Protein-Associated Deoxyribonucleic Acid Strand Breaks in L1210 Cells Treated with the Deoxyribonucleic Acid Intercalating Agents 4′-(9-Acridinylamino)methanesulfon-*m*-anisidide and Adriamycin†

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ABSTRACT: The DNA intercalating agents 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (*m*-AMSA) and adriamycin were studied by using filter elution methods to measure DNA single-strand breaks (SSB's), DNA–protein cross-links (DPC's), and double-stranded breaks (DSB's) in mouse leukemia L1210 cells. Both compounds produced SSB's and DPC's at nearly 1:1 ratios. The SSB's and DPC's were shown to be localized with respect to each other; this was inferred from the finding that filter assays based on protein adsorption completely prevented the elution of the DNA single-strand segments between SSB's. In the case of *m*-AMSA, which produces relatively high frequencies of DNA lesions, the possibility that a protein bridges across the SSB was excluded by alkaline sedimentation studies. Both compounds also produced DSB's, but the SSB/DSB ratios differed; the SSB/DSB ratios increase in the following order: ellipticine < adriamycin < *m*-AMSA < X-ray [results of this paper combined with those of Ross, W. E., & Bradley, M. O. (1981) *Biochim. Biophys. Acta* (in press)]. The *o*-AMSA isomer is much less cytotoxic than *m*-AMSA and did not produce protein-associated strand breaks. The simplest model to explain the results is that a protein becomes covalently bound to either the 3′ or the 5′ termini of the intercalator-induced strand breaks. At moderately cytotoxic doses, *m*-AMSA yielded much larger frequencies of protein-associated SSB's than did adriamycin. *m*-AMSA-induced protein-associated SSB's saturated at approximately 60 000 per cell over a concentration range in which *m*-AMSA uptake by the cells was proportional to the drug concentration. *m*-AMSA was found to enter and exit from cells very rapidly at 37 °C; protein-associated SSB's and DSB's also appeared and disappeared rapidly. At reduced temperature, however, the appearance and disappearance of protein-associated SSB's could be blocked while *m*-AMSA entry and exit still occurred. The saturation behavior and temperature dependence suggest that the formation and disappearance of protein-associated strand breaks is enzymatic. The simplest hypothesis is that the linked protein is a nuclease, such as a topoisomerase, which becomes bound to one terminus of the strand break it produces. It is proposed that topoisomerases producing SSB's and DSB's are stimulated to different degrees by different intercalators.

Deoxyribonucleic acid intercalating agents appear to stimulate in mammalian cells the formation of DNA strand breaks that are associated in some specific manner with DNA–protein cross-links (Ross et al., 1978, 1979a). Two chemically very different intercalators, ellipticine and adriamycin, were found to produce single-strand breaks (SSB's) and DNA–protein cross-links (DPC's) in approximately a 1:1 ratio, and evidence indicated that the two types of DNA lesions were localized relative to each other. Compounds that bind to DNA by nonintercalative mechanisms or that inhibit macromolecular synthesis by other means did not produce protein-associated strand breaks.

Some, or in the case of ellipticine perhaps all, of the SSB's may actually be double-strand breaks (DSB's) (Ross & Bradley, 1981). (Unless otherwise stated, we consider a DSB to consist of two SSB's located on opposite strands sufficiently close so that there are too few intervening base pairs to maintain the continuity of the double strand.) The SSB/DSB ratio was found to be substantially lower for ellipticine than for adriamycin (Ross & Bradley, 1981), suggesting that the two types of breaks arise by different mechanisms.

The intercalator effect in mammalian cells was hypothesized to involve the covalent linkage of a protein to one terminus of each SSB (Ross et al., 1979a). It was proposed that the linked proteins may be endonucleases, perhaps topoisomerases, that generate the SSB's and/or DSB's.

Another class of DNA intercalating agents, chemically very different from either adriamycin or ellipticine, is the 9-anilinoacridines (Waring, 1976; Cain et al., 1978). Some of these compounds, most notably *m*-AMSA (Figure 1), have impressive antitumor activity in animals and man (Cain & Atwell, 1974; Cain et al., 1974; Wilson, 1973; Issell, 1980; Legha et al., 1979, 1980; Benjamin et al., 1980; Rivera et al., 1980). The *o*-AMSA isomer, in which the methoxy group is on the 3′ instead of the 2′ position, is inactive, even though this isomer does intercalate in purified DNA (Waring, 1976). There has been no previous indication as to whether these compounds intercalate in DNA within cells. An effect on DNA in the cell, however, has been reported in the form of single-strand breaks produced by *m*-AMSA but not by *o*-AMSA (Burr-Furlong et al., 1978; Ralph, 1980).

In the present work, using mouse leukemia L1210 cells, we find that *m*-AMSA gives a particularly clear demonstration of protein-associated DNA breaks and lends itself to a detailed study of these entities. *m*-AMSA and *o*-AMSA were studied in comparison with adriamycin.

Materials and Methods

Cells and Radioactive Labeling. L1210 mouse leukemia cells were grown in a suspension culture in RPMI 1630 medium supplemented with 15% fetal calf serum or 10% horse serum plus penicillin and streptomycin. Stock cultures were maintained in static bottles without antibiotics and were used to initiate suspension cultures. Cultures utilized to assess drug effects were in exponential growth phase with a doubling time of 13–15 h.

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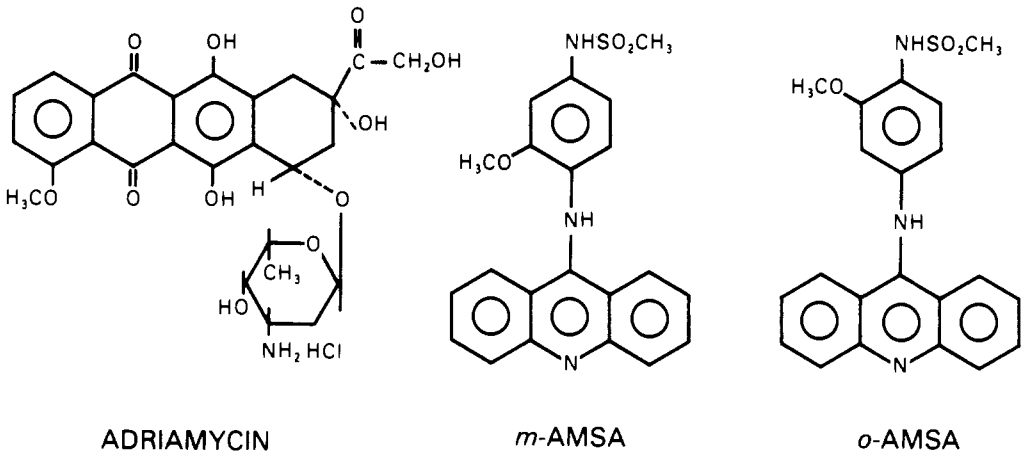

FIGURE 1: Structures of adriamycin, *m*-AMSA, and *o*-AMSA.

Cellular DNA was radioactively labeled in exponentially growing cells by incubation with [2-^{14}C]thymidine (0.01 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. The radioactive label was removed by centrifugation prior to drug treatment or irradiation.

Drugs and Drug Treatment. *m*-AMSA base (NSC 249992), obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, was dissolved in dimethyl sulfoxide at 0.01 M and stored frozen. Pure *o*-AMSA base (SN 12735) was a generous gift from Dr. Bruce F. Cain, Aukland Division Cancer Society of New Zealand. This was also dissolved at 0.01 M in dimethyl sulfoxide and stored frozen. Adriamycin (NSC 123127) was dissolved just prior to use in sterile, glass-distilled water at 1 mg mL^{-1}. All drug treatments were for 1 h at 37 °C, and the drug was removed by centrifugation and resuspension at least twice.

4′-(9-[^{14}C]Acridinylamino)methanesulfon-*m*-anisidide ([^{14}C]-*m*-AMSA) (19.6 mCi mmol^{-1}) was synthesized by SRI International, Menlo Park, CA, and was obtained through the Chemical Resources Section, National Cancer Institute.

Cell Survival. Cells were treated as above and assayed for colony-forming ability in soft agar by the method of Chu & Fisher (1968).

Alkaline Elution Assays. The alkaline elution methodology has been described in detail by Kohn (1979) and Kohn et al. (1981).

DNA–Protein Cross-Linking. Drug-treated [^{14}C]thymidine-labeled cells were placed in iced tubes and X irradiated with 3000 R to introduce approximately 2.7 single-strand breaks per 10^6 nucleotides into the DNA. The cells were then combined with an equal number ($\sim 5 \times 10^5$) of untreated, [^{3}H]thymidine-labeled cells which concurrently received 3000 R in iced medium, and the cell mixture was then deposited on 2-μm polyvinyl chloride filters (Millipore, Bedford, MA) by gentle suction. Cell lysis was effected with 2 M NaCl, 0.2% Sarkosyl, and 0.04 M Na_2EDTA, pH 10.0 (5 mL), and the residual lysis solution was removed by washing the filter with 0.04 M EDTA, pH 10.0 (3 mL). DNA elution was then carried out with tetrapropylammonium hydroxide–EDTA at pH 12.1. Fractions (6 mL) were collected at 3-h intervals and mixed with 10 mL of Aquassure (New England Nuclear, Boston, MA) for scintillation counting. The 3000 R X-ray dose separates DNA into two components, that which elutes rapidly and that which does not elute due to its association with protein which adheres to the filters. The degree to which the filter retention of DNA from drug-treated cells exceeds that from untreated cells is a measure of drug-induced, DNA–protein cross-linking. This retention is designated r_0 for untreated cells and r for drug-treated cells. DNA–protein cross-link frequency (P_x) was calculated by using the bound-to-one-terminal model of Ross et al. (1979a), as follows:

$$P_x = [(1 - r)^{-1} - (1 - r_0)^{-1}]P_B \quad (1)$$

where P_B is the break frequency produced by the administered X-ray dose (3000 rad equiv or 2.7 breaks per million nucleotides).

DNA Single-Strand Breaks. Drug-treated [^{14}C]thymidine-labeled cells were mixed with an equal number of ^{3}H-labeled internal standard cells (see below). The cells were deposited on a polycarbonate filter (2-μm pore diameter, Nucleopore Corp., Pleasanton, CA) in a Swinnex-25 filter holder (Millipore Corp., Bedford, MA) and lysed with 2% sodium dodecyl sulfate (BDH Biochemicals Ltd., Poole, England), 0.1 M glycine, and 0.025 Na_2EDTA, pH 10 (5 mL); this solution was allowed to flow out by gravity. Two milliliters of the same solution containing 0.5 mg mL^{-1} proteinase K was then introduced into the upper chamber of the filter holder, and the eluting solution (tetrapropylammonium hydroxide–EDTA–0.1% sodium dodecyl sulfate, pH 12.1) was placed in the reservoir mounted on the filter unit (Kohn et al., 1981). For the high-sensitivity assay, elution was carried out at 0.03–0.04 mL min^{-1}, and fractions were collected at 3-h intervals. For the low-sensitivity assay (which was required to assay high DNA break frequencies), elution was at 0.12–0.16 mL min^{-1}, and fractions were collected at 5-min intervals. The [^{3}H]DNA internal standard cells received 300 R of X irradiation in the high-sensitivity assay and 1000 R in the low-sensitivity assay.

DNA single-strand break frequency was calculated on the basis of first-order elution kinetics (Kohn et al., 1976), as follows:

$$P_{BD} = \frac{\log (r_1/r_0)}{\log (R_0/r_0)} P_{BR} \quad (2)$$

where P_{BR} is the DNA break frequency produced by the X-ray (300 or 1000 rad equiv), r_1 are the DNA retentions in drug-treated and control ^{14}C-labeled cells, and R_0 is the DNA retention of ^{14}C-labeled cells coirradiated with the ^{3}H-labeled internal standard cells. Retention of [^{14}C]DNA 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.

By use of this method, calibration experiments were performed in which the rad cells received various doses of X-ray

at ice temperature. Internal standard cells (^{3}H labeled) were used as described above. The high-sensitivity assay yielded the regression line

$$\log r = 0.0224 - 1.5X \quad (3)$$

where r is ^{14}C retention at a ^{3}H retention of 0.35 and X is the dose in kilorads (19 determinations between 0 and 0.7 krad; correlation coefficient = −0.99). The low-sensitivity assay yielded the regression line

$$\log r = 0.0368 - 0.028X \quad (4)$$

where r is the ^{14}C retention at ^{3}H retention of 0.60 (20 determinations between 0 and 5 krad; correlation coefficient = −0.99).

The SSB frequencies produced by the drug can be estimated from the rad equivalent values (i.e., dose of X-ray producing an equivalent elution rate) by multiplying by 0.9×10^{-9} rad^{-1} nucleotide^{-1} (Kohn et al., 1976). This calculation is valid, however, only if the drug-induced breaks are randomly distributed, as indicated by a nearly first-order dependence of DNA retention on elution time (Kohn, 1979).

DNA Double-Strand Breaks. DNA double-strand breaks were measured essentially as described by Bradley & Kohn (1979) and by Ross & Bradley (1981). A total of 2.5×10^5 [^{14}C]DNA-labeled cells was deposited on a polycarbonate filter (2-μm pore diameter, Nucleopore Corp.). A 2-mL mixture of $NaDodSO_4$ lysis solution [2% sodium dodecyl sulfate ($NaDodSO_4$), 0.02 M EDTA, and 0.1 M glycine, pH 9.6] containing 0.5 mg mL^{-1} proteinase was rapidly layered on the filter followed by the elution solution of tetrapropylammonium hydroxide–EDTA–0.1% $NaDodSO_4$, pH 9.6, which was pumped through the filter at approximately 2 mL h^{-1}, and fractions were collected at 3-h intervals for 15 h. (No ^{3}H-labeled internal standard cells were used in this assay since the elution of DNA was found to depend upon the number of cells loaded onto the filter.) The end point of the assay was the fraction of the DNA retained after 10 h of elution (r). In agreement with Ross & Bradley (1981), r decreased linearly (not logarithmically) with the X-ray dose. Calibration assays yielded the regression line

$$r = 0.84 - 0.083X \quad (5)$$

where X is the X-ray dose in kilorads (56 determinations between 0 and 10 krad; correlation coefficient = −0.97). The slope of this line is equal to that reported by Ross & Bradley (1981). The calibration line was used to quantify drug-induced DSB's in terms of the X-ray dose, giving an equivalent effect in this assay.

Alkaline Sucrose Sedimentation. L1210 cells in exponential growth phase were incubated with either [2-^{14}C]thymidine (0.2 μCi mL^{-1}) or [*methyl*-^{3}H]thymidine (1.0 μCi mL^{-1}) overnight at 37 °C. The radioactive label was removed by centrifugation 4 h prior to drug treatment, and the cells were again centrifuged and resuspended in fresh medium just prior to drug treatments. Cell lysates were prepared on top of alkaline sucrose gradients by gently pipeting 50 μL of a cell mixture containing 4×10^4 [^{14}C]DNA test cells (*m*-AMSA treated or X-irradiated) plus 4×10^4 [^{3}H]DNA internal standard cells (1000 R) into 0.4 mL of 0.55 M NaCl plus 0.45 M NaOH. Lysates were incubated for 1 h at room temperature prior to centrifugation.

Linear alkaline sucrose gradients were prepared from stock solutions containing (a) 4% sucrose, 0.9 M NaCl, 0.05 M Na_2EDTA, and 0.2 M NaOH and (b) 20% sucrose, 0.9 M NaCl, 0.05 M Na_2EDTA, and 0.3 M NaOH. The gradients in cellulose nitrate tubes were centrifuged in a Beckman SW-40 Ti rotor at 9500 rpm, 20 °C, for 16.5 h ($\omega^2 t = 6.177 \times 10^{10}$ rad^2 s^{-1}). The gradients were fractionated from the top by displacement with a solution of 60% sucrose and 1 M NaCl. Fractions (0.5 mL) were collected. DNA sedimenting to the bottom of the tube was recovered by cutting off the tube bottom and washing it with 1 mL of 0.1 M NaOH and 0.3% Sarkosyl. The number average molecular weights in sedimentation experiments were computed as described by Dingman & Kakunaga (1976).

Transport Studies. Exponentially growth L1210 cells were concentrated to approximately 10^7 mL^{-1} in RPMI 1630 medium plus 15% fetal calf serum. After incubation with radioactively labeled drug, 1 mL of the cells was layered above 0.6 mL of silicone oil (Versilube F50, General Electric Co., Waterford, NY) in a microcentrifuge tube and centrifuged for 1 min at 12000*g*. The bottom of the tube was cut off and placed in a liquid scintillation counting vial. The cell button was dispersed in 1.5 mL of phosphate-buffered saline and solubilized by addition of 1.5 mL of 0.4 M NaOH and incubation overnight at 37 °C. The sample was prepared for scintillation counting by adding 3 mL of water and 10 mL of Aquassure (New England Nuclear; 30 mL of glacial acetic acid was added per 4 L).

The amount of extracellular fluid trapped in the cell pellet was estimated by the use of [^{14}C]inulin (New England Nuclear) to be approximately 1.3 μL/10^7 cells. Small corrections were applied on the basis of this value and the determined radioactivity in the supernatant.

This technique is an adaptation of the method of Vistica (1979), whose help in setting up the method is gratefully acknowledged.

Results

DNA Single-Strand Breaks. The ability of the drugs to induce DNA single-strand breaks (SSB) was assayed by alkaline elution as described under Materials and Methods. The assays utilized proteinase K to eliminate DNA–protein cross-links (DPC).

Adriamycin produced nearly first-order elution kinetics with respect to time (Figure 2A) and concentration (Figure 3), indicating the formation of randomly distributed SSB's. When proteinase K was omitted from the assay procedure, however, there was no increse in the elution rate above that of untreated controls, suggesting that DNA-linked protein molecules were associated with the strand breaks (Ross et al., 1979a). This is very different from the case of X-ray, in which proteinase K has little effect on the alkaline elution rates (Ewig & Kohn, 1978).

The DNA elution curves produced by *m*-AMSA (Figure 2B) are similar in shape to those produced by adriamycin or X-ray. When the assay was carried out without proteinase K, *m*-AMSA produced no significant increase in the DNA elution rate (Figure 2B). Hence the SSB's resulting from treatment with *m*-AMSA are probably hidden by DNA-linked protein, as previously reported for other intercalating agents (Ross et al., 1979a).

In the case of *o*-AMSA, a biologically inactive isomer, concentrations 200-fold greater than those of *m*-AMSA were required to produce comparable increases in the slopes of the DNA elution curves (Figure 2C). The increased DNA elution rates produced by *o*-AMSA occurred even when assayed without proteinase K, indicating that most of the SSB's are free of protein.

At the high concentrations of *o*-AMSA that were required to produce moderate increases in DNA elution rates, a substantial part of the DNA (up to 45% at the highest dose) eluted

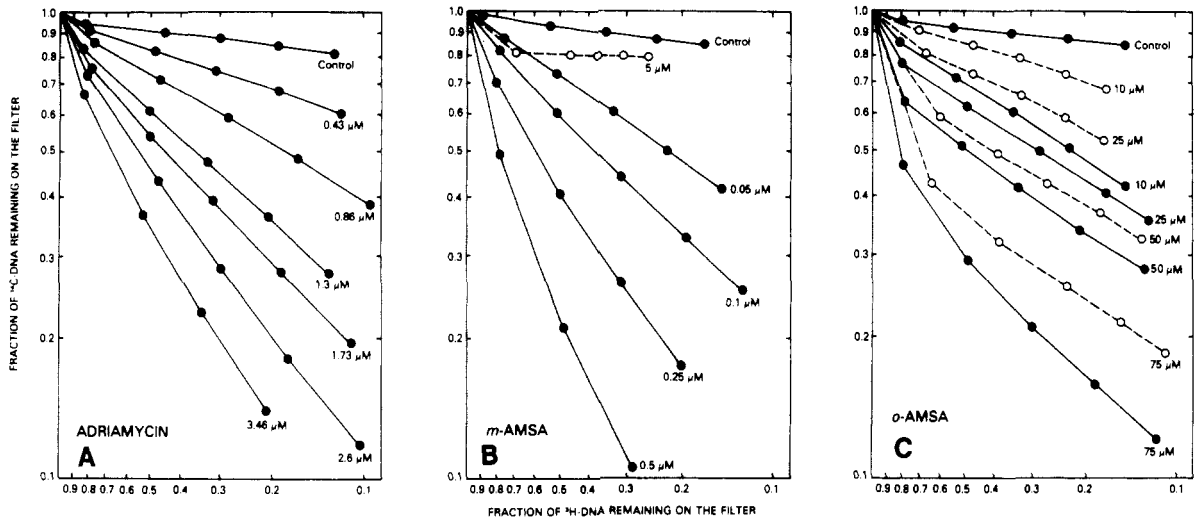

FIGURE 2: DNA alkaline elution kinetics for cells treated with various concentrations of adriamycin, *m*-AMSA, or *o*-AMSA for 1 h. Assays performed without proteinase K (open symbols and dashed lines). Assays performed with proteinase K (filled symbols and solid lines). Horizontal axis is essentially a corrected time scale based on elution of internal standard cells (^{3}H labeled, 300-R irradiated) (see Materials and Methods).

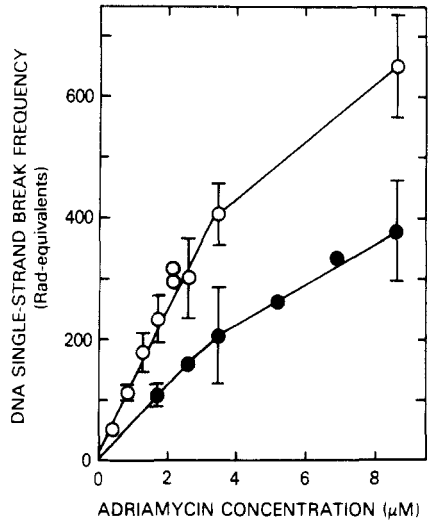

FIGURE 3: Dependence of SSB frequency on adriamycin concentration following 1-h drug exposures. Cells were washed at 4 (●) or at 37 °C (○) (see text) and then assayed by alkaline elution using the proteinase K method at a pump speed of 2 mL h^{-1}.

as a rapid component in the first fraction (Figure 2C). This probably represents degraded DNA from dying cells (Kohn, 1979). Rapid eluting components were also seen in the results with high concentrations of adriamycin and *m*-AMSA, but the magnitude was usually less than 20% of the cell DNA. In estimating SSB frequencies, the rapid eluting components were ignored; however, the essential results were not thereby altered.

The SSB frequency (in proteinase assays) produced by a 1-h treatment with adriamycin was approximately proportional to the drug concentration (Figure 3). Two sets of experiments differing in whether the cells were chilled rapidly by dilution with 5–10 volumes of iced medium before centrifugation or whether the centrifugation and washing were conducted at 37 °C (which delayed cell chilling 15–20 min) were performed. The SSB frequency was about twice as high as when the cells were washed at 37 °C than when washed at 4 °C (Figure 3). This difference is at least in part accounted for by the continued action of adriamycin after cell washing at 37 °C (Figure 8); apparently, adriamycin does not rapidly wash out of the cells. The difference, however, seems too large to be explained solely by the additional drug action time, and it may be that the temperature of centrifugation has an effect that we do not understand. This question, however, was not explored further.

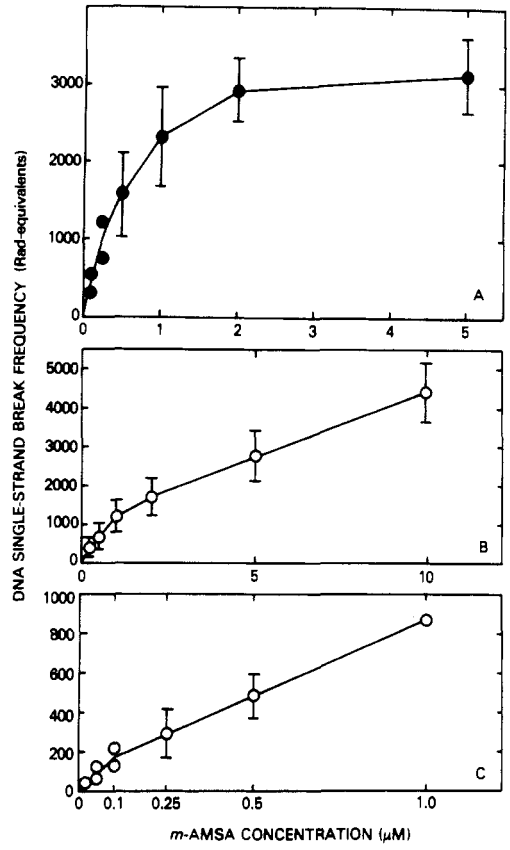

FIGURE 4: Dependence of SSB frequency on *m*-AMSA concentration following 1-h drug exposures. Cells were washed at 4 (A) or 37 °C (B, C) (see text) and then assayed by alkaline elution using the proteinase K method at a pump speed of 8 (A, B) or 2 mL h^{-1} (C). (Error bars) Standard deviation of at least three independent experiments.

In both sets of experiments, however, the SSB frequency was proportional to the adriamycin concentration up to 3.5 μM (2 μg/mL) (Figure 3). At higher concentrations, the efficiency for single-strand breakage decreased slightly.

In contrast to the case of adriamycin, the SSB frequency as a function of the *m*-AMSA concentration showed saturation above 2 μM (Figure 4A). In these experiments, the cells were washed at 4 °C. In experiments conducted with washing at 37 °C (Figure 4B,C), SSB frequencies tended to be lower, and saturation was not observed. This latter behavior can be

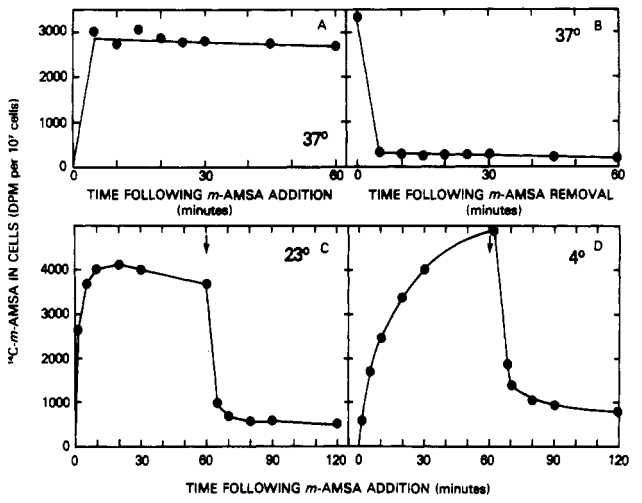

FIGURE 5: Kinetics of uptake and egress of [^{14}C]-*m*-AMSA. L1210 cells (10^7 mL^{-1}) were incubated with 0.5 μM (0.01 μCi mL^{-1}) [^{14}C]-*m*-AMSA for 60 min at 37 °C (A). (B) Egress after cells were washed at 37 °C. (C) Uptake and egress at 23 °C. (D) Uptake and egress at 4 °C. (Arrow) Time of drug removal.

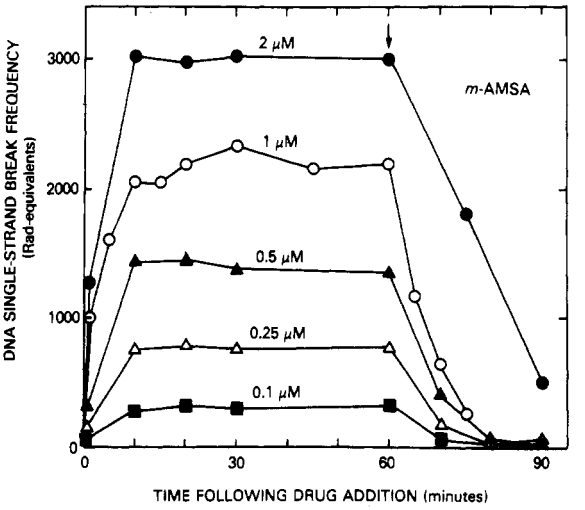

FIGURE 6: Kinetics of formation and disappearance of SSB's assayed by alkaline elution using proteinase K in L1210 cells exposed to the indicated concentrations of *m*-AMSA for 60 min. At 60 min (arrow), the drug was removed by rapid centrifugation and resuspension of cells in fresh medium.

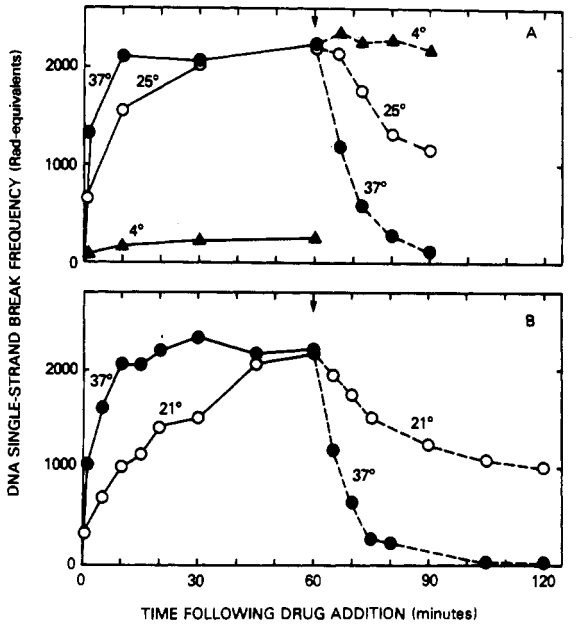

FIGURE 7: Formation and disappearance of SSB's in L1210 cells at the indicated temperature following addition of 1 μM *m*-AMSA. Drug removed by rapid centrifugation at arrow.

accounted for by the rapid washout of *m*-AMSA and the reversibility of the stand breaks, as will be seen in Figures 5 and 6. The SSB frequencies attainable with *m*-AMSA were much greater than those with adriamycin.

Uptake of m-AMSA. The uptake of *m*-AMSA into the cells was strictly proportional to the drug concentration and showed no leveling off at concentrations up to 7.5 μM, at which point the uptake was 5000 dpm/10^7 cells, corresponding to 1.15×10^{-17} mol cell^{-1} (μM *m*-AMSA)$^{-1}$. Hence, the saturation of DNA strand breakage, noted in Figure 4, is not due to saturation of drug uptake.

The drug is taken up very rapidly and reaches an equilibrium intracellular level within 5 min at 37 °C (Figure 5A) or at 23 °C (Figure 5C). At 4 °C, uptake is slower but still approaches a similar level by 60 min (Figure 5D). Whether the level reached depends to some extent on temperature was not established in these experiments.

The drug also exits from the cells very rapidly after the cells are washed (Figure 5). Exit was complete within 5 min at 37 °C, required about 10 min at 23 °C, and about 20 min at 4 °C. Small residual amounts of *m*-AMSA were retained in the cells, but the significance of this remains to be studied.

Kinetics of Formation and Resealing of Single-Strand Breaks. At 37 °C, *m*-AMSA-induced SSB's appear rapidly and reach a concentration-dependent plateau within 10 min (Figure 6). The height of the plateau exhibited saturation behavior, as previously seen in Figure 4A. After removal of the drug by washing the cells in warm medium, single-strand breaks disappear rapidly (Figure 6); the half-time of disappearance was 5–15 min.

The rates of appearance and disappearance of *m*-AMSA-induced SSB's are strongly dependent on temperature (Figure 7). At 20–25 °C, the rates were reduced, but the plateau reached was the same. At 4 °C, however, there was little or no formation of SSB's for at least 60 min (Figure 7A), even though the drug was still taken up by the cells (Figure 5D). Moreover, SSB's, once formed (at 37 °C), did not reseal at 4 °C (Figure 7A), even though 80% of the drug was rapidly washed out of the cells (Figure 5D).

When cells were kept at 4 °C for 1 h, either to prevent SSB formation in the presence of *m*-AMSA or to prevent resealing after drug removal, a subsequent shift to 37 °C then resulted in SSB formation or resealing with kinetics similar to those seen without the cold delay (data not shown). Hence, the temperature reduction to 4 °C does not disrupt the machinery required for the formation or resealing of SSB's but only inhibits its functioning.

In contrast to *m*-AMSA, adriamycin produced a linear increase in SSB's with time during a 1-h drug exposure (Figure 8). In some experiments, there was a brief lag of 1–10 min preceding the linear rise in breaks. After removal of adriamycin from the external medium by washing the cells, the SSB frequency continued to rise for 15 min and then appeared to level off. Even 24 h after the cells were washed, however, the SSB frequency was still approximately 50% of that present at the time of drug removal (data not shown).

The formation of SSB's by adriamycin was even more strongly temperature dependent than in the case of *m*-AMSA, as SSB formation was almost stopped at 25 °C (Figure 9).

Equivalence between Single-Strand Breaks (SSB's) and DNA–Protein Cross-Links (DPC's). A characterization noted by Ross et al. (1979a) of the protein-associated DNA single-strand breaks produced by the intercalating agents

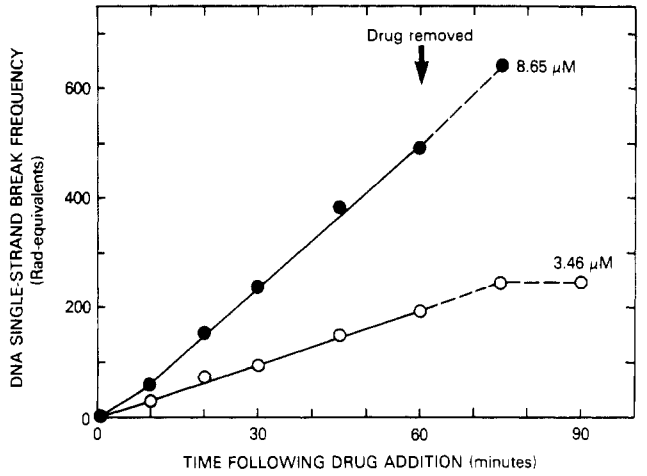

FIGURE 8: Kinetics of formation of SSB's in the presence of 3.46 μM (2 μg/mL) or 8.65 μM (5 μg/mL) adriamycin, assayed by alkaline elution using proteinase K. Drug was removed at 60 min (arrow) by centrifugation and resuspension of cells in fresh medium.

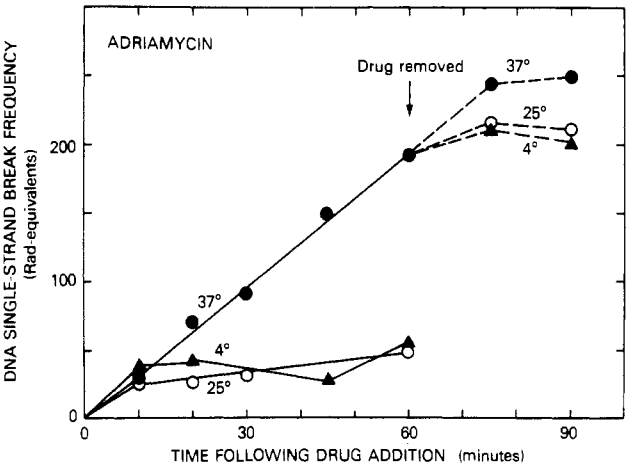

FIGURE 9: Temperature dependence of SSB formation by adriamycin. Cells were incubated with 3.46 μM adriamycin at 37 (●), 25 (○), or 4 °C (▲). After 60 min (arrow), the cells were washed and resuspended in fresh medium at 37 (●), 25 (○), or 4 °C (▲).

adriamycin and ellipticine was the near equivalence of the single-strand break and DNA–protein cross-link frequencies. *m*-AMSA provided a further test of the generality of this equivalence for chemically diverse intercalating agents.

DNA–protein cross-link frequencies were determined as described by Ross et al. (1979a), using the bound-to-one-terminus model (see Materials and Methods).

The SSB/DPC ratio for adriamycin was found to be 1.3 (median of 13 determinations); nearly all of the data values (Figure 10A) were encompassed within the range 0.94–1.70. These values are consistent with the previous results of Ross et al. (1979a).

The SSB/DPC ratios for *m*-AMSA were measured over the wide range of lesion frequencies that could be covered for this drug. SSB's were measured in two ranges in separately calibrated assays (see Materials and Methods), and the results for the two ranges are plotted in Figure 10B,C. The SSB/DPC ratios were 1.29 (1.05–2.07) and 1.29 (0.83–1.82), respectively. Although the median ratios slightly exceeded 1.0, this divergence is within the possible error of the assays.

In the above experiments, the drug was removed by washing the cells at 37 °C. Because of the possible effect of the washing procedure, discussed in connection with Figures 3 and 4, the experiments with adriamycin and *m*-AMSA were repeated by using the cold wash procedure. Data similar to that

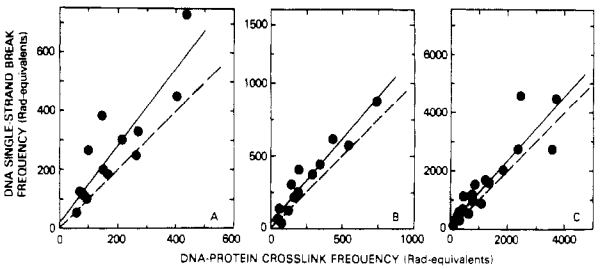

FIGURE 10: Relative production of single-strand breaks and DNA–protien cross-links. SSB's were assayed by alkaline elution using the proteinase K method. DPC's were assayed by alkaline elution without proteinase K. SSB's and DPC's were assayed on aliquots of the same cell suspension. The broken line is theoretical for equal frequencies of SSB's and DPC's. The solid line is calculated by linear regression. (A) Adriamycin; (B) *m*-AMSA (low dose range); (C) *m*-AMSA (high dose range).

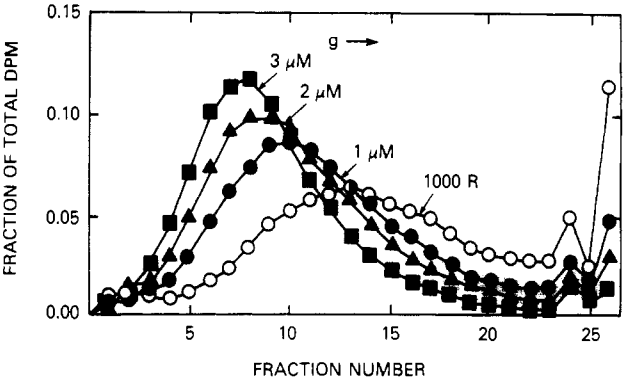

FIGURE 11: Alkaline sucrose gradient sedimentation of cells treated with 1000 R (○) or for 1 h with *m*-AMSA at 1 (●), 2 (▲), or 3 μM (■).

shown in Figure 10A,C were obtained, with SSB/DPC ratios for adriamycin being 1.09 (0.77–1.47) (median and range of five determinations) and for *m*-AMSA being 1.22 (0.99–1.41) (nine determinations).

Although the estimated SSB frequencies tend to exceed the estimated DPC frequencies by 10–30%, the discrepancy could be due to an underestimate of DPC's, due to the assumption that the adsorption of DNA-linked proteins to the filters is 100% efficient. If only 70–90% of the DNA-linked protein molecules were trapped by the filters, the calculated SSB and DPC frequencies would be equal.

In a kinetic experiment analogous to the experiments illustrated in Figure 6, DPC's appeared and disappeared in parallel with SSB's following treatment with *m*-AMSA (data not shown).

DNA-Linked Protein Does Not Bridge the Strand Break. One way in which a 1:1 ratio of DPC's to SSB's could completely hide the SSB's (in assays not utilizing a proteolytic enzyme) is by bridging of the strand break by a protein molecule linked to DNA on both sides of the break. This possibility was tested by alkaline sedimentation studies. Figure 11 shows that the *m*-AMSA-induced strand breaks were clearly detected in alkaline sucrose gradient sedimentation in which no proteolytic enzyme was used.

The difference between alkaline sedimentation and alkaline elution in regard to the detectability of *m*-AMSA-induced DNA strand breaks was verified by using the same pH 13.0 sucrose solution both in alkaline sedimentation and in alkaline elution assays of *m*-AMSA-treated cells. When proteinase K was used, the usual increase in DNA elution rate was seen (data not shown). In the absence of proteinase K, there was no increase of DNA elution relative to untreated cells. Hence, a protein prevents elution of the DNA under the same con-

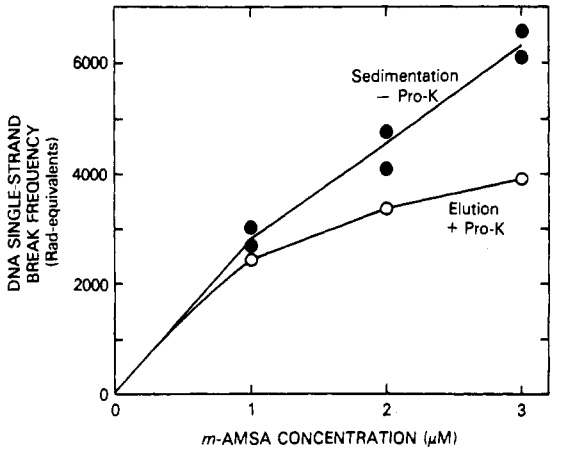

FIGURE 12: Single-strand break frequencies due to 1-h treatments with *m*-AMSA, assayed by alkaline sedimentation (●) or alkaline elution (○). The cell lysates were treated with proteinase K in the alkaline elution assays but not in the alkaline sedimentation assays. The two types of assays were performed on aliquots of the same cell suspension. After drug exposure, the cells were washed by centrifugation at 4 °C.

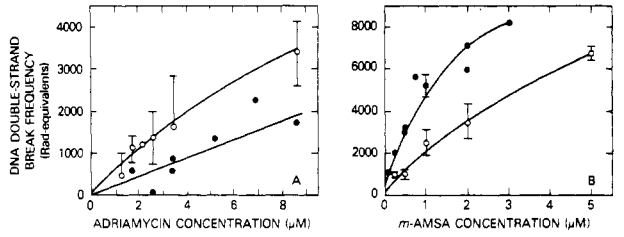

FIGURE 13: Dependence of DSB frequency on adriamycin (A) or *m*-AMSA (B) concentration in L1210 cells exposed to the drug for 1 h. DSB frequency is expressed in DSB rad equivalents, i.e., the X-ray dose (in rads) that would give an equivalent DNA retention. The drug was removed by washing cells at either 37 (open circles) or 4 °C (closed circles).

ditions that show reduced DNA sedimentation.

The SSB frequencies by alkaline sedimentation (carried out without proteinase) were in reasonable agreement with those determined concurrently by alkaline elution using proteinase K (Figure 12).

Double-Strand Breaks (DSB). The DNA elution assay for DSB's was performed at pH 9.6, which is below the pH required to separate strands (see Materials and Methods). The frequency of drug-induced DSB's was expressed as the dose of X-ray producing an equivalent DSB frequency, as measured by the pH 9.6 elution assay. The units of DSB frequency are denoted "DSB rad equivalents" in order to distinguish them from "SSB rad equivalents", which are defined as the X-ray dose producing an equivalent SSB frequency, as measured by the pH 12.1 elution assay.

The dependence of DSB frequency on *m*-AMSA and adriamycin concentrations are shown in Figure 13. Two sets of data differing in whether drug exposure was terminated by rapid chilling (closed circles) or by washing the cells at 37 °C (open circles) are shown. The DSB frequencies produced by adriamycin and *m*-AMSA tended to level off somewhat with increasing drug concentrations (Figure 13).

The kinetics of formation and resealing of *m*-AMSA DSB's were similar to those for SSB's (Figure 14). Both types of breaks increased rapidly to a plateau, although the time required to reach the plateau seemed to be somewhat greater for DSB's than for SSB's. The resealing of DSB's and SSB's after drug removal appeared to be equally rapid.

The drug-induced DSB and SSB frequencies were compared in relation to the effectiveness of X-ray in producing each type

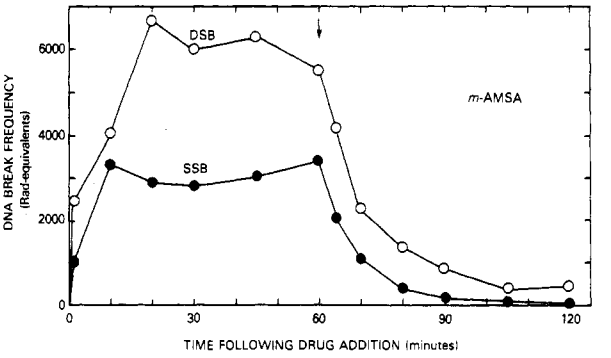

FIGURE 14: Kinetics of formation and resealing of SSB's and DSB's in L1210 cells exposed to 1 μM *m*-AMSA. After 60-min exposure to the drug, the cells were centrifuged and suspended in drug-free medium (arrow).

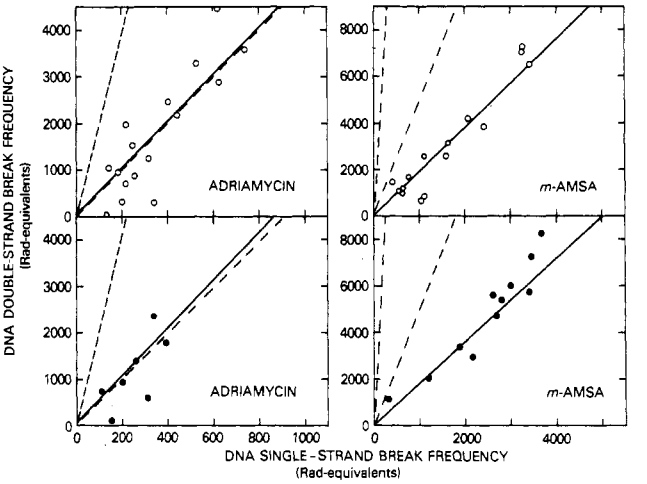

FIGURE 15: Relative production of DNA double-strand breaks and single-strand breaks by adriamycin (A) and *m*-AMSA (B). The frequency of each type of break is expressed as the X-ray dose required to produce the same frequency of that type of break. The solid line represents the median value for the ratio of measured DSB/SSB for the values given. The broken lines represent the estimated medians for measured DSB/SSB which would be obtained if all measured SSB were in fact DSB ($s/d = 0$ in eq 7) for k_{RS}/k_{RD} between 10 and 40 (see text). The drug was removed by washing cells at either 37 (open circles) or 4 °C (closed circles).

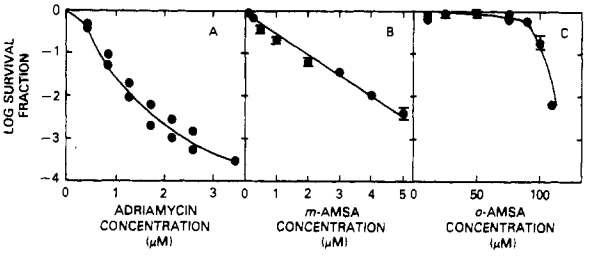

FIGURE 16: Survival of colony-forming ability of L1210 cells treated for 1 h with adriamycin (A), *m*-AMSA (B), or *o*-AMSA (C).

of break (Figure 15). The frequency of each type of drug-induced break is expressed as the X-ray dose that would produce the same frequency of that type of break, and is expressed in rad equivalent units. We did not attempt to determine absolute DSB frequencies because of the difficulty in obtaining reliable neutral sedimentation data in the relevant molecular weight range. The drug-induced SSB/DSB ratios can, however, be expressed in units normalized so as to set the SSB/DSB ratio for X-ray equal to 1. In these normalized units, denoted [SSB]/[DSB], the ratios for adriamycin and *m*-AMSA were 0.19 ± 0.04 and 0.53 ± 0.09, respectively (Table I). Thus adriamycin produced a distinctly lower

Table I: Estimates of True Single-Strand to Double-Strand Break Ratios

drug	expt	[SSB]/[DSB][a]	no. of determinations	s/d $k_{RS}/k_{RD}=10$	s/d $k_{RS}/k_{RD}=40$
m-AMSA	1[b]	0.52 ± 0.09[c]	10	3.2 ± 0.9	19 ± 4
	2	0.53 ± 0.09	10	3.3 ± 0.9	19 ± 4
adriamycin	1	0.20 ± 0.04	13	0.0 ± 0.4	6.0 ± 1.8
	2	0.19 ± 0.04	6	−0.1 ± 0.4	5.6 ± 1.6

[a] Symbols: [SSB], measured SSB frequency in SSB rad equivalents; [DSB], measured DSB frequency in DSB rad equivalents; *s*, frequency of true SSB (not including those arising from DSB); *d*, true DSB frequency; k_{RS} and k_{RD}, efficiencies of SSB and DSB production per roentgen of X-ray; *s*/*d* calculated according to eq 7. [b] Two sets of determinations are reported. In experiment 1, cells were washed at 37 °C; in experiment 2, cells were washed at 4 °C. [c] Median of the indicated number of determinations ± range of 70% of the determinations.

SSB/DSB ratio than *m*-AMSA, and both drugs produced lower ratios than X-ray.

Relation between DNA Breaks and Cell Survival. The survival curve for adriamycin shows a concave curvature, which could be due to heterogeneity of cell sensitivity or to saturation of drug uptake (Figure 16A). *m*-AMSA shows a simple first-order survival curve (Figure 16B). *o*-AMSA produced little loss of viability up to very high concentrations (80 μM). At concentrations above 80 μM, there was a precipitous fall in survival, accompanied by gross morphologic changes in the cells (the other two drugs did not produce microscopically visible changes in the cells at the end of that 1-h treatment period).

It is apparent that for a given frequency of either SSB's or DSB's, adriamycin reduces colony survival much more than does *m*-AMSA. The can be seen by comparing Figures 3, 4, and 13 with Figure 16, and the conclusion is independent of which of the curves in Figures 3, 4, and 13 one choses to use in making the comparison. This observation, however, must be considered in conjunction with the finding that the effect of adriamycin on DNA persists whereas the effect of *m*-AMSA rapidly reverses.

Discussion

Protein-Associated Strand Breaks. The DNA intercalating agent *m*-AMSA produced high frequencies of protein-associated DNA strand breaks at doses having relatively low cytotoxicities. This permitted a more detailed study of intercalator-specific DNA breakage than was previously reported (Ross et al., 1978, 1979a).

In the current work, as in the previous study (Ross et al., 1979a), SSB's and DPC's were measured by alkaline (pH 12.1) elution procedures; DSB's were measured by filter elution pH 9.6 (Bradley & Kohn, 1979; Ross & Bradley, 1981). The experimental and theoretical bases of these assays will be briefly reviewed in the context of the interpretation of the current results.

SSB's, which reduce the length of the DNA single strands that are released in alkali, allow the DNA to pass more rapidly through a filter. This assay was calibrated relative to the independently determined efficiency for SSB production by X-ray, which was estimated by akaline sedimentation to be 0.9×10^{-9} SSB nucleotide^{-1} rad^{-1} (Kohn et al., 1976). A given increase in alkaline elution rate produced by the drug was gauged in terms of the X-ray dose (in rads) that would give a similar increase in elution rate. The "rad equivalent" measure of SSB's can, if desired, be converted to an estimate of absolute lesion frequency by multiplying by the factor 0.9 $\times 10^{-9}$ SSB nucleotide^{-1} rad^{-1}. In this assay, the effect of DNA-linked proteins is eliminated by the use of proteinase K.

The assay of DPC's is based on the use of filters which efficiently adsorb proteins under alkaline conditions and which retain those DNA single-strand fragments that are covalently linked to protein (Kohn & Ewig, 1979). A known distribution of DNA single-strand fragment lengths is produced by exposing the cells to a suitable dose of X-ray. From the fraction of the DNA retained on the filter, the DPC frequency can be calculated on the basis of simple probability theory (Kohn & Ewig, 1979; Ross et al., 1979a). DPC frequencies can be calculated on the basis of either of two simple hypotheses: (1) binding of protein molecules to one terminus (3′ or 5′) of each SSB or (2) binding of protein molecules randomly along the DNA strands. The bound-to-one-terminus model gives estimates for DPC frequencies about 2 times as high as those given by the random model (Ross et al., 1979a). In the current work, the bound-one-to-terminus model indicated that adriamycin and *m*-AMSA produced nearly equal frequencies of DPC's and SSB's. The random model would estimate that these drugs produced more SSB's than DPC's. However, this model can be excluded by the following consideration. If there were at least as many SSB's as DPC's, the random model would predict that in the DPC assay, at least 25% of the DNA should consist of single-strand fragments that by chance have escaped binding to any protein molecules [from eq 1 of Kohn & Ewig (1979)]. This quantity of free DNA should have been easily detected in alkaline elution assays that do not employ proteinase K or X-ray. Such assays, however, showed no increase in DNA elution rate. Therefore, the DNA-linked protein molecules must be associated in some manner with the single-strand breaks.

An alternative to the bound-to-one-terminus model would be binding to both termini, so as to produce a protein bridge across the strand break. This hypothesis was tested for *m*-AMSA, which produced large enough SSB frequencies to permit alkaline sedimentation measurements of DNA single-strand molecular weights without greatly exceeding cytotoxic drug doses. These measurements were carried out at 1–3 times the D_0 dose (dose giving 37% survival of colony formation). In the case of adriamycin, the SSB frequency in this range of dosage relative to cytotoxicity was too low for quantitative measurement by alkaline sedimentation. Alkaline sedimentation of *m*-AMSA-treated cells clearly showed the presence of SSB's, and the estimated SSB frequencies were, if anything, somewhat greater than the estimates by alkaline elution performed concurrently in the same experiments. Whereas the alkaline elution rate was increased if proteinase K was used, alkaline sedimentation was not greatly affected by proteinase K in a suitable lysis medium. A bridging protein would have held the single strands together across a break and would have prevented the reduction in sedimentation in alkaline sucrose. The possibility that the bridging protein dissociated in the alkaline sedimentation medium is eliminated by the finding that when the alkaline sucrose solution was used in alkaline elution, the DNA was retained on the filter in the absence of proteinase and released when proteinase was used. Hence, the protein bridge model is excluded.

The simplest hypothesis to explain these results is that the intercalator-induced DNA strand breaks bear a protein covalently linked to either the 3′ termini or the 5′ termini of the breaks. The linked protein could be an enzyme, such as a topoisomerase, which produces a strand break and becomes covalently bound to the 5′-phosphoryl terminus of the break

(Tse et al., 1980). This action could release the strain placed on the DNA by intercalator-induced helix unwinding.

Double-Strand Breaks (DSB). The alkaline (pH 12.1) elution assays do not distinguish SSB's from DSB's: a DSB would be detected as two SSB's. The "neutral" (pH 9.6) elution assay for DSB's, described by Bradley & Kohn (1979), has recently been applied by Ross & Bradley (1981) to the intercalating agents ellipticine, adriamycin, and actinomycin D; the arguments supporting the contention that the assay measures DSB's have been summarized in these two papers. It has been substantiated that the eluted DNA is double stranded and that double-strand cuts produced by restriction endonucleases produce DNA elution under these conditions. The assay, however, would also detect lesions consisting of a pair of separated SSB's on opposite DNA strands when there is inadequate base pairing in the intervening DNA segment to hold the double strand intact. This could occur if a pair of SSB's is separated by only a few base pairs or if the intervening DNA segment has undergone strand separation. The latter might occur in cell nuclei as a result of local DNA distortion due to intercalation (Center, 1979). In the current work, these cases would not have been distinguished; for the purpose of this discussion, the concept "DSB" can be considered to be enlarged to include these cases.

In contradistinction to the alkaline (pH 12.1) elution assay of SSB's which has been calibrated relative to alkaline sedimentation, the "neutral" (pH 9.6) elution assay of DSB's cannot be checked directly against neutral sedimentation measurements, because the DSB frequency ranges that can be measured by the two methods are widley different. The neutral elution assay used was sensitive to the range of DNA damage produced by 2–8 kR of X-ray. Neutral sedimentation assays, on the other hand, require X-ray doses over 10 kR to yield quantifiable molecular weight distributions, and variable results have been reported (Dugle et al., 1976; Lehmann & Stevens, 1977; Lennartz et al., 1975; Veatch & Okada, 1969). The neutral elution assay was therefore calibrated relative to X-ray under the assumption that DNA elution in this assay depends on DSB frequency and that DSB frequency is proportional to X-ray dose. The SSB and DSB frequency determinations thus were expressed relative to the X-ray dose giving the same frequency of the corresponding type of break.

As previously noted by Ross & Bradley (1981), the calibration curve shows a linear dependence of DNA retention on X-ray dose. The slope of the calibration line was nearly the same as that found by Ross & Bradley. We do not know why DNA retention in the DSB assay is linearly dependent on DSB frequency, whereas in the SSB assay it is the logarithm of the DNA retention that is linearly dependent on SSB frequency.

In order to estimate the absolute break frequencies, it would be necessary to know the break frequencies produced per unit dose of X-ray. The DSB frequency produced by X-ray has been difficult to determine because of the requirement for very high X-ray doses to avoid sedimentation anomalies. Reported values for the ratio of X-ray-induced SSB to DSB in mammalian cells, measured by alkaline and neutral sedimentation, have been in the range 10–40 (Dugle et al., 1976; Lehmann & Stevens, 1977; Lennartz et al., 1975; Veatch & Okada, 1969).

In addition, one must take into account the fact that the SSB frequency measured by alkaline assays includes SSB's arising from DSB's (each DSB gives rise to two SSB) as well as true SSB's (those not arising from DSB's). It is desirable to make this distinction, because DSB's and true SSB's probably are formed by different mechanisms (e.g., different enzymes). The relationship between measured and true break frequencies is described by

$$k_{RS}[SSB] = s + 2d$$
$$k_{RD}[DSB] = d \qquad (6)$$

where [SSB] is the SSB frequency (in SSB rad equivalents) determined by the alkaline assay, [DSB] is the measured DSB frequency in DSB rad equivalent, s is the absolute frequency of SSB, excluding those arising from DSB, d is the absolute DSB frequency, k_{RS} is the SSB frequency produced per unit X-ray dose, as measured by the alkaline assay, and k_{RD} is the DSB frequency produced per unit X-ray dose. (The brackets are used to denote the frequencies of SSB's or DSB's measured in terms of the X-ray dose producing an equivalent effect in the corresponding assay). Therefore, the ratio of the true SSB to DSB is

$$\frac{s}{d} = \left(\frac{k_{RS}}{k_{RD}}\right)\left(\frac{[SSB]}{[DSB]}\right) - 2 \qquad (7)$$

By use of this equation, the true SSB/DSB ratios (s/d) were calculated from the measured ratios, [SSB]/[DSB], for both the lowest and highest values of k_{RS}/k_{RD} that have been reported (Table I). We conclude that the true ratio of single- to double-strand breaks (s/d) produced by *m*-AMSA is between 3 and 25, but in any case less than the ratio produced by X-ray. The s/d ratio for adriamycin is between 0 and 7, but in any case less than the ratio for *m*-AMSA. If the correct value of k_{RS}/k_{RD} is close to 10, it would seem that adriamycin may produce only double-strand breaks (lower broken lines in Figure 15).

The recent results of Ross & Bradley (1981), however, show that another intercalating agent, ellipticine, produces an even lower single- to double-strand break ratio than does adriamycin. If, as suggested by these authors, ellipticine produces DSB's almost exclusively, then k_{RS}/k_{RD} must be at least 20, and adriamycin must produce SSB's as well as DSB's. Our results and those of Ross & Bradley show that there are wide differences between the SSB/DSB ratios produced by different intercalating agents, in the order *m*-AMSA > adriamycin > ellipticine. These differences must constitute an important clue to the origin of these breaks. If the breaks are enzymatically induced, one possibility is the intercalators differ widely in their relative stimulation of topoisomerases producing single-strand vs. double-strand cuts (Cozzarelli, 1980; Liu et al., 1980).

It is uncertain whether or not DSB's are protein associated. The main finding that bears on this question is that alkaline elution assays without proteinase K failed to detect any strand breaks. If there are many SSB's relative to DSB's, as in the case of *m*-AMSA, then DSB's not associated with protein might have gone undetected in the alkaline assay without proteinase. On the other hand, if ellipticine produces mainly DSB's, then these must be protein associated, because the breaks are not revealed by the alkaline assay without proteinase (Ross et al., 1978).

Kinetics. The enzymatic origin of protein-associated DNA strand breaks was supported by kinetic evidence for saturation behavior and for strong temperature dependence. The two drugs, *m*-AMSA and adriamycin, however, exhibited major kinetic differences.

The saturation of SSB frequency was clearly seen with *m*-AMSA but did not occur with adriamycin. In the case of *m*-AMSA, break frequency saturated at 2 μM drug, whereas drug entry into the cells was proportional to drug concentration

up to at least 7.5 μM. The saturation level for *m*-AMSA was approximately 56 000 SSB's per cell (assuming 2×10^{10} DNA nucleotides per cell and SSB saturation at 3100 rad equiv = 2.8 SSB per million nucleotides).

In the case of adriamycin, on the other hand, SSB frequencies increased proportionately to drug concentrations up to 3.5 μM and did not saturate even at 9 μM, which is far above the pharmacologic range for cell killing. The highest frequency of single-strand breaks achieved, however, was only 0.6 per million nucleotides. Hence, if the breaks have the same origin as in the case of *m*-AMSA, saturation would not have been expected at these levels.

The entry of *m*-AMSA into the cells was very rapid and reached a plateau within 5 min at 37 °C; SSB's and DPC's increased to a plateau within 10 min at 37 °C. Entry of adriamycin into L1210 cells, although it may be slower, has been reported to reach a plateau within 30 min (Bachur, 1975); strand breaks and DNA–protein cross-links, however, increase steadily for at least 6 h (Ross et al., 1978). Thus the effect on intracellular DNA is prompt in the case of *m*-AMSA but slowly cumulative in the case of adriamycin. The rate of the adriamycin effect may be limited at the intracellular DNA binding step or at a subsequent step.

m-AMSA and adriamycin differed markedly in the reversibility of their effects. *m*-AMSA effluxed rapidly from cells, and the SSB's, DPC's, and DSB's disappeared within 20 min at 37 °C. Adriamycin appears to efflux slowly by an active transport mechanism (Skovsgaard, 1977, 1978; Inaba & Johnson, 1978). This may account for the observed persistence of the adriamycin-induced DNA effects.

For both drugs, the temperature dependence of DNA strand breakage was much greater than the temperature dependence of cellular uptake. Although the entry of *m*-AMSA into cells was slowed at 4 °C, the amount of drug in the cells after 60 min was similar to that obtained at 37 °C; however, there was little or no DNA strand breakage at 4 °C. The uptake of adriamycin into Ehrlich ascites cells was reported to be only slightly dependent on temperature in the range 0–37 °C (Skovsgaard, 1978). The production of SSB's by adriamycin, however, was highly temperature dependent (Figure 9).

The rapid disappearance of protein-associated strand breaks after removal of *m*-AMSA may be due either to the action of a rapid DNA repair mechanism or to the resealing phase of a topoisomerase reaction. In either case, the plateau seen in Figure 8 must represent a steady state at which protein-associated breaks are formed and removed at equal rates.

Relation to Cytotoxicity. Ross et al. (1979a,b) had noted that when adriamycin was compared with ellipticine at doses producing similar frequencies of protein-associated strand breaks, adriamycin was much more cytotoxic. This suggested that protein-associated strand breaks could not be the major cause of cell killing. We now find an analogous result in the comparison of adriamycin with *m*-AMSA. However, we find that the adriamycin effects on DNA persist for many hours whereas the *m*-AMSA effects are rapidly reversible. This reopens the question of the relation between protein-associated strand breaks and cell killing.

Do protein-associated strand breaks tend to kill cells, or are they a protective mechanism that counteracts the effects of intercalators on DNA torsion? Although there is no adequate evidence against the former, the latter possibility would fit with the proposed involvement of topoisomerase enzymes. Changes in DNA torsion would be expected as a result of DNA helix unwinding by intercalation (Waring, 1970). The unwinding angle per intercalated molecule is 8° for daunomycin (Quigley et al., 1980), 20.5° for *m*-AMSA (Waring, 1976), and 17° for ellipticine (Kohn et al., 1975) relative to a value of 26° for ethidium. Adriamycin, which is 14-hydroxydaunomycin, should produce a smaller unwinding angle per intercalated molecule than *m*-AMSA, ellipticine, or ethidium and therefore may produce less effect on DNA torsion and perhaps less topoisomerase action.

In a study of the effect of *m*-AMSA on the cell cycle, Tobey et al. (1978) and Deaven et al. (1978) noted that the most prominent effect was on cells that had completed DNA synthesis just before drug addition and were in the early G2 phase of the cell cycle. After drug removal, there was a delay of 8 h before onset of mitosis, and the mitotic chromosomes showed many abnormalities. In the G2 phase, chromatin may be undergoing a rearrangement in preparation for chromosome condensation. Either the torsional effect of intercalation or the diversion of topoisomerase enzymes might interfere with this process.

Tobey et al. (1978) also found that cells that are in the S phase at the time of exposure to *m*-AMSA continue DNA replication after drug removal and subsequently tend to become arrested in the G2 phase. It is possible that intercalator-induced DNA torsion or diversion of topoisomerases during the S phase can produce lasting defects in replicated DNA. This could interfere with signals to initiate mitosis or produce the chromosome defects that were observed in those cells that did enter mitosis. The alternative possibility that the protein-associated strand breaks represent DNA damage is not excluded, however, since known DNA damaging agents, such as bleomycin (Tobey, 1972) and chloroethylnitrosoureas (Tobey & Crissman, 1975), also tend to arrest cells in the G2 phase.

Problem of o-AMSA. The *o*-AMSA isomer binds to purified DNA and produces the same unwinding angle as *m*-AMSA (Waring, 1976). Although the relative DNA binding constants have not been reported, there is no reason to expect a marked difference between the two isomers. The derivative lacking the methoxy group also produces the same DNA unwinding angle (Waring, 1976) and retains antitumor activity (Cain et al., 1976). Hence, the lack of antitumor activity and low cytotoxicity of *o*-AMSA appear to derive from the presence of the methoxy group specifically at the ortho position, where it could be involved in a hydrogen bond with the $NHSO_2CH_3$ group. Our finding that *o*-AMSA is 30–90 times less potent than *m*-AMSA in killing L1210 cells and at least 200 times less potent in producing SSB's suggests that *o*-AMSA does not reach the DNA target in cells. We can propose no explanation to account for this. Our data indicate that when high enough concentrations of *o*-AMSA are used to produce DNA breaks, these are largely not protein associated. The mechanism of cell killing by high concentrations of *o*-AMSA is probably different from that of *m*-AMSA.

Alternative Mechanisms. It has been proposed that anthracyclines such as adriamycin produce DNA breakage by a free radical mechanism (Bachur et al., 1979). This mechanism does not account for our findings, however, because protein-associated breaks were more prominent with *m*-AMSA and ellipticine, which are less apt than adriamycin to generate free radicals. Furthermore, it would be difficult to explain the wide differences between intercalators in single- to double-strand break production.

Gowans et al. (1980) and Levin et al. (1981) have recently reported that certain adriamycin derivatives that do not bind to purified DNA nevertheless produce protein-associated DNA breaks in cells. These findings require further study in order

to determine whether adriamycin may be metabolically generated in cells. Also, the possibility has not been excluded that the derivatives might bind to DNA in chromatin.

At high doses of various intercalating agents, extensive DNA breakage is sometimes seen that is not associated with DNA–protein cross-linking and that may be a secondary consequence of severe cytotoxicity (W. E. Ross and K. W. Kohn, unpublished data). Some previous reports of DNA breaks induced by actinomycin (Elkind, 1971; Pater & Mak, 1974), anthracyclines (Schwartz, 1976), and ellipticines (Paoletti et al., 1979) may have this origin. DNA breaks not associated with DPC's might in some cases also arise by free-radical mechanisms. The dosage used in the current work, however, was limited to a range over which DNA breaks not associated with DPC's were absent or undetectable.

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