of PYM, MSM, and MCM were not significant. The unwinding angle of ACM (11.1°) differs significantly from those of ADM and CMM ($p < 0.001$) and differs slightly, but significantly, from those of PYM, MSM, and MCM ($p < 0.05$).

Effect of Ionic Strength on Unwinding Angles. Relative to the unwinding angle of EtBr (26°), the unwinding angle of ADM did not change significantly with decreasing ionic strength. The unwinding angle of CMM decreased slightly from 13.1° to 12.0° ($p < 0.01$) with decreasing ionic strength, while the unwinding angle of MCM increased slightly from 10.3° to 11.6° ($p < 0.05$) with decreasing ionic strength.

Discussion

Intercalative interactions with DNA have been previously demonstrated for the prototypic anthracyclines, DNM and ADM (Kersten et al., 1966; Pigram et al., 1972; Zunino et al., 1977). In this study, ADM and five newer anthracycline analogues, CMM, PYM, MSM, MCM, and ACM, also showed intercalative interactions with DNA as indicated by their abilities to unwind and rewind ccc PM-2 DNA in a manner characteristic of intercalators. The stronger binding affinity of the anthracyclines for native calf thymus DNA, as compared with heat-denatured DNA, suggests a dependence on the double strandedness of the DNA for binding and, thus, is also consistent with the importance of intercalation in the DNA-binding interactions of these anthracyclines.

The increased binding affinities with decreasing ionic strength, which were demonstrated by fluorescence quenching studies with ADM, CMM, and MCM (Table II), are in agreement with previous findings with ADM (Zunino et al., 1977). The number of binding sites of the three anthracyclines per nucleotide of DNA may also have increased slightly with decreasing ionic strength. In addition, larger numbers of molecules of ADM, CMM, and MCM were bound to DNA in lower ionic strength medium. The large magnitude of the increases in the K_{app}'s, in addition to the increases in these other parameters with decreasing ionic strength, attests to the importance of electrostatic forces in the interactions of these anthracyclines with DNA. Calculations of values for m' show that approximately 0.6–0.7 ion pair appears to be involved in complexes of ADM, CMM, and MCM with ccc PM-2 DNA. The number of ion pairs involved in these interactions is probably more nearly one. One reason for the m' values being less than 1 may be that the value for ψ which was used was derived for linear DNA rather than closed circular DNA. However, our calculations from the report of Zunino et al. (1980) on the interaction of ADM with calf thymus DNA also show that approximately 0.7 ion pair is involved in a complex of ADM with calf thymus DNA.

Studies in our laboratory on several anthracyclines have demonstrated that when Scatchard analyses are performed over a broad concentration range, the curves are nonlinear (Bloomfield et al., 1974). However, like other laboratories studying anthracyclines, we have compared the characteristics of anthracyclines by using the linear portion of the curves. In the experiments presented on superhelical DNA, we studied a narrower concentration range, i.e., the linear portion of the Scatchard analyses. We have chosen to do this for several reasons. First, within the concentrations studied, the DNA is unwound and rewound. The use of higher concentrations is less relevant because the conformational changes of superhelical DNA induced by anthracyclines are induced by lower concentrations, and the conformation of the DNA at these higher concentrations is unknown. Moreover, high concentrations of anthracyclines may induce breakage of superhelical DNA, complicating analyses (Mong et al., 1980). Such an approach results in K_{app} and n_{app} values slightly lower than if the broader concentration range is employed. However, we believe that the values presented are the most relevant because they represent concentrations inducing conformational changes in ccc PM-2 DNA and they can be compared to the published results for other anthracyclines (Zunino et al., 1972; DuVernay et al., 1979a).

Unwinding angles in buffers of varying ionic strengths have been studied previously to investigate the amount of nonintercalative binding which contributes to the overall binding of several acridines and quinoline derivatives to DNA (Jones et al., 1980). Although there has been considerable interest in defining the nature of anthracycline–DNA interactions (Waring, 1970; Zunino et al., 1972; Gabbay et al., 1976; DuVernay et al., 1979a), studies of the unwinding angles of anthracyclines in buffers of varying ionic strengths have not been reported.

Jones et al. (1980) have shown that if the unwinding angle of an intercalator, relative to that of EtBr, is independent of ionic strength, then nonintercalative interactions contribute to the DNA binding of that intercalator and EtBr to similar extents.

In this study, the unwinding angle of ADM did not change significantly with decreasing ionic strength, and the unwinding angles of CMM and MCM showed changes of very small magnitude (13.1°–12.0° and 10.3°–11.6°, respectively) with decreasing ionic strength, relative to the unwinding angle of EtBr (26°). Thus, nonintercalative interactions contribute to the DNA binding of ADM, CMM, MCM, and EtBr to similar extents. ADM, CMM, and MCM were selected for this study, because they are representative of three different mechanistic classes of anthracyclines (DuVernay et al., 1981).

In earlier studies, Waring (1970) proposed that the lower unwinding angles of the anthracyclines and a number of other intercalacators, as compared with the unwinding angle of EtBr, might be the result of a greater amount of competitive nonintercalative binding. Since the present study has shown that nonintercalative binding contributes to the DNA binding of several anthracyclines and EtBr to similar extents, it is more likely that the lower unwinding angles of the anthracyclines result from some other mechanism, such as a difference in the

drug–DNA intercalation complex. Among other possibilities, the anthracyclines may achieve only partial insertion between base pairs upon intercalation into DNA, or the mechanism of intercalation of anthracyclines may differ fundamentally from the mechanism of intercalation of EtBr. Structural differences in the intercalation complex have also been proposed to explain the low unwinding angles of quinoline derivatives relative to that of EtBr (Jones et al., 1980).

The unwinding angles which were exhibited by the anthracyclines at high ionic strength seem to show some correlation with structures, suggesting that differences in the structures of the anthracyclines may dictate differences in the extent of distortion of the DNA helix which results from intercalation. ADM and CMM, which differ only in two positions on the aglycon and have daunosamine as their amino sugar, showed higher unwinding angles (13.1°) at high ionic strength than PYM (10.5°), MSM (10.3°), and MCM (10.3°), which bear the markedly different pyrromycinone aglycon and a dimethylamine on their amino sugar. From the present study, it is not possible to tell whether the difference in the unwinding angles of these two structural groups results from differences in the aglycon, differences in the amino sugar, or a combination. It has been reported previously that substitutions on the amino sugar strongly affect the DNA-binding ability of many anthracyclines (Zunino et al., 1972; Gabbay et al., 1976).

The lack of change in the unwinding angle of PYM with additional 2-deoxyfucose residues on the glycosidic side chain (MSM and MCM) at high ionic strength demonstrates that the presence of second and third sugars on the anthracycline side chain does not affect the drug's ability to distort DNA upon intercalation. Thus, it is likely that these sugars do not sterically hinder the intercalation of anthracyclines into DNA duplexes.

The small, but significant, difference between the unwinding angle of ACM at high ionic strength (11.1°) and those of PYM, MSM, and MCM (10.3°–10.5°) could result from either one or both of the structural differences between ACM and MCM. It is difficult to ascertain whether the slightly greater unwinding capacity of ACM results from the lack of a hydroxyl moiety at position 1 of the aglycon or from the presence of cinerulose, rather than 2-deoxyfucose, as the terminal sugar.

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Lipid Hydrolyses Catalyzed by Pancreatic Cholesterol Esterase. Regulation by Substrate and Product Phase Distribution and Packing Density†

Santhoor G. Bhat and Howard L. Brockman*

ABSTRACT: The role of oleic acid in the regulation of the hydrolysis of cholesteryl oleate in lipid films at the air–buffer interface was investigated by using initial rate techniques. A small quantity of enzyme is rapidly adsorbed to substrate-containing films; however, a much greater, although slower, adsorption occurs if oleic acid is present. The rate constant for the slow adsorption is independent of the phase distribution of cholesteryl oleate but is markedly dependent upon both the concentration of oleic acid head groups and the acyl chain packing density in the film. Adsorption is controlled by two ionizable groups, one of which may be the carboxyl group of oleic acid. In contrast to adsorption, catalysis by the surface excess of enzyme is pH independent between 5.5 and 7.5 and is relatively specific for substrate in the monolayer phase. The second-order rate constants for the hydrolysis of cholesteryl oleate in the monolayer phase and the interfacial layer of the double-layer phase are 27 and 2 $cm^2\ s^{-1}\ fmol^{-1}$. These results indicate that adsorption and catalysis occur at functionally, if not physically, distinct sites on the protein. The adsorption of enzyme to a hydrolysis product, oleic acid, constitutes a form of product activation which presumably helps keep it at the interface during intraluminal fat digestion. The catalytic properties of the adsorbed enzyme suggest that substrate specificities determined for cholesterol esterase in complex reaction systems may largely reflect the availability of substrate in the appropriate physical state at the lipid–water interface.

Cholesteryl esters in tissues and serum reside primarily in bulk lipid deposits such as arterial lipid droplets [e.g., see Smith et al. (1967)] and lipoproteins [e.g., see Keim (1979)]. Based on physical studies in model systems (Janiak et al., 1974; Smaby & Brockman, 1981b), it is likely that a small fraction of cholesteryl esters are present in one of two surface phases at the lipid–water interface (Smaby & Brockman, 1981a). The hydrolysis of these esters is a prerequisite for cholesterol utilization in tissues [e.g., see Kritchevsky (1972)] as well as for its absorption in the intestine (Treadwell & Vahouny, 1968). Little is known, however, about the regulation of this reaction, particularly with regard to the interfacial structure.

In a recent study of the properties of the hydrolysis reaction, equilibrium could be achieved over only a relatively narrow range of pH (Bhat & Brockman, 1981). This kinetic deficiency reflects the properties of the water-soluble catalyst employed, porcine pancreatic cholesterol esterase (EC 3.1.1.13). However, it does not reveal if in this two-phase reaction system the lack of catalysis results from a separation of enzyme and substrate or catalytic inactivity of the protein. At the optimum pH, adsorption of enzyme to films consisting of products and reactants does occur and was shown to be a time-dependent, saturable process. In contrast to these results, the human pancreatic cholesterol esterase in the absence of bile salts exhibited negligible activity toward cholesteryl esters and other substrates in films at the air–water interface (Lombardo et al., 1980).

Aside from species differences, the data suggested that this apparent discrepancy in results may be related to the presence of oleic acid at the interface. For porcine pancreatic lipase–colipase acting on phospholipid–triglyceride emulsions, oleic acid decreases the acceleration period for catalysis (Borgström, 1980). Mechanistic studies have shown that this involves the binding of fatty acid to colipase, enhancing its affinity for lipase 100-fold (Larsson & Erlanson-Albertsson, 1981). The potential regulatory significance of these product activation effects prompted us to investigate the role of oleic acid in cholesterol esterase adsorption to interfaces and the subsequent catalytic reaction. Their relationships to lipid packing density and interfacial composition were of particular interest because these parameters determine the amount and phase distribution of cholesteryl esters at the lipid–water interface (Smaby & Brockman, 1981a,b). For simplification of the interpretation of the results, all experiments were, unless noted, confined to the monolayer and monolayer–double-layer coexistence regions of the cholesteryl oleate–oleic acid phase diagrams where no bulk lipid phases are present. The results of these studies using initial rate techniques applied to surface films indicate that adsorption and catalysis are independently regulated and that the rate constants for these processes may be useful as probes

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