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## Purification and Characterization of *Plasmodium berghei* DNA Topoisomerases I and II: Drug Action, Inhibition of Decatenation and Relaxation, and Stimulation of DNA Cleavage

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**ABSTRACT:** It has recently been suggested that topoisomerases could be important targets for drugs used in several diseases. This prompted us to purify and characterize the topoisomerases I and II present in the erythrocytes of protozoan parasites of the genus *Plasmodium*, the causative agent of malaria, in order to later use these enzymatic systems in antimalarial drug assays. The topoisomerases were purified from *Plasmodium berghei*, a parasite of mouse red cells. The *Plasmodium* topoisomerase II consists of two subunits with a molecular weight of about 160K. The enzyme is ATP- and  $Mg^{2+}$ -dependent. The conditions for the reactions of relaxation, unknotting, decatenation, and catenation were found to be similar to those observed with enzymes from other eukaryotic cells. The *Plasmodium* topoisomerase I is a monomeric enzyme with a  $M_r$  of 70K–100K. It is ATP-independent and  $K^+$ - or  $Na^+$ -dependent.  $Mg^{2+}$  is not required for relaxation but stimulates the reaction. Topoisomerase II was more sensitive to drug action than topoisomerase I. The most active drugs were the ellipticine derivatives. The antimalarial drugs, currently used in human clinical therapy, were poor inhibitors. Some antitumoral drugs stimulated the double-stranded DNA cleavage activity of *Plasmodium* topoisomerase II, like that of mammalian topoisomerases II. Antimalarial drugs had no stimulating activity. It is therefore suggested that *Plasmodium* topoisomerases are not good targets for antimalarial drugs.

The DNA topoisomerases are enzymes that catalyze the concerted breakage and rejoining of the DNA backbone. Two categories of enzymes have been found in a variety of organisms. The type I topoisomerases transiently cut and then reseal one DNA strand, while the type II enzymes cut and reseal both strands at the same time, so that the linking number changes by steps of one for the former enzymes and by steps of two for the latter enzymes (Brown & Cozzarelli, 1979). The bacterial topoisomerases have been the best studied enzymes, and some of their biological properties are now well established. The DNA gyrase in particular, a type II enzyme, has been shown to be involved in DNA replication, gene expression, and DNA recombination and repair (Cozzarelli, 1980; Gellert, 1981; Drlica, 1984; Wang, 1985).

If the in vitro properties of eukaryotic topoisomerases of various sources are well characterized, their biological functions are, as yet, not well-known. However, recent results seem to indicate that these enzymes may be involved in fundamental biological processes (DiNardo et al., 1984; Ryoji & Worcel, 1984; Glikin et al., 1984; Noguchi et al., 1983; Jazwinski & Edelman, 1984; Goto & Wang, 1984; Fleischmann et al., 1984; Wang, 1985). It has also been shown that the level of topoisomerase II activity is increased during rat liver regeneration after partial hepatectomy (Duguet et al., 1983) and in rapidly proliferating cells such as tumor cells (Riou et al., 1985). Other studies have shown that reactions catalyzed by topoisomerases II extracted from rat liver and trypanosomes are inhibited by various drugs at low concentrations (Douc-Rasy et al., 1983a, 1984). Recent studies have also shown that type II topoisomerases are stimulated to cleave DNA in vitro or in vivo by some antitumor compounds (Chen et al., 1984; Tewey et al., 1984; Pommier et al., 1984a,b). This suggests

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that topoisomerases may be good targets for drugs in rapidly proliferating cells. In certain parasitic diseases, a rapid multiplication of parasites is observed in the host. This is the case of protozoan parasites of the genus *Plasmodium*, the causative agent of malaria in human beings and in other vertebrates. The *Plasmodium* undergoes a complex evolutive cycle inside the red cells of its host and proliferates very rapidly since more than 70% of the red cells are infested within a few days. Numerous antimalarial drugs have been proposed for human therapy, but the parasites are often resistant (Peters, 1984).

These findings prompted us to purify and characterize type I and II topoisomerases from *Plasmodium* in order to use these enzymatic systems in antimalarial drug assays. We demonstrate the inhibitory effects of several drug series in some of the reactions catalyzed by *Plasmodium berghei* type I and II topoisomerases. We also show the stimulation by some chemical agents of the double-stranded DNA cleavage by type II topoisomerase.

#### MATERIALS AND METHODS

**Isolation of Parasites.** *Plasmodium berghei* (strain sensitive to chloroquine provided by Dr. J. Le Bras, Institut de Médecine et d'Epidémiologie Tropicale, Paris) was inoculated in Swiss female mice (25–30 g) by intraperitoneal injection and maintained until a 70–80% parasitemia was reached, as determined by microscopic examination of Giemsa-stained blood smears. The infected blood was collected by cardiac puncture in the presence of heparin. The blood from 50 infested mice was centrifuged (6 min at 600g), and the collected cells were washed twice with cold isotonic solution containing 0.15 M NaCl–5 mM sodium phosphate buffer, pH 7.2. The buffy coat was discarded, and the red cell pellet was resuspended in the same isotonic buffer and filtered twice through cellulose fibers to eliminate monocytes. The resulting suspension was layered on Ficoll-Hypaque (Pharmacia Fine Chemicals) (v/v) and centrifuged for 30 min at 300g in order to discard the lymphocytes. After being washed, the packed red cells were resuspended in an equal volume of isotonic buffer containing 0.2% (w/v) saponin, 1 mM PMSF,<sup>1</sup> 4 µg/mL aprotinin, 10 mM β-mercaptoethanol, and 0.5 mM dithiothreitol and incubated with gentle stirring for 20 min at 37 °C. The liberated parasites were centrifuged for 6 min at 2500g and then washed twice with the same isotonic buffer containing protease inhibitors. The final washing was carried out in the extraction buffer: 50 mM Tris-HCl, pH 7.9, 4 mM EDTA, 10 mM β-mercaptoethanol, 0.5 mM dithiothreitol, 4 µg/mL aprotinin, and 1 mM PMSF.

**Preparation of Crude Extract.** The parasites (1.0–1.5 g of wet weight cells) resuspended in the extraction buffer were broken by Dounce homogenization. After 15–20 min at 0 °C, the nuclei were lysed by slow addition of a 5 M NaCl solution while stirring, until a 1 M NaCl final concentration was obtained. After being stirred again for 10 min at 0 °C, the nucleic acids were precipitated by slow addition of solid

poly(ethylene glycol) (PEG 6000) (6% w/v final concentration). The mixture was maintained for 45 min at 0 °C with occasional stirring and then centrifuged at 15000g for 45 min. The PEG supernatant was used for further purification.

**Topoisomerase Assays.** The enzymatic activities of topoisomerases I and II were monitored through the different purification steps. Two assays, the decatenation of kinetoplast DNA (kDNA form I) from *Trypanosoma cruzi* for the detection of topoisomerase II and the relaxation of supercoiled DNA for the detection of topoisomerase I, were routinely used. For the decatenation, the kDNA substrate, prepared as previously described (Riou & Gutteridge, 1978), was used at a concentration of 0.10 µg in 20 µL of a solution containing 10 mM Tris-HCl, pH 7.9, 100 mM KCl, 10 mM MgCl<sub>2</sub>, 1 mM ATP, 0.5 mM dithiothreitol, 0.5 mM EDTA, and 15 µg/mL BSA. For the relaxation assay, the substrate was supercoiled DNA (0.1 µg) obtained from the replicative form of bacteriophage fd or from simian virus 40 (SV40), and the reaction mixture contained 100 mM KCl without ATP to detect topoisomerase I and with 0.5 mM ATP to detect topoisomerase II. We also tested purified topoisomerase II for its unknotting activity. Double-stranded knotted DNA was isolated from tailless P4 phage capsids according to the method of Liu et al. (1981) and used in a reaction mixture similar to that used for decatenation. The reaction mixtures were incubated for 30 min at 37 °C and stopped by addition of SDS. Samples were applied on horizontal 1% agarose slab gels for relaxation, 2% for decatenation, and 0.7% for unknotting in 36 mM Tris, 30 mM NaH<sub>2</sub>PO<sub>4</sub>, and 1 mM EDTA, pH 7.7, and run for 2 h at room temperature at 6 V/cm. The gels were stained with ethidium bromide and photographed under UV light (Riou et al., 1983).

The degree of decatenation was estimated on the basis of the migration, in agarose gels, of the 1.45-kb minicircles liberated from the kDNA network (Douc-Rasy et al., 1983a). The negative photographs of the gels were scanned with the laser densitometer Ultrosan (LKB), and the peak areas were evaluated. One unit of topoisomerase II activity is defined as the amount of enzyme that converts the kDNA network into minicircles under the conditions of the assay (Douc-Rasy et al., 1983a). The relaxation activity of topoisomerase I was assayed as described above. One unit of topoisomerase I activity is defined as the amount of enzyme that relaxes supercoiled fd phage DNA completely under standard assay conditions.

**Purification.** All operations were performed at 4 °C unless otherwise indicated. The purification of topoisomerases I and II was performed from the same PEG supernatant. The PEG supernatant (68 mL) was loaded onto a phosphocellulose (P11) column (1.5 × 10 cm) equilibrated with 50 mM Tris-HCl, pH 7.9, 0.2 M NaCl, 0.5 mM EDTA, 0.5 mM dithiothreitol, 10 mM β-mercaptoethanol, 4 µg/mL aprotinin, and 1 mM PMSF. The column was washed with the same buffer (20 mL), and the enzyme preparation was eluted with 110 mL of a 0.2–1 M potassium phosphate (pH 7.0) linear gradient in solution G containing 20% w/v glycerol, 10 mM β-mercaptoethanol, and 1 mM PMSF.

**(A) Topoisomerase II.** Fractions 6–9 of the phosphocellulose column, eluted between 0.35 and 0.45 M potassium phosphate, contained fully decatenated kDNA. They were pooled (8 mL), diluted twice in solution G, and loaded onto a hydroxyapatite column (0.9 × 1.5 cm) equilibrated with 0.2 M potassium phosphate, pH 7.0, in solution G. The column was washed with the same buffer, and the proteins were eluted stepwise with 1.5 mL of 0.4, 0.55, 0.65, and 1.0 M potassium

<sup>1</sup> Abbreviations: kDNA, kinetoplast DNA; PEG, poly(ethylene glycol); SDS, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride; EtdBr, ethidium bromide; EtdDi, ethidium dimer; AcrEtdDi, acridine-ethidium heterodimer; EI, ellipticine; EI<sup>m</sup>, ellipticinium; NMHE, 2-CH<sub>3</sub>-9-OH-EI<sup>m</sup>; IsoEI<sup>m</sup>, isoellipticinium; 10-OCH<sub>3</sub>-2-CH<sub>3</sub>-7H-pyrido[4,3-c]carbazolium; IsoEI<sup>m</sup>Di, isoellipticinium dimer; mAMSA, 4'-(9-acridinylamino)methanesulfon-*m*-anisidine; VM26, 4'-demethylepipodophyllotoxin 6-thenylidene-β-D-glucoside; VP16, 4'-demethylepipodophyllotoxin 6-ethylidene-β-D-glucoside; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin.



phosphate. Two active fractions of 0.5 mL contained fully decatenated kDNA (HP fraction). They were dialyzed against conservation buffer (20% w/v glycerol, 0.1 M Tris-HCl, pH 7.9, 10 mM  $\beta$ -mercaptoethanol, 1 mM EDTA) for 4 h and then against the same buffer containing 50% w/v glycerol. The dialyzed fractions were stored at  $-20^{\circ}\text{C}$ .

**(B) Topoisomerase I.** The proteins of fractions 12–18 of the preceding phosphocellulose column, eluted between 0.45 and 0.7 M potassium phosphate, pH 7.0, contained relaxed supercoiled DNA in the absence of ATP. The fractions were pooled (14 mL) and diluted in solution G in order to obtain a molarity of 0.2 in potassium phosphate. The proteins were chromatographed in an hydroxyapatite column ( $0.9 \times 1.5$  cm) equilibrated with 0.2 M potassium phosphate in solution G, pH 7.0. The proteins were eluted stepwise with 1.5 mL of 0.4, 0.55, 0.65, 0.80, and 1.0 M potassium phosphate. The most active fractions were eluted between 0.5 and 0.7 M potassium phosphate. The fractions were pooled (3 mL), dialyzed against 0.1 M KCl, and chromatographed on a double-stranded DNA-cellulose column ( $0.4 \times 2$  cm) (Alberts & Herrick, 1971). The topoisomerase I was eluted at about 0.5 M KCl. The enzyme was dialyzed for 4 h against conservation buffer and then against the same buffer containing 50% glycerol (w/v). The dialyzed fractions were stored at  $-20^{\circ}\text{C}$ .

**Other Techniques.** Chromatography, glycerol gradients, and SDS-polyacrylamide slab gel electrophoresis of proteins were performed as previously described (Riou et al., 1983). The cellular DNA content was determined by a fluorescence assay using bis(benzimidazole) (Hoechst 33258) (Labarca & Paigen, 1980). The protein concentrations were measured by the method of Bradford (1976).

**Inhibition of Decatenation, Unknotting, and Relaxation by Drugs.** Drug assays were performed to observe their possible inhibitory effects on the relaxation reaction catalyzed by topoisomerase I and on the decatenation and unknotting reactions catalyzed by topoisomerase II. The SV40 DNA or the DNA of the replicative form of bacteriophage fd purified in a CsCl-ethidium bromide gradient was used in the relaxation assay. The kinetoplast DNA (kDNA) of *Trypanosoma cruzi* and the P4 phage DNA were used as substrates for decatenation and unknotting, respectively. Supercoiled DNA ( $0.2 \mu\text{g}$ ), kDNA ( $0.1 \mu\text{g}$ ), or P4 DNA ( $0.1 \mu\text{g}$ ) was incubated for 15 min at  $37^{\circ}\text{C}$  with suitable concentrations of the drugs before enzyme addition.

**Double-Stranded DNA Cleavage Assay.** *Plasmodium* type II topoisomerase was incubated at  $37^{\circ}\text{C}$  for 10 min with various drug concentrations. The reaction mixture (20  $\mu\text{L}$ ) contained 20 mM Tris-HCl, pH 7.9, 50 mM KCl, 10 mM  $\text{MgCl}_2$ , 0.5 mM ATP, 0.5 mM EDTA, 0.5 mM dithiothreitol, 15  $\mu\text{g}/\text{mL}$  BSA, and 5  $\mu\text{g}/\text{mL}$  supercoiled SV40 DNA. The reaction was stopped by the addition of 5  $\mu\text{L}$  of 5% SDS, 4 mg/mL proteinase K, 0.02% bromophenol blue, and 25% glycerol. The mixture was incubated for 45 min at  $50^{\circ}\text{C}$  (Chen et al., 1984), and the DNA was electrophoresed. The reversion of the double-strand cleavage reaction by saline was performed as described by Liu et al. (1983).

**Drugs.** The ellipticine derivatives were provided by Dr. J. B. Le Pecq, Laboratoire de Physicochimie Macromoléculaire, Institut Gustave Roussy, Villejuif, and by Dr. B. P. Roques, University René Descartes, Paris. Their structure and DNA binding constants have been described by Le Pecq et al. (1974), Pelaprat et al. (1980a,b), and Paoletti et al. (1980). mAMSA was kindly provided by Dr. B. Baguley (Auckland Medical School, New Zealand) and halofantrine, a new antimalarial drug (Cosgriff et al., 1982), by Dr. Le Bras (Institut de



FIGURE 1: Analytical ultracentrifugation at equilibrium in neutral CsCl gradient of the DNA extracted from *Plasmodium berghei*. *Micrococcus luteus* DNA was used as DNA density marker (density  $\rho = 1.731 \text{ g}/\text{cm}^3$ ). The assay was performed in a L5 65 Beckman ultracentrifuge, equipped with a UV scanner system (Rotor AN-F, at 40000 rpm for 36 h at  $25^{\circ}\text{C}$ ).



FIGURE 2: Elution of topoisomerase II activity from a phosphocellulose column. The fractions (2  $\mu\text{L}$ ) were assayed for enzymatic activity by measuring kDNA decatenation in the presence of 1 mM ATP, 150 mM KCl, and 15 mM  $\text{MgCl}_2$ .

Table I: Partial Purification of Type II Topoisomerase from *Plasmodium berghei*<sup>a</sup>

purification steps	total protein (mg)	total act. (units)	sp act. (units/mg of protein)	activity recovered (%)
nuclear extract	100	ND	ND	
PEG supernatant	60	$6.8 \times 10^4$	$1.13 \times 10^3$	100
phosphocellulose column	1.18	$1.2 \times 10^4$	$1.02 \times 10^4$	18
hydroxyapatite column (HP fraction)	0.12	$2.5 \times 10^3$	$3.8 \times 10^4$	4

<sup>a</sup> The enzyme purification was carried out using  $1.6 \times 10^{10}$  infected red blood cells. The DNA content of the starting material was 3.9 mg, as evaluated by fluorometric assay (Labarca & Paigen, 1980).

Médecine et d'Epidémiologie Tropicale, Paris). The other drugs were commercially available.

## RESULTS

**Characterization of Topoisomerase II.** The analysis of *Plasmodium* DNA in a neutral CsCl gradient at equilibrium revealed the presence of a unique DNA band with a buoyant density of  $1.683\text{--}1.684 \text{ g}/\text{cm}^3$ , as expected (Figure 1) (Gutteridge et al., 1971; Dore et al., 1980). The parasitic fraction did not seem to be significantly contaminated by mouse leukocytes.

The topoisomerase II activity was evaluated on the basis of its ability to decatenate the complex network of *T. cruzi* kDNA, composed of about 25000 minicircles of 1.45 kb (Kayser et al., 1982). When the minicircles were liberated by type II topoisomerase, two to three main bands, corresponding to minicircles of different superhelicity, were observed after migration in a 2% agarose slab gel (Figure 2). The decatenation reaction was not affected by the presence of type I topoisomerase (Miller et al., 1981; Riou et al., 1985) but was partly reduced by the presence of inhibitors or, more likely, by a catenating protein present in the partially purified enzyme of the PEG supernatant and phosphocellulose fractions. This inhibitory effect on the decatenation could be overcome by increasing the concentration of KCl to 150 mM and of  $\text{Mg}^{2+}$  to 15 mM (Figure 2). A typical *Plasmodium* topoisomerase II purification is shown in Table I. The active fractions of the second chromatography column (HP fraction) were pooled

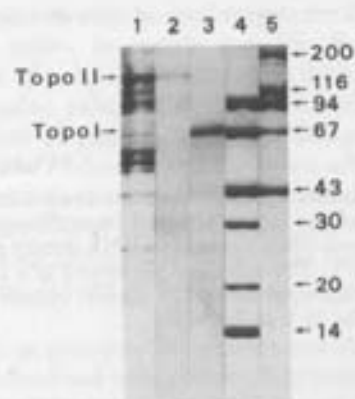


FIGURE 3: Analysis of type I and type II DNA topoisomerases from *Plasmodium* by electrophoresis in a 5–20% gradient SDS-polyacrylamide slab gel: (Lane 1) DNA topoisomerase II (active fractions of the phosphocellulose column); (lane 2) DNA topoisomerase II from the HP fraction; (lane 3) type I DNA topoisomerase from DNA-cellulose chromatography; (lanes 4 and 5) molecular weight markers purchased from Pharmacia and Bio-Rad shown in kilodaltons.



FIGURE 4: Conditions for the decatenation (A) and unknotting (B) reactions catalyzed by *Plasmodium* type II topoisomerase (3 units). Electrophoretic analysis, in a 2% (A) or 0.7% (B) agarose slab gel, of the DNA products after enzymatic reaction with the HP fraction. (A) (Lanes 1–5) Decatenating assay using 0.1 µg of kDNA, as described under Materials and Methods: no enzyme (lane 1); enzyme (lane 2), dATP (1 mM) instead of ATP (lane 3); no ATP (lane 4); no Mg (lane 5). (B) (Lanes 6–9) Unknotting assay using 0.1 µg of knotted DNA from tailless P4 phage capsids as described under Materials and Methods: no enzyme (lane 6); enzyme (lane 7); no ATP (lane 8); no Mg (lane 9).

and used for biochemical studies and drug assays. Attempts to further purify the topoisomerase by chromatography on DNA-cellulose led to a considerable loss of enzymatic activity, the enzyme being very labile.

**Molecular Weight of Topoisomerase II.** The electrophoresis on a SDS-polyacrylamide gel of the most active fractions of the hydroxyapatite column revealed the presence of a major protein band with an apparent molecular weight of about 160 000 (Figure 3). Several bands of weak intensity, with lower molecular weights, were also observed. When scanned with the laser densitometer Ultrosan, the 160K band represented about 70% of the protein bands observed in the gel. When fraction HP was sedimented in a glycerol gradient (Riou et al., 1985), the decatenating activity was detected in a protein fraction of about 11 S (mean value of three experiments). After gel filtration in Sephacryl S-300 (Riou et al., 1985), the topoisomerase revealed a Stokes radius of about 66 Å (mean value of two experiments) (Andrews, 1970). When this value was combined with that found as sedimentation coefficient and a partial specific volume of 0.725 cm<sup>3</sup>/g was assumed for protein, a molecular weight of approximately 299 000 was found for the native enzyme according to the method of Siegel and Monty (1966). A comparison of the two molecular weight values (299 000 and 160 000) suggests that the native enzyme

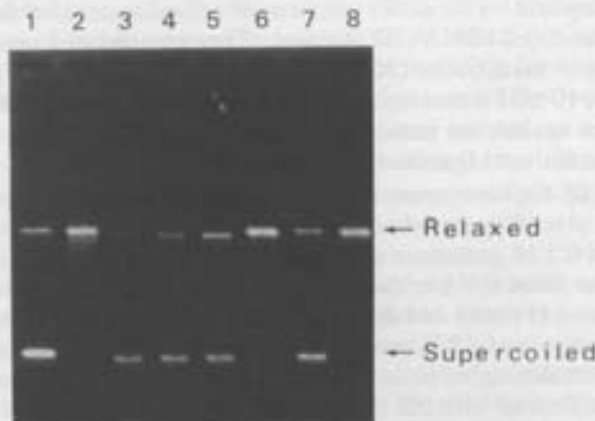


FIGURE 5: Relaxation of positive superhelical DNA by *Plasmodium* topoisomerases I and II. To prepare positive supercoiled DNA, fd phage DNA (lane 1) was relaxed by *Plasmodium* topoisomerase I, and ethidium bromide (2 µg/mL) was added (Liu et al., 1979). The DNA returned to the relaxed state after removal of the ethidium bromide during electrophoresis (lane 2). When positively supercoiled DNA was relaxed by *Plasmodium* topoisomerase II (lane 3) or I (lane 4), it returned to the relaxed state by the subsequent removal of ethidium bromide. For comparison, the action of calf thymus topoisomerase II, which also catalyzes the relaxation of positively superhelical DNA, and of *Micrococcus luteus* DNA gyrase, which does not in the absence of ATP, is shown (lanes 5 and 6, respectively). When ATP was added to the relaxed DNA, the DNA gyrase induced negative superturns (lane 7) while the *Plasmodium* topoisomerase II did not (lane 8).

Table II: Purification of Type I Topoisomerase from *Plasmodium berghei*<sup>a</sup>

purification steps	total protein (mg)	total act. (units)	sp act. (units/mg of protein)	activity recovered (%)
nuclear extract	100	ND	ND	
PEG supernatant	17.4	6.15 × 10 <sup>5</sup>	3.5 × 10 <sup>5</sup>	100
phosphocellulose column	1.72	1.2 × 10 <sup>5</sup>	2.35 × 10 <sup>4</sup>	19
hydroxyapatite column	0.4	8 × 10 <sup>4</sup>	8 × 10 <sup>4</sup>	13
DNA-cellulose	0.06	2.2 × 10 <sup>4</sup>	3.5 × 10 <sup>5</sup>	3.6

<sup>a</sup> The enzyme purification was carried out with 8.2 × 10<sup>8</sup> infected red blood cells.

is, like other eukaryotic enzymes, a dimer (Wang, 1985).

**Requirements for Topoisomerase II Activity.** The requirements of the *Plasmodium* type II topoisomerase for the decatenation of kDNA (Figure 4A) and the unknotting of P4 tailless phage duplex DNA (Figure 4B) were quite similar to those observed for eukaryotic topoisomerases II: Mg<sup>2+</sup> (10–15 mM) and ATP (0.5–1 mM) were required and could be substituted by Mn<sup>2+</sup> (1 mM) and dATP (1 mM), respectively. UTP, GTP, and TTP were ineffective. KCl optimized the reaction at concentrations of 100–150 mM for decatenation and unknotting. Similar results were obtained with NaCl. The optimum pH for the enzyme reaction was between 7.5 and 8.2. The enzyme relaxed negatively as well as positively supercoiled DNA and was not able to induce negative superturns like gyrase (Figure 5). However, some relaxation reaction did occur in the absence of ATP, while stimulated by the presence of ATP (results not shown). The enzyme fully catenated supercoiled DNA molecules in the presence of ATP and a condensing agent (Krasnow & Cozzarelli, 1982) (data not shown).

**Characterization of Topoisomerase I.** A typical topoisomerase I purification from *Plasmodium* is shown in Table II. The purified topoisomerase I, obtained after DNA-cellulose chromatography, was used in enzyme assays. The *Plasmodium* enzyme was able to relax supercoiled DNA without any high-energy cofactor. Mg<sup>2+</sup> (10 mM), as well

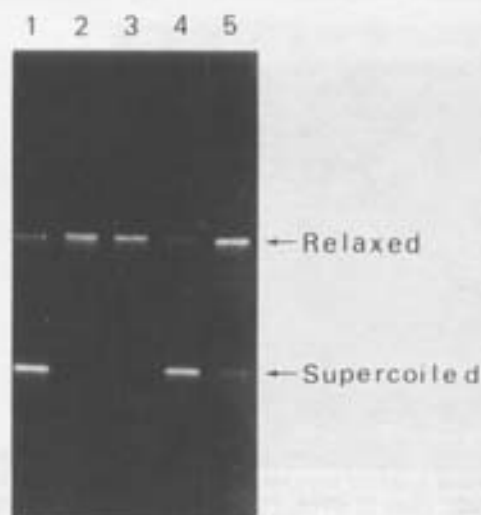


FIGURE 6: Activation of the relaxation activity of *Plasmodium* type I DNA topoisomerase by Mg and spermidine: (lane 1) supercoiled fd DNA as control; (lane 2) DNA + enzyme (0.2  $\mu$ L) and 10 mM  $Mg^{2+}$ ; (lane 3) DNA + enzyme (0.2  $\mu$ L) and 5 mM spermidine; (lane 4) DNA + enzyme (0.2  $\mu$ L) and no  $Mg^{2+}$  and no spermidine; (lane 5) DNA + enzyme (2  $\mu$ L) and no  $Mg^{2+}$  and no spermidine.

as 5 mM spermidine, stimulated the reaction by about 10-fold (Figure 6). The relaxation depended on the salt concentration of the reaction mixture. The *Plasmodium* enzyme exhibited an optimal relaxation activity at concentrations of KCl or NaCl between 100 and 125 mM. The enzyme relaxed negatively, as well as positively, supercoiled DNA molecules (Figure 5).

The electrophoresis of the purified enzyme on SDS-polyacrylamide gel revealed the presence of a major band with an apparent  $M_r$  of 70 000 (Figure 3). When the enzyme was sedimented in a glycerol gradient, the relaxation activity was detected in protein fractions corresponding to 5.8 S. After gel filtration of the phosphocellulose fractions in Sephacryl S-300, the topoisomerase revealed a Stokes radius of about 43.5 Å. From the measured values for the sedimentation coefficient and the Stokes radius, the native molecular weight of topoisomerase I was evaluated at 104 000. There is a discrepancy between this value and the value obtained by SDS-polyacrylamide gel electrophoresis. This may be due to a partial degradation during the procedure of purification for electrophoretic analysis, as has been already described (Liu & Miller, 1981; Martin et al., 1983). Thus, it is suggested that the *Plasmodium* topoisomerase I is a monomer.

**Drug Inhibition of Decatenation Reaction Catalyzed by *Plasmodium* Type II Topoisomerase.** Intercalating and nonintercalating drugs were assayed for their effects on the decatenation reaction catalyzed by type II topoisomerase. Some of these drugs are used in antimalarial, trypanocidal, antitumoral, and antimicrobial chemotherapy. The inhibition of the decatenation reaction can be analyzed directly by electrophoresis in agarose slab gels: the decatenation is inhibited when the minicircle band disappears from the gel. We have tested several ellipticine derivatives (Tables III and IV) and compared their inhibitory effects on decatenation with those of other intercalating agents widely used in many fields as chemical models or therapeutic agents, such as ethidium, acriflavin, chloroquine, adriamycin, and mAMSA. All these intercalating drugs inhibited the decatenation at various concentrations (Table V) (Figure 7). We also tested homodimers of ethidium, acridine, and isoellipticinium and a heterodimer of ethidium and acridine because dimeric molecules may have a higher affinity for DNA than monomers, de-

Table III: Structure of Ellipticine Derivatives

derivatives			
	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>
El	H	H	H
9-NH <sub>2</sub> -El	NH <sub>2</sub>	H	H
9-Br-El	Br	H	H
9-OH-El	OH	H	H
9-OCH <sub>3</sub> -6-CH <sub>3</sub> -El	OCH <sub>3</sub>	CH <sub>3</sub>	H
2-CH <sub>3</sub> -9-OH-El <sup>m</sup> (NMHE)	OH	H	CH <sub>3</sub>
2,6-(CH <sub>3</sub> ) <sub>2</sub> -El <sup>m</sup> (I <sup>-</sup> )	H	CH <sub>3</sub>	CH <sub>3</sub>
2,6-(CH <sub>3</sub> ) <sub>2</sub> -9-OH-El <sup>m</sup> (Cl <sup>-</sup> )	OH	CH <sub>3</sub>	CH <sub>3</sub>

Table IV: Structure of Isoellipticinium (7H-Pyrido[4,3-c]carbazole) Derivatives

derivatives				
	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>	R <sub>4</sub>
IsoEl <sup>m</sup> Di <sup>a</sup> (1)	OCH <sub>3</sub>	H	(CH <sub>2</sub> ) <sub>2</sub> -1-piperidyl, Cl <sup>-</sup>	H
IsoEl <sup>m</sup> (2)	OCH <sub>3</sub>	CH <sub>3</sub>	CH <sub>3</sub>	CH <sub>3</sub>
IsoEl <sup>m</sup> Di (3)	OCH <sub>3</sub>	CH <sub>3</sub>	(CH <sub>2</sub> ) <sub>2</sub> -1-piperidyl, Cl <sup>-</sup>	CH <sub>3</sub>
IsoEl <sup>m</sup> (4)	OCH <sub>3</sub>	H	(CH <sub>2</sub> ) <sub>2</sub> -1-piperidyl, Cl <sup>-</sup>	CH <sub>3</sub>
IsoEl <sup>m</sup> Di (5)	OCH <sub>3</sub>	H	(CH <sub>2</sub> ) <sub>2</sub> -1-piperidyl, Cl <sup>-</sup>	CH <sub>3</sub>
IsoEl <sup>m</sup> (6)	OCH <sub>3</sub>	CH <sub>3</sub>	CH <sub>3</sub>	H

<sup>a</sup> This compound is used in antitumoral therapy under the name of ditercalium.

Table V: Inhibitory Concentrations ( $\mu$ M) of Intercalating Agents on Topoisomerase II Decatenating and Topoisomerase I Relaxing Activities

drug	decatenation	relaxation
El	26	170
9-NH <sub>2</sub> -El	38	
9-Br-El <sup>a</sup>	31	
9-OH-El	7 <sup>b</sup>	170
9-OCH <sub>3</sub> -6-CH <sub>3</sub> -El	17	
2-CH <sub>3</sub> -9-OH-El <sup>m</sup> (NMHE)	7 <sup>b</sup>	35
2,6-(CH <sub>3</sub> ) <sub>2</sub> -El <sup>m</sup> (I <sup>-</sup> )	12	125
2,6-(CH <sub>3</sub> ) <sub>2</sub> -9-OH-El <sup>m</sup> (Cl <sup>-</sup> )	8 <sup>b</sup>	150
mAMSA	77	
adriamycin	4	
EtdBr	25	
Acr	35	180
EtdDi	2	
AcrDi	3	20
EtdAcrDi	5	
IsoEl <sup>m</sup> Di (ditercalium) (1)	5 <sup>b</sup>	
IsoEl <sup>m</sup> (2)	6	
IsoEl <sup>m</sup> Di (3)	3	
IsoEl <sup>m</sup> (4)	12	
IsoEl <sup>m</sup> Di (5)	6	
IsoEl <sup>m</sup> (6)	21	

<sup>a</sup> 9-Bromoellipticine is not an intercalating agent (Le Pecq et al., 1974). <sup>b</sup> When these drugs were tested in the unknotting assay, the inhibitory concentrations were the same.

pending on the chain linking of the intercalating groups (Gauguin et al., 1978; Roques et al., 1979). The decatenating activity was strongly inhibited by dimers. The effects of some ellipticine derivatives were also tested on the unknotting activity



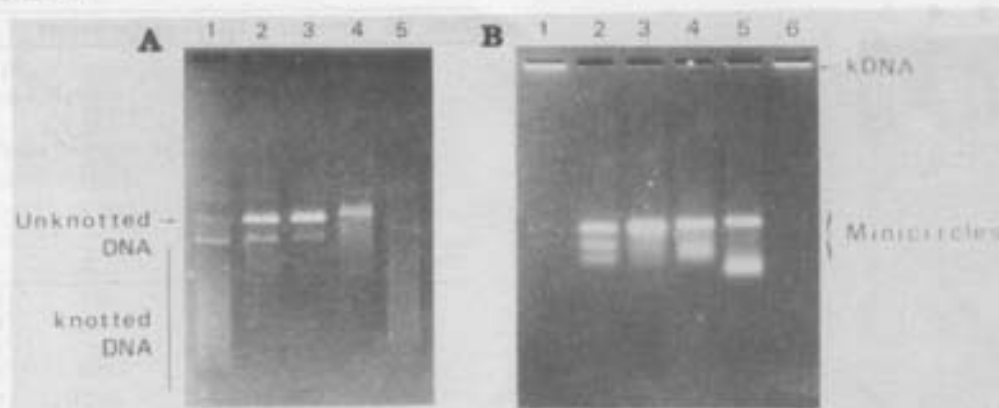


FIGURE 7: Inhibition of the unknotting (A) and the decatenating (B) activities of *Plasmodium* type II topoisomerase by the ellipticin derivative 2,6-(CH<sub>3</sub>)<sub>2</sub>-9-OH-EI<sup>+</sup> (Cl<sup>-</sup>) (Table III). The kDNA decatenating and P4 DNA unknotting activities were monitored as described under Materials and Methods. (A) (Lane 1) Knotted P4 DNA control, appearing in the gel as a smear; (lane 2) DNA + *Plasmodium* type II topoisomerase (100 units/mL) (the P4 DNA was unknotted and migrated as a band); (lanes 3–5) identical with lane 2 + ellipticin (1  $\mu$ g/mL, lane 3; 2.5  $\mu$ g/mL, lane 4; 5  $\mu$ g/mL, lane 5). The weak DNA band (lanes 1–3) migrating more rapidly than unknotted DNA is linear P4 DNA. (B) (Lane 1) kDNA control; (lane 2) kDNA + *Plasmodium* type II topoisomerase (100 units/mL); (lanes 3–6) identical with lane 2 + ellipticin (0.1  $\mu$ g/mL, lane 3; 0.5  $\mu$ g/mL, lane 4; 1  $\mu$ g/mL, lane 5; 2.5  $\mu$ g/mL, lane 6). The ellipticin derivative inhibits the decatenation and unknotting

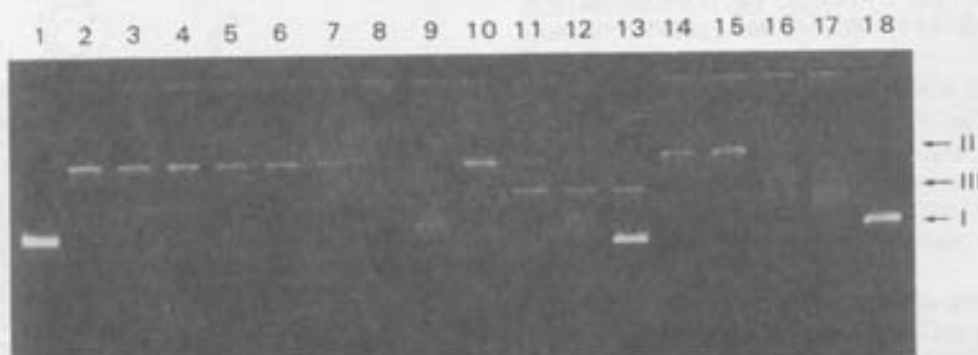


FIGURE 8: Stimulation of DNA cleavage by *Plasmodium* type II topoisomerase. The reaction mixtures (20  $\mu$ L), containing 5  $\mu$ g/mL supercoiled SV40 DNA and 500 units/mL topoisomerase, were incubated for 10 min at 37  $^{\circ}$ C in the presence of various concentrations of chloroquine or mAMSA and analyzed by 1.2% agarose slab gel electrophoresis. (Lane 1) DNA control; (lane 2) DNA + topoisomerase; (lanes 3–9) identical with lane 2 + chloroquine (0.5  $\mu$ g/mL, lane 3; 1  $\mu$ g/mL, lane 4; 3  $\mu$ g/mL, lane 5; 12  $\mu$ g/mL, lane 6; 25  $\mu$ g/mL, lane 7; 50  $\mu$ g/mL, lane 8; 100  $\mu$ g/mL, lane 9); (lanes 10–13) identical with lane 2 + mAMSA (1  $\mu$ g/mL, lane 10; 5  $\mu$ g/mL, lane 11; 10  $\mu$ g/mL, lane 12; 50  $\mu$ g/mL, lane 13). Reversion of DNA breakage by salt: 0.5 M NaCl added for 15 min at 37  $^{\circ}$ C before SDS and proteinase K. (Lanes 14–18) (Lane 14) As in lane 2; (lanes 15–18) as in lanes 10–13.

of *Plasmodium* topoisomerase II; their inhibitory concentrations were found to be close to those observed on the decatenating activity (Figure 7, Table V). Antimalarial drugs and specific inhibitors of DNA gyrase inhibited poorly topoisomerase II. Some trypanocidal drugs were more active (Table VI).

**Double-Stranded DNA Cleavage by *Plasmodium* Type II Topoisomerase.** It has recently been shown that a purified type II topoisomerase of eukaryotic origin forms a tight complex with DNA (Sander & Hsieh, 1983; Liu et al., 1983), as previously shown for DNA gyrase (Gellert, 1981). Upon protein denaturation, breaks occur in the protein-linked DNA. Some intercalating and nonintercalating drugs stimulate the formation of topoisomerase II–DNA complexes detected by gel electrophoresis under suitable protein denaturing conditions (Nelson et al., 1984; Tewey et al., 1984; Chen et al., 1984). We have tested our type II topoisomerase to see whether the enzyme was able to induce, like other enzymes, the cleavage of double-stranded DNA and whether it could be stimulated by some drugs under the experimental conditions previously described (Nelson et al., 1984). The results of Figure 8 show that the *Plasmodium* type II topoisomerase produced few DNA breaks under our assay conditions. However, the production of breaks was highly stimulated by the presence of some drugs in suitable concentrations. The DNA intercalating drug mAMSA (Figure 8) and the ellipticine derivative

Table VI: Inhibitory Concentrations ( $\mu$ g/mL) of DNA Gyrase Inhibitors, Antimalarial Agents, and Trypanocidal Drugs on *Plasmodium* Topoisomerase II Decatenating and Topoisomerase I Relaxing Activities

drug	relaxation	decatenation
nalidixic acid		500
pefloxacine	500	250
coumermycin		125
novobiocin	1000*	500 (80% of inhibition)
clorobioicin	500	200 (90% of inhibition)
quinine		500 (60% of inhibition)
chloroquine	1000*	500*
primaquine	1000*	500*
sulfadoxine		500 (50% of inhibition)
halofantrine	1000*	250
mefloquine		500 (90% of inhibition)
berenil	100	75
hydroxystilbamidine	250	100 (50% of inhibition)
radanil	500*	500*
lampit	500*	500*

\* Not inhibitory for the concentration quoted.

NMHE, as well as the nonintercalating drugs epipodophyllotoxins (VM26 and VP16) (data not shown), induced DNA breakage as revealed by the presence of a band of form III DNA in the gel after electrophoresis. mAMSA, VM26, and VP16 at a concentration of 20–50  $\mu$ g/mL and NMHE at a concentration of 1  $\mu$ g/mL cleaved about 20, 30, 30, and

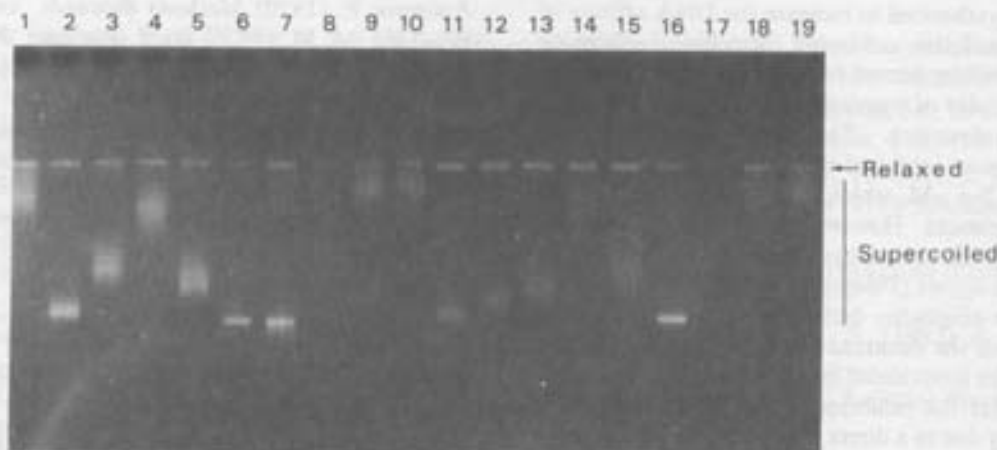


FIGURE 9: Inhibition of the relaxation activities of *Plasmodium* topoisomerases I and II by the ellipticin derivative 2,6-(CH<sub>3</sub>)<sub>2</sub>-9-OH-Elm (Cl). Electrophoretic analysis in 1.2% agarose slab gels containing 18 µM chloroquine, as previously described (Douc-Rasy et al., 1983a, 1984). (Lane 1) fd DNA control (0.2 µg); (lane 2) DNA + DNA topoisomerase I (3 units); (lanes 3-9) DNA + 0.25, 0.5, 1, 2.5, 5, 25, and 50 µg/mL ellipticin (the relaxation by topoisomerase I was inhibited by 50 µg/mL of the drug); (Lane 10) fd DNA + type II topoisomerase (3 units); (lanes 11-19) same as lane 10 + 0.1, 0.25, 0.5, 1.0, 2.5, 5, 7.5, 10, and 25 µg/mL ellipticin (the relaxation by topoisomerase II was completely inhibited by 10 µg/mL of the drug).

10% of the DNA, respectively, as evaluated by densitometric analysis of negative photographs of the gels (laser densitometer Ultrosan LKB). When the drug concentrations were increased, mAMSA or VM26 induced the cleavage of the same proportion of DNA while NMHE inhibited the cleavage at 5 µg/mL (data not shown). The cleavage induced by mAMSA and NMHE was due to a stimulation of complex formation since its reversion was obtained by salt addition, as shown in Figure 8 (lanes 14-18). The DNA cleavage was Mg-dependent. ATP (0.5 mM) stimulates the reaction. KCl was inhibitory at 150 mM. In contrast, the antimalarial drugs chloroquine, halofantrine, primaquine, and mefloquine did not significantly stimulate the double-strand cleavage of DNA (Figure 8).

**Effect of Drugs on Relaxation Reaction Catalyzed by *Plasmodium* Topoisomerase I.** The inhibition by nonintercalating agents of the relaxation catalyzed by the topoisomerase I can be analyzed in agarose gels. Table VI shows the inhibitory effects of such drugs on the relaxation. The inhibition was observed at relatively high drug concentrations. In contrast, the inhibition of relaxation by intercalating drugs cannot be detected in neutral agarose gels but in gels containing an unwinding agent. Chloroquine was used at a concentration of 18 µM, as previously described (Douc-Rasy et al., 1984). The effect of several drugs is shown in Figure 9. The topoisomers of the bacteriophage fd DNA control appear as a series of more than 10 bands (Figure 9, lane 1). The uppermost band (lane 2) is due to nicked DNA. When topoisomerase I was added to the reaction mixture, relaxed DNA appeared, under our assay conditions, as a supercoiled DNA band (lane 2). When the drug was added to the reaction mixtures, the resulting DNA presented various band distributions, depending on the drug concentration. To analyze the molecular form of the DNA without drug interference, the drug was extracted with butanol. Lanes 3-9 show the drug effects. An inhibitory effect was present when the DNA band distribution remained like that of the control DNA, as in lanes 9, 18, and 19. These results, as well as similar data obtained with other drugs, are shown in Tables V and VI.

#### DISCUSSION

We have purified DNA topoisomerases I and II from different cellular extracts of *Plasmodium berghei*. Four experiments were performed with  $(0.8-3.0) \times 10^{10}$  parasites (1-1.5 g of wet weight cells). Several problems were en-

countered during the *Plasmodium* topoisomerase II purification. Assays could only be carried out with small quantities of fresh parasites. It seemed, indeed, important to start with living parasites rather than frozen cells in order to reduce enzyme inactivation during the purification steps. Enzyme degradation occurred very rapidly, which was probably due to proteolysis, although several protease inhibitors were included in all buffers. The instability of the enzyme preparations was mostly observed after the second chromatography column, and we were unable to further purify topoisomerase II. However, addition of BSA (1 mg/mL) to the hydroxyapatite fractions permitted to obtain an enzyme stable for several months. We were, nevertheless, able to extract from *Plasmodium* a type II topoisomerase satisfactorily pure. The degree of contamination of the enzyme preparations by the type I enzyme was relatively weak, as judged by the difference in the inhibitory effect of drugs observed with the two types of enzyme (see Figure 9). The problem of the inactivation of topoisomerase I during its purification was not as great as that of topoisomerase II, and the enzyme could be prepared with a relatively high degree of purity.

We have purified from *Plasmodium* a topoisomerase that can fully decatenate the complex network of kDNA from trypanosomes, a reaction that necessitates the double-stranded DNA passing activity of topoisomerase II, which suggests that our enzyme most likely belongs to the category of type II topoisomerase. We have used the decatenating assay to follow the enzyme purification because it is not affected by the presence of a contaminating type I topoisomerase (Riou et al., 1985) and because kDNA can easily be obtained from trypanosomes. However, in one of our four experiments, we were not able to detect any decatenating effect in the PEG supernatant, probably due to the presence of an inhibitor or of an excess of catenating factor even after dilution (Riou et al., 1985). From the results presented in this paper, it would seem that the *Plasmodium* topoisomerases have enzymatic properties quite similar to those purified from other eukaryotic cells (Wang, 1985). However, the ATP requirement for relaxation by topoisomerase II was not as great as that for decatenation and unknotting. A similar observation has also been recently reported for the topoisomerase II from calf thymus (Halligan et al., 1985).

The *Plasmodium* type II topoisomerase has been shown to be sensitive to intercalating agents such as ellipticine derivatives, which present a high affinity for DNA. Dimeric mol-



ecules have been synthesized to increase the DNA affinity of the intercalating moieties, rendering them bis-intercalating agents. These molecules proved to be potent inhibitors of the strand-passing activity of topoisomerase II in the course of decatenation or unknotting. The ethidium, acridine, and isoellipticinium dimers inhibited the decatenating activity at concentrations of 2–6  $\mu$ M, which is lower than those of the corresponding monomers. However, under the ionic strength and pH conditions of the assays, these derivatives act as mono-intercalating agents (Pelaprat, 1980a,b). Furthermore, the affinity of the ellipticine derivatives is modified by the conditions in which the decatenation is performed, and the number of molecules intercalated into DNA remains unknown. It seems likely that the inhibitory effect of the ellipticine derivatives is partly due to a direct effect on drug intercalation into DNA, but an effect on the enzyme is not excluded. The ellipticine derivatives are more potent inhibitors of the reactions catalyzed by the *Plasmodium* type II topoisomerase than of those catalyzed by the type I enzyme. Similar results have already been described for enzymes originating from various sources (Douc-Rasy et al., 1983a, 1984). An exception is that of the type I topoisomerase of *T. cruzi*, which is inhibited by 2,6-dimethyl-9-hydroxyellipticinium at the same concentration that inhibits the type II enzyme (Douc-Rasy et al., 1983a,b). This suggests that intercalation cannot, alone, explain the inhibition of the reactions catalyzed by the topoisomerases. As previously shown for other eukaryotic type II topoisomerases, the inhibitors of DNA gyrase have no effect on the *Plasmodium* type II topoisomerase activity. Antimalarial drugs are weak inhibitors of the reactions catalyzed by topoisomerase II.

It has recently been demonstrated that several antitumor drugs, DNA-intercalating as well as DNA-nonintercalating drugs, were able to stimulate the formation of cleavage complexes with an increase in DNA breaks (Nelson et al., 1984; Tewey et al., 1984; Chen et al., 1984). DNA damage such as double-stranded DNA cleavage is important for the economy of the cell, particularly in rapidly growing cells such as malaria parasites. It seemed, therefore, of interest to test in vitro this *Plasmodium* topoisomerase II activity. The results show that, like the mammalian enzymes, the *Plasmodium* topoisomerase II presents a DNA breakage activity that is stimulated by some antitumoral drugs. In contrast, antimalarial drugs do not stimulate significantly the cleaving activity of topoisomerase II. Although the *Plasmodium* strain used to purify DNA topoisomerase was sensitive in vivo to chloroquine, this drug, like other antimalarial drugs, is a poor in vitro inhibitor of the reactions catalyzed by the topoisomerases. Thus, it seems likely that their in vivo action on the parasite is due to another biochemical process.

**Registry No.** 1, 80145-11-5; 2, 100466-25-9; 3, 100466-39-5; 4, 100466-26-0; 5, 100466-40-8; 6, 72250-44-3; Et, 519-23-3; 9-NH<sub>2</sub>-Et, 54779-53-2; 9-Br-Et, 18073-34-2; 9-OH-Et, 51131-85-2; 9-OCH<sub>3</sub>-6-CH<sub>3</sub>-Et, 18073-32-0; NMHE, 58337-34-1; 2,6-(CH<sub>3</sub>)<sub>2</sub>-Et (I<sup>+</sup>), 63315-02-6; 2,6-(CH<sub>3</sub>)<sub>2</sub>-9-OH-Et (Cl<sup>+</sup>), 58337-36-3; mAMSA, 51264-14-3; EtdBr, 1239-45-8; Acr, 260-94-6; EtdDi, 89160-73-6; AcrDi, 70114-94-2; EtdAcrDi, 100466-41-9; DNA topoisomerase, 80449-01-0; adriamycin, 25316-40-9; nalidixic acid, 389-08-2; pefloxacin, 70458-92-3; coumermycin, 78040-85-4; novobiocin, 78040-85-4; clorobiocin, 39868-96-7; quinine, 130-95-0; chloroquine, 54-05-7; primaquine, 90-34-6; sulfadoxine, 2447-57-6; halofantrine, 69756-53-2; mefloquine, 53230-10-7; berenil, 908-54-3; hydroxystilbamidine, 495-99-8; radanil, 22994-85-0; lampit, 23256-30-6.

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