Degree of DNA Unwinding Caused by the Binding of Aclacinomycin A

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Received May 13, 1997; accepted July 7, 1997

The effect of drug binding on the geometry of DNA duplex (plasmid pBR322) has been examined using topoisomerase I relaxation followed by gel electrophoresis. The binding of one molecule of aclacinomycin A was found to cause an unwinding of the DNA double helix by an angle of 8±2° in aqueous solution at 37 °C. The unwinding angle of daunomycin was 12±2°, and that of ethidium bromide 15±3°. To determine the unwinding angle, precise determination of the equilibrium constant of drug–DNA binding-dissociation reaction in the same buffer as that for the topoisomerase reaction (at 37 °C) was indispensable. This determination was made by ultraviolet absorption measurement of the same plasmid–drug system, followed by a Scatchard plot and analysis using McGhee–von Hippel's excluded site model. For the aclacinomycin–pBR322 system, the binding constant (K) was 7.2×10^4 M^{-1}, and the number of base pairs in the single site of drug binding (n) was 4.0. For daunomycin–pBR322, $K=7.1 \times 10^4$ M^{-1} and $n=3.4$, and for ethidium–pBR322, $K=4.0 \times 10^4$ M^{-1} and $n=3.3$. On the basis of these experimental results, the possible role of the sugar moieties of these antitumour drugs, as well as that of intercalating chromophores, was discussed.

Key words unwinding of DNA duplex; aclacinomycin; plasmid topoisomer; daunomycin; ethidium

A functioning of DNA always involves an intermolecular interaction with another molecule, and such an interaction always causes a deformation of the geometrical structure of DNA. One of the important parameters defining such a geometrical deformation is the angle of unwinding of DNA duplex. In a free DNA duplex in aqueous solution (B-DNA), a base-pair is reached from the next-door base-pair by rotating it around the helical axis by an angle of 36° (on average), as well as a simultaneous translation by 0.34 nm along the helix axis.[1] If B-DNA deforms into A-DNA, the angle is reduced from 36° to 32.7° [2]; if it deforms into Z-DNA, the angle is reduced from 36° to −30°.[3] The purpose of our present series of studies is to determine such an unwinding angle in B-DNA produced by a drug binding.

The method we adopted for determining the unwinding angle consists of i) incubation of a mixture of closed circular DNA duplex + drug, ii) addition of topoisomerase I to it and further incubation, iii) extraction of DNA from it, and iv) determination of the number of superhelical turns of the extracted DNA by gel electrophoresis. This is a modification of the method of Fisher and coworkers, used for their DNA unwinding assay of bleomycin bithiazole analogues.[4] We have attempted to bring the method into a quantitative analysis, as will be detailed below.

We have chosen aclacinomycin A (Fig. 1) as the subject. Aclacinomycin A was found in a culture of *Streptomyces galilaeous* twenty years ago,[5] and used for more than ten years as an antitumour drug with low cardiac toxicity now known worldwide. Its activity is believed to be related with its binding to DNA, but the detailed mode of binding is not yet clear. Does its binding cause an unwinding of DNA duplex, and if so how much? This is the primary question we attempted to answer in our present investigation.

For comparison, we chose two additional drugs as subsidiary subjects, daunomycin and ethidium bromide (Fig.1). Daunomycin is another anthracycline antibiotic widely used in cancer chemotherapy. The crystal structure of daunomycin–d(CGTACG)$_2$ complex has been determined by X-ray diffraction analysis at 1.2 Å resolution.[6] It is important to learn whether the complex structure in the crystal is maintained when the complex is brought from the crystal into solution. Ethidium bromide is a well-known trypanocydal dye, which can strongly bind to DNA, and which is widely used for visualizing DNA in electrophoresis gel. The binding of ethidium bromide to DNA has been studied for many years by a variety of experimental techniques under many conditions.[7]

Theoretical Background

Unwinding Angle The topological properties of closed-circular duplex DNA are defined by the relationship,

$$\alpha=\beta+\tau \quad (1)$$

Here, α is the linking number of the closed-circular duplex.

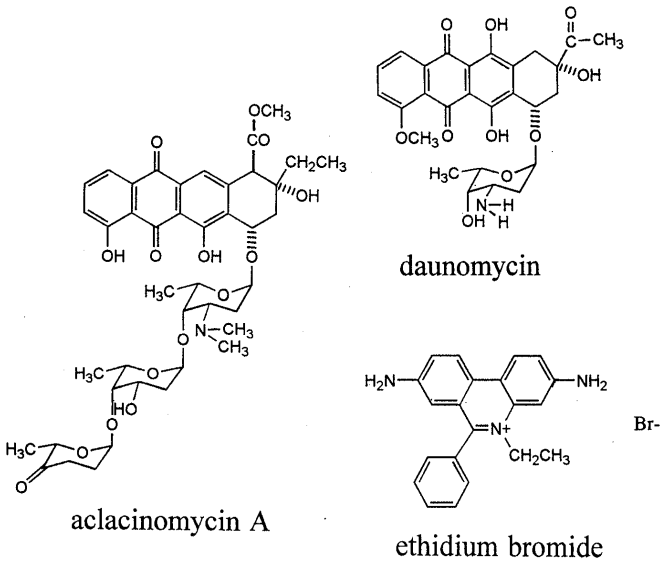

Fig. 1. Structure of Aclacinomycin A, Daunomycin and Ethidium Bromide

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This is the number of times one strand is linked through the other, and is a fixed integral number in the absence of strand scission. β (twisting number) is the number of helical turns, and τ (writhing number) is the number of superhelical turns. β is given by

$$\beta=\frac{N}{h} \tag{2}$$

where N is the total number of base-pairs involved in the closed-circular duplex, and h is the number of base-pairs involved in one pitch of the DNA helix. Equation 1 is now rewritten as

$$\alpha=\frac{N}{h}+\tau \tag{3}$$

Let us define θ as an angle of clockwise rotation viewed along the local duplex axis in order to reach a base pair from the adjacent base pair located closer to the viewer. Thus

$$\theta=\frac{360}{h}\ (^\circ) \tag{4}$$

or, using Eq. 3,

$$\theta=\frac{360(\alpha-\tau)}{N}\ (^\circ) \tag{5}$$

What we would like to know is the difference of the angles $\Delta\theta$ before (θ_0) and after (θ) the drug binding, namely,

$$\Delta\theta=\theta-\theta_0=\frac{360(\alpha-\tau)}{N}-\frac{360(\alpha-\tau_0)}{N}=\frac{360\Delta\tau}{N}\ (^\circ) \tag{6}$$

where $\Delta\tau=\tau_0-\tau$ is the difference in the number of superhelical turns before (τ_0) and after (τ) the drug binding. The effect of drug binding on the total angle of the base pair rotation of the entire closed-circular duplex is

$$\Delta\Theta=\Theta-\Theta_0=360\Delta\tau \tag{7}$$

If the number of drug molecules bound to one closed-circular duplex DNA molecule is m, the desired value of the unwinding angle (ϕ) due to one drug molecule should be

$$\phi=\frac{\Delta\Theta}{m}=\frac{360\Delta\tau}{m} \tag{8}$$

How to Measure the Change ($\Delta\tau$) in Writhing Number Caused by a Drug Binding The mobility of a DNA molecule in an electrophoresis depends primarily upon its molecular weight. For closed-circular duplex DNA molecules with the same molecular weight, however, the mobility depends upon the number (τ) of their superhelical turns. A drug binding to a closed-circular duplex does not change the linking number (α), but it may change the helical parameter h, and therefore β, and hence τ (see Eqs. 2 and 1, above). If the change in the writhing number ($\Delta\tau$) is estimated here, the desired unwinding angle ϕ is known by Eq. 8 above.

The change in τ caused by a drug binding can be estimated by an electrophoresis, if the buffer contains a proper amount of drug molecule everywhere, so that the DNA molecule is kept in equilibrium with the proper drug concentration all the time during the electrophoresis. This might be possible in principle, but is not practical. Instead, it is practical to store the $\Delta\tau$ value (caused by an equilibrium drug binding) in the DNA molecule itself by the use of topoisomerase. Suppose a closed-circular duplex with α_0, β_0 and τ_0 is brought into equilibrium with a proper concentration of drug, and that its parameters change as $\alpha_0\rightarrow\alpha_0$ (this should not change), $\beta_0\rightarrow\beta$, and $\tau_0\rightarrow\tau$. Let us then suppose that a topoisomerase action is imposed here to relax the circular duplex all the way, so that the writhing number is brought into zero. This must cause the following changes:

For circular duplex (O) without drug:

$$\tau_0\rightarrow 0,\quad \beta_0\rightarrow\beta_0,\quad \alpha_0\rightarrow\alpha_0-\tau_0.$$

For circular duplex (W) with drug:

$$\tau\rightarrow 0,\quad \beta\rightarrow\beta,\quad \alpha_0\rightarrow\alpha_0-\tau.$$

Next, suppose that the drug and topoisomerase are both removed and the duplex is purified. This procedure results in the following parameters. For circular duplex (O): linking number remains as $\alpha_0-\tau_0$, twisting number remains as β_0, and therefore writhing number remains zero. Note that $\tau_0=\alpha_0-\beta_0$, here. For circular duplex (W): linking number remains at $\alpha_0-\tau$, twisting number changes to β_0 (because drug has been removed), and writhing number changes to $\tau_0-\tau$ (so that twisting number + writhing number are equal to linking number). Thus, the desired $\Delta\tau=\tau_0-\tau$ must be equal to the writhing number of this circular duplex (W), which can be readily determined by electrophoresis of this duplex sample.

Fluctuation of the Linking Number In the topoisomerase reaction mentioned above the writhing number (τ) of the circular duplex becomes zero ($\tau=0$). This is considered to be true, however, only in an average. At room temperature, not every topoisomer necessarily has $\tau=0$; some have $\tau=1$, some have $\tau=-1$; a few even have $\tau=2$ or $\tau=-2$. This is because the free energy differences among these topoisomers are not great, so that the topoisomerase causes a Boltzmann distribution through thermal fluctuation. This Boltzmann distribution can be predicted through the known values of elastic constants of the DNA duplex.

Young's modulus of DNA duplex has been estimated to be $E=2.7\times10^8\ \mathrm{Nm}^{-2}$, from experiments on the light scattering, viscosity, and sedimentation.[7,8] Suppose that here is a long cylinder with radius r. If a part of this cylinder, with length l, is bent so that it has curvature R, this part should have free energy higher than that when it is straight by

$$V_b=\frac{1}{2}E\frac{1}{4}\pi r^4 l R^{-2} \tag{9}$$

Since $\theta=l/R$ is the bent angle,

$$V_b=\frac{1}{2}\left(E\frac{1}{4}\pi r^4 l^{-1}\right)\theta^2 \tag{10}$$

The parenthesized portion of the right-hand side of this equation represents the bending force constant k_b of the cylinder. For DNA duplex cylinder, E is known as given above, and $r=10$ Å. By putting $l=3.4$ Å,

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$$k_b = 2.7 \times 10^8 \times (1/4) \times 3.14 \times (10 \times 10^{-10})^4/(3.4 \times 10^{-10}) = 6.2 \times 10^{-19}\ \mathrm{Nm/rad} \quad (11)$$

is the bending force constant of one base pair of DNA duplex. By the use of this force constant, the free energy difference of DNA duplex with N base pairs, having writhing number τ, from that having τ_0, is now evaluated as

$$V_b = \frac{1}{2} k_b \left(\frac{2\pi \Delta\tau}{N}\right)^2 N \quad (12)$$

where $\Delta\tau = \tau - \tau_0$. Therefore, the number of super-helices with τ is given, from the number of super-helices with τ_0, by multiplying

$$\omega(\tau - \tau_0) \propto \exp(-V_b/k_B T) = \exp\left[-\frac{1}{2} k_b \frac{4\pi^2}{N} (\tau - \tau_0)^2/k_B T\right] \quad (13)$$

where k_B is the Boltzmann constant ($1.38 \times 10^{-23}\ \mathrm{J\,K^{-1}}$), and T is the temperature (K).

On the other hand, the torsional force constant k_t of DNA duplex was estimated to be 4.2×10^{-19} Nm/rad from a Raman spectroscopic measurement[9] and also from some fluorescence decay experiments.[10,11] This leads to the number of DNA molecules having twisting number β, which is deviated by $\beta - \beta_0$ from standard value β_0. This is

$$\omega(\beta - \beta_0) \propto \exp\left[-\frac{1}{2} k_t \frac{4\pi^2}{N} (\beta - \beta_0)^2/k_B T\right] \quad (14)$$

Finally, let us now estimate Boltzmann distribution among the topoisomers produced by the topoisomerase through the relation,

$$\alpha - \alpha_0 = \beta - \beta_0 + \tau - \tau_0 \quad (15)$$

The result is

$$\omega(\alpha - \alpha_0) \propto \exp\left[-\frac{1}{2} \frac{k_b k_t}{k_b + k_t} \frac{4\pi^2}{N} (\alpha - \alpha_0)^2/k_B T\right] \quad (16)$$

By putting $n = 4362$, $T = 303$ K, and $\alpha - \alpha_0 = 0, \pm 1, \pm 2$, *etc.*, the Boltzmann distribution can be visualized as shown in Fig. 2.

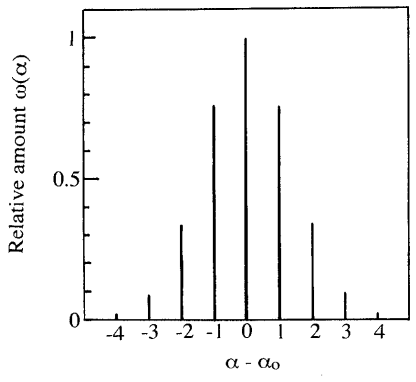

Fig. 2. Boltzmann Distribution of Topoisomers with Different Linking Numbers of Plasmid pBR322 DNA (with 4362 Base Pairs) at 30 °C, Calculated on the Assumption that Bending Force Constant $k_b = 6.2 \times 10^{-19}$ Nm/rad and Torsional Force Constant $k_t = 4.2 \times 10^{-19}$ Nm/rad

α_0 is the linking number of the most stable topoisomer. The ordinate gives the relative amount $\omega(\alpha)$ of topoisomers having the linking number α, which is greater than α_0 by $\alpha - \alpha_0$.

Number of Drug Molecules Bound to One Closed Circular DNA Duplex To determine the unwinding angle ϕ through Eq. 8, we need to evaluate not only $\Delta\tau$ but also m, the number of drug molecules bound to the one closed circular DNA duplex in question. In other words, we need to evaluate equilibrium constant of the drug+DNA solution in the same buffer and at the same temperature as those in which our topoisomerase experiment is made.

In an extreme simplification, the reaction system now in question can be expressed as follows:

$$\text{free drug} + \text{free site on DNA} = \text{bound drug} \quad (17)$$

The equilibrium constant K is given by

$$K = \frac{[\text{bound drug}]}{[\text{free drug}] \times [\text{free site}]} \quad (18)$$

where [free drug] *etc.* mean the concentrations of free drug *etc.* This is rewritten as

$$K = \frac{L_b}{(n^* D - L_b) L_f} \quad (19)$$

where L_f is the concentration of the free drug, L_b is the concentration of the bound drug, D is the concentration of plasmid base pair, and n^* is the number of sites per base pair. Let us introduce binding ratio γ defined as

$$\gamma = \frac{L_b}{D} \quad (20)$$

so that $L_b = \gamma D$. Then, $K = \gamma/(n^* - \gamma) L_f$ and therefore

$$\frac{\gamma}{L_f} = K(n^* - \gamma) \quad (21)$$

The plot of γ/L_f against γ is called a Scatchard plot. On the basis of this simplified theory[1,2] the plot should result in a straight line, and the inclination should give the equilibrium constant K. As was pointed out by McGhee and von Hippel,[13] however, Eq. 21 involves a serious defect in enumerating the number of free sites along the DNA duplex. Here, the number of free sites is assumed to be always proportional to the number of unoccupied base pairs. Suppose, for example, that one aclacinomycin molecule occupies four base pairs on its binding to DNA (this is actually the case, as will be shown later). Suppose next that two aclacinomycin molecules are bound to the same DNA duplex with a gap in which three base pairs are left unbound. It is clear that in this gap the free site must be zero, instead of 3/4. If the gap contains six base pairs, then the number of free sites here must be three, instead of 6/4. McGhee and von Hippel[13] properly estimated the probability P_g of occurrence of the gap containing g base pairs, and then the number of free sites. They finally derived the following equation, to replace Eq. 21:

$$\frac{\gamma}{L_f} = K(1 - n\gamma)\left[\frac{1 - n\gamma}{1 - (n-1)\gamma}\right]^{n-1} \quad (22)$$

where n is the number of base pairs occupied by one drug molecule bound to DNA duplex. When γ/L_f was plotted against γ (Scatchard plot) using this equation it came out as a curved line instead of a straight line.

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Experimental

Materials Some of the samples of plasmid pBR322 DNA were prepared using a QIAGEN plasmid kit (QIAGEN GmbH (Max-Volmer-Straße 4 40724 Hilden, Germany)). This kit was purchased through Funakoshi Co. Some of the samples of plasmid pBR322 DNA were purchased from Takara Shuzo Co. These two kinds of samples did not show any difference as far as our experiments were concerned. Topoisomerase I from calf thymus was purchased from Takara Shuzo Co., and used without further purification. Aclacinomycin A hydrochloride was kindly supplied by Dr. Tomio Takeuchi (Institute of Microbial Chemistry, Tokyo) and Dr. Hiroshi Tone (Sanraku-Ocean Co., Ltd.). Daunomycin and ethidium bromide were purchased from Sigma Co.

The drug plus plasmid reactions were always produced in a buffer, which is proper for topoisomerase I activity measurement. This buffer contains 35 mM Tris–HCl (pH = 8.0), 72 mM KCl, 5 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 5 mM spermidine, and 0.01% bovine serum albumin. Let us call this buffer-T.

Absorption Spectroscopic Measurements The drug was dissolved in buffer-T, 3500 μl of this solution was then placed in a cuvette, and this was mounted on a Shimadzu automatic recording spectrometer UV-2200A. A proper amount of DNA solution was added to the drug solution, and the spectroscopic measurement was made after stirring. The temperature of the solution was kept at 37 °C using a Taitec EL-8F Coolnit Bath.

Electrophoresis Analysis A proper amount of plasmid pBR322 DNA was dissolved in buffer-T, and a proper amount of drug was added (see below). The mixture solution was incubated at 37 °C for 10 min to complete the binding reaction. Next, the proper amount (12 units for 100 μl of the mixture solution) of topoisomerase was added, and the solution was incubated at 37 °C for 2 h, to cause a complete relaxation of the supercoiled plasmid (with drug bound). Then, the drug and enzyme were removed through phenol extraction, and DNA was isolated by ethanol precipitation. The isolated DNA was then subjected to 1% agarose gel electrophoresis. The buffer used here was TBE (90 mM Tris–borate and 2 mM EDTA). The experiment was done by imposing 40V, at room temperature and for 20 h. After that, the gel was stained with ethidium bromide and subjected to photography.

The mobility of pBR322 DNA is related to its writhing number τ. The relation was examined in detail using agarose gel containing various amounts of chloroquine. It was found that pBR322 DNA with $\tau = +1, +2, \cdots$ had a slightly greater mobility than pBR322 DNA with $\tau = -1, -2, \cdots$, respectively.

Results

Aclacinomycin A Aclacinomycin A was dissolved in buffer-T up to 2.3×10^{-5} M. Its absorption spectrum showed a peak at 514 nm (see Fig. 3). When 2.5 μl of 2.04×10^{-2} M pBR322 DNA solution was added to 3500 μl of the aclacinomycin A solution, the peak height at 514 nm was lowered. The addition of 2.5 μl DNA solution was repeated 21 times until the total addition reached 52.5 μl. Each time the absorption spectrum of the mixture solution was recorded. As seen in Fig. 3, the 514 nm peak lowered gradually, and absorbance at 450 nm increased. During these spectral changes, an isosbestic point appeared at 460 nm. This fact indicates that the spectra are caused by only two components, namely free drug and one type of bound drug. The spectrum of pure bound drug was recorded for a solution obtained by adding 2.5 mg of pBR322 DNA to 3500 μl of 2.3×10^{-5} M aclacinomycin A solution (curve b of Fig. 3).

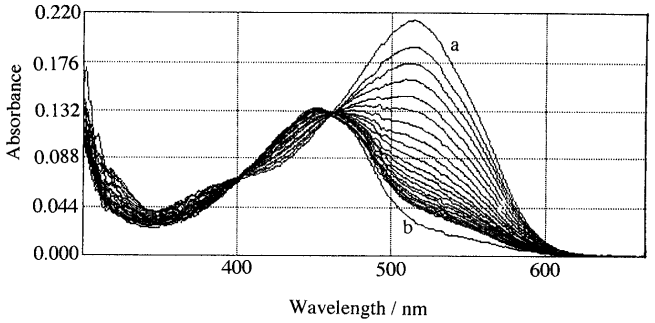

Fig. 3. Absorption Spectra of Aclacinomycin A in Buffer-T at 37 °C

a, aclacinomycin A 2.3×10^{-5} M only. (Curves with no labels): solutions consist of 3500 μl of 2.3×10^{-5} M aclacinomycin A + 2.5 μl $\times J$ of 2.04×10^{-2} M (in base pairs) of pBR322 DNA, where $J = 1$—21. b, aclacinomycin A 2.3×10^{-5} M (3500 μl) + 2.5 mg of pBR322DNA.

By examining the 514 nm peak height, the concentrations of free drug and bound drug (L_f and L_b, see "Theoretical Background") were evaluated for each of the mixture solutions. Then, the binding ratio γ was calculated (Eq. 20), and γ/L_f was plotted against γ (Scatchard plot). The result is shown in Fig. 4. Equation 22 was used to search for the most probable set of K (equilibrium constant) and n (the number of base pairs occupied by one drug molecule). As can be seen in Fig. 4, a McGhee–von Hippel curve fitted the experimental plot when it was assumed that $K = 72000\ \mathrm{M}^{-1}$ and $n = 4.0$.

Figure 5 shows the results of our electrophoresis experiments on aclacinomycin A. The purified native pBR322 DNA was found to be highly super-coiled, containing a small amount of relaxed circular duplex (lane No. 1). The super-coiled pBR322 DNA was completely relaxed by the action of topoisomerase I (lane No. 2). This relaxed pBR322 DNA, however, showed a distribution of τ value from +5 to −1, instead of showing the single value $\tau = 0$. The range of distribution (± 3) is just what was predicted from the known values of elastic constants of DNA duplex (see "Theoretical Background," see also Fig. 2). The fact that the central peak value of τ is +2 instead of 0 is considered to be due to differences in temperature and solvent in our topoisomerase incubation from those in our electrophoresis experiment. The temperature was 37 °C in the former and 20 °C in the latter; solvent was buffer-T in the former and TBE buffer in the latter.

Sample No. 3 in our electrophoresis experiment came from the topoisomerase solution containing 5.89×10^{-7} M

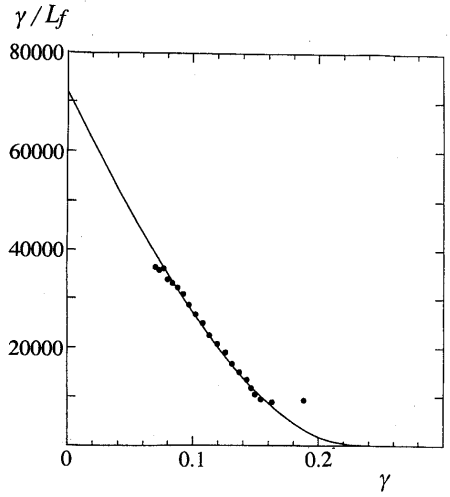

Fig. 4. A Scatchard Plot (●) of Aclacinomycin A Bound to Plasmid pBR322 DNA

Solid line shows a theoretical curve on the basis of Eq. 22, in which it was assumed that $K = 72000\ \mathrm{M}^{-1}$ and $n = 4.0$.

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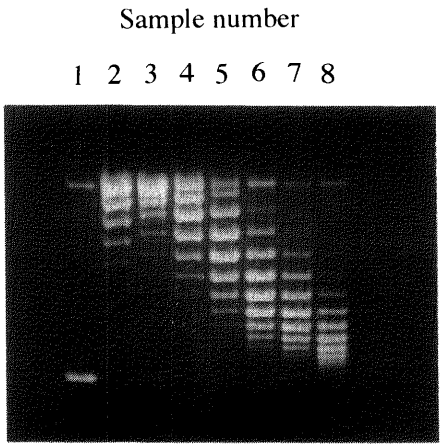

Fig. 5. Electrophoresis Analysis of Topoisomers of pBR322 DNA, Produced by an Aclacinomycin A Binding and the Topoisomerase Relaxation, Followed by the Removal of the Drug and Enzyme

1, Purified intact pBR322 DNA; 2, completely relaxed pBR322 DNA. No drug was added. 3—8, aclacinomycin A had been added before topoisomerase was introduced. DNA concentration = 1.52×10^{-5} M. Drug concentrations: 3, 5.89×10^{-7} M; 4, 1.18×10^{-6} M; 5, 1.77×10^{-6} M; 6, 2.36×10^{-6} M; 7, 2.95×10^{-6} M; 8, 3.54×10^{-6} M.

Table 1. Angle of Unwinding of DNA Double Helix Caused by Aclacinomycin A Binding as Derived from Writhing Number and Equilibrium Constant

Sample No.	C_T [a]	C_B [b]	m [c]	τ [d]	$\Delta\tau$ [e]	ϕ [f]
3	5.89×10^{-7}	3.05×10^{-7}	87	+3—−4	2±0.5	8.2±2.1
4	1.18×10^{-6}	6.05×10^{-7}	174	+1—−5	4±0.5	8.3±1.0
5	1.77×10^{-6}	8.98×10^{-7}	258	0—−7	5.5±0.5	7.7±0.7
6	2.36×10^{-6}	1.19×10^{-6}	340	−2—−10	8±1	8.5±1.1
7	2.95×10^{-6}	1.47×10^{-6}	421	−3—−11	9±1	7.7±0.9
8	3.54×10^{-6}	1.74×10^{-6}	500	−6—−13	11.5±0.5	8.3±0.4

a) C_T is the total concentration of aclacinomycin A (M). *b*) C_B is the concentration of aclacinomycin A bound to DNA (M). *c*) m is the number of aclacinomycin A molecules bound to one pBR322 (closed circular DNA duplex) molecule. *d*) τ is the writhing number of pBR322 DNA, which comes from the topoisomerase + pBR322 DNA solution containing a given amount of aclacinomycin A (see Fig. 5). *e*) $\Delta\tau$ is the change of writhing number of pBR322 DNA caused by the aclacinomycin A binding (see "Theoretical Background"). *f*) ϕ is the unwinding angle of pBR322 DNA due to one aclacinomycin A molecule, calculated by Eq. 8.

aclacinomycin A as well as 1.52×10^{-5} M DNA (buffer-T, at 37 °C). Because the equilibrium constant K is known to be 72000 M^{-1}, the concentration of the bound drug can be calculated as 3.05×10^{-7} M. This means that the number of drug molecules bound per one pBR322 DNA molecule is $m=87$ (Table 1). From the electrophoresis analysis, $\Delta\tau = |\tau_0 - \tau| = 2$. By substituting these values for m and $\Delta\tau$ in Eq. 8, the unwinding angle is calculated to be 8° (Table 1).

A similar experiment was repeated by increasing the concentration of aclacinomycin A stepwise. As is seen in Fig. 5, $\Delta\tau$ value also increased stepwise. The calculated m value also rose, but the calculated unwinding angle remained at 8° (see Table 1). Thus, our conclusion is that one molecule of aclacinomycin A bound to DNA duplex causes an unwinding of the helix by 8±2°.

Daunomycin In Fig. 6, absorption spectra of daunomycin + pBR322 DNA mixture solution (in buffer-T at 37 °C) are shown. From the peak height measurement at 580 nm a Scatchard plot, shown in Fig. 7, was derived. A

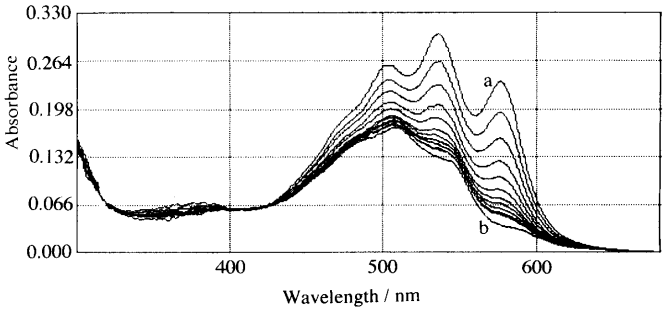

Fig. 6. Absorption Spectra of Daunomycin in Buffer-T at 37 °C

a, daunomycin 2.5×10^{-5} M only. (Curves with no labels): Solutions consist of 3500 μl of 2.5×10^{-5} M of daunomycin + 5 μl × J of 2.04×10^{-2} M (in base pairs) of pBR322 DNA, where $J = 1$—10. b, Daunomycin 2.5×10^{-5} M (3500 μl) + 1.7 mg of pBR322 DNA.

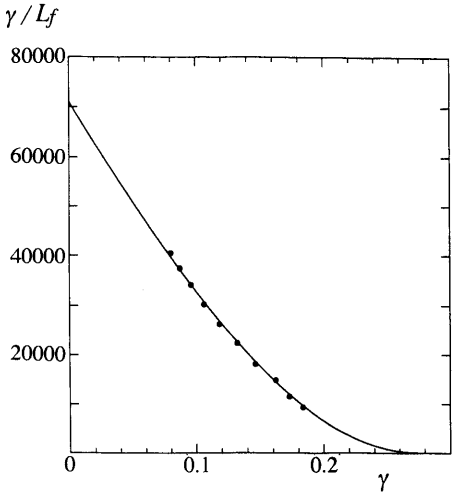

Fig. 7. A Scatchard Plot (●) of Daunomycin Bound to Plasmid pBR322 DNA

Solid line shows a theoretical curve on the basis of Eq. 22, in which it was assumed that $K = 71000\ M^{-1}$ and $n = 3.4$.

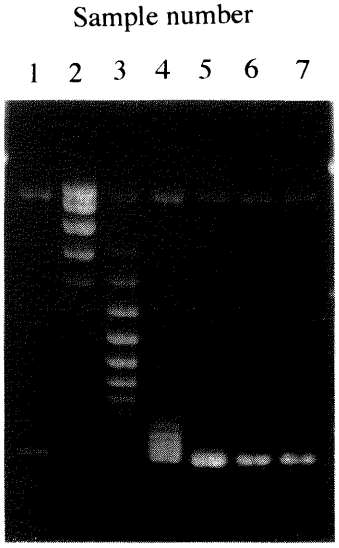

Fig. 8. Electrophoresis Analysis of Topoisomers of pBR322 DNA, Produced by a Daunomycin Binding and the Topoisomerase Relaxation, Followed by the Removal of the Drug and Enzyme

1, Purified intact pBR322 DNA; 2, completely relaxed pBR322 DNA. No drug was added. 3—7, daunomycin had been added before topoisomerase was introduced. DNA concentration = 1.52×10^{-5} M. Drug concentrations: 3, 1.77×10^{-6} M; 4, 3.55×10^{-6} M; 5, 5.32×10^{-6} M; 6, 7.09×10^{-6} M; 7, 8.87×10^{-6} M.

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McGhee–von Hippel curve fitted the plot when K was assumed to be 71000 M^{-1} and n to be 3.4. Figure 8 shows the results of our electrophoresis experiments of the pBR322 DNA produced by a binding of daunomycin, topoisomerase relaxation, and removal of the drug and enzyme. The $\Delta\tau$ value was estimated for each sample, and the unwinding angle was concluded to be $12\pm2°$ by Eq. 8.

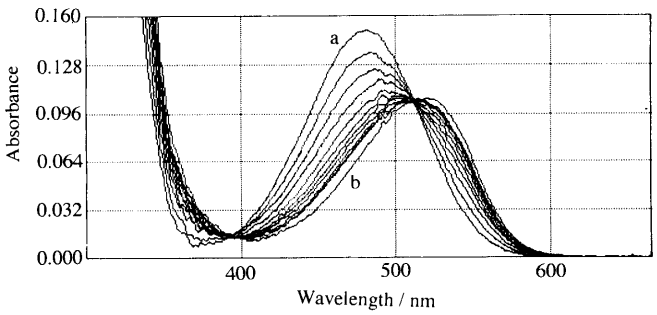

Fig. 9. Absorption Spectra of Ethidium Bromide in Buffer-T at 37 °C

a, ethidium bromide 2.58×10^{-5} M only. (Curves with no labels): Solutions consist of 3500 μl of 2.58×10^{-5} M + 5 μl $\times J$ of 2.04×10^{-2} M (in base pairs) of pBR322 DNA, where $J=1$—10. b, ethidium bromide 2.58×10^{-5} M (3500 μl) + 1.4 mg of pBR322 DNA.

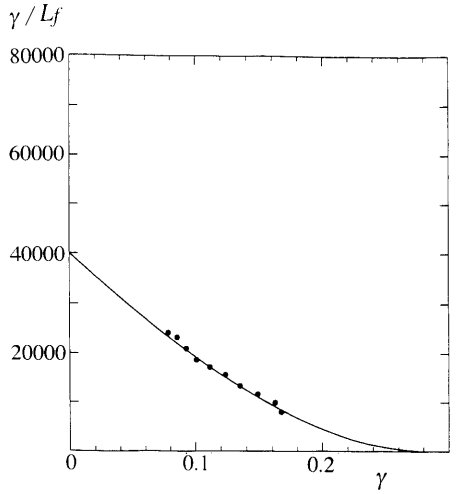

Fig. 10. A Scatchard Plot (●) of Ethidium Bromide Bound to Plasmid pBR322 DNA

Solid line shows a theoretical curve on the basis of Eq. 22, in which it was assumed that $K=40000\ M^{-1}$ and $n=3.3$.

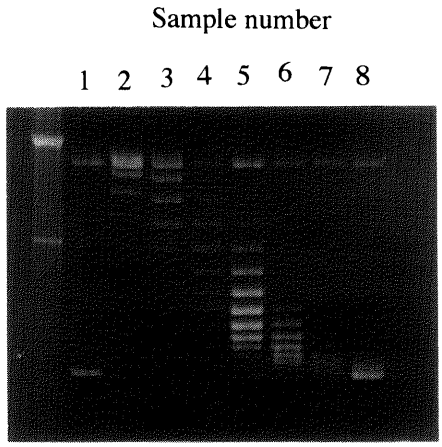

Fig. 11. Electrophoresis Analysis of Topoisomers of pBR322 DNA, Produced by an Ethidium Bromide Binding and the Topoisomerase Relaxation, Followed by the Removal of the Drug and Enzyme

1, Purified intact pBR322 DNA; 2, completely relaxed pBR322 DNA. No drug was added; 3—8, ethidium bromide had been added before topoisomerase was introduced. DNA concentration = 1.52×10^{-5} M. Drug concentrations: 3, 6.02×10^{-7} M; 4, 1.20×10^{-6} M; 5, 1.81×10^{-6} M; 6, 2.41×10^{-6} M; 7, 3.01×10^{-6} M; 8, 3.61×10^{-6} M.

Ethidium Bromide A similar procedure was applied to a series of ethidium bromide + pBR322 DNA mixture systems. Absorption spectra are shown in Fig. 9, Scatchard plot is shown in Fig. 10, and the results of electrophoresis experiments are shown in Fig. 11. The unwinding angle of DNA helix caused by the binding of one ethidium bromide molecule has been determined to be $15\pm4°$.

Discussion

The main conclusions of our present experiments are summarized in Table 2, where the unwinding angle (ϕ) in DNA duplex caused by the binding of each of the three drugs is listed. The value of ϕ was obtained from $\Delta\tau$ and m through Eq. 8, and m was found from the drug/DNA equilibrium constant, K. It should be emphasized that both of the K and $\Delta\tau$ values were determined in the same buffer (buffer-T) and at the same temperature (37 °C). This is most important because, in general, K depends greatly upon the solvent conditions, and unless K is determined in the same solvent as that for the $\Delta\tau$ determination, the proper value of m for ϕ determination (through Eq. 8) cannot be learned.

To demonstrate how the contents of buffer affect the DNA–drug interaction, we show below the equilibrium constants (K) of ethidium bromide–salmon sperm DNA interaction determined in various solvents:

35 mM Tris–HCl, pH = 8.0	$K=1.1\times10^{6}\ M^{-1}$
35 mM Tris–HCl, pH = 8.0 + 72 mM KCl	$3.0\times10^{5}\ M^{-1}$
35 mM Tris HCl, pH = 8.0 + 72 mM KCl + 5 mM $MgCl_2$	$6.0\times10^{4}\ M^{-1}$
35 mM Tris HCl, pH = 8.0 + 72 mM KCl + 5 mM $MgCl_2$ + 5 mM DTT	$5.6\times10^{4}\ M^{-1}$
35 mM Tris–HCl, pH = 8.0 + 72 mM KCl + 5 mM $MgCl_2$ + 5 mM DTT + 5 mM spermidine	$5.0\times10^{4}\ M^{-1}$
35 mM Tris–HCl, pH = 8.0 + 72 mM KCl + 5 mM $MgCl_2$ + 5 mM DTT + 5 mM spermidine + 0.01% bovine serum albumin	$4.0\times10^{4}\ M^{-1}$

Thus, the greater is the ionic strength, the lower is the K value.[14] MacGregor *et al.*[15] found that $K=3.5\times10^{5}\ M^{-1}$ in 20 mM Tris–HCl + 100 mM NaCl + 1 mM EDTA (pH = 7.2) at 20 °C, and this K value is close to our value $K=3.0\times10^{5}\ M^{-1}$ found in 35 mM Tris–HCl + 72 mM KCl. It has also been shown that the effect of Mg^{2+} seems

Table 2. Three Parameters, K (Equilibrium Constant), n (Number of Base Pairs, Occupied by the Drug Molecule Bound to DNA) and ϕ (Angle of Unwinding) Determined for the Binding Reactions of Three Drugs to pBR322 DNA (in Buffer-T at 37 °C)

	K (M^{-1})	n	ϕ (°)
Aclacinomycin A	72000	4.0	8 ± 2
Daunomycin	71000	3.4	12 ± 2
Ethidium bromide	40000	3.3	15 ± 4

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especially great, while the effects of DTT, spermidine, and serum albumin seem relatively small. For the aclacinomycin–DNA system, Fritzsche and Walter[16] gave $K=(7.58\pm2.15)\times10^6\,\mathrm{M}^{-1}$, for example. Unfortunately, however, their solvent is greatly different from buffer-T, and therefore we should not use this value for our estimation of ϕ value.

It would be of great interest to find out here whether the unwinding angle (ϕ) itself (besides the equilibrium constant K) depends upon the ionic strength as well as upon other factors of the solvent. Unfortunately, however, such an attempt was not successful by our present method. In a medium with higher ionic strength, for example, the topoisomerase activity has been found to be inhibited. The solvent condition for this enzyme seems to be quite narrow.

A number of investigators have given contradictory conclusions about the ϕ value of the ethidium–DNA system. According to an X-ray crystallographic study of Fuller and Waring,[17] ϕ was 12°, and Vinograd *et al.*[18] also concluded that $\phi=12°$ on the basis of their alkaline titration. Wang,[19,20] however, reported that ϕ should be as high as 26°. Keller[21] also gave $\phi=26\pm2°$ on the basis of his electrophoresis experiment. This same author[21] used the m value he obtained through his "buoyant-separation" experiment, and this is a subject of debate. It has not yet been established whether it is appropriate to use for the m value in Eq. 8. It should be pointed out here again that the use of a proper K value is the most critical condition to reach a significant ϕ value. If we use the K value given by Bresloff and Crothers,[22] for example, in combination with our $\Delta\tau$ value, ϕ comes out to be 26° through Eq. 8. We have to claim, however, that $\phi=15\pm4°$ through the proper value of K, *i.e.*, $40000\,\mathrm{M}^{-1}$.

Pachter *et al.*[23] reported that DNA unwinding angle ϕ was 13° for adriamycin, which is very similar to daunomycin (position C14 CH_3 of the latter is replaced by CH_2OH in the former), on the basis of their viscosity and fluorescence measurements. This value of ϕ was determined on an assumption that the unwinding angle of DNA by ethidium is 26°. As detailed above, the ϕ value for ethidium must actually be smaller than 26°, and therefore, ϕ for daunomycin would also be smaller than 13°. We propose that $\phi=12\pm2°$ in the present study. It is interesting that, according to an X-ray analysis[24] of the daunomycin + $d(CGTACG)_2$ crystal, ϕ is found to be 7°, much smaller than what has been found in buffer-T. This is important data for future clarification of the effects of crystalline force field, base sequence, and length of DNA upon the detailed molecular structure of the DNA duplex.

Finally, we propose that aclacinomycin A molecule binds to DNA duplex with equilibrium constant $K=72000\,\mathrm{M}^{-1}$ and causes an unwinding of the duplex by $8\pm2°$ in buffer-T at 37 °C. This means that what was proposed by Pachter *et al.*[23] ($\phi=11.1\pm0.2°$) is probably an overestimated value. The proposed unwinding angle (8°) for aclacinomycin A with a trisaccharide moiety is smaller than that (12°) for daunomycin, with monosaccharide moiety, and the latter is smaller than that (15°) for ethidium, with no saccharide moiety. This finding suggests that the sugar portion of drug reduces the unwinding angle. This suggestion raises a desire to examine (by our present method) anthracyclines with the same chromophore but different numbers of amino-sugars. The unwinding of the DNA duplex is one of the key steps in DNA replication and transcription. Any of the quantitative data of the duplex unwinding must be useful in elucidating the mechanics and dynamics of these genetic phenomena. Aiming at such a goal, we have examined Hoechst 33258 and chromomycin A_3, both of which have been suggested to make groove binding, instead of intercalation, to DNA duplex. Details will be published in our next report.

Acknowledgments We wish to express our sincere thanks to Professor Kazuhisa Sekimizu (Kyushu University) and Professor Yoshimi Maeda (Iwaki-Meisei University) for their valuable advice during our study. Our thanks are also due to Dr. Kazuhiro Kanda (Iwaki-Meisei University) for his kind advice in our programming of the calculations of equilibrium constant (K). Messrs. Yoshihiro Miyazaki, Takahiro Ishii, Yutaka Yoshida, and Naomi Kato helped us in our experiments.

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