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Effects of DNA-binding drugs on T4 DNA ligase

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A number of DNA intercalating and externally binding drugs have been found to inhibit nick sealing, cohesive and blunt end ligation, AMP-dependent DNA topoisomerization and EDTA-induced DNA nicking mediated by bacteriophage T4 DNA ligase. The inhibition seems to arise from drug-substrate interaction so that formation of active DNA–Mg^{2+}-AMP-enzyme complex is impaired while assembled and active complexes are not disturbed by drug binding to the substrate.

INTRODUCTION

DNA ligases have been found in a variety of sources. These enzymes catalyze the synthesis of the phosphodiester bond between adjacent 3′-hydroxyl and 5′-phosphoryl termini in nicked duplex DNA. The enzymes from T4 and T7 phages and from yeast, *Drosophila*, mammalian and plant cells [1–5] use ATP as coenzyme. Those from *Escherichia coli* and *Bacillus subtilis* use NAD^+ [6]. DNA ligases play essential roles in DNA replication, repair and recombination [7–9]. DNA ligases possess an AMP-dependent DNA topoisomerase activity, therefore representing a new class of enzymes, the closing/nicking-closing enzymes [10–11]. This property has also suggested a mechanism of action similar to that of DNA topoisomerases, i.e. that ligation occurs through two separate steps: (i) formation of an enzyme intermediate by transfer of an adenylate group from the coenzyme to a lysine residue of the enzyme; (ii) phosphodiester bond formation inside a complex involving DNA, Mg^{2+}, AMP and ligase.

During recent studies on the effects of anthracyclines on DNA tertiary structure we have found that T4 DNA ligase is resistant to several intercalating anthracyclines but sensitive to those bearing a free amino group on the sugar [12]. This observation suggested that the ability of anthracyclines to inhibit DNA ligation is not a simple consequence of their intercalation within the DNA molecule. In this study we have now more deeply investigated the effects of a typical anthracycline, doxorubicin, and of other intercalating and non-intercalating drugs, on the action of T4 DNA ligase. For this purpose we used different types of substrates and paid special attention to the different steps of the DNA ligation reaction, to the associated AMP-dependent DNA topoisomerase activity and finally to the EDTA-induced enzyme-dependent DNA nicking.

MATERIALS AND METHODS

Polynucleotide ligation assays

Nicked circular or linear substrate DNA containing either sticky or blunt ends was incubated with DNA ligase in the following reaction buffer (20 μl): 66 mM-Tris/HCl, pH 7.6, 6.6 mM-$MgCl_2$, 1 mM-dithioerythritol, 0.7 mM-ATP plus the desired drug concentration. After incubation at the required temperature, reactions were stopped by addition of EDTA (20 mM final concentration). Ligated samples were analysed on 1% agarose gels made in Tris/acetate/EDTA (TAE) buffer (40 mM-Tris acetate, 2 mM-EDTA, 18 mM-NaCl, final pH 8). Gels were run at 1.4 V/cm for 14 h at 25 °C in the same buffer. Staining and destaining of gels and analysis of picture negatives were performed as described elsewhere [13]. DNA ligase activity was also measured by the assay described by Weiss *et al.* [14], using calf thymus DNA as substrate.

Inhibition of [^{3}H]poly(dA-dT) ligation was determined as previously described [12]. One unit of T4 DNA ligase was considered the amount of enzyme activity converting 100 nmol of poly(dA-dT) to an exonuclease III-resistant form within 30 min at 30 °C. At concentrations used in the present work none of the tested compounds caused breakdown of the ligated polymer on boiling for 3 min. Some of the tested drugs also inhibited the exonuclease III: in order to overcome this situation we optimized the conditions for studying the inhibition of ligation by utilizing as substrate poly(dA-dT) preparations averaging 60 nucleotides in length and an excess amount of exonuclease III.

Enzyme adenylation

T4 DNA ligase (1 unit) was incubated at 37 °C for 10 min in the same reaction mixture (10 μl) utilized for DNA ligation except that [^{3}H]ATP was present at a concentration of 2.3 μM (specific radioactivity 5.8×10^4 d.p.m./pmol). The reaction was stopped by adding 20 μl of a solution containing 10 mM-EDTA and 150 μg of bovine serum albumin/ml, then 25 μl of the reaction mixture was spotted onto Whatman GF/C filters and batch-washed with trichloroacetic acid, dried and counted.

Materials

Naturally supercoiled pAT153 DNA was purified on CsCl and sucrose gradients as described by Maniatis *et al.* [15]; blunt and sticky end substrates were obtained by restriction of pAT153 DNA with *Nru*I and *Eco*RI,

Abbreviations used: *R*-Ida, (7*R*,9*R*)-idarubicin; DDH-Epi, 3′-deamino-4-demethoxy-3″-hydroxyepirubicin; DAPI, 4′,6-diamidino-2-phenylindole.

respectively; nicked circular pAT153 DNA substrate was prepared by pancreatic DNAase I treatment according to Depew & Wang [16]. Relaxed, covalently closed pAT153 was prepared as described by Montecucco & Ciarrocchi [10]. [^{3}H]Poly(dA-dT) was prepared as previously described [12]. [^{32}P]DNA was prepared according to Weiss *et al.* [14]. Doxorubicin, (7*R*,9*R*)-idarubicin (*R*-Ida) and the 3′-deamino-4-demethoxy-3″-hydroxy-epirubicin (DDH-Epi) were kindly supplied by Dr. F. Arcamone, Farmitalia-Carlo Erba. Ethidium bromide and 4′,6-diamidino-2-phenylindole (DAPI) were purchased from Sigma Chemical Co.; agarose was a Bio-Rad Laboratories product. Bacteriophage T4 DNA ligase was purchased either from Toyobo, Bethesda Research Laboratories or Boehringer, and nucleases were from Boehringer.

RESULTS AND DISCUSSION

Inhibition of T4 DNA ligase by ethidium bromide, DAPI, doxorubicin and (7*R*,9*R*)-idarubicin

We have recently shown that bacteriophage T4 DNA ligase is resistant to several intercalating anthracycline derivatives but is inhibited by other intercalating anthracyclines bearing a free amino group on the sugar [12]. The suggestion that the inhibitory properties of effective drugs are not a simple consequence of intercalation is here further supported by new findings with another unrelated intercalating agent, ethidium bromide, and more interestingly with the externally binding, non-intercalating drug DAPI. In fact, we observed that ethidium bromide, contrary to previous findings using [^{32}P]oligo(dT)$_{10}$·poly(dA) as substrate [17], is an inhibitor of T4 DNA ligase (Figs. 1 and 2*a*). The DNA binding drug DAPI [18] is also a good inhibitor of poly(dA-dT) ligation catalysed by the T4 enzyme (Fig. 1*a*). However, contrary to what we observed with ethidium bromide and the inhibiting anthracycline doxorubicin, DAPI is a less effective inhibitor when natural DNA is used as a substrate (Fig. 1*c*). When poly(dA-dT) is used as substrate, the higher inhibitory efficiency of DAPI might be ascribable to its ability to externally bind AT sequences, where it probably interacts within the narrow groove of the double helix [19]. On the contrary, it has not been possible to determine the inhibitory activity of DAPI by the assay of Weiss *et al.* [14] (Fig. 1*b*) because of its strong interference with the alkaline phosphatase utilized in this assay [14]. *R*-Ida, a non-intercalating, weak externally binding anthracycline derivative [12], is always ineffective (Figs. 1*a* and 1*b*). In our previous work

Table 1. Effect of DNA-binding drugs on poly(dA-dT) ligation catalysed by T4 DNA ligase.

	Ligation	
	ID_{50} (μM)*	*K*†
Ethidium bromide	4.3±0.9	0.23
DAPI	2.4±0.03	6.9×10^{-2}

* Drug concentration inhibiting 50% ligation of poly(dA-dT), obtained from *K* values.

† Coefficient of I^3 in eqn. (1) (μM^{-3}); for ethidium bromide, coefficient of *I* in eqn. (2) (μM^{-1}).

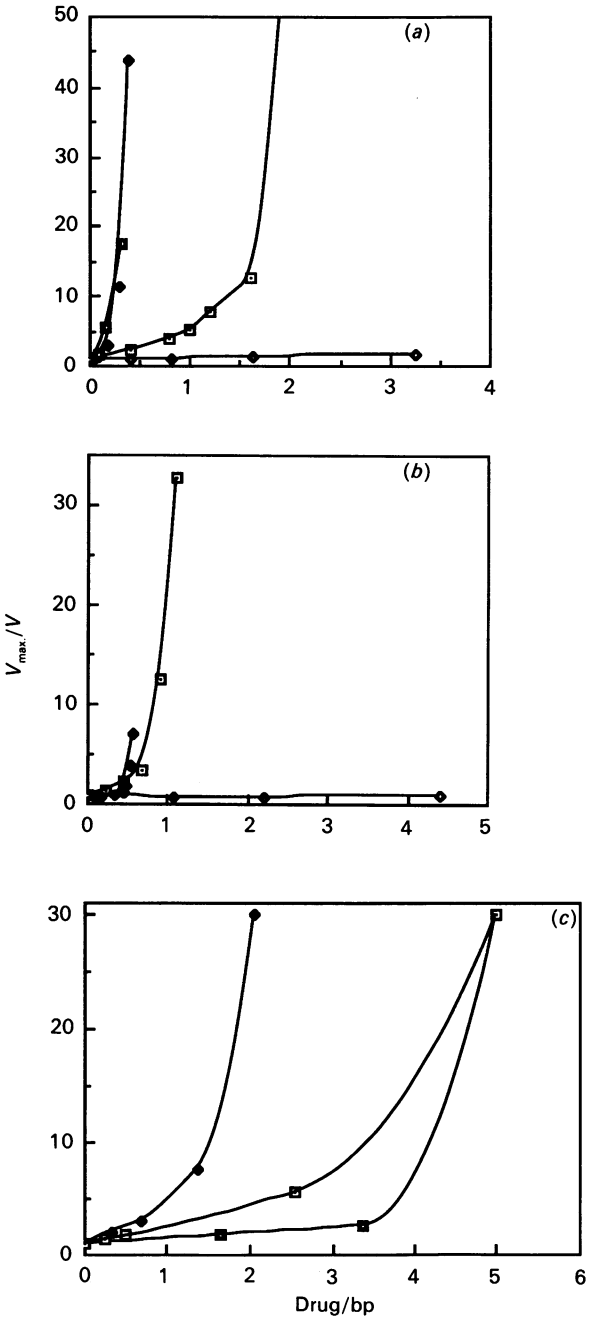

Fig. 1. Inhibition of T4 DNA ligase activity by DNA-binding drugs as determined: (*a*) by poly(dA-dT) circularization, (*b*) by nick sealing in DNA according to Weiss *et al.* [14], and (*c*) by DNA circle-ligation assay.

⊡, Ethidium bromide; ◆, doxorubicin; ■, DAPI; ◇, *R*-Ida. (*a*) Inhibition of [^{3}H]poly(dA-dT) ligation was monitored as a decrease in the conversion of the substrate to an exonuclease III-resistant form. [^{3}H]Poly(dA-dT) bp concentration was 25 μM. (*b*) Inhibition of nicked DNA ligation by ethidium bromide, doxorubicin and *R*-Ida was monitored as a decrease in the conversion of [^{32}P]phosphomonoesters in nicked DNA to a form resistant to phosphatase. DNA bp concentration was 227 μM. (*c*) Inhibition of DNA circle-ligation by ethidium bromide, doxorubicin and DAPI was monitored as accumulation of unligated, nicked substrate by the assay of circularization of pancreatic DNAase I-treated plasmid DNA. DNA bp concentration was 10 μM.

the inhibition of T4 DNA ligase by anthracycline derivatives bearing an amino group on the sugar was found to be described by a cubic function of drug concentration [12]. The same cubic function:

$$V_{max.}/V = KI^3 + 1 \quad (1)$$

where I is the inhibitor concentration, $V_{max.}$ is the initial velocity in absence of drug, V the initial velocity in the presence of the drug and K is a constant, appears now to describe the inhibition of poly(dA-dT) ligation by DAPI (Fig. 1*a*) as well. In the case of ethidium bromide, again tested on the synthetic polynucleotide, the best function seems to be:

$$V_{max.}/V = KI + 1 \quad (2)$$

up to a drug/bp ratio of 0.8, covering approximately

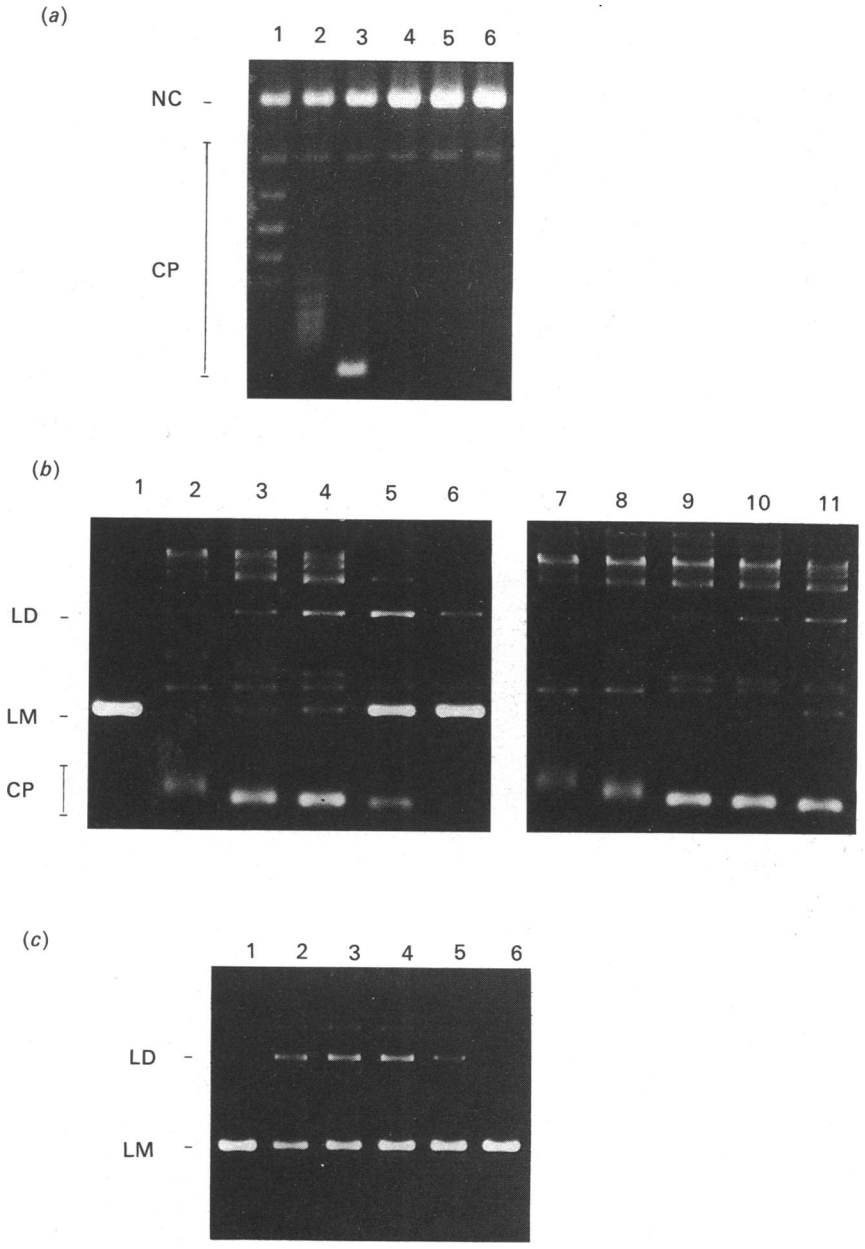

Fig. 2. Inhibition of DNA ligation by ethidium bromide using different DNA substrates

(*a*) Pancreatic DNAase I-treated pAT153 DNA (nicked circle). The DNA substrate (20 μM as bp) was incubated at 0 °C for 10 min in the presence of 0, 8, 16, 25 and 33 μM-ethidium bromide in the reaction mixtures of lanes 1–5, respectively. Lane 6, unligated substrate. (*b*) *Eco*RI-digested pAT153 DNA (63 μM as bp). The linear substrate was incubated with 0.1 unit of T4 DNA ligase in the presence of 0, 13, 25, 51 and 77 μM-ethidium bromide in the reaction mixtures of lanes 2–6, respectively; with 2.4 unit and in the presence of identical concentrations as before in lanes 7–11. Incubation was at 15 °C for 30 min. Lane 1, unligated substrate. Electrophoresis was as usual except that 13 μM-chloroquine was present in gel and buffer for better resolution of circular DNA products. (*c*) *Nru*I-digested pAT153 DNA (63 μM as bp). The linear substrate was incubated with 0, 13, 25, 51 and 77 μM-ethidium bromide in reaction mixtures of lanes 2–6, respectively. Incubation was done at 15 °C for 16 h. Lane 1, unligated substrate. NC, nicked circles; CP, circularized products; LD, linear dimer; LM, linear monomer.

80% of the inhibition. Beyond these values the function is no longer linear (Fig. 1*a*).

K values for ethidium bromide and DAPI obtained by least-squares methods applied to eqns. (1) and (2) are listed in Table 1. The findings with doxorubicin and DAPI are surprising even considering that polynucleotide binding by both intercalating and externally binding drugs might be of co-operative type. In fact, studies on DNA unwinding by anthracyclines done by comparable techniques had shown the existence of a linear relationship between drug concentration and DNA unwinding. This had suggested that the amount of bound drug is linearly proportional to drug concentration.

The comparison of inhibition data using poly(dA-dT) or nicked DNA as substrates suggests that all tested drugs are more effective in inhibiting the ligation of the synthetic substrate. The substrate composition and/or structure seems to affect not only the inhibitory property of DAPI but to a lesser extent also the behaviour of all tested drugs.

The results presented in Fig. 1 have been obtained by means of three different assays that measure the covalent joining of polynucleotide chains. Each kind of assay contains different amounts of polynucleotide substrate. In addition all drugs considered in the experiments of Fig. 1 bind to DNA. Therefore to compare the results obtained with various drug concentrations, we opted for a representation where such concentrations are expressed as the ratio of drug concentrations versus polynucleotide substrate concentrations.

Effect of ethidium bromide on the ligation of substrates with cohesive and blunt ends

To investigate further the mechanism of inhibition of DNA ligation, we then compared the effect of ethidium bromide on the ligation of DNA molecules with cohesive and blunt ends. In the experiment shown in Fig. 2(*b*), *Eco*RI-digested pAT153 DNA was the substrate and two different amounts of enzyme (0.1 and 2.4 units/assay) were challenged with identical, increasing concentrations of ethidium bromide. Inhibition of DNA ligation was observed in both cases, but complete inhibition was detected only in the presence of the lower enzyme concentration. Inhibition of the lower amount of enzyme was deduced from: (*a*) progressive reduction in dimension of ligated linear products, (*b*) reduction in the amount of circular products, and (*c*) accumulation of unreacted substrate. However, addition of the higher amount of enzyme almost completely overcame the inhibition. Under these conditions a limited inhibition of ligation is evidenced from the measurable reduction in the overall dimension of ligated products and from initial accumulation of unreacted substrate at the highest drug concentration. These observations suggest that effective drugs do not act by substrate subtraction.

The experiment of Fig. 2(*c*), where *Nru*I-digested pAT153 DNA was the substrate, proved that ethidium bromide can completely prevent blunt end ligation as well.

Doxorubicin and DAPI (at a drug-to-bp ratio higher than 5) were also found capable of preventing ligation of substrates utilized in the experiments of Fig. 2 (results not shown).

Lack of inhibition of the T4 DNA ligase adenylation

The first step of ligation, the DNA ligase adenylation, can be easily determined by incubating the enzyme with [^{3}H]ATP in the absence of polynucleotide substrate. None of the drugs tested so far were found to inhibit this step of reaction even at drug concentrations almost 100 times higher than those found to affect the joining reaction (Fig. 3). We concluded that these drugs do not directly interact with the enzyme. For their action on DNA ligase these drugs probably need to bind to DNA.

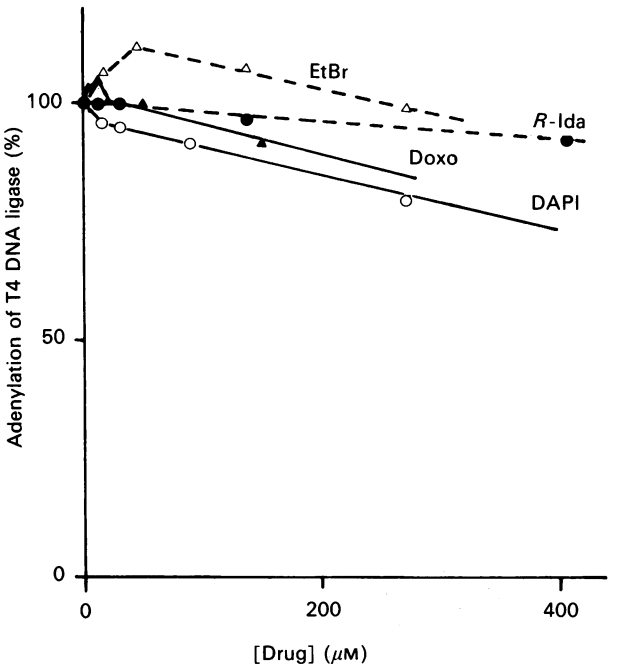

Fig. 3. T4 DNA ligase adenylation in the presence of DNA-binding drugs

Enzyme adenylation was followed as trichloroacetic acid-precipitable radioactivity after incubation of 1 unit of enzyme with [^{3}H]ATP for 10 min at 37 °C. The data obtained with ethidium bromide are in close agreement with those reported by Kalisch & van de Sande [17]. Abbreviations: EtBr, ethidium bromide; Doxo, doxorubicin.

Therefore, the ability of such different drugs to inhibit the DNA joining activity of bacteriophage T4 DNA ligase can neither be ascribed to intercalation *per se* nor to an interaction with the enzyme itself.

Inhibition of the AMP-dependent DNA relaxing activity

We then investigated whether drugs affecting DNA ligation are also capable of interfering with the AMP-dependent DNA topoisomerase activity of T4 DNA ligase [10]. To this purpose the ability of T4 DNA ligase to relieve superhelical tension from the substrate was analysed in the presence of two anthracycline derivatives: the first, doxorubicin, is an inhibitor of DNA ligation; the second, DDH-Epi, is inactive against DNA ligase but effective as DNA intercalator [12]. When a population of relaxed molecules of plasmid pAT153 was exposed to increasing concentrations of these two anthracycline derivatives, only doxorubicin inhibited the reaction while DDH-Epi was found to be inactive at concentrations known to introduce a similar degree of superhelicity at the same temperature (Fig. 4). Upon exposure to increasing drug concentrations, the initial relaxed substrate

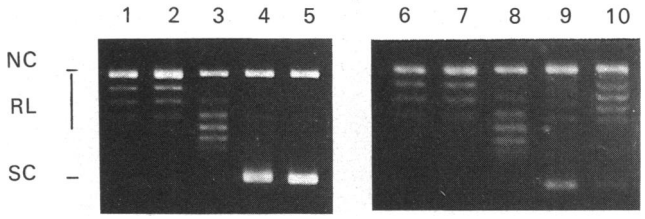

Fig. 4. AMP-dependent relaxation of positively supercoiled DNA

The T4 DNA ligase ability to relax supercoiled substrates (pAT153, 154 μM as bp) was monitored in the presence of 1.5, 3 and 9 μM-DDH-Epi in experiments of lanes 3, 4 and 5, respectively, and of 0.5, 1.5 and 4.5 μM-doxorubicin in lanes 8, 9 and 10, respectively. Added drugs were removed by butanol extraction just before loading the samples onto the gel. Lanes 1 and 6, original substrate; lanes 2 and 7, substrate incubated in the presence of ligase and AMP but in the absence of added drugs.

becomes increasingly more positively supercoiled under the conditions of reaction and gel analysis utilized for these experiments. T4 DNA ligase is capable of relieving both negative and positive supertwists [10], exactly as the *E. coli* enzyme [20]. Therefore, DNA molecules relaxed by DNA ligase in the presence of intercalated drugs would appear negatively supercoiled upon drug removal. If the relaxation reaction were inhibited by an added drug, the substrate would remain relaxed upon drug removal. From the results presented in Fig. 4 DDH-Epi appears ineffective as inhibitor of DNA relaxation while very effective as an intercalator. The substrate becomes in fact progressively supercoiled (Fig. 3, lanes 2–5). On the contrary, while low concentrations of doxorubicin allow part of the substrate to become supercoiled (Fig. 3, lanes 8–9), higher drug concentrations favour the accumulation of unreacted substrate that no longer becomes supercoiled (Fig. 3, lanes 9–10). Doxorubicin (10 μM) completely inhibits DNA relaxation. Interestingly, the reaction products in the presence of doxorubicin are either fully supercoiled or completely relaxed, suggesting that the enzyme, in the presence of inhibitory drug, works even more processively than in the absence of drug [10]. The more processive mode of action of the T4 enzyme in the presence of doxorubicin recalls the increased processivity of *Micrococcus luteus* DNA topoisomerase I acting on a u.v.-damaged substrate [13]. It is also interesting that in this last case inhibition of DNA relaxation was observed. Although both anthracycline derivatives utilized in the experiments of Fig. 4 are good DNA intercalating and unwinding agents, doxorubicin is the only effective inhibitor of DNA ligation [12]. Thus the specific inhibition of the AMP-dependent DNA relaxation by doxorubicin further supports our hypothesis that the stepwise DNA relaxing activity is strictly associated with the DNA ligase activity [10].

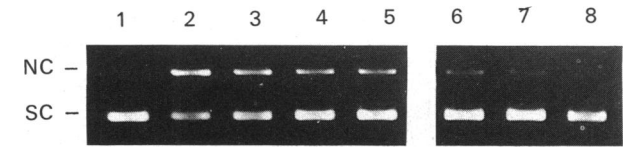

Fig. 5. Titration of DNA–Mg^{2+}–AMP–ligase complexes

Complexes were allowed to form at 0 °C by mixing 100 ng of naturally supercoiled pAT153 with 1 unit of T4 DNA ligase in the usual reaction mixture. Active complexes were titrated by EDTA addition (20 mM) which induces DNA nicking at the complex level. Lane 1, supercoiled substrate; lane 2, EDTA-induced, ligase-dependent nicking; 3, 4 and 5, EDTA-induced nicking in the presence of 1, 1.5 and 2.2 μM-DDH-EPi, respectively; lanes 6, 7 and 8, EDTA-induced nicking in the presence of 1, 1.5 and 2.2 μM-doxorubicin. SC, supercoiled substrate; NC, nicked product.

Inhibition of formation of active DNA-enzyme complexes

Doxorubicin may inhibit the stepwise relaxation of DNA by preventing the formation of the DNA–Mg^{2+}–AMP–ligase complex. This interpretation would be in perfect agreement with all the results so far described. It is possible to study the formation of these complexes specifically in the presence of an excess of enzyme by blocking the reaction with EDTA at 0 °C [10]. Under these conditions added EDTA titrates the fraction of complexes in which the phosphodiester bond is transiently opened, inducing the formation of nicked substrate [10]. In Fig. 5 we present the results of an experiment in which the formation of such complexes was allowed to take place in the presence of increasing concentrations of doxorubicin. Complexes were monitored as EDTA-induced nicking. Even in this case doxorubicin was found very effective in preventing the formation of active complexes while DDH-Epi was much less effective at the same concentrations.

CONCLUSIONS

The results of the present study on T4 DNA ligase inhibition by DNA binding drugs can therefore be summarized as follows: (i) the first step of reaction, the enzyme adenylation, is not the target of the examined drugs; (ii) drug binding on the substrate is an important factor in inhibiting the joining activity of DNA ligase, while (iii) intercalation is not strictly necessary; (iv) formation of EDTA-sensitive protein–DNA complexes is affected, while (v) active ligase–DNA complexes are not impaired by the presence of the drugs.

In conclusion, our observations strongly suggest a model of DNA ligase inhibition in which drug molecules bound to the substrate prevent DNA ligase–AMP–Mg^{2+}–DNA complex formation.

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