$= k^-$, it has the characteristic equation

$$(k^+ + \lambda)[(k^- + \lambda)^n - (k^-)^n] - (k^+)^n[(k^- + \lambda)^n - (k^-)^n] - (k^+)^n(k^-)^n = 0$$

which can be reduced to

$$(1 + \lambda/k^+)^{-n} + (1 + \lambda/k^-)^{-n} = 1$$

and solved implicitly for λ/k^+ for assumed values of n and k^+/k^-. For large values of n, λ/k^+ and λ/k^- become very small, and the characteristic equation can be written in exponential notation:

$$\exp(-n\lambda/k^+) + \exp(-n\lambda/k^-) = 1$$

which can be solved for assumed values of k^+/k^-, e.g.

$$k^+/k^- = 1 \qquad \lambda/k^+ = (1/n) \ln 2$$

$$k^+/k^- = 2 \text{ or } \tfrac{1}{2} \qquad \lambda/k^+ = (1/n) \ln [2/(5^{1/2} - 1)]$$

Registry No. Qβ replicase, 9026-28-2.

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Formation and Rejoining of Deoxyribonucleic Acid Double-Strand Breaks Induced in Isolated Cell Nuclei by Antineoplastic Intercalating Agents†

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ABSTRACT: The biochemical characteristics of the formation and disappearance of intercalator-induced DNA double-strand breaks (DSB) were studied in nuclei from mouse leukemia L1210 cells by using filter elution methodology [Bradley, M. O., & Kohn, K. W. (1979) *Nucleic Acids Res. 7*, 793-804]. The three intercalators used were 4′-(9-acridinylamino)-methanesulfon-*m*-anisidide (*m*-AMSA), 5-iminodaunorubicin (5-ID), and ellipticine. These compounds differ in that they produced predominantly DNA single-strand breaks (SSB) (*m*-AMSA) or predominantly DNA double-strand breaks (ellipticine) or a mixture of both SSB and DSB (5-ID) in whole cells. In isolated nuclei, each intercalator produced DSB at a frequency comparable to that which is produced in whole cells. Moreover, these DNA breaks reversed within 30 min after drug removal. It thus appeared that neither ATP nor other nucleotides were necessary for intercalator-dependent DNA nicking–closing reactions. The formation of the intercalator-induced DSB was reduced at ice temperature. Break formation was also reduced in the absence of magnesium, at a pH above 6.4 and at NaCl concentrations above 200 mM. In the presence of ATP and ATP analogues, the intercalator-induced cleavage was enhanced. These results suggest that the intercalator-induced DSB are enzymatically mediated and that the enzymes involved in these reactions can catalyze DNA double-strand cleavage and rejoining in the absence of ATP, although the occupancy of an ATP binding site might convert the enzyme to a form more reactive to intercalators. Three inhibitors of DNA topoisomerase II—novobiocin, nalidixic acid, and norfloxacin—reduced the formation of DNA strand breaks. These findings are consistent with the hypothesis that intercalator-induced DNA breakage results from the DNA cleavage action of a DNA topoisomerase II.

DNA intercalators produce strand breaks in cellular DNA (Ross et al., 1978; Zwelling et al., 1981, 1982a,d). The formation of these breaks tends toward saturation at high drug concentration and is temperature dependent (Zwelling et al., 1981, 1982d). The DNA breaking activity, with biochemical properties similar to those of intercalator-induced DNA scission, can be reconstituted in a cell-free system by means of a 0.35 M NaCl extract of isolated nuclei (Filipski et al., 1983). These characteristics have suggested that the formation of intercalator-induced DNA breaks is mediated by enzymes associated with chromatin. In addition, several aspects of this intercalator-induced DNA breakage have suggested that the

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activities of DNA topoisomerases might be involved: (1) the breaks were found to be associated with apparently covalently linked protein (Ross et al., 1978, 1979) which prevented DNA strand swiveling (Pommier et al., 1984a); (2) there were concomitant changes in DNA linking number (Pommier et al., 1984b); and (3) an inhibitor of DNA topoisomerases, novobiocin, inhibited the restoration of DNA linking number and reduced the rate of break resealing after 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (*m*-AMSA) removal (Pommier et al., 1984b).

Two types of DNA topoisomerases have been characterized in bacteria and mammalian cells (Cozzarelli, 1979; Gellert, 1981). Type I DNA topoisomerases act by means of transient DNA single-strand breaks (SSB) and can break and rejoin DNA repeatedly without any cofactor to supply energy. Type II DNA topoisomerases differ from type I in that they act by means of transient double-strand breaks (DSB) and in that some of the reactions that they catalyze require a DNA-dependent hydrolysis of ATP (Cozzarelli, 1979; Gellert, 1981). As intercalators produce both DNA single- (SSB) and double-strand breaks (DSB) in cells (Ross & Bradley, 1981; Ross & Smith, 1982; Zwelling et al., 1981, 1982a,d), the breaks could result from the action of either type I or type II DNA topoisomerases.

Intercalators can form DNA breaks also in isolated nuclei (Filipski & Kohn, 1982; Pommier et al., 1982) but at frequencies that are lower than in whole cells (Pommier et al., 1982). Since the ATP concentration of isolated nuclei is well below that usually necessary for energy-dependent topoisomerase II activity, it seemed possible that the relative reduction in break formation in isolated nuclei vs. whole cells was due to reduced production of DSB by ATP-dependent type II DNA topoisomerase but normal production of SSB by type I DNA topoisomerase.

The production of DSB by DNA intercalators in isolated nuclei was therefore investigated. Intercalating drugs from three chemical classes were employed. All three had been found to produce both SSB and DSB in whole cells, but the relative frequency of each type of scission varied from drug to drug. Ellipticine produced a greater DSB/SSB ratio than the anthracycline derivative 5-iminodaunorubicin (5-ID) (Zwelling et al., 1982a,d), which in turn produced a greater DSB/SSB ratio than the acridine derivative 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (*m*-AMSA) (Zwelling et al., 1981). All three agents produced DSB in isolated nuclei, and the decreased frequency of DNA scission detected in nuclei as compared with whole cells could not be explained by the selective diminution of DSB formation in isolated nuclei. The isolated nuclei system permitted further biochemical characterization of the intercalator-induced DNA double-strand breaking–rejoining activity. In particular, we found that the reaction was temperature, pH, magnesium, and salt dependent and that three inhibitors of DNA topoisomerase II—novobiocin, nalidixic acid, and norfloxacin (Gellert, 1981)—partially inhibited the formation of DNA breaks induced by intercalators in isolated nuclei. Although ATP was not required for either the breaking or the resealing reaction, both ATP and the nonhydrolyzable analogues of ATP enhanced intercalator-induced DSB production. These data, taken together with our previous work (Pommier et al., 1984a,b) are consistent with the involvement of type II DNA topoisomerases in the intercalator-induced DNA breaking–rejoining reactions.

Materials and Methods

Cells and Radioactive Labeling. L1210 mouse leukemia cells were grown in suspension culture in RPMI 1630 medium supplemented with 15% fetal calf serum. Stock cultures were maintained in static bottles without antibiotics and were used to initiate suspension cultures. Cultures used to assess drug effects were in exponential growth phase with a doubling time of 13–15 h.

Cellular DNA was radioactively labeled in exponentially growing cells by incubation with [2-^{14}C]thymidine (0.02 μCi mL^{-1}) or with [*methyl*-^{3}H]thymidine (0.1 μCi mL^{-1}, 10^{-6} M unlabeled thymidine added) for 20 h at 37 °C (New England Nuclear, Boston, MA). In all cases, radioactive label was removed by centrifugation prior to drug treatment, irradiation of the cells, or preparation of nuclei.

Isolation of L1210 Cell Nuclei. The procedure of nucleus isolation has been described previously (Filipski & Kohn, 1982). L1210 mouse leukemia cells were centrifuged and resuspended in nucleus buffer [150 mM NaCl, 1 mM KH_2PO_4, 5 mM $MgCl_2$, 1 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), and 0.1 mM dithiothreitol, pH 6.4] at 4 °C. These cells were centrifuged again and resuspended in $^{1}/_{10}$th volume of ice-cold nucleus buffer; $^{9}/_{10}$th volume of 4 °C nucleus buffer containing 0.3% Triton X-100 was then added and the mixture incubated for 10 min at 4 °C. The nuclei were pelleted by centrifugation (1200 rpm for 5 min) and resuspended in nucleus buffer at 37 °C. Nuclei were examined microscopically after being stained with trypan blue to confirm their permeability.

Drug Treatments and Cell Irradiation. 4′-(9-Acridinylamino)methansulfon-*m*-anisidide (*m*-AMSA) (NSC 249992) and ellipticine hydrochloride (NSC 71795) were obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, NCI. *m*-AMSA was dissolved in dimethyl sulfoxide at 10 mM, and ellipticine was dissolved in 10 mM HCl at 2.5 mM. 5-Iminodaunorubicin (5-ID) (NSC 254681) was a gift from Dr. Robert I. Glazer, Applied Pharmacology Section, Laboratory of Medicinal Chemistry and Biology, NCI, who obtained the compound from Dr. E. Acton, Stanford Research Institute. 5-ID was dissolved in glass-distilled water at 1 mM. *m*-AMSA and 5-ID were stored frozen in stock solutions. Ellipticine stock solutions were kept refrigerated in plastic tubes. Adenosine 5′-triphosphate (ATP) (Sigma Chemicals, St. Louis, MO), 5′-adenylyl imidodiphosphate (AMPPNP) (Boehringer Mannheim), and adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS) were dissolved immediately before use in nucleus buffer (pH 6.4). Novobiocin and coumermycin A_1 were purchased from Sigma Chemicals (St. Louis, MO) and dissolved in nucleus buffer immediately before use. Nalidixic acid (Sigma Chemicals), oxolinic acid (Warner-Lambert, Parke Davis, Ann Arbor, MI), and norfloxacin (a gift from Dr. Hans Gadebush, Merck Sharp & Dohme, Rahway, NJ) were dissolved first in NaOH as stock solutions and then in nucleus buffer whose pH was kept at 6.4.

Drug treatments were performed for 30 min at 37 °C unless otherwise indicated. ATP analogues and topoisomerase II inhibitors were incubated with nucleus buffer for 5 min before the addition of intercalators. All drug treatments were stopped by a 1:50 dilution of treated nuclei in iced drug-free nucleus buffer unless otherwise stated.

Isolated nuclei or L1210 cells at a concentration between $1 \times 1.5 \times 10^6$ and 10^6 cells mL^{-1} in iced nucleus buffer were irradiated with either a ^{137}Cs or a 200-kV X-ray source as described previously (Kohn et al., 1981). Nuclei or cells were maintained at ice temperature until they were assayed by alkaline elution.

Alkaline Elution Assays. The alkaline elution methodology has been described in detail in previous publications (Kohn et al., 1981; Zwelling et al., 1981).

DNA Double-Strand Breaks. DNA double-strand breaks (DSB) were measured by a modification of the method of Bradley & Kohn (1979). Approximately 2.5×10^5 [^{14}C]-thymidine-labeled nuclei or cells were deposited on a polycarbonate membrane filter (2-μm pore diameter; Nucleopore Corp., Pleasanton, CA) in a Swinnex 25 filter holder (Millipore Corp., Bedford, MA). Elution was performed with tetrapropylammonium hydroxide–ethylenediaminetetraacetic acid (EDTA)–0.1% sodium dodecyl sulfate (SDS), pH 9.6, layered above 2 mL of lysis solution (0.1 M glycine, 0.025 M Na_2EDTA, and 2% SDS, pH 10.0, plus 0.5 mg mL^{-1} proteinase K). Elution was carried out with a peristaltic pump to control the flow rate at a pump speed of 0.03–0.04 mL min^{-1} (2 mL h^{-1}), and fractions were collected at 3-h intervals for 15 h. The fraction of [^{14}C]DNA retained on the filter was plotted vs. the time of elution. The similarity between the curves describing the fraction of DNA eluted with time for DNA from drug-treated nuclei and those for that from X-irradiated nuclei allowed the expression of the intercalator-induced DSB in "DSB rd-equiv". The retention of DNA from drug-treated nuclei or cells at 10 h of elution was used as a measure of DSB frequency by comparing the retention of drug-treated nuclei or cells with a standard curve generated by assays performed with X-irradiated nuclei.

DNA Single-Strand Breaks. Treated (or untreated control) [^{14}C]thymidine-labeled nuclei (approximately 5×10^5) were mixed with an equal number of [^{3}H]thymidine-labeled cells which had received no drug treatment and had been irradiated to serve as internal standard cells (Kohn et al., 1981). Nuclei and cells were deposited on polycarbonate membrane filters and lysed with 0.1 M glycine, 0.025 M Na_2EDTA, and 2% SDS, pH 10.0, plus 0.5 mg mL^{-1} proteinase K. Elution was performed with tetrapropylammonium hydroxide–EDTA–0.1% SDS, pH 12.1, by using a peristaltic pump to control the flow rate. For the high-sensitivity assay, elution was carried out at a pump speed of 0.03–0.04 mL min^{-1} (2 mL h^{-1}), and fractions were collected at 3-h intervatls for 15 h as described previously (Kohn et al., 1981). For the low-sensitivity assay (which was required to quantify DNA single-strand break frequencies exceeding 600 rd-equiv), elution was carried out at a pump speed of 0.12–0.16 mL min^{-1}, and fractions were collected at 5-min intervals for 30 min (Zwelling et al., 1981). The [^{3}H]DNA internal standard cells received 300 rd of X irradiation in the high-sensitivity assay and 1000 rd in the low-sensitivity assay. Single-strand break frequency was calculated as follows:

$$\text{break frequency} = \frac{\log (r_1/r_0)}{\log (R_0/r_0)} P_B \qquad (1)$$

where P_B is the DNA break frequency produced by X-ray (300 or 1000 rd-equiv) and r_1, r_0, and R_0 represent the retention (Kohn et al., 1981) of DNA from drug-treated, untreated, and 300- or 1000-rd-treated [^{14}C]thymidine-labeled cells, respectively. Retention was evaluated at the time corresponding to retention of 0.35 of the [^{3}H]DNA in the high-sensitivity assay or 0.60 of the [^{3}H]DNA in the low-sensitivity assay. The exact choice of this end point was not critical since the elution kinetics were nearly first order with respect to time following all drug treatments. Results are expressed in terms of the X-ray dose that would produce an equivalent elution in the DSB or SSB assays, and the corresponding units are designated "DSB rd-equiv" or "SSB rd-equiv".

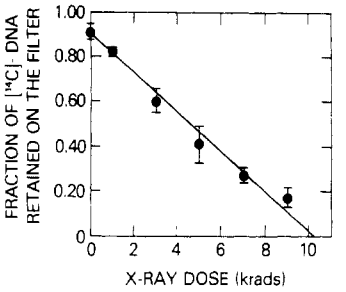

FIGURE 1: X-ray calibration curve of DNA double-strand breaks in isolated nuclei. L1210 cells were labeled with [^{14}C]thymidine. Nuclei were isolated and irradiated with the indicated X-ray doses in ice. Elution assays were performed with proteinase K (see Materials and Methods), and the fraction of [^{14}C]thymidine-labeled DNA retained on the filters after 10 h of elution was used as the end point.

The ratio of the frequency of true single-strand breaks (s) to the frequency of double-strand breaks (d) was calculated by the formula (Zwelling et al., 1981)

$$\frac{s}{d} = \frac{k_{RS}[SSB]}{k_{RD}[DSB]} - 2 \qquad (2)$$

where s includes only those single-strand breaks that are not part of a double-strand break. k_{RS} and k_{RD} are the measured single- and double-strand breaks produced per rad of X-ray. [SSB] is the apparent drug-induced single-strand break frequency, expressed as the dose of X-ray that would produce a similar effect; the units are SSB rd-equiv. [DSB] is the analogous value for double-strand breaks and is in units of DSB rd-equiv. The SSB frequency per rad has been estimated in L1210 cells to be $k_{RS} = 2.7 \times 10^{-12}$ rd^{-1} dalton^{-1} (Kohn et al., 1976). DSB frequencies at low X-ray doses in mammalian cells are more difficult to determine. A recent estimate in Ehrlich ascites cells by means of very low speed neutral density gradient sedimentation is $k_{RD} = (11.7 \pm 2) \times 10^{-14}$ rd^{-1} dalton^{-1} (Blöcher, 1982). We will make the assumption that k_{RD} is similar in L1210 cells and in Ehrlich ascites cells, and we adapt the value $k_{RS}/k_{RD} = (2.7 \times 10^{-12})/(11.7 \times 10^{-14}) = 23$; this estimate is subject to an experimental error of ±30%. Thus, from eq 2, if a drug produced solely double-strand breaks, s would equal 0, and the measured values for [SSB]/[DSB] would be 0.09.

m-AMSA Content in Nuclei. The technique used has been described previously (Vistica, 1979; Zwelling et al., 1982b). The DNA of exponentially growing L1210 cells was labeled with [^{3}H]thymidine. Nuclei whose DNA had been labeled with [^{3}H]thymidine were isolated, concentrated to approximately 10^7 mL^{-1} in nucleus buffer, and treated with a mixture consisting of 0.5 μM nonradiolabeled *m*-AMSA and 0.5 μM 4′-(9-[^{14}C]acridinylamino)methanesulfon-*m*-anisidide ([^{14}C]-*m*-AMSA) (19.6 mCi mmol^{-1}) (SRI International, Menlo Park, CA) (final [^{14}C]-*m*-AMSA concentration 0.01 μCi mL^{-1}) at 37, 22, or 4 °C. The *m*-AMSA content in isolated nuclei was determined by the [^{14}C]-*m*-AMSA/[^{3}H]thymidine-labeled DNA ratio obtained by counting the radioactivity associated with the nuclei after their sedimentation through silicone oil (Zwelling et al., 1982b).

Results

X-ray Calibration Curve for DNA Double-Strand Breaks. The fraction of the DNA from untreated nuclei retained on the filter after 10 h of elution was greater than 0.90, in agreement with the results of DSB assays of whole cells (Bradley & Kohn, 1979). Hence, the isolation of nuclei did not produce detectable DNA DSB (Figure 1).

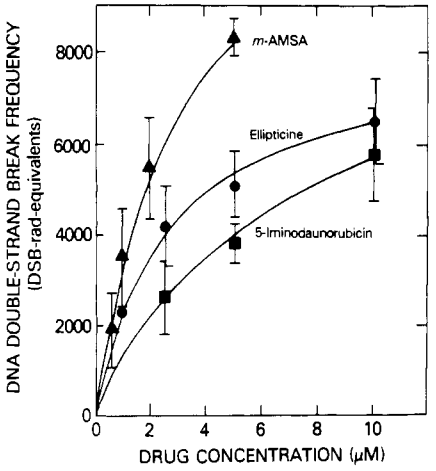

FIGURE 2: Dependence of DSB frequency on *m*-AMSA, 5-ID, or ellipticine concentration. Cells were labeled with [^{14}C]thymidine. Nuclei were isolated and treated for 30 min at 37 °C with the indicated concentrations of *m*-AMSA (▲), 5-ID (■), or ellipticine (●). DSB were then assayed by filter elution using proteinase K. Error bars represent the standard deviation of at least three independent experiments.

Table I: DNA Double-Strand Breaks (DSB rd-equiv) Produced by *m*-AMSA, 5-ID, or Ellipticine in Cells (RPMI 1630 Medium, pH 7.1) and in Isolated Nuclei (Nucleus Buffer, pH 6.4)[a]

	m-AMSA, 2 μM	5-ID, 10 μM	ellipticine, 10 μM
cells	6750	7550	7650
nuclei	6650	7550	6300

[a] Drug treatments (30 min at 37 °C) were performed in parallel in whole cells and in isolated nuclei.

X irradiation of isolated nuclei decreased the fraction of DNA retained on the filters proportionally to the administered X-ray dose (Figure 1). The assay yielded the regression line $r = 0.903 - 0.088x$, where r is the fraction of the DNA retained on the filter after 10 h of elution and x is the administered X-ray dose in kilorads (57 determinations between 0 and 9 krd; correlation coefficient = −0.98). This equation is similar to that reported for DNA elution in this assay using whole L1210 cells (Ross & Bradley, 1981; Zwelling et al., 1981). This calibration line was used to quantify the frequency of drug-induced DSB expressed in DSB rd-equiv, i.e., the X-ray dose giving equivalent 10-h retention in this assay.

DNA Double-Strand Breaks Produced by m-AMSA, 5-ID, and Ellipticine. *m*-AMSA, 5-ID, and ellipticine produced DSB in isolated nuclei (Figure 2). The frequency of DSB produced by *m*-AMSA, 5-ID, or ellipticine was dependent upon drug concentration, and *m*-AMSA produced the highest DSB frequencies.

DNA double-strand breaks produced by *m*-AMSA, 5-ID, or ellipticine in whole cells vs. isolated nuclei are compared in Table I. The observed DSB frequencies produced in cells are in agreement with previous data (Zwelling et al., 1981, 1982a,d) and were similar to those produced in isolated nuclei.

Kinetics of Formation and Disappearance of Intercalator-Induced DNA Double-Strand Breaks. At 37 °C, *m*-AMSA, 5-ID, and ellipticine formed DSB rapidly in isolated nuclei (Figure 3). After 10 min of exposure of the nuclei to the drugs, a plateau was reached, the height of which increased with intercalator concentration, as shown in Figure 3 for the case of ellipticine. Removal of the drugs was followed by a rapid disappearance of the DSB (Figure 3).

In the case of *m*-AMSA, the kinetics of this disappearance were similar to those seen in whole cells (Zwelling et al., 1981).

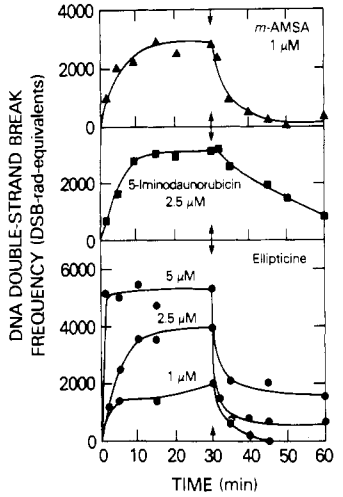

FIGURE 3: Kinetics of formation and resealing of DNA double-strand breaks in isolated nuclei exposed to 1 μM *m*-AMSA, 2.5 μM 5-ID, or 1, 2.5, or 5 μM ellipticine. Drug treatments were performed at 37 °C. After a 30-min exposure to the drug, the nuclei were diluted by a 1:50 dilution in drug-free nucleus buffer at 37 °C (arrows). At the indicated times, DSB were assyed by filter elution using proteinase K. *m*-AMSA (▲), upper panel; 5-ID (■), middle panel; ellipticine (●), lower panel.

On the other hand, in the case of ellipticine, the DSB reversed rapidly in isolated nuclei, whereas they persisted after removal of whole cells from drug-containing medium (Zwelling et al., 1982d). The persistence of ellipticine-induced DNA breaks in cells, however, was likely to be secondary to the retention of this lipophilic compound within the cell membranes (Ross & Smith, 1982). In isolated nuclei, the absence of intact cell membranes and cytoplasmic components might preclude such a depot effect. The kinetics of 5-ID break formation and resealing in isolated nuclei (Figure 3) were similar to what they were in cells (Zwelling et al., 1982a), although the plateau of break formation was reached more rapidly in isolated nuclei than in whole cells. Perhaps 5-ID enters cells more slowly than *m*-AMSA (Figure 3) (Zwelling et al., 1982b), but the absence of intact cell membranes in isolated nuclei removes a barrier which limits 5-ID influx in whole cells.

Relative Production of DNA Double-Strand Breaks and DNA Single-Strand Breaks. Aliquots of the same nucleus suspensions were assayed simultaneously for single-strand breaks (SSB) and DSB. The frequency of each type of drug-induced DNA break was expressed in SSB rd-equiv or DSB rd-equiv, i.e., as the X-ray dose that would produce the same frequency of that type of break (Figure 4). The apparent [SSB]/[DSB] frequency ratios for 5-ID and ellipticine were similar. In the case of *m*-AMSA, [SSB]/[DSB] was clearly lower than those values for the two other drugs.

By use of eq 2 (see Materials and Methods), the ratios of intrinsic single- to double-strand breaks (s/d) were estimated from the measured [SSB]/[DSB] ratios (Table II). s/d for *m*-AMSA was between 2 and 5.4. Thus, at least some, and probably most, of the *m*-AMSA-induced DNA breakage was single stranded. In the case of 5-ID or ellipticine, negative values were obtained. Such negative values could arise if k_{RS}/k_{RD} for X-rays in isolated nuclei was actually greater than the assumed value of 23. The values for ellipticine were slightly lower than those for 5-ID and may correspond to exclusive DSB production. These results support the conclusion that all three intercalators studied produced a larger number of DSB per SSB than did X-ray and that the intercalator-induced DSB did not arise from randomly distributed SSB, some close enough on opposite strands to produce DSB.

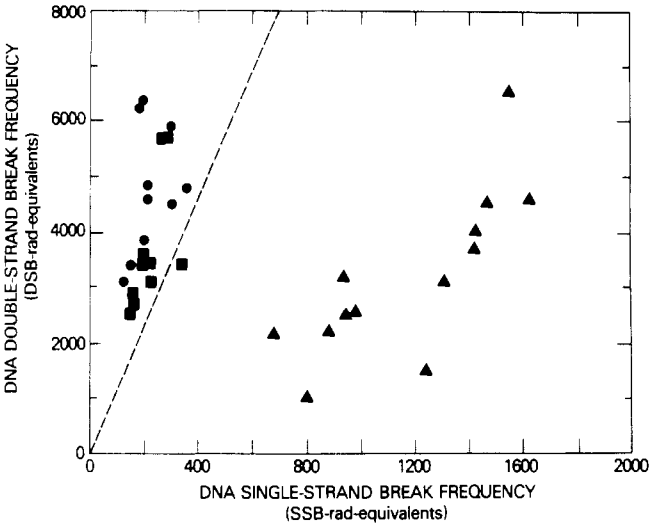

FIGURE 4: Relative production of DNA double-strand breaks and DNA single-strand breaks by *m*-AMSA (0.5–2 μM) (▲), 5-ID (2.5–10 μM) (■), or ellipticine (2.5–10 μM) (●) in isolated nuclei. Nuclei were treated with the indicated drugs for 30 min at 37 °C. Aliquots of the same nucleus suspension were assayed concurrently for SSB and DSB. The broken line represents the relationship that would be expected if all measured DSB were derived from opposed SSB, on the basis of an assumed X-ray-induced DSB/SSB ratio of 1/23.

Table II: Estimates of True Single-Strand to Double-Strand Break Ratio in Isolated Nuclei Treated with DNA Intercalating Drugs[a]

drug treatment	[SSB]/[DSB]	no. of determinations	*s*/*d*
m-AMSA, 1 μM	0.39 ± 0.11[b]	6	2.0
m-AMSA, 2 μM	0.32 ± 0.05	4	5.4
μ-AMSA, 5 μM	0.22 ± 0.25	2	3.4
5-ID, 2.5 μM	0.58 ± 0.003	3	−0.7
5-ID, 5 μM	0.060 ± 0.006	4	−0.6
5-ID, 10 μM	0.065 ± 0.029	3	−0.5
ellipticine, 2.5 μM	0.045 ± 0.006	3	−1.0
ellipticine, 5 μM	0.054 ± 0.021	4	−0.8
ellipticine, 10 μM	0.042 ± 0.010	3	−1.0

[a] Abbreviations: [SSB], measured SSB frequency in SSB rad-equivalents; [DSB], measured DSB frequency in DSB rad-equivalents; *s*, frequency of intrinsic SSB (not including those arising from DSB); *d*, DSB frequency; *s*/*d*, calculated according to eq 2 by assuming K_{RS}/K_{RD} = 23. [b] Mean ± 1 SD of the indicated number of independent determinations.

Ellipticine and 5-ID apparently produced almost exclusively DSB, whereas *m*-AMSA produced both single- and double-strand breaks.

Temperature Dependence. Treatment of isolated nuclei for 30 min at reduced temperatures resulted in the production of fewer DSB than produced at 37 °C (Figure 5). At 4 °C, *m*-AMSA produced little or no DSB. This was not due to decreased drug content, since the association of [^{14}C]-*m*-AMSA with isolated nuclei was even higher at 4 °C than at 22 or 37 °C (Figure 6). Hence, the process which produces DSB has one or more temperature-sensitive steps.

In contrast to *m*-AMSA or 5-ID, ellipticine showed a lack of temperature dependence between 22 and 37 °C (Figure 5). This was true over a range of ellipticine concentrations (Figure 7).

pH Dependence. The production of DSB by *m*-AMSA, 5-ID, and ellipticine in isolated nuclei decreased by 65–90% as the pH was raised from 6.4 to 7.1 (Figure 8). Above this range of pH values, the DNA of nuclei acquired DSB even in the absence of any drug treatment. The decreasing effect

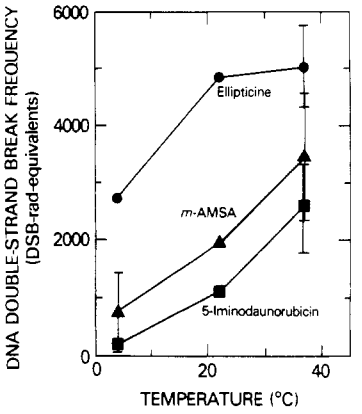

FIGURE 5: Dependence of DSB formation on temperature. Nuclei were treated for 30 min at the indicated temperature with 1 μM *m*-AMSA (▲), 2.5 μM 5-ID (■), or 5 μM ellipticine (●). DSB frequency was assayed by filter elution using proteinase K. Error bars represent the standard deviation of at least three independent determinations.

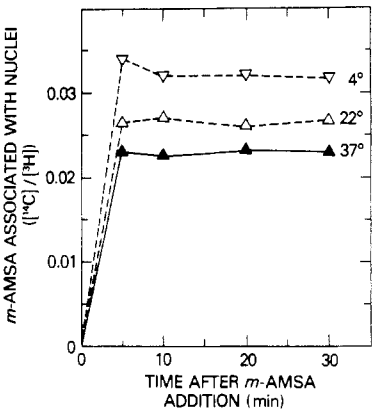

FIGURE 6: Dependence of the nuclear content of [^{14}C]-*m*-AMSA on temperature. Cellular DNA was labeled with [^{3}H]thymidine. Nuclei were isolated and incubated with 1 μM (0.01 μCi mL^{-1}) [^{14}C]-*m*-AMSA for 30 min at 37 (▲), 22 (△), or 4 °C (▽). The nuclear content of *m*-AMSA was estimated by the [^{14}C]-*m*-AMSA/[^{3}H]thymidine-labeled DNA ratio at various times after drug treatment.

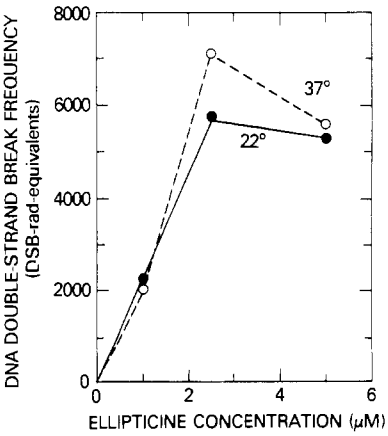

FIGURE 7: Dependence of ellipticine-induced DSB on temperature. Nuclei were treated for 30 min at either 37 (○) or 22 °C (●) with the indicated concentrations of ellipticine. DSB frequency was assayed by filter elution assays with proteinase K.

with increasing pH was probably not due to simply a selective effect by an acid form, relative to a base form, of the drugs, since the DSB produced by all three drugs decreased similarly with pH, although their pK_a's are different [for *m*-AMSA, pK_a = 7.6 (Ferguson & Denny, 1980); for ellipticine, pK_a = 5.8 (Le Pecq et al., 1974)].

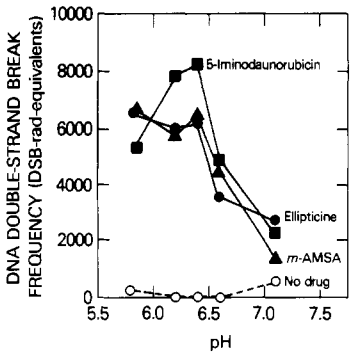

FIGURE 8: Dependence of DSB formation on pH. Nuclei were treated for 30 min at the indicated pH with 2 μM *m*-AMSA (▲), 10 μM 5-ID (■), or 10 μM ellipticine (●). DSB frequency was assayed by filter elution with proteinase K. At each pH, untreated nuclei were assayed for DSB (○), and the DSB frequency produced by *m*-AMSA, 5-ID, and ellipticine at each pH value was corrected for the DSB frequency measured in untreated nuclei at this same pH.

Table III: DNA Double-Strand Breaks (DSB rd-equiv) Produced by *m*-AMSA, 5-ID, or Ellipticine in the Presence or Absence of Mg^{2+} [a]

	m-AMSA, 2 μM	5-ID, 10 μM	ellipticine, 10 μM
no Mg^{2+}	0	0	0
2 mM Mg^{2+}	5250	4300	3800

[a] Nuclei were isolated as described under Materials and Methods but were resuspended in nucleus buffer containing 0.5 mM spermidine and either 2 mM $MgCl_2$ or no $MgCl_2$. Drug treatments were for 30 min at 37 °C.

Dependence of Intercalator-Induced DSB on Mg^{2+} Concentration. Intercalator-dependent DSB frequencies were maximum between 2 and 5 mM $MgCl_2$ (Figure 9). The omission of $MgCl_2$ from the nucleus buffer resulted in the appearance of endogenous DSB, which rendered the interpretation of the intercalator-dependent DNA double-strand breaking activity difficult. This problem was circumvented by adding 0.5 mM spermidine to the nucleus buffer. Under these conditions, spermidine protected nuclei from endogenous DNA breaks in the absence of Mg^{2+} (Table III) and did not alter by itself the intercalator-dependent DNA double-strand breaks. In the absence of Mg^{2+}, none of the three intercalators studied produced DSB in isolated nuclei (Table III). In addition, when no Mg^{2+} and 10 mM EDTA instead of EGTA were contained in the nucleus buffer, no detectable DSB were observed either in the untreated or in the intercalator-treated nuclei (Figure 9).

Dependence of m-AMSA-Induced DSB on NaCl Concentration. Modifications of NaCl concentration are known to affect chromatin structure and the association of nuclear proteins with DNA. NaCl concentrations between 50 and 150 mM did not affect greatly the production of DSB by 2 μM *m*-AMSA in isolated nuclei (Figure 10); however, between 200 and 400 mM, a sharp reduction was observed. At 600 mM NaCl, isolated nuclei exhibited DSB in the absence of *m*-AMSA treatment (Figure 10).

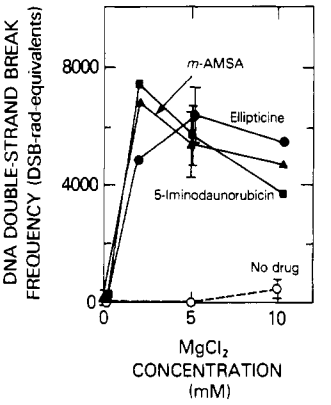

FIGURE 9: Dependence of DSB formation on magnesium concentration. Nuclei were treated at the indicated $MgCl_2$ concentration with 2 μM *m*-AMSA (▲), 10 μM 5-ID (■), or 10 μM ellipticine (●). EDTA (10 mM) was present in nucleus buffer when $MgCl_2$ was absent. DSB frequency was assayed by filter elution with proteinase K. At each $MgCl_2$ concentration, untreated nuclei were assayed for DSB (○). The DSB frequency produced by *m*-AMSA, 5-ID, and ellipticine at each $MgCl_2$ concentration was corrected for the DSB frequency measured in untreated nuclei at this same $MgCl_2$ concentration. Error bars represent the standard deviation of at least three independent determinations.

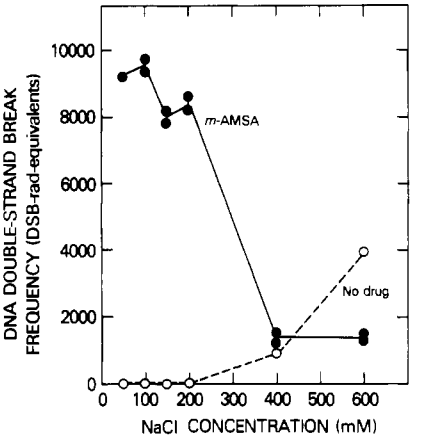

FIGURE 10: Dependence of DSB formation on NaCl concentration. Nuclei were treated at the indicated NaCl concentrations with 2 μM *m*-AMSA (●). DSB frequency was assayed by filter elution with proteinase K. At each NaCl concentration, untreated nuclei were assayed for DSB (○). The DSB frequency produced by *m*-AMSA at each NaCl concentration was corrected for the DSB frequency measured in untreated nuclei at this same NaCl concentration (two independent experiments).

Effects of Added ATP and ATP Analogues on Intercalator-Induced DSB. The nonhydrolyzable ATP analogues AMPPNP (0.5 mM) and ATPγS (0.5 mM) did not reduce the formation of DSB by intercalators (Table IV). The concentrations used were much greater than the ATP concentration measured in isolated nuclei (<2 nmol/10^9 nuclei; determined by Dr. David Cooney, Laboratory of Chemical Pharmacology, NCI). It is therefore likely that ATP hy-

Table IV: DNA Double-Strand Breaks (DSB rd-equiv) Produced by Intercalators in the Presence of ATP or Analogues[a]

ATP or analogue	no treatment	*m*-AMSA, 2 μM	5-ID, 10 μM	ellipticine, 10 μM
none	0	4881 ± 948[b]	4789 ± 1191	6097 ± 272
ATP (0.5 mM)	110[c]	6007 ± 896	5884 ± 1061	6233 ± 1458
AMPPNP (0.5 mM)	700	6810 ± 728	6013 ± 925	7735 ± 1108
ATPγS (0.5 mM)	1300	>9000	>9000	>9000

[a] Nuclei were isolated as described under Materials and Methods but were resuspended in nucleus buffer (pH 6.4) either containing no drug or containing ATP, AMPPNP, or ATPγS. Intercalator treatments were started after 5 min in these different conditions and were for 30 min at 37 °C. [b] Mean ± 1 SD of at least three independent determinations. [c] Mean of two independent determinations.

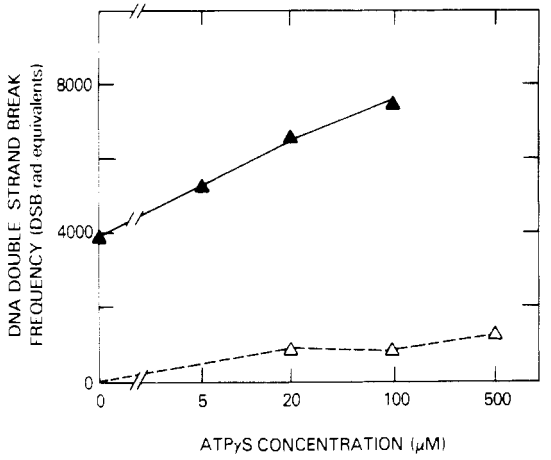

FIGURE 11: Potentiating effect of the ATP analogue adenosine 5'-*O*-(3-thiotriphosphate) (ATPγS) on *m*-AMSA-induced DSB. Isolated nuclei were treated first with various concentrations of ATPγS for 5 min, after which 2 μM *m*-AMSA was added to the reaction mixture for 30 min at 37 °C. Reactions were stopped by a 50-fold dilution of treated nuclei in drug-free nuclei buffer at 0 °C. In the same experiments, DSB frequencies produced by ATPγS alone (△; dashed line) and *m*-AMSA in the presence of various concentrations of ATPγS (▲; solid line) were measured.

Table V: DNA Double-Strand Breaks (DSB rd-equiv) Produced by Intercalators in the Presence of Topoisomerase Inhibitors[a]

inhibitor	no treatment	*m*-AMSA, 2 μM	5-ID, 10 μM	ellipticine, 10 μM
none	0	4778 ± 720[b]	4638 ± 818	6057 ± 710
novobiocin (0.5 mM)	79[c]	2595 ± 252	2142 ± 643	1948 ± 460
coumermycin (0.2 mM)	629	5124 ± 241	5375 ± 578	5584 ± 769
nalidixic acid (2 mM)	2512	3690 ± 900	3858 ± 970	6997 ± 705
oxolinic acid (0.2 mM)	393	6025 ± 538	6623 ± 129	5883 ± 1488
norfloxacin (1 mM)	1974	2307 ± 212	2169 ± 411	5883 ± 1488

[a] Nuclei were isolated as described under Materials and Methods but were resuspended in nucleus buffer (pH 6.4) either containing no drug or containing novobiocin, coumermycin A_1, nalidixic acid, oxolinic acid, or norfloxacin. Intercalator treatments were started after 5 min in these different conditions and were for 30 min at 37 °C. [b] Mean ± 1 SD of at least three independent determinations. [c] Mean of two independent determinations.

drolysis is not required for the formation of intercalator-induced DNA cleavage. Exogenous ATP (0.5 mM) enhanced slightly but significantly the intercalator effects (Table IV). An even larger increase was observed with the nonhydrolyzable ATP analogues (Table V and Figure 11). In addition, both AMPPNP and ATPγS induced DNA cleavage in isolated nuclei (Table IV and Figure 11). These results show that the activity (enzyme) which cleaves DNA in the presence of intercalators is enhanced by ATP, AMPPNP, and ATPγS and suggest that the DNA breaking activity of the putative enzyme(s) is activated by the binding of adenyl moieties.

Effect of Topoisomerase Inhibitors. Pretreatment of isolated nuclei for 5 min with novobiocin (0.5 mM), nalidixic acid (2 mM), or norfloxacin (1 mM) followed by a 30-min treatment with intercalators reduced the production of DSB (Table V). In the case of nalidixic acid, a lower concentration (200 μg mL^{-1} = 0.86 mM) had a smaller effect (Figure 12). However, the inhibition was still present and observed not only in the intercalator-induced DSB but also in the intercalator-induced SSB, which suggests that both reactions could be

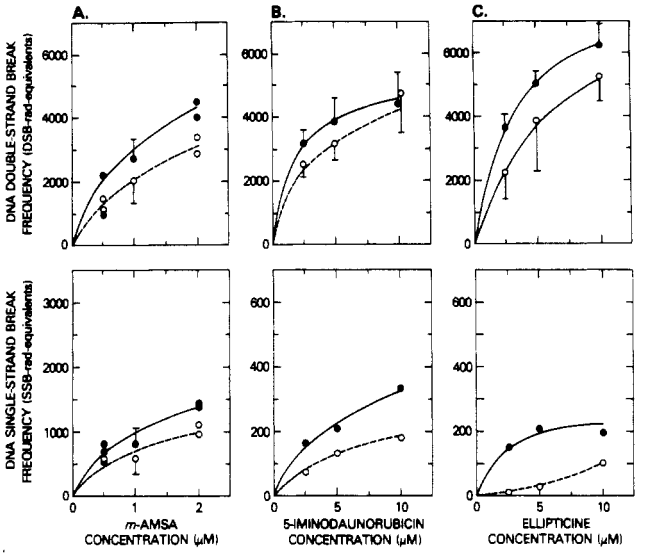

FIGURE 12: Effects of nalidixic acid on DNA strand breaks produced by *m*-AMSA (A), 5-ID (B), or ellipticine (C). Isolated nuclei were treated either only with *m*-AMSA, 5-ID, or ellipticine (●) or with *m*-AMSA, 5-ID, or ellipticine in the presence of nalidixic acid (200 μg mL^{-1}) (○) for 30 min at 37 °C. Nalidixic acid was added to the nuclei 5 min before *m*-AMSA, 5-ID, or ellipticine addition. Error bars represent the standard deviation of at least three independent determinations.

mediated by similar enzymes. In addition to their inhibitory effect on intercalator-induced DNA breaks, nalidixic acid and norfloxacin produced by themselves a significant yield of DSB (Table V), which implies that the effect of intercalators in the presence of these inhibitors was even smaller than in their absence. Neither coumermycin A_1 nor oxolinic acid had a significant effect (Table V). However, higher concentrations of these two drugs could not be used because of solubility limitations in the nucleus buffer used at pH 6.4. It is thus possible that coumermycin and oxolinic acid could affect the intercalator-induced DNA cleavage at higher concentrations, as did the other topoisomerase II inhibitors.

Discussion

It has been demonstrated that DNA intercalating agents produce both single-strand and double-strand DNA breaks in cells (Ross & Bradley, 1981). The mechanism of formation of double-strand breaks is not simply the proximity of two single-strand breaks on opposite strands (Ross & Bradley, 1981; Zwelling et al., 1981, 1982a,d). Both kinds of strand breaks have been postulated (Ross & Bradley, 1981) to result from the action of DNA topoisomerase.

In the present study, we used isolated nuclei to characterize the intercalator-induced DNA double-strand breaks further and in a more controlled fashion than in whole cells. The properties of intercalator-induced DNA breaks could thus be compared with those of DNA topoisomerases. The biochemical conditions under which eukaryotic type II topoisomerases catalyze DNA cleavage have recently been published (Liu et al., 1983). Both topoisomerase II mediated and intercalator-induced DNA cleavages exhibited similar temperature, pH, magnesium, and salt requirements. That DSB could form in isolated nuclei irrespective of the presence of ATP inhibitors shows that cytoplasmic components, free nucleotides, and ATP hydrolysis were not required for these reactions, as they are for other forms of DNA break resealing, e.g., DNA repair. In addition, DSB resealed in isolated nuclei. This is in agreement with previous studies showing that neither the DNA polymerase inhibitor arabinocytidine (Minford et al., 1983) nor the poly(adenosine diphosphoribose) synthetase inhibitor

3-aminobenzamide (Pommier et al., 1984b; Zwelling et al., 1982c) affected the resealling of intercalator-induced DNA single-strand and double-strand breaks. Thus, the resealing of intercalator-induced DNA double-strand breaks appears to occur at the DNA breaking site without base loss and ATP hydrolysis. The enhancing effect of ATP and ATP analogues on intercalator-induced DNA cleavage suggests that ATP binding stimulates the intercalator-sensitive enzymes to bind and break DNA. This result is analogous to what is observed when ATP analogues are added to the DNA gyrase–oxolinic acid reaction (Sugino et al., 1978; Morrison et al., 1980) and shows a similar ATP influence (but not ATP requirement) for the DNA cleavage induced by both DNA gyrase and the intercalator-sensitive enzyme (possibly topoisomerase II).

The inhibitory effect of novobiocin, nalidixic acid, and norfloxacin on the intercalator-dependent DNA double-strand break reaction is consistent with the involvement of type II topoisomerases in the DNA cleavage induced by intercalators (Gellert, 1981; Osheroff et al., 1983). Since nalidixic acid affected the formation of both single- and double-strand breaks, it could be that both kinds of breaks result from the interaction of intercalating drugs with DNA and a single species of DNA topoisomerase.

During the DSB formation and resealing cycle of topoisomerase II, there must be intermediate states in which only one DNA strand is cut. The binding of an intercalator to the DNA–topoisomerase complex may selectively trap the complex in a state in which either one or both DNA strands are cut. The relative amount of complex trapped in the single-cut or double-cut state may depend on the stereochemistry of the intercalator (Ross & Bradley, 1981). The different states of the complex may be in equilibrium with each other, and the equilibrium may be affected by the selective reversible binding of intercalator. The frequencies of SSB and DSB that are observed probably show what existed at the instant that the system was fixed by the addition of detergent.

These results taken together suggest that type II topoisomerases are responsible for the intercalator-induced DNA breaks. Recently this conclusion has been confirmed more directly by using an extract from L1210 cell nuclei isolated in a way similar to that described in the present paper. The intercalator-dependent DNA–protein cross-linking activity copurified with topoisomerase II and this last enzyme was shown to produce DNA double-strand breaks in the presence of *m*-AMSA (Minford et al., 1984).

Acknowledgments

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Registry No. *m*-AMSA, 51264-14-3; 5-ID, 72983-78-9; ellipticine, 519-23-3.

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