Excision of 7-Bromomethylbenz[*a*]anthracene–DNA Adducts in Replicating Mammalian Cells†

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ABSTRACT: The excision of 7-bromomethylbenz[*a*]anthracene–DNA adducts was studied in two cell lines (HeLa S-3 and Chinese hamster V-79 379A). In both cell lines, carcinogen-modified adenine residues were excised more readily than the modified guanine residues and the percentage of the total products excised decreased after treatment with higher concentrations of carcinogen. At the highest concentrations used in the Chinese hamster cells, neither DNA synthesis nor excision was detected. The lowest concentration used for these cells permitted almost 100% survival and all the DNA was replicated in a 30-h interval even though 50% of the initial damage was still present. The two- to threefold lower sensitivity of the Chinese hamster cells (compared with the Hela cells) to the carcinogen is attributed to this capacity for replication of DNA on a damaged template since the two cell lines' capacities for excision of the chemical damage were found to be comparable.

The deficiency in repair of UV[1] damage in xeroderma pigmentosum fibroblasts (Cleaver, 1968) and the lack of excision of certain alkylated bases in carcinogen-susceptible organs in animals (Goth and Rajewsky, 1974) suggest that the efficacy of excision repair processes may determine the carcinogenic potential or site specificity of agents which damage cellular DNA. Many mechanistic details of these repair processes have yet to be defined and the experiments reported herein were designed to yield more information on the specificity, completeness, and kinetics of the excision repair of chemical damage in DNA of growing mammalian cells.

In the present study, DNA was damaged by reaction with the carcinogen, 7-bromomethylbenz[*a*]anthracene (Dipple and Slade, 1971; Roe et al., 1972) which reacts on the amino groups of nucleic acid bases (Dipple et al., 1971) giving products which are chemically stable under physiological conditions (Lieberman and Dipple, 1972). Excision was then monitored in a human cell line (HeLa S-3) and a Chinese hamster cell line (V-79 379A) which exhibit widely different sensitivities to the toxic effects of carcinogenic nitroso compounds (Roberts et al., 1971a; Plant and Roberts, 1971).

Experimental Procedure

[^{3}H]-7-Bromomethylbenz[*a*]anthracene was prepared at a specific radioactivity of 3.72 Ci per mmol as previously described (Dipple et al., 1971). The growth of Chinese hamster V-79 379A cells, HeLa S3 cells, and the determination of survival curves were as described by Roberts et al. (1971a).

Suspension Cultures. Cell suspensions (5–7 × 10^5 cells per mL) were grown in medium containing 5-bromo-2′-deoxyuridine (5 μg/mL) for 2 h prior to treatment with a 0.001 volume of a freshly prepared solution of the radioactive carcinogen in dry acetone. They were then maintained at 37 °C for a further 2.5 h after which the cells were collected by centrifugation and resuspended in fresh medium containing 5-bromo-2′-deoxyuridine. Aliquots of the suspension (200 mL) were removed at this time (3 h after adding the carcinogen) and at various times thereafter, and the cells were collected by centrifugation and washed twice in saline and the cell pellets were frozen. Subsequently, these pellets were lysed in a solution containing sodium triisopropylnaphthalenesulfonate, butan-2-ol, sodium 4-aminosalicylate, and sodium chloride (Kirby and Cook, 1967) and extracted with an equal volume of phenol–*m*-cresol–8-hydroxyquinoline reagent (Kirby, 1965). The aqueous layer was reextracted with a 0.5 volume of phenol reagent and DNA was then precipitated with 2-ethoxyethanol. The DNA was dissolved in 0.01 M sodium phosphate buffer (pH 7.1) and purified by centrifugation in CsCl solution (8.5 g of CsCl plus 6.5 mL of DNA solution) at 42 000 rpm for 40 h (fixed angle Ti 50 rotor).

V-79 Monolayer Cultures. Confluent monolayers were grown in 5-bromo-2′-deoxyuridine-containing medium and treated as above. One 150-cm^2 plastic bottle was harvested by scraping off the cells at each time point for each carcinogen concentration employed. DNA was isolated from these pellets by lysis of the cells in Triton X-100 and of the nuclei in 0.5% sodium dodecyl sulfate as described by Lieberman and Dipple (1972). The final lysate (1 mL) was added to cesium chloride solution (7.75 mL) prepared by dissolving CsCl (85 g) in 0.01 M phosphate buffer (pH 7.1) (55 mL) and centrifuged as above.

Neutral gradients were pumped up from the bottom and collected in 18–20 fractions and all fractions containing DNA (detected by optical density) were pooled and dialyzed against distilled water at 4 °C to yield a solution of purified DNA.

Estimation of Excision of Carcinogen-Modified Residues from DNA. The method used was essentially that described by Roberts et al. (1971b). An aliquot of the purified DNA solution was subjected to centrifugation in CsCl solution at pH 12.5 [6.5 g of CsCl plus 4.5 mL of DNA solution on 0.1 M Na_2HPO_4–NaOH buffer adjusted to pH 12.5 (Painter and Cleaver, 1967)] at 45 000 rpm for 40 h (fixed angle Ti 50 rotor). Approximately 40 fractions were obtained from each gradient and, after dilution with water (0.5 mL), the optical density of each fraction at 260 nm was recorded. An aliquot

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[1] Abbreviation used: UV, ultraviolet.

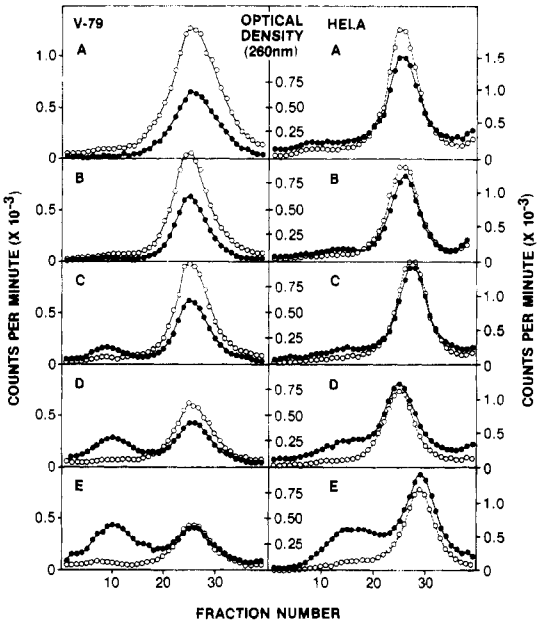

FIGURE 1: Alkaline CsCl density gradient centrifugation (Roberts et al., 1971b) of DNA isolated from cells after a 3-h treatment with 0.2 μM [^{3}H]-7-bromomethylbenz[*a*]anthracene (A), after a further 3 h (B), 9 h (C), 18 h (D), and 30 h (E). Gradients were pumped out from the bottom and collected in about 40 fractions. (O—O) cpm; (●—●) optical density 260 nm.

(0.5 mL) from each fraction was diluted with water (0.5 mL) and radioactivity was determined by liquid scintillation counting using PCS solubilizer (Amersham/Searle).

Specificity of Excision of Carcinogen-DNA Adducts. Samples of the purified DNA preparations were enzymically digested to mixtures of deoxyribonucleosides (Rayman and Dipple, 1973b) and subjected to chromatography on Sephadex LH-20 eluted with methanol as previously described (Dipple et al., 1971). Fractions (5 mL) were collected in scintillation vials and evaporated to dryness, and radioactivity was then determined by liquid scintillation counting using PCS solubilizer (10 mL).

Reaction of Carcinogen with Isolated DNA. DNA was isolated from cell pellets of untreated HeLa and V-79 cell suspensions by the Kirby phenol procedure described under Suspension Cultures. Solutions of these DNAs (0.25 mg/mL) in 0.02 M sodium phosphate buffer, pH 7.0, were treated with a 0.1 volume of acetone solution of [^{3}H]-7-bromomethylbenz[*a*]anthracene (1.5 μM). After 15 min the DNAs were precipitated, washed, and the extents of reaction and product distributions were determined, all as previously described (Rayman and Dipple, 1973a).

Results

The object of this study was to measure directly the kinetics, completeness, and specificity of excision of chemical damage from the DNA of mammalian cells after various extents of reaction with a radioactive chemical carcinogen. Excision was measured by monitoring the specific radioactivity of the least dense DNA peak in alkaline cesium chloride density gradients containing DNA from cells treated with radioactive carcinogen and grown in the presence of 5-bromo-2′-deoxyuridine. DNA synthesized after carcinogen treatment was, therefore, denser than the original DNA, separable from it in the alkaline gradients, and did not interfere with the measurement of excision (Roberts et al., 1971b).

Extent and Rate of Overall Excision. Extensive excision of radioactive products occurred in suspension cultures of both Chinese hamster V-79 cells and HeLa cells exposed to 0.2 μM [^{3}H]-7-bromomethylbenz[*a*]anthracene (Figure 1). It can also be seen, from the relative amounts of light and heavy DNA, that the hamster cells synthesized more DNA during the course of these experiments than did the HeLa cells. In order to examine the response of the excision process to different extents of DNA reaction, similar experiments at various concentrations of carcinogen were undertaken (Table I). The extents of reaction with DNA at the arbitrary zero time point (3 h after treatment) in these studies were dose related. Thus, for HeLa cells the extents of reaction were 11.1 and 29.4 μmol per mol of DNA phosphorus after exposure to carcinogen at 0.2 and 0.6 μM. For V-79 cells the extents of reaction were 7.2, 14.6, and 169 μmol per mol of DNA phosphorus after 0.1, 0.2, and 1.8 μM treatments.

TABLE I: Percentage Excision of Radioactive Adducts from the DNA of Cells in Suspension Culture Exposed to [^{3}H]-7-Bromomethylbenz[*a*]anthracene at Various Concentrations.[a]

	HeLa		V-79		
Time (h)	0.2 μM	0.6 μM	0.1 μM	0.2 μM	1.8 μM
3	15	10	16	17	4
9	21	13	25	22	−9
18	29	16	44	31	1
30	37	25	52	47	4

[a] Cells were treated and DNA isolated, purified, and analysed as described under Experimental Procedure. The specific radioactivities of the light peaks in alkaline cesium chloride gradients (Figure 1) were computed and expressed as a percentage of the zero time specific radioactivity. The percentage excision is the difference between these numbers and 100%.

It is clear from Table I that the fraction of the total initial damage which is excised decreases with increasing doses of carcinogen. Nevertheless, the absolute amount of damaged residues excised (given by percentage excised multiplied by the initial extent of reaction) does increase with dose at the lower end of the dose range studied. At the highest doses used in both cell lines (1.8 μM in hamster, 0.6 μM in HeLa), no heavy peak was detected in the alkaline gradients, indicating that DNA synthesis was completely inhibited. Nevertheless, excision was apparent in the HeLa cell culture, but not in the hamster cells under these conditions (Table I).

This inhibition of excision at high carcinogen concentrations and examination of excision over a longer time period were studied in hamster V-79 cells grown on surfaces. Replicate cultures were treated with a range of [^{3}H]-7-bromomethylbenz[*a*]anthracene concentrations and excision was monitored over a 21.5-h period (Table II). Again, the percentage of the total adducts excised decreased with increasing carcinogen concentrations until at a 1.0 μM concentration excision was no longer detectable.

In the extended time course study, replicate cultures were exposed to 0.125 μM [^{3}H]-7-bromomethylbenz[*a*]anthracene and excision was monitored over a 70-h period (Figure 2). Most of the observed excision occurred in the first 21.5 h (64%). At 24 h, the remaining cultures were re-fed with medium containing 5-bromo-2′-deoxyuridine. Nevertheless, it can be seen that at 70 h newly synthesized DNA was apparently appearing in the light peak on the gradient, indicating that the bromodeoxyuridine supply had been exhausted. Excision at this time point (80%) is obviously overestimated, but excision at the 46-h

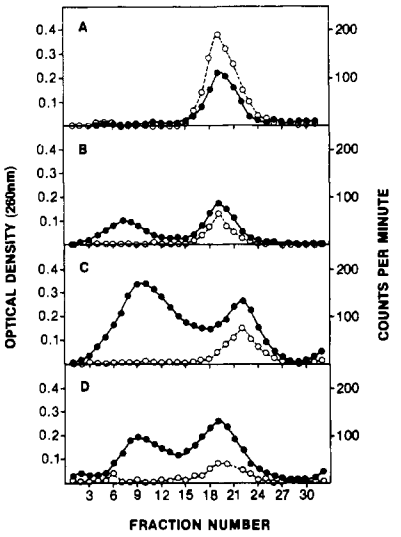

FIGURE 2: Alkaline CsCl density gradients of DNA isolated from V-79 cells grown on surfaces after a 2.5-h exposure to 0.125 μM 7-bromomethylbenz[*a*]anthracene (A), after a further 21.5 h (B), 46 h (C), and 70 h (D). (○—○) cpm; (●—●) optical density.

TABLE II: Excision of Radioactive Adducts from the DNA of V-79 Cells Cultured on Surfaces.

Concn of [^{3}H]-7-Bromomethylbenz[*a*]anthracene (μM)	Adducts Excised in 21.5 h (%)
0.125	64
0.25	32-47[a]
0.50	13-30[a]
1.0	~8

[a] The UV background was unusually high in one of the alkaline gradients. The actual extent of excision is within the range given since this was calculated by using highest and lowest possible values for background subtraction.

time point (70%) should be reasonably precise. In any event, it is clear from Figure 2 that some carcinogen adducts are still present in the hamster cell DNA even at 70 h after treatment.

Specificity of Excision. In each of the experiments with the suspension cultures described above, a portion of the purified DNA was enzymatically degraded to a mixture of deoxyribonucleosides and the radioactive products were separated and quantitated by chromatography on Sephadex LH-20 (Dipple et al., 1971) as illustrated for the HeLa cells in Figure 3. The excision of the well-characterized N^6-(benz[*a*]anthracenyl-7-methyl)deoxyadenosine and N^2-(benz[*a*]anthracenyl-7-methyl)deoxyguanosine residues could then be examined individually. In all cases in which excision was apparent it appeared that the modified adenine residues were preferentially excised. This is summarized in Table III where it can be seen that the ratio of the residual adenine product to the residual guanine product decreases during the course of the excision process.

The chromatography experiments yield information on the proportions of the residual adenine and guanine products in the DNA, and the alkaline CsCl gradients give the relative specific radioactivities of the original DNA at various times. Combination of these two sets of data provides an indication of the relative rates of removal of these individual products

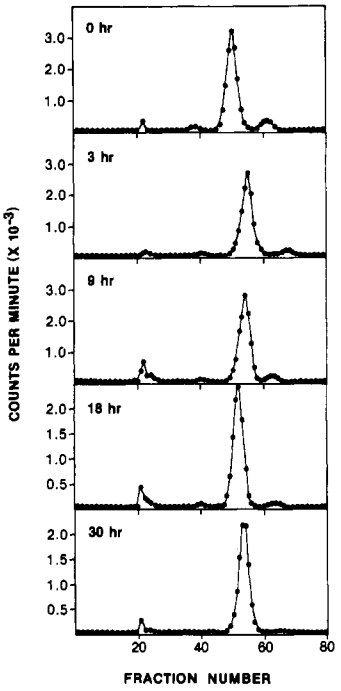

FIGURE 3: Column chromatograms of nucleoside products from DNA isolated from HeLa cells at various times after a 3-h exposure to 0.2 μM [^{3}H]-7-bromomethylbenz[*a*]anthracene. DNA was degraded enzymically (Rayman and Dipple, 1973b) and then eluted from Sephadex LH-20 with methanol (Dipple et al., 1971). The last two peaks eluted from the chromatogram have been identified as N^2-(benz[*a*]anthracenyl-7-methyl)-2′-deoxyguanosine and N^6-(benz[*a*]anthracenyl-7-methyl)-2′-deoxyadenosine, respectively, and the peak beginning in fraction 40 has been tentatively identified as N^4-(benz[*a*]anthracenyl-7-methyl)-2′-deoxycytidine (Dipple et al., 1971).

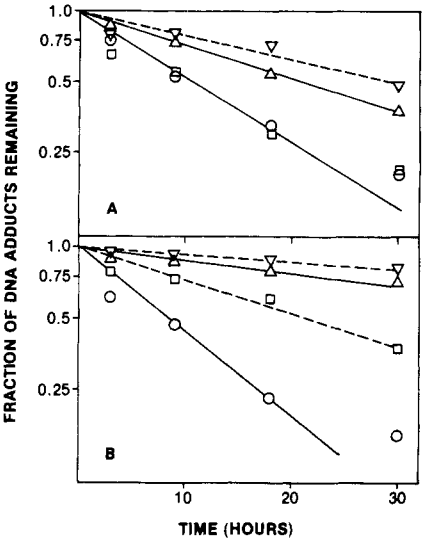

FIGURE 4: Fraction of initial products present in DNA remaining at various times. (A) Chinese hamster V-79 cells; (▽- - -▽ and □—□) guanine and adenine products remaining after exposure to 0.2 μM 7-bromomethylbenz[*a*]anthracene; (△—△ and ○—○) guanine and adenine products remaining after exposure to 0.1 μM 7-bromomethylbenz[*a*]-anthracene. (B) HeLa cells; (▽- - -▽ and □- - -□) guanine and adenine products remaining after exposure to 0.6 μM 7-bromomethylbenz[*a*]-anthracene; (△—△ and ○—○) guanine and adenine products remaining after exposure to 0.2 μM 7-bromomethylbenz[*a*]anthracene.

from the DNA. These data are presented in a semilogarithmic plot in Figure 4. The half-life for removal of modified guanine residues is approximately twice that for removal of modified adenine residues in V-79 cells, while there is an approximately

TABLE III: Ratio of Amount of Adenine Product/Amount of Guanine Product Remaining in DNA at Various Times after Exposure to Various Concentrations of [^{3}H]-7-Bromomethylbenz[*a*]anthracene.[a]

	HeLa		V-79			
Time (h)	0.2 μM	0.6 μM	0.1 μM	0.2 μM	0.6 μM	1.8 μM
0	0.15	0.16	0.19	0.22	0.23	0.25
3	0.10	0.13	0.16	0.18	0.21	0.21
9	0.08	0.13	0.14	0.15	0.15	0.25
18	0.04	0.11	0.11	0.09	0.14	0.24
30	0.03	0.07	0.10	0.10	0.13	0.24

[a] DNA was degraded and the radioactive products separated as described under Experimental Procedure and Figure 3. The counts in each of the last two radioactive peaks were summed and the ratio calculated.

fourfold difference in these rates in the HeLa cells. There is no obvious indication of a selective effect of the higher doses of carcinogen on the excision of either of these products but the data do not justify a firm conclusion on this point.

The differences in the product ratios for V-79 cells at zero time (Table III) are intriguing and the fact that they increase with increasing concentrations of carcinogen might suggest that some appreciable excision occurs during the 3-h treatment time. It is not clear why the initial ratios for HeLa and hamster cells are so different. When isolated DNA from each cell line (0.25 mg/mL) was exposed to [^{3}H]-7-bromomethylbenz[*a*]-anthracene (0.15 μM), comparable extents of reaction (31.7 and 33.7 μmol per mol of DNA phosphorus for hamster and HeLa DNA, respectively) and comparable product ratios (deoxyadenosine product:deoxyguanosine product was 0.27 for hamster and 0.28 for HeLa DNA) were obtained.

Cell Survival. In order to relate these observations to the toxic effect of 7-bromomethylbenz[*a*]anthracene on hamster V-79 and HeLa S-3 cells, survival of these cells after exposure to various concentrations of this agent was also examined. Although the current study demonstrates that excision is at least comparable in both cell lines, the HeLa cells were more sensitive to the toxic action of 7-bromomethylbenz[*a*]anthracene than were the hamster cells as was found previously for *N*-methyl-*N*-nitrosourea and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Roberts et al., 1971a). The survival curves are characterized by the D_0 values (i.e., the dose increment required to reduce survival by $1/e$ on the exponential part of the curve) of 0.09 and 0.038 μM for hamster and HeLa cell, respectively, and by the D_Q values (i.e., the intercept of the extrapolation of this linear portion of the curve on the *x* axis) of 0.12 and 0.08 μM for hamster and HeLa cells, respectively.

Discussion

Direct measurements were made of the disappearance of chemically stable damage from DNA in growing cells using a method which separates newly synthesized DNA from DNA present at the time of treatment.

In all experiments, the most rapid rate of excision was seen during the early time intervals and at all time points examined some residual radioactivity was present in the DNA. The fraction of the initial products excised decreased with increasing doses of carcinogen and at the very high doses used in the hamster cells (1.0 and 1.8 μM) excision was no longer detectable. The total amount of adducts excised did increase with dose at the lower end of the dose range studied and these data are consistent with other manifestations of excision repair. Thus, repair replication (Roberts et al., 1971b) and unscheduled DNA synthesis (Lieberman et al., 1971), following treatment with a variety of agents, increase with dose and then decrease. Inhibition of excision repair in vivo by high doses of carcinogen has also been reported recently (Kleihues and Margison, 1976).

In both of the cell lines examined here carcinogen–adenine residues were excised more readily than the carcinogen–guanine residues (Table III). It is clear from Figure 3 that 30 h after exposure of HeLa cells to 0.2 μM 7-bromomethylbenz[*a*]anthracene, essentially only carcinogen–guanine adducts remain in the DNA. The preferential excision of these carcinogen–adenine residues was first observed in nondividing human lymphocytes (Lieberman and Dipple, 1972), and more recently Kirtikar et al. (1975) found a similar phenomenon in in vitro studies with DNA modified by 7-bromomethyl-12-methylbenz[*a*]anthracene and an endonuclease II preparation from *E. coli*. The basis for this preferential excision of the adenine adducts is not known. Although these adducts presumably lie within the wide groove of the DNA helix whilst the guanine adducts lie in the narrow groove, endonuclease II releases 3-methyladenine from the narrow groove of DNA more rapidly than it releases O^6-methylguanine from the wide groove (Kirtikar and Goldthwait, 1974). 3-Methyladenine is also lost more rapidly from rat liver DNA (Craddock, 1973) and from various mouse tissues (Frei and Lawley, 1975). Furthermore, it does not hold that modified adenine residues are always excised more readily than modified guanine residues since the converse was reported by Venitt and Tarmy (1972) after treatment of *E. coli* with 7-bromomethylbenz[*a*]anthracene.

The similarities between the findings in mammalian cells and the in vitro studies with endonuclease II are particularly interesting since this enzyme releases carcinogen-modified bases through enzymatic cleavage of the glycosidic bond (Kirtikar and Goldthwait, 1974; Kirtikar et al., 1975) and does not exhibit any endonuclease action on UV-irradiated DNA. This type of enzyme activity could, therefore, be part of an alternative excision repair system to that believed to be responsible for the excision of UV-induced thymine dimers (Lindahl, 1976). The present studies may be relevant to this point since rodent cells are generally far less effective in the excision of thymine dimers than are human cells (Trosko and Kasschau, 1967; Regan et al., 1968; Cleaver, 1974), although both the cell lines examined here exhibited comparable capacities for the excision of this chemical damage.

Complete excision of all products was not observed in any of the studies presented here, though it is now clear that some of the individual products can be completely excised (Figure 3). It is also apparent that complete excision of damage is not a prerequisite for DNA replication and subsequent cell survival. For example, at a concentration of carcinogen (0.1 μM), which permits almost 100% survival of the Chinese hamster

cells, all of the DNA has replicated in a 30-h period (data not shown but compare with Figure 1E at 0.2 μM) whilst only 50% of the carcinogen adducts have been excised (Table I). This clearly shows that some other repair mechanism which can circumvent the lesions present in DNA during DNA synthesis is present in the hamster cells. Moreover, differences in the capacities of the HeLa and hamster cells for this latter type of repair (Plant and Roberts, 1971) presumably largely account for their observed two- to threefold difference in sensitivity to the toxic effects of 7-bromomethylbenz[*a*]anthracene since the minor differences in their excision repair capacities observed here are insufficient to account for this.

Finally, it is of interest that recent studies have indicated that the widely distributed environmental carcinogen, benzo[*a*]pyrene, mimics the bromomethylbenz[*a*]anthracenes in reacting, after metabolic activation, with the amino group of guanine in nucleic acids (Weinstein et al., 1976). The studies reported here are probably relevant, therefore, not only to the directly acting aralkylating carcinogens, but also to the polycyclic hydrocarbon carcinogens in general.

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