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Studies on Interaction of Anthracycline Antibiotics and Deoxyribonucleic Acid: Geometry of Intercalation of Iremycin and Daunomycin†

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ABSTRACT: The structure of iremycin [10-(α-L-rhodosaminyl)-γ-rhodomycinone] hydrochloride has been confirmed by ^1H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy. We studied the interaction of iremycin and the related compound daunomycin with DNA by transient electric dichroism and by sedimentation analysis of supercoiled closed duplex DNA. The apparent length increase of sonicated calf thymus DNA (150 ± 20 base pairs) in 2.5 mM sodium cacodylate buffer (pH 7) at 12 °C was determined to be 0.40 ± 0.02 nm/bound iremycin, which is significantly higher than the apparent length increase induced by daunomycin (0.31 ± 0.02 nm/bound drug). The Cu(II) complex of iremycin with a metal/drug ratio of 0.7 induces a length increase of DNA of 0.44 ± 0.02 nm/added drug. The alignment of the iremycin chromophore with respect to the DNA helix axis was determined from the electric dichroism of the complex. The tilt (long axis) and twist (short axis) of the chromophore are both 28 ± 4°, whereas for daunomycin the long axis is perpendicular to the helix axis and the short axis is twisted by about 25°. Intercalation of iremycin between DNA base pairs is supported by unwinding of the supercoiled closed duplex form of pBR 322 plasmid DNA from *Escherichia coli*. In 2.5 mM sodium cacodylate buffer at pH 7 and at 25 °C, the unwinding induced by iremycin is 15.0 ± 1.5°/bound drug. Under identical conditions daunomycin shows on unwinding angle of 15.4 ± 1.5°. The superhelical density of pBR 322 DNA ($\bar{\sigma}_0$) was determined to be −0.087 ± 0.002 at standard conditions (0.2 M NaCl, 37 °C).

Recently, the anthracycline antibiotic iremycin (IM)[1] was obtained by selection and characterization of an interspecific recombinant phenotype obtained by hybridization experiments with mutants of various *Streptomyces* species blocked in antibiotic production (Schlegel & Fleck, 1980; Schlegel et al., 1980). The antibiotic IM possesses antimicrobial and cytostatic activity (Schlegel et al., 1979), and IM has been identified by Ihn et al. (1980) as 10-(α-L-rhodosaminyl)-γ-rhodomycinone (I) on the basis of ^1H and ^{13}C NMR, mass spectrometry, infrared and UV–vis spectroscopy, and circular dichroism. The structure of IM is closely related to the antitumor drugs daunomycin (DM, II) and adriamycin (AM,

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[1] Abbreviations: IM, iremycin; DM, daunomycin; NMR, nuclear magnetic resonance; EB, ethidium bromide.

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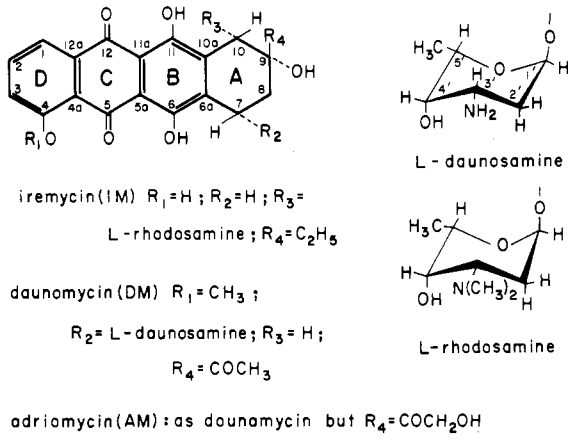
Chart I



III). Independently of Ihn et al. (1980), we investigated the hydrochloride of IM by ^1H and ^{13}C NMR spectroscopy. The results reported here fully support their structure (I) of IM (see Chart I).

It is generally accepted that the planar chromophores of anthracycline antibiotics are intercalated between DNA base pairs (Neidle, 1979). Recently, an X-ray diffraction study of an oligodeoxynucleotide–daunomycin intercalation complex has been reported (Quigley et al., 1980).

In this paper we report comparative studies of the intercalation of IM and DM by three independent methods: (i) the elongation of linear duplex DNA as a consequence of drug intercalation, (ii) the unwinding action of intercalating drugs on superhelical closed circular DNA, measured by sedimentation analysis in an analytical ultracentrifuge, and (iii) electric dichroism studies of chromophore orientation. A companion paper explores the interaction of daunomycin with DNA in more detail (Chaires et al., 1981).

Materials and Methods

Drugs. Iremycin hydrochloride was isolated and purified as described elsewhere (Ihn et al., 1980). The purity was checked by thin-layer chromatography and NMR spectroscopy. Daunomycin hydrochloride was either a gift from Farmitalia (Italy) or purchased from Sigma Chemical Co. EB was from Serva, Heidelberg, Federal Republic of Germany; both samples were used without further purification.

DNA. Sonicated and fractionated calf thymus DNA with an average length of 150 ± 20 base pairs was used in the electric dichroism experiments. The method of sonication and fractionation of DNA has been described elsewhere (Hogan et al., 1978). The spectrophotometric titrations were carried out with sonicated calf thymus DNA of approximately 750 base pair length. The sedimentation experiments were done with pBR 322 plasmid DNA from *Escherichia coli*, which has a chain length of 4362 base pairs (Sutcliffe, 1978). As judged by sedimentation analysis, the preparation contained two components, namely, the supercoiled closed duplex form (cdDNA) and the nicked relaxed circular form (ncDNA).

Sedimentation. The plasmid DNA was extensively dialyzed against 2.5 mM sodium cacodylate buffer (pH 7). For most experiments, fresh DNA–drug solutions were prepared by 1:1 dilution (by weight) of the DNA stock solution with appropriate aliquots of pure buffer and of drug in the same buffer, yielding a final DNA concentration of 4.5×10^{-5} M in base pairs. A few samples were rerun after addition of small increments of drug [method 2 of Waring (1970)]. Boundary sedimentation experiments were performed by running the An-F rotor with three double-sector cells at 28 000 rpm at temperatures close to 25 °C in a Beckman Model E Spinco analytical ultracentrifuge equipped with a monochromator, a photoelectric scanning system, and a multiplex attachment. Sedimentation coefficients obtained from the least-squares slopes of the log r vs. t plots were corrected for the temperature dependence of viscosity but not for buoyancy.

Spectrophotometric Titration. Fresh stock solutions of the drugs were prepared by dissolving the drug in 2.5 mM sodium cacodylate buffer (pH 7). The stock solution was added in 50-μL increments to a calf thymus DNA solution of $\sim 4.5 \times 10^{-5}$ M in base pairs in the same buffer. The cell (optical path length 2 cm) and the stirrer were hydrophobized to suppress drug adsorption on the glass surface.

The spectra were recorded on a Specord UV–vis spectrophotometer, Carl Zeiss, Jena, German Democratic Republic, coupled on-line to a process computer. The fraction of bound drug, C_B, was calculated from the measured absorbance A:

$$A/d = \epsilon_f(C_0 - C_B) + \epsilon_B C_B$$

where d is the optical path length, C_0 and C_B are the total and bound drug concentrations, respectively, and ϵ_f and ϵ_B are the molar extinction coefficients of the free and bound drug, respectively. The fraction of bound IM was calculated with $\epsilon_f = 13\,600$ M^{-1} cm^{-1} and $\epsilon_B = 6700$ M^{-1} cm^{-1} for IM at 495 nm (H. Schütz, E. Stutter, and A. Walter, unpublished results). Similarly, $\epsilon_f = 11\,500$ M^{-1} cm^{-1} and $\epsilon_B = 6429$ M^{-1} cm^{-1} for DM at 487 nm (Schütz et al., 1979) were used to calculate the fraction of bound DM.

NMR Experiments. A 30 mM solution of IM·HCl dissolved in a 1:2 acetone-d_6–D_2O mixture was used for the NMR spectra. A Bruker HX-270 spectrometer at Yale University (Department of Chemistry) was run at 270 MHz for the ^1H NMR spectra and at 67.9 MHz for the ^{13}C NMR spectra, ^1H broad-band noise-decoupled spectra, and "off-resonance" spectra. Further ^1H NMR spectra of a 30 mM solution of IM·HCl dissolved in dimethyl-d_6 sulfoxide were recorded at 100 MHz by a Jeol FXQ-100 spectrometer (The Johns Hopkins University, School of Hygiene and Public Health) and at 360 MHz by a Bruker WH-360 spectrometer (Mid-Atlantic NMR Facility located at the University of Pennsylvania, Philadelphia, PA). The HDO peak in the ^1H NMR spectra was suppressed by a special pulse sequence.

Transient Electric Dichroism. This method was used as described previously (Hogan et al., 1978; Dattagupta et al., 1978; Hogan et al., 1979a). Appropriate amounts of a stock solution of drug dissolved in 2.5 mM sodium cacodylate buffer (pH 7) were added to a solution of sonicated calf thymus DNA that was dialyzed against the same buffer. The DNA concentration varied between 1.27×10^{-4} and 3×10^{-4} M in base pairs. The experiments were carried out at 12 °C. The field strength was varied between 8.5 and 32 kV cm^{-1} to extrapolate the dichroism to infinite field.

Results

Structure of Iremycin. We studied both ^1H and ^{13}C NMR spectra of IM·HCl independently of the work done with the IM base by Ihn et al. (1980). The results (Tables I and II) fully support the structure suggested by Ihn et al. (1980). Some interesting features are briefly described. The protonation of the sugar dimethylamino group of IM·HCl is expected to affect the NMR lines of the neighboring nuclei with respect to the lines of the free base, i.e., H3′ and *N*-methyl groups in the ^1H spectra as well as C3′ and *N*-methyl carbons in the ^{13}C NMR spectra.

The only significant difference of the IM·HCl ^1H NMR spectrum (Table I) to the corresponding spectrum of the IM

Table I: 360-MHz ^{1}H NMR Spectra of Iremycin Hydrochloride[a]

δ	multiplicity, J (Hz)	intensity	assignment
1.04	t (7.5)	3	H14
1.23	d (6.3)	3	H6′
1.6–2.1	m	8	H13, H7, H8, H2′, H2″
2.72	s	6	N-Me$_2$
2.77	dd	1	H3′
3.87	m	1	H4′
3.96	q (6.3)	1	H5′
4.88	s	1	H10
5.35	m (width 7.5)	1	H1′
7.41	dd (6.3, 2.2)	1	H3
7.88	t (6.3)	1	H1
7.87	dd (6.3, 2.2)	1	H2

[a] 3×10^{-2} M IM·HCl dissolved in dimethyl-d_6 sulfoxide at 65 °C. Chemical shift values (δ) relative to tetramethylsilane.

Table II: 67.9-MHz ^{13}C NMR Spectrum of Iremycin Hydrochloride[a]

δ	multiplicity	assignment	δ	multiplicity	assignment
191.6	s	C5	72.0	s	C9
186.7	s	C12	70.5	d	C10
162.8	s	C4	68.0	d	C5′
158.7	s	C11	65.3	d	C4′
156.7	s	C6	63.0	d	C3′
142.6	s	C10a	42.0	d	N-Me$_2$
138.7	s	C6a	41.6	q	N-Me$_2$
137.6	d	C2	31.9		C13
134.3	s	C12a	28.1	t	C2′
125.5	d	C3	27.2	t	C7
120.4	d	C1	21.9	t	C8
116.6	s	C4a	17.3	q	C6′
111.6	s	C5a	7.4	q	C14
96.4	d	C1′			

[a] 3×10^{-2} M IM·HCl dissolved in an acetone-d_6/D_2O mixture, 1:2, at 25 °C. Chemical shift values (δ) relative to tetramethylsilane.

base dissolved in $CDCl_3$ [cf. the data reported by Ihn et al. (1980)] is the strong shift of the N-dimethyl signal (IM·HCl, 2.72 ppm; IM base, 2.12 ppm). This downfield shift is a consequence of the protonation of the nitrogen of the N-dimethyl group of IM·HCl.

On the other hand, the ^{13}C NMR spectra of IM base ($CDCl_3$ solution) and IM·HCl (acetone-d_6–D_2O, 1:2) differ only in one signal by more than 1.7 ppm. The C3′ lines are found at 59.7 ppm for the IM base and at 63.0 ppm for IM·HCl. C3′ is the carbon primarily affected by the protonation of the N-dimethyl group. Rather surprisingly, the difference of the methyl signals of the N-dimethyl group is negligible: the chemical shifts are 42.0 ppm for the IM base and 41.6 and 42.0 ppm for IM·HCl. Interestingly, the equivalence of the methyl carbons is lost in the hydrochloride.

Length Increase of DNA by Anthracycline Drugs. As pointed out by Hogan et al. (1979a), transient electric dichroism is a very sensitive method to determine the apparent DNA length increase induced by intercalating drugs with a precision of ±10%. The rise time τ^r of the field-induced orientation of the rod-shaped DNA is approximately proportional to the third power of the DNA length (Hogan et al., 1978) if no major change in helix diameter or of the mechanism of orientation is assumed.

We used the technique of transient electric dichroism to compare the drug-induced length increase of DNA by IM and DM. Furthermore, we measured the length increase induced by a chelate complex of Cu(II) and IM with a metal/drug ratio of 0.7. The latter experiment is of interest in view of the reduced cardiotoxicity of anthracycline antibiotics when chelated with divalent or trivalent metal ions (Gosalvez et al., 1978, 1979).

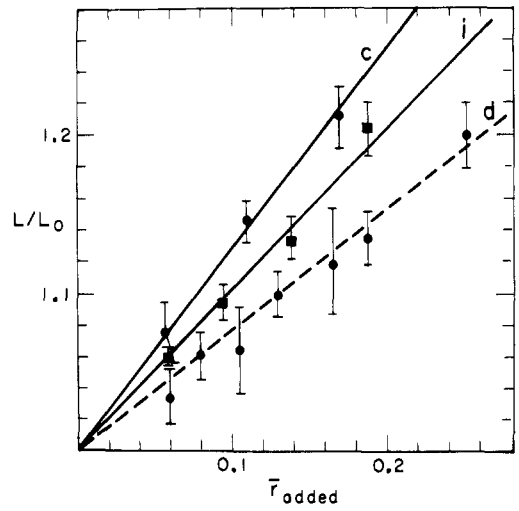


FIGURE 1: Drug-induced length increase L/L_0 of calf thymus DNA vs. r. r is the number of bound drug molecules per DNA base pair in the case of the drugs daunomycin and iremycin but the number of added drug molecules per DNA base pair in the case of the Cu(II)–iremycin complex. DNA is dissolved in 2.5 mM sodium cacodylate buffer, pH 7. The dichroism is measured at 12 °C and an applied field of 10 kV, which corresponds to a field strength of 8.5 kV cm^{-1}. Abbreviations: d, daunomycin; i, iremycin; c, Cu(II)–iremycin. Metal ion/drug ratio 0.7.

Table III: Comparison of Some Experimental Data of DNA Complexes of the Two Anthracycline Antibiotics Daunomycin and Iremycin

	daunomycin	iremycin
L_r/L_0, DNA length increase (nm/bound drug molecule)	0.306 ± 0.020	0.399 ± 0.020
θ_1, chromophore tilt (deg)	3	29
θ_2, chromophore twist (deg)	25	25
ϕ, unwinding angle (deg/bound drug)	15.4 ± 1.5	15.0 ± 1.5

The results are shown in Figure 1. The apparent length increase of DNA by drug intercalation increases in the order DM < IM < IM–Cu(II) (Table III). The length increase of DNA induced by IM is greater than the highest value of an intercalator measured hitherto, which was actinomycin D with 0.39 nm/added drug (Hogan et al., 1979a). The effect of the IM–Cu(II) complex with 0.44 nm/added drug even surpasses the length increase of IM of 0.40 nm. The most remarkable result, however, is the higher value of the DNA length increase of IM compared with that of DM, which is only 0.31 nm/added drug.

Twist and Tilt of Anthracycline Chromophore with Respect to DNA Helix Axis. The dichroism spectra of the DNA complexes of IM and DM are shown in Figure 2. The amplitude (reduced dichroism) of the spectrum is converted from the measured reduced dichroism $\rho = (A_\parallel - A_\perp)/A$ to the angle α between the transition moment and the axis of orientation (the DNA helix axis) by the equation (O'Konski et al., 1959)

$$\rho_i = (3/2)(3 \cos^2 \alpha_i - 1)\Phi_E \qquad (1)$$

where Φ_E is the fractional orientation, equal to the ratio ρ/ρ_∞ of the measured dichroism to the dichroism at perfect orientation, achieved in the limit of infinite field.

The dichroism ρ_∞ at infinite field was determined by extrapolation of a plot of ρ vs. $1/E$, the reciprocal of the field, to the limit $1/E = 0$. A typical plot is shown in Figure 3. The

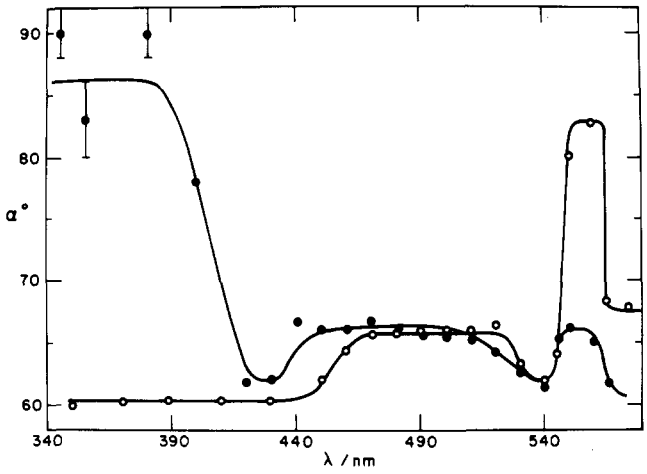


FIGURE 2: Dichroism spectra of drug–DNA complexes: (○) iremycin–DNA, r = 0.112, and (●) daunomycin–DNA, r = 0.12. Conditions as in Figure 1. The angle α between the transition moment and the DNA helix axis was calculated from the measured reduced dichroism ρ by the equation $\rho = (3/2)(3\cos^2\alpha - 1)\phi$, with $\phi = 0.37$, taken from Figure 3.

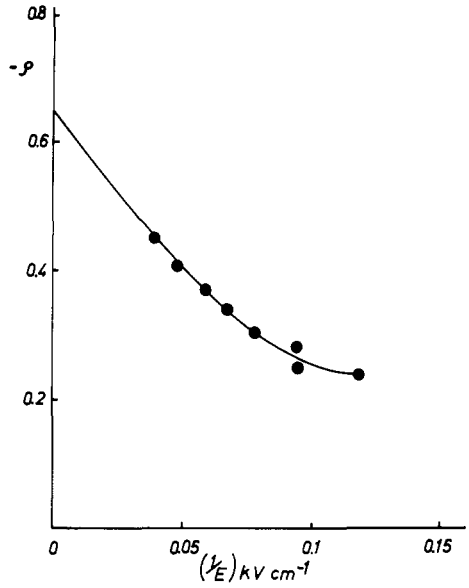


FIGURE 3: Field extrapolation for the iremycin–DNA complex, r = 0.112, at 500 nm. Other conditions as in Figure 1.

justification of this extrapolation procedure has been extensively discussed in previous papers (Hogan et al., 1978, 1979a,b). This procedure has been criticized (Sokerov & Weill, 1979), but we consider that the alternative model proposed by those authors is unsatisfactory since it cannot account for the values of $\rho_\infty = -1.5$ found experimentally for some intercalation complexes (Hogan et al., 1979b). In addition the potential function proposed by Sokerov and Weill seems unrealistic because it produces a discontinuity in the torque exerted on a molecule at right angles to the field.

From eq 23 given in Rill (1972), the tilt angle θ_1 and the twist angle θ_2 of the chromophore relative to the DNA helix axis [for a quantitative definition of the tilt of the long axis and twist of the short axis, cf. eq 23 in Rill (1972) and Hogan et al. (1979a)] can be calculated from the measured dichroism if the directions of at least two transition moments with respect to the long (y) and short (x) axes of the chromophore and their displacements from the helix axis are known.

Gabbay et al. (1976) have assigned the anthracycline long-wavelength transition at approximately 460 nm to a short-axis transition $L^1_b \leftarrow A$ and the short-wavelength

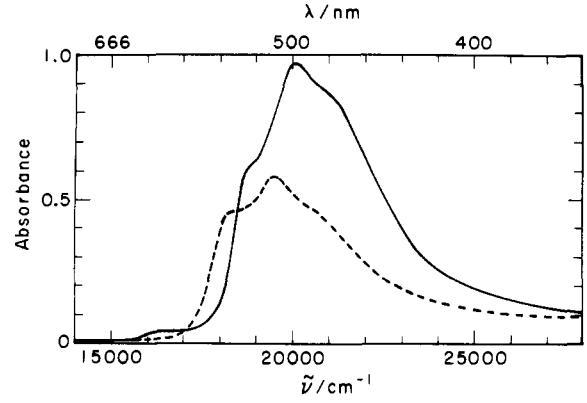


FIGURE 4: Absorption spectra of iremycin (solid line), $C_{IM} = 1.5 \times 10^{-5}$ M, and the iremycin–DNA complex (broken line), $C_{DNA} = 3.6 \times 10^{-4}$ M in base pairs. Milieu conditions as in Figure 1.

transition at approximately 340 nm to a long-axis transition $L^1_a \leftarrow A$, referring to the paper of Sidman (1956). The absorption spectrum of IM ($C = 1.5 \times 10^{-5}$ M) shows absorption maxima at 205, 235, 254, 295, 467, 494, and 528 nm. The three low-energy transitions are shifted to 478, 505, and 540 nm, respectively, when IM is bound to DNA (Figure 4). Similar low-energy shifts were observed for other anthracycline antibiotics as a consequence of their binding to DNA (Gabbay et al., 1976).

Under the reasonable assumption that the strong absorption bands between 295 and 540 nm are assigned to π–π^* transitions, all these transition moments are aligned in the plane of the chromophore, primarily along long and short chromophore axes. Hence, according to Figure 2, both axes of the IM chromophore are inclined by about $\alpha_i = 62 \pm 4°$ relative to the DNA helix axis, yielding tilt and twist angles of 28 ± 4°. The sharp maximum at 555 nm in the dichroism spectrum (Figure 2) of IM indicates a transition with a transition moment aligned more perpendicular ($\alpha_i = 83.0°$) to the helix axis than the transition moments of all other transitions at shorter wavelengths. This suggests a minor off-axis transition of a moderately tilted chromophore.

For daunomycin, the α value between 340 and 390 nm is about 86 ± 4° whereas for iremycin the α value in this range is about 60°. There is similar contrast in α values in the wavelengths between 545 and 565 nm, where IM has an α value close to 83° and DM shows a value close to 65°. On the assumption that Gabbay's (Gabbay et al., 1976) assignment of the major short-axis transition moment (450–520 nm) is correct, both IM and DM show the same orientation of that axis. For the long axis (340–400 nm), IM is ~30° away from perpendicularity whereas the DM long axis is almost perpendicular to the helix axis.

DNA Unwinding Angle of Iremycin Resembles That of Daunomycin. The results of the sedimentation velocity titration experiments on pBR 322 DNA with iremycin are shown in Figure 5A, together with the effect of the reference substance ethidium bromide (EB) on the sedimentation coefficient of the same DNA. It can be seen that IM promotes removal and reversal of the supercoils of cdDNA in the fashion accepted as a diagnostic feature of an intercalation process (Waring, 1970, 1971). At the same time, upon addition of IM, ncDNA exhibits the classical small decrease in s_{20} (Figure 5A) characteristic of intercalation to nicked circular (Waring, 1970, 1971; Bauer & Vinograd, 1971) and linear (Lerman, 1961) DNA molecules.

With respect to the position of the minimum of the s_{20} vs. r_t curve, the behavior of IM differs from that of EB by a factor

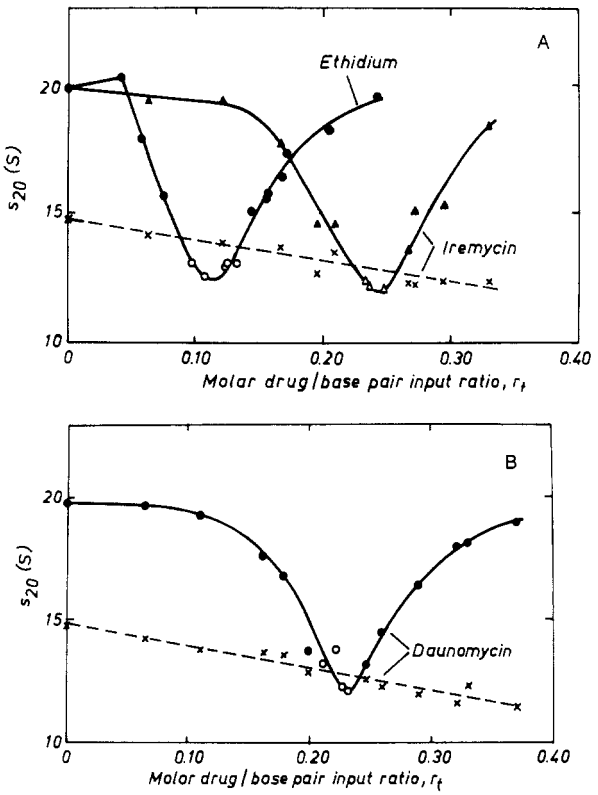


FIGURE 5: (A) Effect of ethidium bromide and iremycin on the sedimentation behavior of pBR 322 DNA in 2.5 mM sodium cacodylate buffer, pH 7. The sedimentation coefficient of superhelical cdDNA molecules in the presence of EB or IM is represented by (●) or (▲), respectively, and that of relaxed ncDNA molecules in the presence of IM by (×). (B) Effect of daunomycin on the sedimentation coefficient of superhelical closed duplex molecules (●) and relaxed nicked circular molecules (×) of pBR 322 DNA. Open symbols (○, △) refer to experiments in which cosedimentation of the two DNA species, cdDNA and ncDNA, with a single unresolved boundary was observed. Total DNA concentration is 30 μg/mL.

Table IV: Critical Input Ratio $(r_c)_t$, Critical Binding Ratio r_c, and DNA Unwinding Angles ϕ of Different Drugs

drug	$(r_c)_t$ (drug molecules/ base pair)	r_c (drug molecules/ base pair)	ϕ (deg)	ϕ/ϕ_{EB}
ethidium bromide	0.115 ± 0.002	0.114 ± 0.002	(26)	(1)
iremycin	0.241 ± 0.003	0.198 ± 0.020	15.0 ± 1.5	0.576 ± 0.058
daunomycin	0.233 ± 0.003	0.192 ± 0.020	15.4 ± 1.5	0.594 ± 0.058

of about 2 (Figure 5B). The input ratios at the equivalence point obtained by a second-order polynomial fitting of the sedimentation data in the vicinity of the minimum (Baase & Johnson, 1979) are collected in Table IV. For estimation of relative helix unwinding angles, these numbers have to be converted to the true equivalence point binding ratios, r_c. Since at the critical drug concentration the binding affinity of cdDNA is equal to that of ncDNA (Bauer & Vinograd, 1971), binding parameters of nicked circular or linear DNA may be used to calculate r_c.

To correct for the binding of EB, we used the neighbor exclusion model (Crothers, 1968; McGhee & von Hippel, 1974) and an association constant of $K = 4 \times 10^6$ M^{-1} as measured by Hogan et al. (1979a) in dichroism buffer (2.5 mM Na^+) at 11 °C. The assumption that this value of K holds also at 25 °C is supported by the results of LePecq & Paoletti (1967), who found the association constant of EB to be independent of temperature (23–37 °C) at low ionic strength.

For IM and DM, the input ratio r_t of added drug molecule per DNA base pair was corrected for the fraction of nonbound drug by spectrophotometric titration as described above (cf. Materials and Methods). The titrations of IM and DM were done under solution conditions very near to those of the sedimentation experiment, yielding corrected values of the ratio r of bound drug molecules per DNA base pair. Corrections to the ethidium values are negligible, justifying our approximation in using the 11 °C binding constant.

The true critical binding ratios r_c obtained in this way are summarized in Table IV. Together with the convenient assumption that the r_c values refer to that mode of binding which brings about the unwinding of DNA, these data allow calculation of the relative unwinding angles for IM and DM. We do not know any investigation in which the unwinding angle induced by EB has been determined at low salt concentration, but it is generally accepted that the unwinding angle of EB is essentially independent of ionic strength (Jones et al., 1980; Baase & Johnson, 1979; Pulleyblank & Morgan, 1975). On the basis of the unwinding angle $\Phi_{EB} = 26°$ (Wang, 1974), the unwinding angle of IM turns out to be 15.0 ± 1.5°, which, within the limits of error, is equal to that of DM at the same ionic strength (Table IV).

Superhelix Density of pBR 322 DNA. The EB titration experiments enabled us to estimate the superhelix density of pBR 322 DNA. From the equation (Waring, 1971)

$$\tau = -N\Phi r_c/360$$

with $N = 4362$ base pairs/pBR 322 molecule (Sutcliffe, 1978), $\Phi = 26°$ for EB (Wang, 1974), and $r_c = 0.114 \pm 0.002$ as determined in this paper, the number of titratable superhelical turns in the absence of drug may be calculated to be $\bar{\tau} = -35.9 \pm 0.6$. The superhelix density that defines the number of turns per 10 base pairs (Bauer, 1978) then amounts to $\bar{\sigma} = -0.0823 \pm 0.0015$ at 2.5 mM Na^+ and 25 °C. Using the quantitative expressions for the temperature and ionic strength dependence of σ given by Bauer (1978), we arrive at $\bar{\sigma}_0 = -0.087 \pm 0.002$ for the titratable superhelix density of pBR 322 DNA at standard conditions (0.2 M NaCl, 37 °C). Quite similar standard superhelix densities have been reported (Bauer, 1978) for the majority of pSM plasmid DNAs from the drug resistance factor R 12 in *E. coli*.

Discussion

Iremycin Is a Strong Intercalator. The evidence for IM intercalation is based upon two independent results, the length increase of rodlike linear duplex DNA (Hogan et al., 1978) and the unwinding of supercoiled closed duplex DNA (Waring, 1970). It should be mentioned, however, that both results can be discussed in terms of DNA kinking, too. The nonintercalating drug irehdiamine A (pregn-5-ene-3,20-diamine) unwinds supercoiled cdDNA (Waring, 1970; Waring & Chisholm, 1972; Waring & Henley, 1975) and produces a monotonous length increase of eukaryotic DNAs (Dattagupta et al., 1978). On the other hand, all physicochemical facts are in excellent agreement with the intercalation of the anthracycline chromophore of the antibiotics DM and adriamycin (Neidle, 1979; Quigley et al., 1980; Chaires et al., 1981), which are both closely related to IM. Therefore, length increase of linear DNA and unwinding of superhelical twisted DNA are regarded as strong evidence for IM intercalation between DNA base pairs.

The unwinding angles of IM and DM are similar (Tables III and IV; Figure 5) and are consistent with the intercalation of the drugs between the DNA bases. Theoretical calculations

(Miller & Pycior, 1979) have suggested two possible intercalation sites for B-DNA, with unwinding angles of 17.3° and 26°. Our observed value of ~15° for IM and DM is lower than either of these estimates, which might be explained by some outside binding to the DNA phosphate groups or by the winding of DNA adjacent to the intercalation site by the polar groups of IM and DM.

While the unwinding behavior of IM and DM is similar, the length increase of DNA as a consequence of drug intercalation is significantly different for these two drugs (Figure 1; Table III). The 0.31 ± 0.02 nm increase per bound drug of DM is in the range of most typical intercalators studied previously (Hogan et al., 1979a). The 0.40 ± 0.02 nm increase per bound drug of IM is higher than the value of actinomycin D (0.39 nm/bound drug), which was the highest of a series of intercalators studied previously (Hogan et al., 1979a).

The unusually large length increase of IM, enhanced by 30% compared to that of DM, is accordingly not reflected in a high unwinding angle but corresponds to a large chromophore tilt of IM relative to the DNA helix axis as discussed below. It turns out that IM is more analogous to actinomycin D than to the closely related DM with respect to chromophore tilt and the length increase of DNA.

Metal Ion–Iremycin Complex Induces an Extremely High Length Increase of DNA. The Cu(II)–IM complex induces a length increase of DNA of 0.44 nm/added drug (Figure 1), which is even higher than the length increase induced by intercalation of IM itself or actinomycin D. The findings support a location of the bulky metal ion at or near the intercalation site. Chelation of Cu(II) can be assumed at the quinone system and at one of the neighboring hydroxyl groups. Thus one can speculate that with ring B intercalated between DNA base pairs, the presence of the bulky metal ion should increase the distance between the DNA base pairs that form the intercalation site.

Orientation of Drug Chromophore in the Complex. If one assumes that the relative orientation of the short (~350 nm)-wavelength and long (~450 nm)-wavelength transition moments are perpendicular to each other and are of the π–π^* type, the angular orientation of the two transition moments defines the spatial geometry of the plane of the chromophore. Assuming further that the 350- and 450-nm transitions are polarized respectively along the long and short chromophore axes, we conclude that the long axis of the drug DM is almost perpendicular to the helix axis whereas that of IM is 30° tipped away from perpendicularity. For the major short-axis transition both DM and IM are about 25° tipped away from perpendicularity.

The puzzling result of strong perturbation of DNA geometry and structure as a consequence of IM intercalation raises the question whether any plausible arguments can be given to explain the behavior of IM, which parallels actinomycin D with respect to chromophore tilt and DNA length increase but is significantly different from the closely related drug DM. On the other hand, the IM intercalation is accompanied by an unwinding of superhelical DNA comparable to that of DM. If we accept the conclusion drawn from NMR (Patel & Canuel, 1978; Phillips & Roberts, 1980) and crystal structure studies (Quigley et al., 1980) on the DM–DNA complex to be applicable in the case of the IM complex, their mode of intercalation should be identical. Rings B and/or C are intercalated and they are identical in both drugs, while rings A and D protrude into grooves.

IM and DM differ (i) in the substitution of ring A (see Chart I) by the amino sugar, which is L-rhodosamine in position 10 for IM and L-daunosamine in position 7 for DM, (ii) in the substituent R_4 at position 9 (IM, C_2H_5; DM, $COCH_3$), and (iii) in position 4 (IM, OH; DM, OCH_3). The substitution of –OH for $-OCH_3$ in position 4 causes negligible or slight increase in drug activity (Henry, 1979). It is hard to imagine how this can be responsible for the different geometric orientation of IM and DM. All these point to the fact that the cyclohexane ring and sugar residue must play an important role in the geometry of orientation. In this respect, the results of crystal structure studies by Quigley et al. (1980) give a direct indication why IM should have different orientation than DM although the rings intercalated are identical.

Quigley et al. (1980) showed that the oxygen atom of $-COCH_3$ at the C9 position of daunomycin is hydrogen bonded via a water molecule to carbonyl O2 of cytosine at the top and that the base–base distance at the C9 side is extended further (at the intercalation site) compared to that at the C4 side. IM lacks this O atom, having $-C_2H_5$ instead of $-COCH_3$ at the C9 position. Moreover, the sugar residue is at position C10 and not at C7 as in DM. If the bulky sugar is present at the top of C9 and lacks the support of a water-bridged H bond, it is not unexpected that the base–base distance at the C9 side can be further extended for the IM complex than for DM. Moreover, the C9 hydroxyl of DM seems to be within H-bonding distance of two atoms of guanine below the ring. Extension of the distance between the base pairs requires tilting of the IM chromophore to reach the distance. Both the position of the sugar residue on the opposite side of the ring and the lack of $-COCH_3$ at C9 may be responsible for the long-axis tilt and greater length increase in the case of IM. At this moment information about the short-axis twist in the DM–DNA crystal is not available to us. But if rings B and C are intercalating (they are identical in both the molecules), the twist can be the same in both the drugs.

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Rate-Determining Folding and Association Reactions on the Reconstitution Pathway of Porcine Skeletal Muscle Lactic Dehydrogenase after Denaturation by Guanidine Hydrochloride†

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ABSTRACT: Reactivation of tetrameric porcine skeletal muscle lactic dehydrogenase after dissociation and extensive unfolding of the monomers by 6 M guanidine hydrochloride (Gdn·HCl) is characterized by sigmoidal kinetics, indicating a complex mechanism involving rate-limiting folding and association steps. For analysis of the association reactions, chemical cross-linking with glutaraldehyde may be used [Hermann, R., Jaenicke, R., & Rudolph, R. (1981) *Biochemistry 20*, 2195-2201]. The data clearly show that the formation of a dimeric intermediate is determined by a first-order folding reaction of the monomers with $k_1 = (8.0 \pm 0.1) \times 10^{-4}$ s^{-1}. The rate constant of the association of dimers to tetramers, which represents the second rate-limiting step on the pathway of reconstitution after guanidine denaturation, was then determined by reactivation and cross-linking experiments after dissociation in 0.1 M H_3PO_4 containing 1 M Na_2SO_4. The rate constant for the dimer association (which is the only rate-limiting step after acid dissociation) was $k_2 = (3.0 \pm 0.5) \times 10^4$ M^{-1} s^{-1}. On the basis of the given two rate constants, the complete reassociation pattern of porcine lactic dehydrogenase after dissociation and denaturation in 6 M Gdn·HCl can be described by the kinetic model

$$4M \xrightarrow{k_1} 4M^* \xrightarrow{\text{fast}} 2D \xrightarrow{k_2} T$$

Reconstitution experiments with oligomeric enzymes have been frequently performed in the past in order to make clear various aspects of the subunit interaction such as the correlation of quaternary structure and biological activity (Jaenicke & Rudolph, 1980). Rate measurements of the reactivation of numerous enzymes after previous denaturation and dissociation often revealed sigmoidal kinetics (Vallee & Williams, 1975; Rudolph et al., 1977a,b; Jaenicke et al., 1979; Zabori et al., 1980; Dautry-Varsat & Garel, 1978), indicating a complex reconstitution mechanism with rate-limiting folding and association steps. Although little information was available regarding the sequence of events and the nature of the intermediate states, the observed sigmoidal kinetics were previously ascribed to a model consisting of two consecutive

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