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Fluorometric Assays for DNA Topoisomerases and Topoisomerase-Targeted Drugs: Quantitation of Catalytic Activity and DNA Cleavage

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SUMMARY

We have introduced the novel application of a simple ethidium fluorescence assay, using covalently closed circular DNA, for the study of topoisomerase-targeted drugs. With the specificity of camptothecin for eukaryotic topoisomerases I and of VM26 for eukaryotic topoisomerases II, the two classes of enzymes can be assayed independently in crude extracts and during purification. These assays are fast, sensitive, and quantitative, have a large sample capacity, and eliminate the need for radioactive materials, filters, and agarose gels. We have demonstrated the use of this fluorescence assay to measure the inhibition of the relaxation and supercoiling activities of purified mammalian topoisomerases I and II and bacterial gyrase by nonintercalating drugs. Similarly, the production of drug-induced topoisomerase-mediated cleavable complexes was readily quantitated with both nonintercalating and intercalating drugs. When inhibition and cleavage with VM-26 were measured concurrently as a function of topoisomerase II concentration, a clear inverse relationship between topoisomerase II inhibition and cleavable complex production was observed. When the physiologically relevant salt K^+L-glutamate$^-$ was used, quantitative relaxation by topoisomerase II was observed up to twice the salt concentration obtained with KCl. The enantiomer K^+D-glutamate$^-$ gave exactly the same results, indicating that the enhancing role of glutamate$^-$ is nonstereospecific.

As our understanding of the physiological importance of DNA topoisomerases in DNA replication, recombination, and transcription has increased over recent years, so has our appreciation for the clinical importance of this class of enzymes as drug targets (reviewed in Refs. 1–3). Eukaryotic topoisomerase I has been identified as the primary target of the antineoplastic alkaloid camptothecin, whereas eukaryotic topoisomerase II is the target of many anticancer agents, including both nonintercalating (e.g., VM-26) and intercalating (e.g., *m*-AMSA) compounds. Gyrase is a target of antibacterial agents of the coumarin and quinolone classes (e.g., novobiocin and oxolinic acid).

Topoisomerases promote topological changes in DNA by transient breaks, due to a protein-DNA covalent intermediate. Effects of antitopoisomerase drugs may be observed by the inhibition of topoisomerase catalytic activities and, in some cases, by the production of drug-stabilized "cleavable complexes" (4). The latter are converted to topoisomerase-linked DNA breaks in the presence of a strong protein denaturant such as SDS or alkali. Conventional assay methods used to study topoisomerases and topoisomerase-targeted drugs are reviewed in Ref. 5. In general, the most commonly used methods include agarose gel electrophoresis, alkaline elution (6, 7), and K-SDS precipitation (8, 9). Agarose gels separate DNA according to their topology and so may be used to follow both catalytic activities and cleavable complex production. Alkaline elution and K-SDS precipitation are designed to follow cleavable complex formation for cell studies, but not catalytic activity.

In this paper we describe the novel use of an ethidium fluorescence assay for studying antitopoisomerase drugs *in vitro*, with an emphasis on anticancer drugs targeted at mammalian topoisomerase II. In contrast to the conventional methods indicated, the ethidium fluorescence method does not require the use of gel electrophoresis, filters, or radioactive materials. In avoiding these time-consuming procedures, it provides rapid and sensitive quantitation of both catalytic activity and production of cleavable complexes. This assay exploits the large fluorescence enhancement of ethidium when it intercalates duplex DNA. The amount of ethidium able to intercalate is determined by the topological state of the DNA molecule. Consequently, as the superhelicity of CCC DNA is altered by a topoisomerase, the amount of ethidium fluores-

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ABBREVIATIONS: VM-26, 4′-demethylepipodophyllotoxin thenylidene-β-D-glucoside; SDS, sodium dodecyl sulfate; DMSO, dimethylsulfoxide; *m*-AMSA, *meta*-amsacrine; CCC DNA, covalently closed circular DNA; BSA, bovine serum albumin; K-Glu, potassium glutamate.

cence changes. The amount of DNA cleavage in a topoisomerase reaction may be determined by heat denaturation of the DNA at pH 12, which results in loss of fluorescence because ethidium cannot intercalate single-stranded DNA.

These assays should find widespread use in the initial screening of new anticancer and antibacterial drugs, as well as the study of the many chemically derived analogues.

Materials and Methods

Drugs and chemical reagents. VM-26 was kindly provided by Dr. R. Hancock, Laval University. Camptothecin and novobiocin were obtained from Sigma Chemical Co. VM-26 was dissolved in DMSO at 1.5 mM. Camptothecin was dissolved in DMSO at 10 mM. Novobiocin was dissolved in 10 mM NaOH at 10 mM. All stock solutions of drugs were filtered through 0.2-μm Nalgene nylon membrane filters and stored frozen at −20°. All other chemicals were of at least analytical grade.

Enzymes and DNA substrates. Topoisomerase II was purified from calf thymus by a combination of the methods described by Schomburg and Grosse (10) and by Drake *et al.* (11). In brief, preparation of the 0.35 M NaCl nuclear extract was followed by chromatography on a Pharmacia Biotechnology, Inc., FPLC system, using Ultrafgel hydroxyapatite (LKB) and MonoQ (Pharmacia) columns. Complete separation of topoisomerase I from topoisomerase II was achieved by a 15–45% glycerol gradient ultracentrifugation. Calf thymus topoisomerase I and *Micrococcus luteus* DNA gyrase were obtained from Bethesda Research Laboratories. One unit of enzyme is defined as the minimum amount required to completely relax or supercoil 1 μg/20 μl PM2 DNA in 30 min at 37°, as determined by the ethidium fluorescence assay. Supercoiled PM2 DNA was isolated as described previously (12). Relaxed PM2 DNA was prepared by relaxing supercoiled PM2 DNA with topoisomerase I, followed by phenol-chloroform extraction. All DNA used was 92–98% covalently closed circles, as judged by the ethidium fluorescence assay.

Assays of calf thymus topoisomerases I and II and DNA gyrase. The standard reaction mixture for assaying the relaxation activity of topoisomerases I and II contained 50 mM Tris·HCl (pH 7.5), 0.2 M K-Glu, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM dithiothreitol, 30 μg/ml BSA, 1 μg/20 μl supercoiled PM2 DNA, and 1 unit/20 μl topoisomerase. ATP (1 mM) was also included for the topoisomerase II assay. Drugs dissolved in DMSO gave final DMSO concentrations of 5% and 10% for topoisomerases II and I, respectively. At these concentrations, DMSO had no effect on drug-free controls. DNA cleavage reaction mixtures were identical, except that 0.1 M K-Glu and 4 units of topoisomerase were used to promote production of cleavable complexes. After 30 min at 37°, 10 μl of the reaction mixture were removed into 1.2 ml of pH 12 ethidium assay buffer (see below), and the fluorescence was measured both before and after heating of the assay tubes for 2 min in a Temp Block (Labline) set at 96°. To the remaining 10 μl of reaction mixture were added 10 μl of a 2× stop solution containing 2% SDS, 10 mM EDTA, 0.1% bromphenol blue, 20% glycerol, and 0.4 mg/ml proteinase K (Boehringer Mannheim). After an additional 30-min incubation at 37°, the reaction products were separated on a 0.7% agarose gel in TBE buffer (89 mM Tris-borate, 2 mM EDTA, pH 8.2) at 1.3 V/cm for 18 hr, stained with 0.5 μg/ml ethidium bromide, and photographed under UV light. For heat reversal of cleavable complexes (13), the reaction was heated to 65° for 10 min before termination of the reaction by addition of the ethidium bromide assay solution of the SDS-containing gel-loading solution.

The standard reaction mixture for assaying the supercoiling activity of DNA gyrase contained 35 mM Tris·HCl (pH 7.5), 20 mM K-Glu, 20 mM $MgCl_2$, 0.1 mM EDTA, 2 mM spermidine·$(HCl)_3$, 1.0 mM ATP, 0.5 mM dithiothreitol, 30 μg/ml BSA, 10% glycerol, 1 μg/20 μl relaxed PM2 DNA, and 1 unit/20 μl gyrase. The reaction was carried out as described for topoisomerases I and II.

Ethidium fluorescence method. The ethidium fluorescence assay used here has been previously described in considerable detail (14, 15). The pH 12 ethidium assay buffer contained 20 mM potassium phosphate (pH 12), 0.5 mM EDTA, and 0.5 μg/ml ethidium bromide. The buffer was kept at room temperature in a light-proof container fitted with a repeat dispenser set at 1.2 ml. A Gilson fluorometer with an excitation filter at 525 nm and an emission filter at 600 nm, thermostated at 25°, was used to measure fluorescence. Typically, 10-μl samples were added to 1.2 ml of the pH 12 ethidium assay buffer, in 12- × 75-mm glass tubes, for fluorescence readings. Before reading of the assay samples, the fluorometer was zeroed with a blank of pH 12 ethidium assay buffer and then standardized to 50 arbitrary fluorescence units with 10 μl of 50 μg/ml calf thymus DNA. For DNA cleavage determinations, the same assay tubes were heated to 96° for 2 min, followed by a 2-min cooling in a room temperature water bath, and the "after-heat" fluorescence readings were taken. All drugs were checked for interference with ethidium fluorescence by measurement of the fluorescence of reaction samples incubated with the drug in the absence of enzyme treatment.

Calculation of percentage of enzyme inhibition and percentage of DNA cleaved. The relaxation of negatively supercoiled PM2 DNA may be followed by the concomitant decrease in ethidium fluorescence, in which a final decrease of 33% corresponds to complete relaxation. The extent of enzyme inhibition may, therefore, be calculated simply from the percentage of fluorescence decrease in the presence of an inhibitor, compared with that of the control drug-free enzyme reaction. Inhibition of gyrase may be calculated similarly, with supercoiling of relaxed PM2 DNA giving a final fluorescence increase of 33%. Calculation of the extent of cleaved DNA is determined from the fluorescence difference between the unheated and heat-denatured DNA, relative to the fluorescence difference when all the DNA is nicked. Camptothecin and VM-26 do not intercalate DNA, but it should be stressed that any drug (especially intercalators) that alters the writhe of CCC DNA would interfere with all topoisomerase assays.

Results

Theoretical aspects of the pH 12 ethidium fluorescence assay. A brief background to the pH 12 ethidium fluorescence assay will enhance an understanding of its use. Originally, an ethidium fluorescence assay at pH 8 was used for synthetic DNAs [e.g., $d(TG)_n \cdot d(CA)_n$], to measure cross-linking of complementary strands (16). If the strands were separable, then no fluorescence was obtained after heat treatment, whereas any fluorescence remaining was due to linked complementary strands. The same assay could not be used for natural duplex DNAs, because heat-denatured DNA, due to intramolecular base-pairing, gave about 50% the fluorescence of duplex DNA. However, at pH 12, short intrastrand base-paired sections of denatured DNA are selectively destabilized, relative to long duplex DNA. It is perhaps surprising that a pH as high as 12 is required and that natural duplexes are stable unless heated, but this is attributed to the stabilizing effect of ethidium intercalation. Although the pH 12 assay was originally devised for measuring cross-linking of linear duplex DNA, it was later found that, unlike linear duplex DNA, CCC DNAs, in which the strands are topologically linked, also renatured spontaneously after a heating step. The difference in fluorescence between the unheated and heat-denatured samples of circular duplex DNA is, therefore, a measurement of the cleaved DNA. As expected, the fluorescence for CCC DNA is less on a per microgram basis, due to topological constraints on ethidium intercalation, than for linear DNA. A maximum positive superhelix density of +0.2 due to the unwinding of the helix by ethidium is obtained under the pH 12 assay conditions. Thus, the fluorescence/μg for CCC DNA also depends on the original

superhelix density, with the more negatively supercoiled the DNA the more fluorescence/μg. PM2 DNA, with the highest titratable superhelix density of −0.12, is, therefore, used for assaying topoisomerases that relax CCC DNA.

Relaxation, supercoiling, and cleavage assays by the ethidium fluorescence method. Figs. 1 and 2 illustrate the time-dependent changes in ethidium fluorescence that occur for typical relaxation and supercoiling assays. As described in Materials and Methods, the total relaxation of negatively supercoiled PM2 DNA is accompanied by a final decrease in fluorescence of 33%. Conversely, introduction of negative supercoils from a relaxed PM2 DNA substrate by gyrase results in a 33% increase in fluorescence, which is 90% of the titratable superhelix density for PM2 DNA ($\sigma = -0.12$). For the purpose of illustrating the fluorescence assay for DNA cleavage, pancreatic DNase was used to introduce "nicks" into negatively supercoiled and relaxed PM2 DNA (Fig. 3). The fluorescence of negatively supercoiled DNA increased by a total of 30% as the DNA became nicked. After heat-denaturation of the DNA at pH 12, a concomitant decrease in fluorescence was observed as the nicked DNA became single-stranded and no longer bound ethidium. Only 2% of the ethidium fluorescence obtained on nicking of all CCC DNA molecules was seen after the heat treatment, possibly due to residual long hairpin duplexes. Fig. 3 also shows that differences in the original superhelical density of the substrate DNA do not interfere with the measurement of DNA cleavage. The fluorescence of relaxed DNA increased by 98% when completely nicked, compared with a 33% increase for supercoiled PM2 DNA ($\sigma = -0.12$). However, the difference between the fluorescence readings before and after the heat treatment at pH 12 reflected only the amount of nicked (open-circular and linear) DNA present, regardless of superhelical density of the intact DNA (Fig. 3B, *inset*).

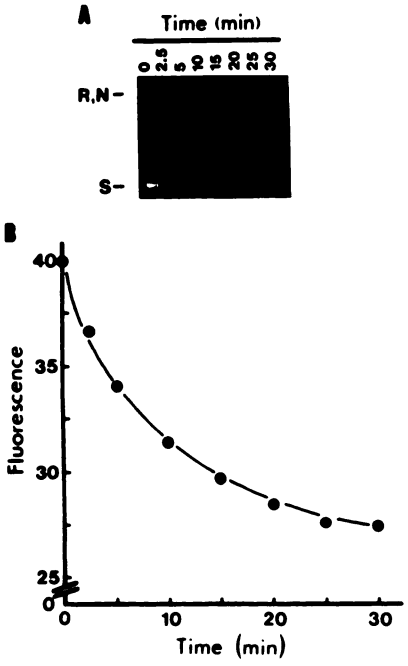

Fig. 1. Relaxation of supercoiled PM2 DNA by calf thymus topoisomerase II. The relaxation of native PM2 DNA by 1 unit of topoisomerase II under standard relaxation conditions was followed over time with agarose gel electrophoresis (A) and the pH 12 ethidium fluorescence assay (B). *Bands R, N,* and *S,* relaxed, nicked, and supercoiled DNA, respectively. Complete relaxation is indicated by a 33% final decrease in ethidium fluorescence, as measured by the fluorescence assay.

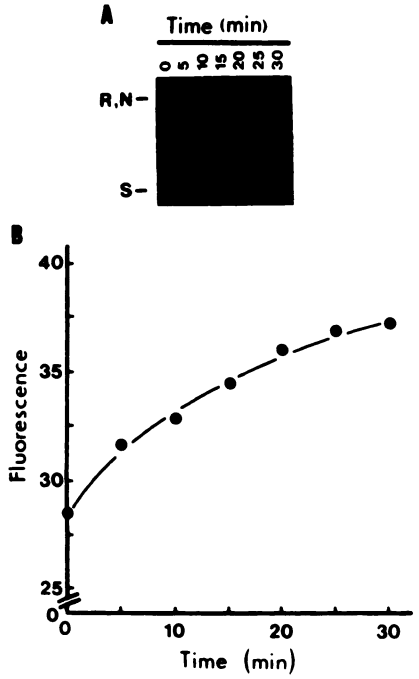

Fig. 2. Supercoiling of relaxed PM2 DNA by *M. luteus* gyrase. The supercoiling of relaxed PM2 DNA by 1 unit of gyrase under standard supercoiling conditions was followed over time by agarose gel electrophoresis (A) and the pH 12 ethidium fluorescence assay (B). *Bands R, N,* and *S,* as for Fig. 1. Complete supercoiling is indicated by a 33% final increase in ethidium fluorescence, as measured by the fluorescence assay.

Glu^- rather than Cl^- has been identified as the physiological counterion to K^+ (17). Many DNA-protein interactions are affected by Glu^- replacing Cl^-. Zwelling *et al.* (18) have demonstrated that DNA topoisomerases I and II retain their enzyme activity *in vitro* over a wider range of ionic strength when K-Glu rather than KCl is used in the reaction. That observation was quantitated by DNA decatenation and cleavage assays using agarose gel electrophoresis and by alkaline elution assays for DNA cleavage. Using the ethidium fluorescence assay, we have quantitated the effects of K-Glu and KCl on the relaxation activity of DNA topoisomerase II (Fig. 4). Similar to Zwelling's results, the enzyme was completely inactive with >250 mM KCl, whereas it remained active until the K-Glu concentration reached 450 mM. In addition, our results were identical whether the potassium salt of L-Glu^- or D-Glu^- (data not shown) was used, indicating that a stereospecific interaction of glutamate with the enzyme, DNA, or water bound to DNA does not contribute to this enhancing effect.

Drug-dependent inhibition with nonintercalating drugs. Enzyme inhibition was studied for mammalian topoisomerases II and I and bacterial gyrase using the antineoplastic drugs VM-26 and camptothecin and the antibacterial drug novobiocin, respectively (Figs. 5–7). Under standard assay conditions (200 mM K-Glu, 1 unit of enzyme), a small amount of DNA cleavage by topoisomerase II was observed with VM-26 (Fig. 5A, *L*). These samples were heat-treated at 65° to reverse the cleavable complexes before removal of samples for the fluorescence assay (13). This eliminates any interference with the quantitation of inhibition. The ethidium fluorescence read-

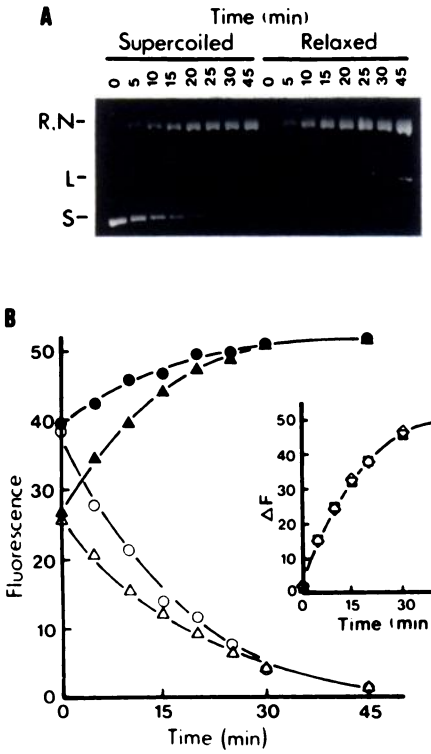

Fig. 3. Cleavage of negatively supercoiled and relaxed PM2 DNA by pancreatic DNase. From a 100-μl sample containing 20 μg of supercoiled PM2 DNA, a 50-μl aliquot was relaxed completely by calf thymus topoisomerase I under standard relaxation conditions. The remaining 50 μl were treated similarly but without topoisomerase. Forty microliters each of the resulting supercoiled (●, ○) and relaxed (▲, △) DNA samples were added to 120 μl of 1.3 ng/ml pancreatic DNase (Boehringer Mannheim) in 50 mM Tris·HCl (pH 8), 5 mM $MgCl_2$, 30 μg/ml BSA. The cleavage reaction was incubated at 37°, while 10-μl samples were taken at various times for agarose gel electrophoresis (A) and the pH 12 ethidium fluorescence assay (B). *Bands R, N,* and *S,* as for Fig. 1; *L,* linear DNA. Ethidium fluorescence assay readings were taken before (●, ▲) and after (○, △) heat-denaturation of the DNA in the pH 12 ethidium assay buffer. *Inset,* absolute difference between the before- and after-heat fluorescence readings (Δ*F*) for the originally supercoiled (□) and relaxed (◇) DNA samples.

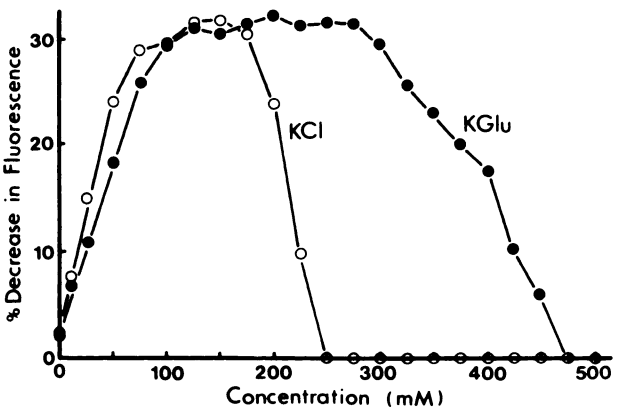

Fig. 4. Effect of various concentrations of K-Glu and KCl on topoisomerase II relaxation activity. Reactions (20 μl each) containing 1 μg of native PM2 DNA with various concentrations of K-Glu (●) or KCl (○) were relaxed by 1 unit of topoisomerase II. After incubation at 37° for 30 min, 10-μl samples were taken for the pH 12 ethidium fluorescence assay. Complete relaxation of the DNA is indicated by a final decrease in fluorescence of about 33%.

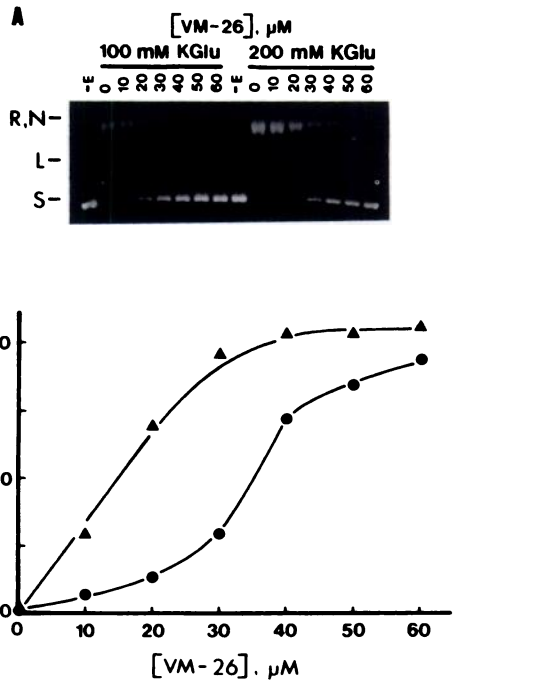

Fig. 5. Inhibition of calf thymus topoisomerase II relaxation activity by VM-26 at 100 mM and 200 mM K-Glu. Reactions (40 μl each) contained standard relaxation conditions with either 100 mM (▲) or 200 mM (●) K-Glu and various amounts of VM-26. After incubation at 37° for 30 min, 10-μl samples were taken for agarose gel electrophoresis (A). *Bands R, N, L,* and *S,* as for Fig. 3. The remaining reaction was heated at 65° for 10 min, to reverse the cleavable complexes, before removal of 10 μl for the ethidium fluorescence assay, from which percentage of enzyme inhibition was quantitated (B).

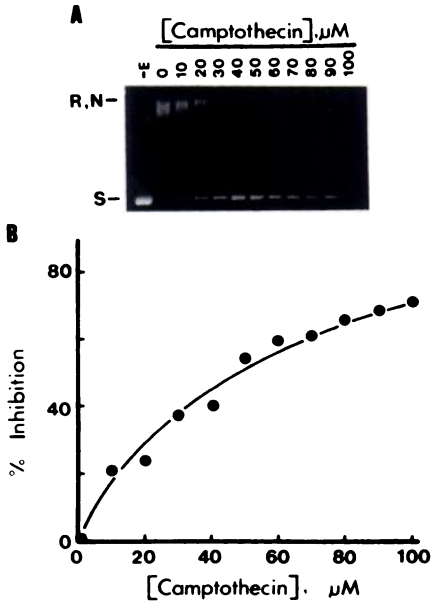

Fig. 6. Inhibition of calf thymus topoisomerase I relaxation activity by camptothecin. Reactions (40 μl each) contained standard relaxation conditions with various amounts of camptothecin. After incubation at 37° for 30 min, 10-μl samples were removed for agarose gel electrophoresis (A) and the pH 12 ethidium fluorescence assay (B). *Bands R, N,* and *S,* as for Fig. 1. Enzyme inhibition was quantitated from the ethidium fluorescence assay.

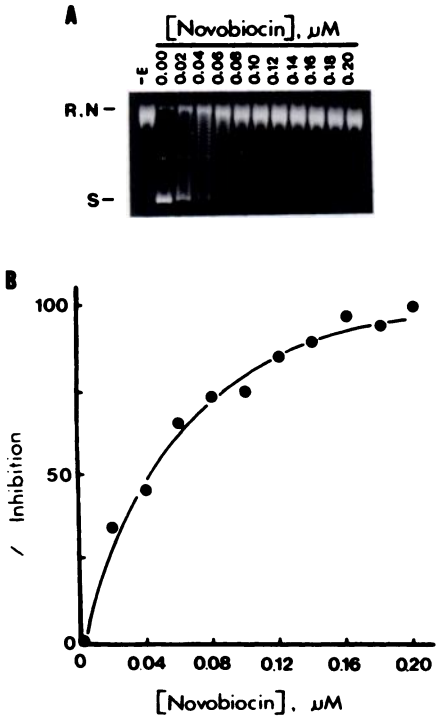

Fig. 7. Inhibition of *M. luteus* gyrase supercoiling activity by novobiocin. Reactions (40 μl each) contained standard supercoiling assay conditions with various amounts of novobiocin. After incubation at 37° for 30 min, 10-μl samples were removed for agarose gel electrophoresis (A) and the pH 12 ethidium fluorescence assay (B). *Bands R, N,* and *S,* as for Fig. 1. Enzyme inhibition was quantitated from the ethidium fluorescence assay.

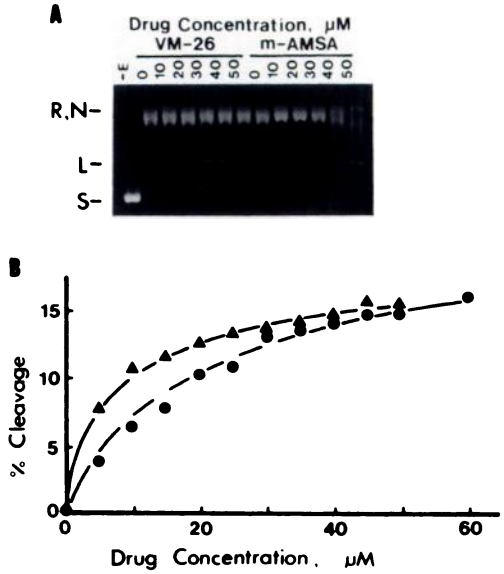

Fig. 8. Calf thymus topoisomerase II-mediated DNA cleavage with VM-26 and *m*-AMSA. Reactions (40 μl each) contained standard cleavage assay conditions, including 4 units of topoisomerase II, and various amounts of VM-26 (●) or *m*-AMSA (▲). After a 30-min incubation at 37°, 10-μl samples were removed for agarose gel electrophoresis (A) and the pH 12 ethidium fluorescence assay (B). *Bands R, N, L,* and *S,* as for Fig. 3. DNA cleavage was quantitated from the fluorescence assay.

ings of the cleavable complex-reversed samples were virtually parallel before and after heat denaturation at pH 12 (data not shown), indicating that very little or no cleaved DNA was present. No detectable DNA breaks were found for topoisomerase I with camptothecin (Fig. 6) or for gyrase with novobiocin (Fig. 7). In 200 mM K-Glu, topoisomerase II was inhibited by 75% with 60 μM VM-26 (Fig. 5B). When the concentration of K-Glu was lowered to 100 mM, there was more enzyme inhibition (84% at 60 μM VM-26) and the drug-dependent inhibition plot changed from a sigmoidal to a hyperbolic shape (Fig. 5B). In contrast, when KCl was used there was more inhibition at a higher concentration of KCl (200 mM) than at a lower concentration (100 mM) with 60 μM VM-26, and both inhibition plots were hyperbolic (data not shown).

Topoisomerase-mediated DNA cleavage with nonintercalating and intercalating drugs. It is possible to quantitate conveniently, by the fluorescence method, the extent of drug-induced topoisomerase-mediated DNA cleavage for both nonintercalating and intercalating drugs. This is demonstrated in Fig. 8, which illustrates DNA cleavage mediated by topoisomerase II in the presence of the nonintercalating drug VM-26 and the intercalating drug *m*-AMSA. At the concentrations used in these experiments, *m*-AMSA did not interfere with the ethidium fluorescence readings, as monitored by a control experiment without enzyme. The DNA breakage by these drugs was dose dependent and showed saturation at higher drug concentrations. In order to obtain significant levels of DNA breakage, an increased amount of enzyme (4 units) was used in this study, compared with that used for the catalytic relaxation and supercoiling assays (1 unit). For the same reason, 100 mM K-Glu instead of 200 mM K-Glu was used in these assays. Percentages of DNA cleavage observed with 4 units of enzyme and 60 μM VM-26 were 16% at 100 mM K-Glu (Fig. 8B) and 11.5% at 200 mM K-Glu (Fig. 9C).

Effects of topoisomerase II concentration on enzyme inhibition and DNA cleavage. The extent of topoisomerase II inhibition and topoisomerase II-mediated DNA breaks in the presence of VM-26 is highly dependent on the amount of enzyme used in an assay (Fig. 9). The assay reaction was analyzed for both enzyme inhibition and DNA cleavage in the presence of a fixed concentration of VM-26 (60 μM) and various amounts of enzyme (0.5–4 units/assay). The results clearly show that, at lower concentrations of topoisomerase II, the predominant effect was enzyme inhibition. In contrast, at higher enzyme levels more DNA cleavage occurred, with less enzyme inhibition (Fig. 9). A similar observation has been made for topoisomerase I with camptothecin, using agarose gels (19). It is also interesting to note that after the heat reversal of cleavable complexes no supercoiled DNA was observed. This may indicate that the cleavable complexes were produced only after the DNA was relaxed. Again, a similar observation has been documented in a study with topoisomerase I and camptothecin (20).

Discussion

In general, the ethidium fluorescence method is a fast, convenient, and quantitative method for topoisomerase assays. The complete results, including any necessary calculations and plotting of the data, may be readily obtained within 1 hr. Agarose gel methods tend to require long electrophoretic runs to achieve good resolution, and densitometric analysis or radiolabeled DNA are necessary for quantitation.

Advantages obtained with the sensitivity and quantitative power of this assay over gel methods may be particularly appreciated in measuring the progressive action of topoisomerases on their DNA substrate. Densitometer scanning of agarose

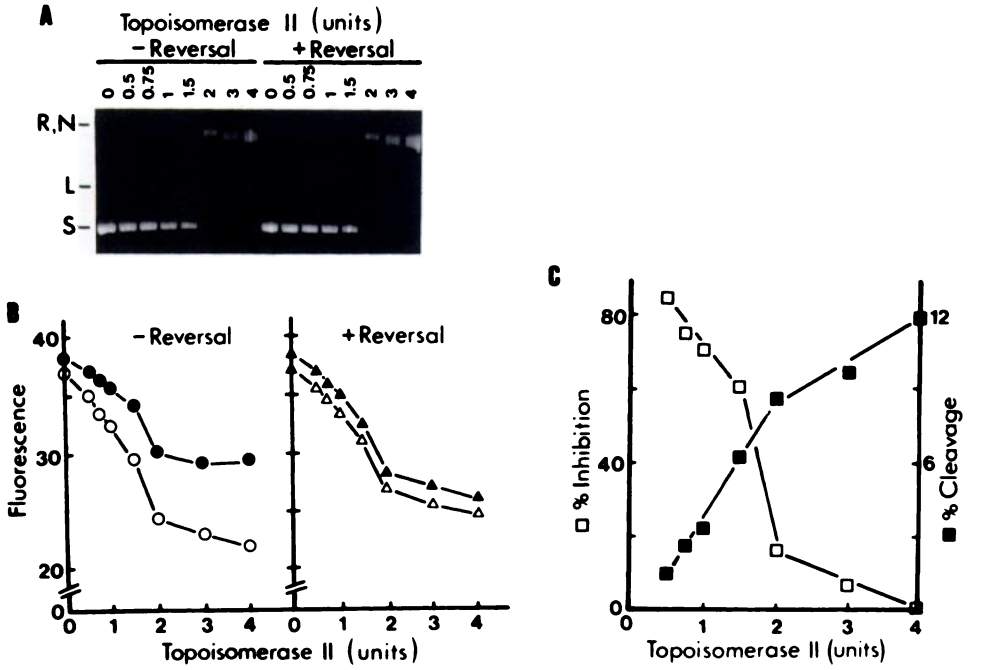

Fig. 9. Topoisomerase II inhibition and topoisomerase II-mediated DNA cleavage with VM-26. Reactions (40 μl each) contained standard relaxation assay conditions with 60 μM VM-26 and various amounts of topoisomerase II. After incubation at 37° for 30 min, 10-μl samples were taken for agarose gel electrophoresis (A) and the pH 12 ethidium fluorescence assay (B and C). The remaining reaction was heat-treated, to reverse the cleavable complexes, before removal of samples for agarose gel electrophoresis and the fluorescence assay, as described above. *Bands R, N, L*, and *S*, as for Fig. 3. Fluorescence was measured both before (●, ▲) and after (○, △) heat-denaturion of the DNA in the pH 12 ethidium assay buffer (B). Enzyme inhibition (□) was calculated from the fluorescence assay of the cleavable complex-reversed samples, whereas DNA cleavage (■) was calculated from the samples that were not cleavable complex reversed (C).

gels can be difficult to quantitate, especially when there are many bands. This is further complicated by the dependence of ethidium intercalation on DNA superhelical density. Consequently, identical amounts of DNA located in separate bands (i.e., different superhelical densities) result in different ethidium fluorescence intensities. The ethidium fluorescence method described here, however, takes advantage of this very fact and so reflects shifts in the average superhelical density of the DNA sample. The net extent of a topoisomerase reaction is, therefore, accurately determined. This method is limited, however, in that it cannot discriminate between processive and distributive modes of topoisomerase action, as revealed by the topoisomer distribution on agarose gels. Both of these assays, however, are based upon the observation of Morgan and Pulleyblank (21) that ethidium bromide will bind selectively at pH 12 to double-stranded DNA in the presence of denatured DNA, which lacks secondary structure at this pH.

The fluorescence assay described here uses a purified system and is not designed to replace cell studies, such as those that use alkaline elution, K-SDS precipitation, or unwinding assays. Nonetheless, other researchers have found that DNA cleavage results using purified enzymes and CCC DNA agree with those from cultured cells (4, 9, 22). Consequently, it may be more practical, in many cases, to use a simple and fast assay method such as that described here. These instances may include procedures that require a large number of samples, such as detailed kinetic studies and the primary screening of compounds for antitopoisomerase activities. In addition, because not all compounds that inhibit topoisomerase activity stabilize a cleavable complex, methods that measure only DNA cleavage are not applicable for studies of such compounds.

We have demonstrated that the ethidium fluorescence method is suitable for assessing the abilities of topoisomerase-targeted drugs both to inhibit topoisomerase catalytic activity and to stimulate topoisomerase-mediated cleavage. Unlike non-intercalating drugs, intercalating drugs may cause problems in catalytic assays. For example, a CCC DNA in which all supercoils were removed by such a drug cannot change its linking number with a topoisomerase. This could be incorrectly interpreted as inhibition of topoisomerase activity. We have previously described a simple rapid (~2 hr) fluorescence assay for measuring the unwinding angle of intercalating drugs with excess topoisomerase (23). This allows for a quick assessment of whether topoisomerase catalytic activity can be quantitated. On the other hand, quantitation of topoisomerase-mediated cleavage is not influenced by the presence of intercalating drugs, because it does not depend on the superhelical density of the DNA as a basis for measurement. In addition, intercalating drugs cause little or no interference with ethidium fluorescence itself, because of the large dilution of the reaction sample by the ethidium assay buffer and the large excess of ethidium compared with drug, as is the case for *m*-AMSA, the intercalating drug used in this study.

Using the ethidium fluorescence assay, we have been able to corroborate the results described previously regarding anion-dependent effects on the topoisomerase activity when K-Glu is substituted for KCl as the salt component of the reaction (18). In addition to providing quantitation of these effects on the catalytic relaxation activity of topoisomerase II (Fig. 4), we have found both quantitative and qualitative differences in VM-26-dependent topoisomerase II inhibition at 100 and 200 mM K-Glu (Fig. 5). The sigmoidal inhibition plot with 200 mM K-Glu indicates cooperative effects or changes in dissociation constants for interaction between VM-26, enzyme, or DNA in the presence of >100 mM K-Glu. Additional kinetic studies would be required to provide further details regarding this observation.

Considerable effort has been dedicated to understanding the mechanism of cell killing by antitopoisomerase drugs and modes of cellular resistance to these drugs. As indicated by the varying and often conflicting results found throughout the literature, these issues, particularly the role of the cleavable complexes, remain unresolved (reviewed in Ref. 1). Recently, two groups have separately examined the dependence of drug-

induced DNA cleavage and cytotoxicity on the cell cycle stage, using chromosomal DNA break frequency as a parameter for drug sensitivity (24, 25). In both of these studies, sensitivity to DNA cleavage was maximal during mitosis, which was when topoisomerase II activity or content was highest, whereas maximal cytotoxicity was observed in S phase, where the least amount of topoisomerase II activity or content was found. In ethidium fluorescence assays with topoisomerase II and VM-26, we have observed a clear inverse relationship between the degree of enzyme inhibition and the extent of DNA breakage (Fig. 9). This result then indicates that in S phase topoisomerase II was maximally inhibited, suggesting that enzyme inhibition may be an important factor contributing to cell death. This is probably an oversimplification, because our observation was made using a purified system and it is difficult to extrapolate to the much more complex situation that exists within the cells. Nonetheless, it does demonstrate the usefulness of the fluorescence method in mechanistic studies. This experiment also shows that both enzyme inhibition and cleavable complex formation become relatively independent of changes in enzyme concentration at higher topoisomerase II levels. Consequently, only at lower enzyme concentrations will changes in the amount of enzyme produce significant changes in the degree of enzyme inhibition and DNA cleavage. It is possible that discrepancies among attempts to correlate the amount of enzyme activity with various drug effects (e.g., enzyme inhibition, DNA breakage, and cytotoxicity) may be a result of differences in the total enzyme levels used in the assays.

In conclusion, the applications of the ethidium fluorescence assay are by no means limited to those presented here. We have also used this method extensively to detect enzyme fractions during the purification of calf thymus topoisomerase II, with excellent results. Because of the large sample-handling capacity, speed, and versatility, it is particularly suitable for screening of topoisomerase inhibitors, kinetic studies such as K_i determinations, drug specificity studies, and mechanistic studies. Future uses may also include enzyme assays of crude cell extracts and DNA cleavage assays in cell studies (26).

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