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# Blocked 5'-termini in the fragments of chromosomal DNA produced in cells exposed to the antitumour drug 4'-[(9-acridinyl)-amino]methanesulphon-m-anisidide (mAMSA)

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## ABSTRACT

Comparison of the sensitivity of DNA isolated from untreated and mAMSA-treated PY815 mouse mastocytoma cells to hydrolysis by E.coli 3'-exonuclease III and phage λ or phage T7 5'-exonucleases show that the fragments of chromosomal DNA produced by mAMSA treatment have free 3'-OH termini and blocked 5'-termini.

## INTRODUCTION

Several anticancer drugs such as adriamycin and 4'- [(9-acridinyl)-amino] methanesulphon-m-anisidide (mAMSA) which intercalate into DNA, induce breaks in the DNA of treated cells at spacings in the range of 10-100 Kb, with concomitant binding of protein to the resulting DNA fragments (1-8). The stability to alkali of the bonds linking DNA and protein suggests that they are covalent, and it has been proposed that they may result from action of a topoisomerase (6, 7, 8). We have earlier presented evidence that the 5'-termini of the DNA fragments from mAMSA-treated PY815 cells are not available for phosphorylation by phage T4 polynucleotide kinase (9). We have now used 5'- and 3'-specific exonucleases to provide more direct evidence that the 5'-termini of these DNA fragments are blocked, whereas the 3'-termini are not, consistent with the presence of a protein linked to their 5'-termini.

## MATERIALS AND METHODS

### Materials

E.coli exonuclease III and phage λ 5'-exonuclease were products of Bethesda Research Laboratories, Md., USA. Phage T7 5'-exonuclease was a kind gift from Dr. M. Cusick. (Methyl-$^3$H)thymidine was from Amersham, UK.

### Preparation of DNA

PY815 mouse mastocytoma cells were grown for 10 h with 0.1 μCi/ml of ($^3$H)-thymidine in DMEM medium with 10% calf serum and 7 mM glutamine. The

culture (400 ml, $4x10^5$ cells/ml) was divided equally and one half was treated with 4 μM mAMSA for 1 h. The cells were recovered by centrifugation, washed with 20 ml ice-cold medium without serum, rapidly mixed with 20 ml of 1 M NaCl, 1% sodium dodecylsulfate, 5 mM EDTA, 50 mM Tris-HCl (pH 7.6), and extracted with 10 ml of water-saturated phenol with gentle rocking at 2° for 30 mins. After centrifugation at 10,000 g for 10 min, the viscous aqueous phase was removed with a cut-off wide-bore Pasteur pipette and reextracted with 5 ml of phenol. After centrifugation to separate the phases, the DNA in the supernatant was precipitated with 2 vols ethanol, spooled onto a glass rod, washed twice with 70% ethanol, 0.1 M Na acetate and redissolved in 10 mM Tris-HCl (pH 7.6), 1 mM EDTA. Pancreatic ribonuclease (preheated at 100° for 5 min) was then added to 10 μg/ml for 30 min at 25° to degrade RNA. Next, 1 ml of 1% sodium dodecylsulfate, 1 M NaCl, 50 mM Tris-HCl (pH 7.6), 5 mM EDTA and 2 ml of phenol solution were added, and the mixture was rocked gently for 15 min. After centrifugation to separate the phases, the supernatant was again extracted with 2 ml of phenol. DNA in the final aqueous supernatant was precipitated with 2 vols ethanol, recovered by centrifugation, washed three times with 20 ml of 70% ethanol, 0.1 M Na acetate, slowly redissolved in 2 ml of 10 mM Tris-HCl (pH 7.6), 1 mM EDTA and stored at -20°. Yields of about 27 $OD_{260}$ were obtained ; the DNA from mAMSA-treated cells was visibly less viscous than the DNA of untreated cells, consistent with sedimentation studies which indicate that mAMSA treatment reduces the molecular weight of PY815 cell DNA to about $2x10^8$ daltons (3). For some experiments DNA in assay mixtures at 0° was fragmented by multiple 3 second bursts in a Branson B12 sonifier, prior to enzyme addition and incubation at 37°.

Radioactivity was determined after precipitating aliquots of DNA solutions or enzyme digests with 100 μg bovine serum albumin in 5% trichloroacetic acid, collection of the precipitates on GF/B glass fiber filters, and measurement of radioactivity in a toluene-based scintillant.

Enzyme assays

a) Exonuclease III. Samples of DNA (1-2 μg; approx. 6,000 cpm) in 50 mM Tris-HCl (pH 8), 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol were incubated with a predetermined excess of exonuclease III (5 units) in a final volume of 0.03 ml at 37°. Aliquots (5 μl) were removed at intervals, and TCA-precipitable radioactivity determined as described.

b) Phage λ 5'-exonuclease. Samples of DNA (5 μg; approx. 25,000 cpm) in 0.1 ml 67 mM glycine (pH 9.7), 2.5 mM $MgCl_2$, 50 μg/ml bovine serum albumin were incubated with 1.5 units of λ exonuclease at 37°. Aliquots (10 μl) were

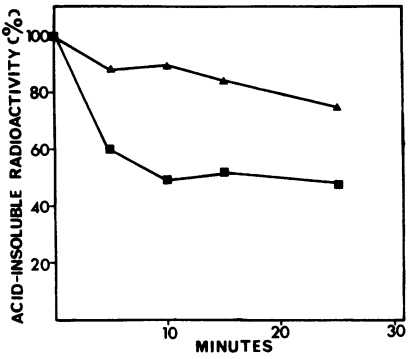


Figure 1. Degradation of DNA from PY815 cells by E. coli exonuclease III. The time zero samples contained 1,000 cpm acid-insoluble radioactivity. ▲—▲ , DNA from untreated cells; ■—■ , DNA from mAMSA-treated cells.

removed at intervals, precipitated, and counted as described.

c) Phage T7 5'-exonuclease. Samples of DNA (5 µg, approx. 25,000 cpm) in 0.1 ml 0.1 M Tris-HCl (pH 8), 20 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol were incubated with T7 5'-exonuclease (10 µl) at 37°. Aliquots (0.01 ml) were removed at intervals, precipitated, and counted. The enzyme, kindly donated by Dr. M. Cusick, originally contained about 1000 units per 0.065 ml. Preliminary tests showed that 7 units of enzyme hydrolysed 50% of sonicated DNA from untreated cells to acid-soluble material within 5 min at 37°, and therefore this excess of enzyme was used for all subsequent assays.

## RESULTS

Fig. 1 compares the rates of degradation by E. coli 3'-exonuclease III of equivalent amounts of DNA from untreated and mAMSA-treated cells. It is clear that the fragmented DNA from mAMSA-treated cells is degraded much more rapidly than DNA of untreated cells, confirming that additional free 3'-OH termini are present. The slow hydrolysis of DNA from untreated cells presumably occurs by enzymic attack at 3'-OH ends of nicks or breaks produced during DNA isolation or manipulation, since the rate and extent of hydrolysis vary with different DNA preparations. However, in parallel preparations, the DNA of mAMSA-treated cells is always more rapidly and extensively hydrolysed than that from untreated cells.

Figs. 2A and 2B compare the hydrolysis of DNA from untreated and mAMSA-treated cells by phage λ 5'-exonuclease. There is no significant difference in the rates of DNA degradation over 4 h; the initial fast rate presumably results from hydrolysis of a small proportion of nicked or broken DNA. When the DNA-containing assay mixtures are sonicated prior to adding enzyme, there is an immediate and rapid degradation of 50% of the DNA,

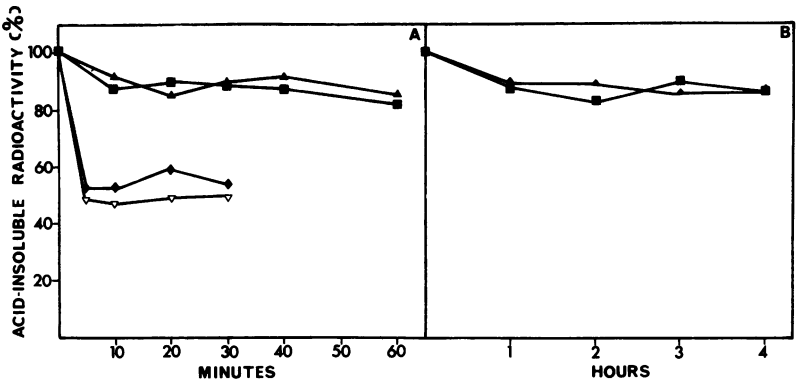


Figure 2. Degradation of DNA from PY815 cells by phage λ 5'-exonuclease. The time zero samples contained 3,000 cpm acid-insoluble radioactivity. ■—■ , DNA from untreated cells; ▲—▲ , DNA from mAMSA-treated cells; ▽—▽ , sonicated DNA from untreated cells, ◆—◆ , sonicated DNA from mAMSA-treated cells.

confirming that the enzyme would be able to rapidly hydrolyse any new 5'-OH termini produced by mAMSA treatment. We conclude that fragmentation of PY815 cell DNA by mAMSA does not produce additional free 5'-OH termini. Since additional free 3'-OH termini are produced by mAMSA treatment, these results suggest that the 5' termini are blocked, possibly by protein bound to the terminal 5'-nucleotide.

We reasoned that the smaller mAMSA-DNA should be less affected than DNA of untreated cells by gentle shear, and subsequently more resistant to exonuclease degradation because of its blocked 5'termini, and therefore examined the effect of gentle shearing of both DNA preparations on their susceptibility to phage λ 5'-exonuclease. DNA in the assay mixture was sheared to a mean fragment length of 10 kbp (as determined by sedimentation in sucrose gradients with a marker of phage DNA) prior to the addition of enzyme, by passage through a hypodermic needle. After shearing, the mAMSA-DNA was less readily degraded by λ 5'-exonuclease than DNA from untreated cells, consistent with its lower sensitivity to shear and the presence of blocked 5'-termini (Fig. 3).

Repetition of the above experiments with T7 5'-exonuclease in place of λ 5'-exonuclease gave essentially identical results, with an initial 15% reduction in acid-insoluble radioactivity over the first 10 min and little further hydrolysis over the next 4 h. Sonicated DNAs were degraded to 50% acid-soluble material within 5 min at 37°.

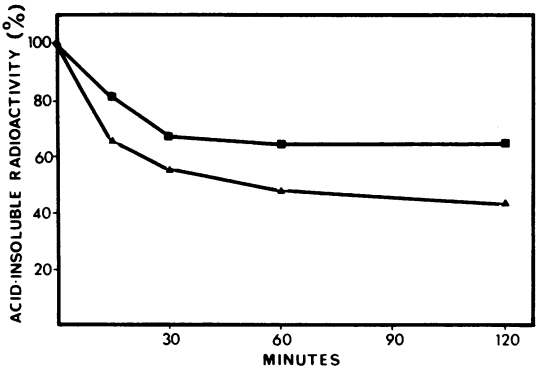


Figure 3. Degradation of sheared PY815 cell DNA by phage λ 5'exonuclease. DNA of mean fragment length 10 kbp from untreated cells ▲——▲ , and from mAMSA-treated cells ■——■ .

## DISCUSSION

These results support our earlier evidence (9) that the 5' termini of the DNA fragments produced in mAMSA-treated PY815 cells are not available for enzymic modification. The present experiments do not directly demonstrate that this inaccessibility is due to linkage of the 5' termini to protein. However, we believe this is possible in view of other evidence (5, 6 8) and of our observations (R. Ralph and R. Hancock, in preparation) that the DNA fragments from mAMSA-treated cells bind to glass fiber filters under the specific assay conditions of Coombs and Pearson (11), and that polypeptides can be isolated from this DNA, but not from DNA of untreated cells, after banding in CsCl. These polypeptides would not appear to be derived from a eukaryotic type I topoisomerase, since this enzyme becomes linked to 3'-OH termini of DNA (12).

The idea that intercalation of mAMSA into chromosomal DNA induces the action of a topoisomerase to relieve torsional constraints is plausible, and is compatible with recent observations (13 and B. Marshall, unpublished observations) that mAMSA can produce reversible DNA breakage in isolated nuclei. There does not appear to be a simple correlation between the frequency of DNA strand breakage and the cytotoxicity of different intercalating drugs (7, 14), suggesting that a more subtle consequence of intercalation and possible topoisomerase action is involved in their lethal action. mAMSA very efficiently induces sister-chromatid exchange in CHO cells at only 0.05 µg/ml, a concentration that does not affect cell survival (15), and in PY815 cells (N. Iranpour, unpublished observations). The frequency of sister-chromatid

exchanges induced by alkylating agents correlates well with reduced survival of CHO cells (16). We therefore suggest that specific sister-chromatid exchanges, in which topoisomerase activity has been implicated (17, 18), may be responsible for the lethal action of mAMSA and other members of this class of drugs.

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