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Repair of O^6-Methyl-Guanine Residues in DNA Takes Place by a Similar Mechanism in Extracts From HeLa Cells, Human Liver, and Rat Liver

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Extracts from HeLa S_3 cells, human liver, and rat liver were found to contain an activity that transfers the methyl group from O^6-methyl-guanine residues in DNA to a cysteine residue of an acceptor protein. The molecular weights of the acceptor proteins in HeLA cells and human liver are 24,000 ± 1,000 and 23,000 ± 1,000, respectively. Assuming that each acceptor molecule is used only once, the average number of acceptor molecules in HeLa cells was calculated to be about 50,000. The extracts also contained 3-methyl-adenine-DNA glycosylase activity and 7-methyl-guanine-DNA glycosylase activity, although the latter activity was not detected in extracts from human liver in our assay system. Thus, the three major alkylation products resulting from the effect of methylating agents, such as *N*-methyl-*N*-nitroso urea, can all be repaired in animal cells. Pretreatment of HeLa cells with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (0.1 μg/ml) strongly reduced the capacity of HeLa cell extracts to repair O^6-methyl-guanine residues, while the activity of three DNA-*N*-glycosylases was essentially unaltered. This inactivation was not caused by a direct methylation of the enzyme by the carcinogen. The results demonstrate that the mechanism of repair of O^6-methyl-guanine residues in DNA is strikingly similar in E coli and animal cells, including humans.

Key words: O^6-methyl-guanine, DNA repair, human cells

Although nitrosoamines can methylate bases in DNA at several sites [1], only alkylation at the O^6-atom of guanine is clearly correlated with the mutagenicity and carcinogenicity of such compounds [2–4]. Damage to bases by methylating agents and damage caused by spontaneous deamination are usually repaired by an excision repair mechanism which is initiated by DNA glycosylases [5]. In contrast, repair of O^6-methyl-guanine residues in DNA of E coli was recently shown to take place by a

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one-step mechanism in which the methyl group is transferred to a cysteine residue of an acceptor protein [6]. A similar repair mechanism was recently reported for mouse liver [7], while somewhat conflicting results have been reported for rat liver [8,9]. Some human cell lines are capable of removing O^6-methyl-guanine residues [10–12], but the repair mechanism has not been investigated.

Here we demonstrate that extracts from HeLa cells, human liver, and rat liver all contain an activity that transfers the methyl group from O^6-methyl-guanine residues to a cysteine residue of a small acceptor protein, which appears to be inactivated in the process.

MATERIALS AND METHODS

N-[methyl-^{3}H]-*N*-nitroso urea ([^{3}H]MNU) (specific radioactivity 1.6 Ci/mmol) was purchased from New England Nuclear. 7-Methyl-guanine (7-MeG) and 3-methyl-adenine (3-MeA) were purchased from Fluka, and *S*-methyl-*L*-cysteine(S-Me-L-cys) from Sigma. O^6-methyl-guanine (O^6-MeG) was a generous gift from Dr. H. Autrup, The National Cancer Institute, Bethesda, Maryland. *N*-methyl-*N'*-nitro-*N*-nitroso-guanidine (MNNG) was purchased from Pfaltz and Bauer, Inc., Stamford, Connecticut. Leucine aminopeptidase was from Sigma, Pronase from Calbiochem, and Proteinase K from Merck. Acrylamide and bisacrylamide were purchased from Eastman, Rochester, New York. Other chemicals were obtained as described earlier [13].

Calf thymus DNA was treated with [^{3}H]MNU as described [7]. Various batches of DNA alkylated in this manner (substrate I) contained 0.22–0.61 μCi of alkylated DNA/mg DNA. To remove *N*-7-[^{3}H]methyl-guanine and *N*3-[^{3}H]methyl-adenine selectively, alkylated DNA was heat treated at neutral pH as described [14]. Batches of alkylated DNA prepared by heat treatment (substrate II) contained 0.025–0.097 μCi alkylated DNA/mg DNA.

Preparation of Cell Extracts

HeLa S_3 cells were grown in suspension cultures as described [15]. All the succeeding steps were carried out at 0–4°C. To prepare a cell extract, $1.2 \cdot 10^9$ cells were homogenized in a Potter-Elvehjem homogenizer in 25 ml buffer A [50 mM Tris-HCl, 1 mM dithiothreitol, and 0.1 mM Na_2 EDTA (pH 7.8)], sonicated, and then centrifuged (20,000 g for 30 min). The supernatant was collected and stored at −20°C in small portions. To prepare rat liver cell extracts, male Wistar rats were killed by decapitation and the liver perfused with saline before homogenization in a Potter-Elvehjem homogenizer (4°C) in 3 vol of buffer A. The homogenate was centrifuged (10,000 g for 5 min). The resulting pellet was suspended in 3 vol of buffer A and sonicated. The supernatant and the sonicated pellet were combined and centrifuged (20,000 for 30 min). The supernatant was stored at −20°C in small portions. Biopsies from human liver were obtained during intraabdominal surgery and extracts prepared as above except that the buffer also contained 1 mM phenylmethylsulfonyl fluoride (PMSF) and 10 mM $NaHSO_3$.

Assay for Repair of Alkylated DNA

Cell extracts were incubated with alkylated DNA substrate I, in buffer A (final vol 150 μl) at 37°C for 0–60 min. The reaction was terminated by adding 15 μl of

2.5 M NaCl and 350 μl of cold ethanol. After storage for at least 60 min at −20°C the tubes were centrifuged (10,000 g for 30 min), the ethanol supernatant collected, and the precipitate hydrolyzed at 70°C in 250 μl of 0.1 M HCl. Methylated purines in ethanol supernatants and acid hydrolysates were separated by descending chromatography on Whatman 3 MM paper for 16 hr in isopropanol/25% NH_3/H_20 (7:1:2). References (3-MeA, 7-MeG, and O^6-MeG) were mixed with the samples and localized as UV absorbing material. After drying, the individual sample lanes were cut transversely into 1-cm pieces, and the radioactivity counted in a scintillation counter; alternatively, only the zones containing the methylated bases were cut out and counted (Fig. 5).

Specific Assay for O^6-Methyl-Guanine–Cysteine Methyl Transferase Activity

The principle of the procedure is that the radioactivity in the methyl group which is transferred to an acceptor protein is made acid/ethanol soluble by digestion with pronase. Cell extracts (5–70 μl) were mixed with buffer A to a final volume of 70 μl and incubated with 5 μl of substrate II (about 2,000 dpm) for 0–60 min at 37°C in an Eppendorf tube. Then pronase (Calbiochem) (50 μg) was added and the incubation continued for 60 min, after which 20 μl of denatured DNA (1 mg/ml), 20 μl of NaCl (1 M), and 250 μl of ice-cold 1% trichloroacetic acid in 80% ethanol were added. The tubes were kept at −20°C for at least 1 hr before centrifugation (15,000 rpm, 10 min at 4°C). Radioactivity was measured in the supernatant by scintillation counting. The degraded acceptor protein is soluble at −20°C in 80% ethanol/1% trichloroacetic acid, while DNA was efficiently precipitated.

Amino Acid Analysis

The reaction mixtures were precipitated with ethanol and hydrolyzed for 24 hr at 110°C in sealed glass ampoules in 1 ml of 6 M HCl. The samples were taken to dryness under vacuum at 20°C, dissolved in 1 ml double-distilled water, dried once more, and then dissolved in 1 ml 0.2 M sodium citrate buffer (pH 2.2). The hydrolyzed material was analyzed on a Joel JLC-6AH amino acid analyzer (Japan Electronic Optics Laboratory, Tokyo, Japan).

Other Methods

Uracil-DNA-glycosylase activity was measured as described earlier [13]. Protein [16] and DNA [17] were quantitated according to standard procedures. Analytical sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (10%) was carried out according to Laemmli [18]. Size markers were phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumine (43,000), carbonic anhydrase (30,000) soybean trypsin inhibitor (20,100), and α-lactalbumin (14,400) from Pharmacia supplied as a kit. DNA-cellulose was prepared according to Litman [19], except that DNA was denatured with NaOH and then neutralized with HCl prior to irradiation.

RESULTS

Alkylation of DNA

DNA was treated with [^{3}H]MNU and methylation of bases analyzed by paper chromatography. As expected [1], the major alkylation products were 7-MeG (~70%), 3-Mea (8–10%), and O^6-MeG, (8–10%). All numbers are relative to total

alkylated DNA. Unidentified alkylation products (~ 10%) remained near the origin of the chromatograms and probably represented methylated pyrimidines and phosphotriesters [1]. The heat treatment removed most of the 3-MeA and 7-MeG, thus yielding a radiolabeled product containing about 70% O^6-MeG, 10–11% 7-MeG, 1–2% 3-MeA, and 15–19% unidentified radioactivity products.

Removal of Alkylated Bases by Cell Extracts

When alkylated DNA (substrate I) was incubated with sufficient amounts of cell extract in the presence of EDTA, O^6MeG was completely removed, while a partial removal of 3-MeA and 7-MeG was observed (Figs. 1A and 2A). While all the 3-MeA and 7-MeG released was recovered as free base in the ethanol supernatant, no O^6-MeG was found in this supernatant (Figs. 1B and 2B). No methylated purines were released when the extracts were heated to 80°C for 10 min prior to incubation. This strongly suggests that 7-MeG and 3-MeA residues are removed by DNA glycosylases [5] while O^6-MeG is removed by another mechanism. In fact, in these experiments the same amount of radioactivity that was removed from O^6-MeG was quantitatively recovered as an increase in radioactivity at the origin of the chromatogram (Figs 1A and 2A), suggesting that the radioactivity was transferred to a macromolecule that was not hydrolyzed in weak acid. When the same type of experiment was performed with substrate II and sufficient extract, we again observed an increase in the amount of radioactivity at the origin of the chromatogram, equal to the amount removed from the O^6-MeG peak. Essentially identical results were obtained with extracts from human liver, except that release of 3-MeA was lower and significant release of 7-MeG was not detected in these experiments. It is, however, possible that our assay is not sensitive enough to detect low activities which might be present in human liver. Table I summarizes the results from several such experiments, including results with extracts from human liver.

Based upon the known specific activity of the methyl group in O^6-MeG, and the assumption that each acceptor molecule is used only once, we have calculated the average number of acceptor molecules in HeLa cells to be about 50,000.

To investigate whether the methyl group of O^6-MeG was transferred to a protein, alkylated, heat-treated DNA (substrate II) was first incubated with the various extracts and the whole reaction mixture was ethanol precipitated. Control experiments showed that essentially no radioactivity was recovered in the ethanol supernatant. The ethanol precipitate was then hydrolyzed and subjected to amino acid analyses as described in Materials and Methods. The radioactivity eluted from the amino acid analyzer at 84 min exactly as did S-Me-L-cys. Identical results were obtained with HeLa cell extracts, human liver, and rat liver extracts (Figs. 3A,B,C). When correcting for the fraction that was reacted with ninhydrin, an amount of radioactivity corresponding to more than 90% of the radioactivity originally present in O^6-MeG in DNA was recovered in *S*-methyl-cysteine. Thus, it is unlikely that a significant portion of the methyl groups is released in the form of methanol [8]. However, since substrate II contained 15–19% unidentified alkylation products, we cannot exclude the possibility that some of the *S*-methyl-cysteine formed may come from methylated pyrimidines or phosphotriesters. Identical results were obtained when the protein was hydrolyzed enzymatically with proteinase K and leucine aminopeptidase as described

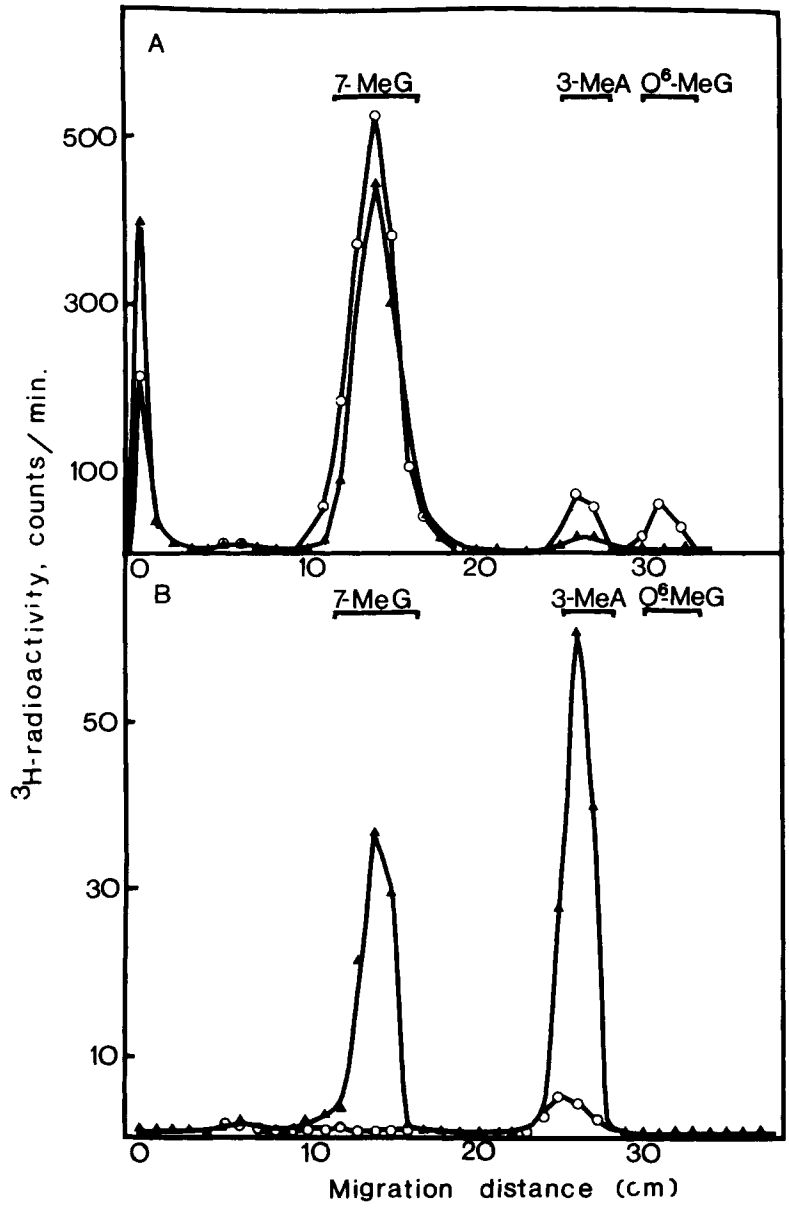

Fig. 1. Removal of alkylated purines from DNA by HeLa cell extracts. Alkylated DNA (18 μg) was incubated with HeLa cell extracts (810 μg) (—▲—), or buffer (—○—). The ethanol supernatant (B) or acid-hydrolyzed pellet (A) was then analyzed by paper chromatography.

[6]. The small amount of radioactivity that eluted around 55–65 min was due to a trace of 7-MeG and possibly some 3-MeA remaining as precipitable material after the incubation, since a large peak was seen in this position when total alkylated DNA was used as a substrate. Also in these experiments a narrow peak was found to elute at 84 min.

Molecular Weight of the Acceptor Protein

To determine the molecular weight of the human acceptor protein, extracts from HeLa cells and human liver were incubated with substrate II to allow radioactive labeling of the acceptor protein by the ^{3}H-methyl-group from O^6-MeG. The reaction mixture was then applied to separate single-strand DNA cellulose columns to remove

TABLE I. Repair of Alkylated DNA*

Substrate	Extract	(μg)	7-MeG released (pmol)	3-MeA released (pmol)	O^6-MeG repaired (pmol)	O^6-MeG present in substrate (pmol)
Substrate I		0	<0.01	<0.01	<0.01	0.16
	HeLa	(27)	ND	0.03	0.02	0.16
	HeLa	(54)	ND	0.05	0.05	0.16
	HeLa	(270)	ND	0.06	0.15	0.16
	HeLa	(810)	0.10	0.19	0.16	0.16
	Rat liver	(1,000)	0.15	0.11	0.16	0.16
	Human liver 1.	(1,050)	<0.01	0.11	0.34	0.34
Substrate II		0	—	—	<0.01	0.21
	Rat liver	(1,000)	—	—	0.10	0.11
	Rat liver	(50)	—	—	0.01	0.21
	liver	(100)	—	—	0.027	0.21
	liver	(250)	—	—	0.055	0.21
	liver	(700)	—	—	0.10	0.21
	Human liver 1.	(24)	—	—	0.014	0.21
	liver	(49)	—	—	0.022	0.21
	liver 1.	(147)	—	—	0.057	0.21
	liver 1.	(343)	—	—	0.11	0.21
	Human liver 2.	(70)	—	—	0.14	0.21
	liver 2.	(140)	—	—	0.15	0.21
	liver 2.	(420)	—	—	0.16	0.21
	Human liver 3.	(130)	—	—	0.20	0.21
	liver 3.	(980)	—	—	0.21	0.21
	Human liver 4.	(178)	—	—	0.05	0.21
	liver 4.	(445)	—	—	0.10	0.21

*Incubation conditions and methods are described in Materials and Methods. All incubations were carried out for 60 min. HeLa and rat liver results are average of four experiments. Results with human liver are average of two experiments. ND, not determined.

unreacted radioactive DNA and also to obtain partial purification of the methylated acceptor protein. The radioactive protein was eluted with 0.50 M NaCl in the equilibration buffer and analyzed by SDS polyacrylamide gel electrophoresis. We found the molecular weights of the acceptor proteins in human liver and HeLa cells to be essentially identical, 23,000 ± 1,000 and 24,000 ± 1,000 respectively (Fig. 4).

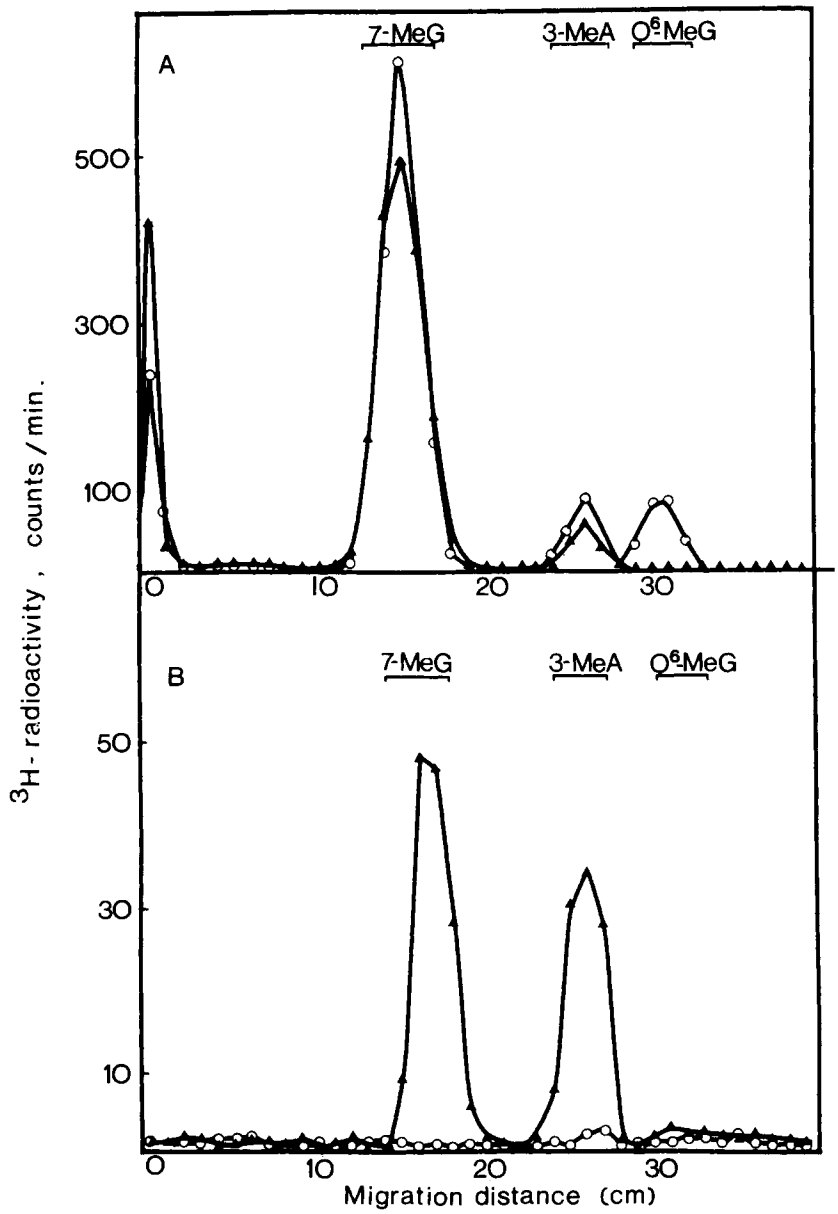

Fig. 2. Removal of alkylated purines from DNA by rat liver extracts. The experiment was identical to that presented in Figure 1 except that a rat liver extract (1,000 μg) was used.

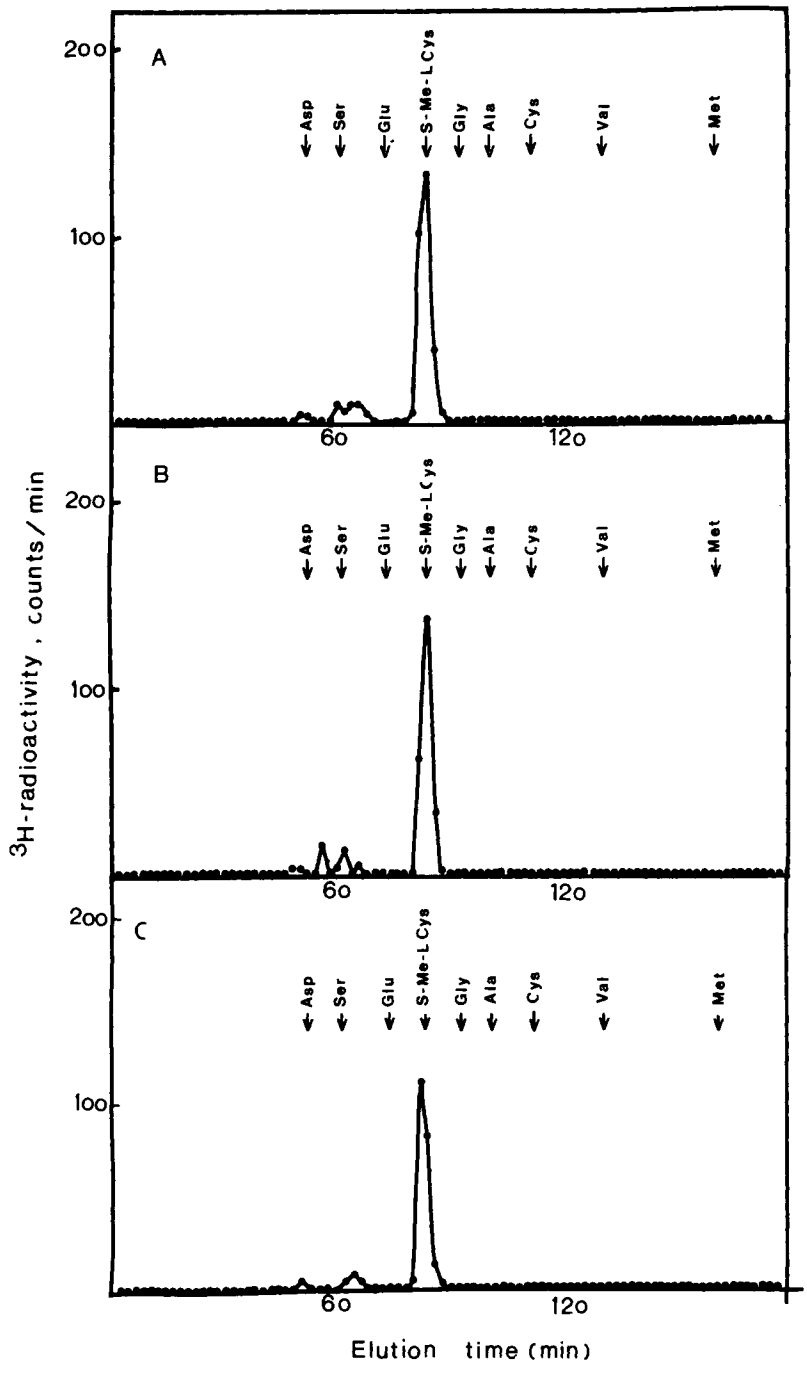

Fig. 3. Transfer of [^{3}H]methyl from the O^6-[^{3}H]methyl-guanine to a cysteine residue. Methylated, heat-treated DNA (substrate II) (90 μg) containing 65% of its [^{3}H]methyl in O^6-[^{3}H]methyl-guanine was incubated for 60 min at 37°C with HeLa cell extract (4.85 mg) (A): rat liver extract (9 mg) (B); or human liver extract (2.8 mg) (C). The ethanol-precipitated material was hydrolyzed in 6 M HCl and analyzed in an amino acid analyzer. The peak positions for marker amino acids were determined in separate runs. Arrows indicate the ninhydrine maxima of unlabeled amino acids. Fractions were collected every 2 min and radioactivity was measured by scintillation counting.

Effect of Pretreatment of HeLa Cells With MNNG on the O^6-Methyl-Guanine Repair Capacity of Cell Extracts

It has been shown both by in vitro and in vivo experiments that the system for removal of O^6-MeG from DNA in E coli is consumed in the reaction [20]. Furthermore, it was recently shown that pretreatment of Raji cells with MNNG (*N*-methyl-*N'*-nitro-*N*-nitroguanidine) strongly reduced the capacity for O^6-MeG removal during subsequent labeling with [^{3}H]MNNG [21]. We were able to demonstrate a strong reduction of the repair capacity in cell extracts prepared from HeLa cells pretreated with MNNG (Fig. 5). Cells in exponential growth were treated with MNNG (0.1 μg/ml) and cell extracts prepared at various times after the pretreatment. We also measured the activity of 3-MeA-DNA-glycosylase, 7-MeG-DNA-glycosylase, and uracil-DNA-glycosylase. The capacity for O^6-MeG removal decreased by about 85% within 3 hr after MNNG pretreatment and subsequently recovered partially. Since the activity of the three DNA glycosylases only dropped by 10–20% during the same interval, it is unlikely that the 85% decrease in O^6-MeG-DNA-cysteine-methyltransferase activity is due to a direct methylation of the protein by MNNG. Furthermore, treatment of HeLa cell extracts with MNNG (0.1 μg/ml) for 1 hr at 37°C

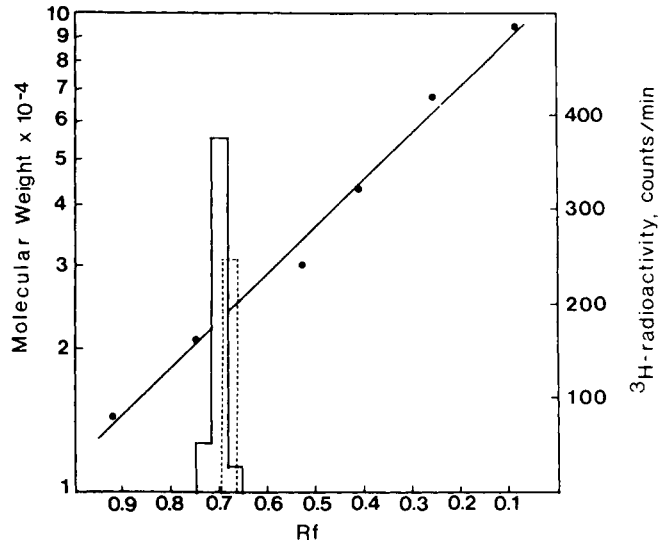

Fig. 4. Determination of the molecular weight of the human liver and HeLa cell methyl-acceptor proteins. Human liver extract (175 μg) and HeLa cell extract (540 μg) were incubated in buffer A with substrate II (2,000 dpm, 0.097 μCi alkylated DNA/mg DNA) for 60 min at 37°C in total volumes of 225 μl and 1100 μl, respectively. The incubations were terminated by adding ice-cold buffer A to a total volume of 2.5 ml. The diluted reaction mixtures were then applied to single-strand DNA cellulose columns (each 10 ml) equilibrated in buffer A. The columns were washed with about 4 vol of buffer A before the methylated acceptor proteins were eluted with 0.5 M NaCl in buffer A. The pooled fractions containing the methylated acceptor proteins were lyophilized, dissolved in 200 μl SDS buffer [18], and dialyzed against the same buffer. SDS polyacrylamide gel electrophoresis (10%) in tube gels was carried out with 3 mA per gel. The gel containing the size markers (●) was stained with Coomassie brilliant blue R-250. The gels containing the methylated acceptor proteins were cut in 4-mm pieces, transferred to scintillation vials, protein-extracted overnight with 0.5 ml Soluene (Packard), and radioactivity was counted in a scintillation counter. ▭, human liver acceptor protein; ⬚, HeLa cell acceptor protein.

inhibited the DNA repair enzymes less than 14%. Our results therefore are consistent with the idea that the system for repair of O^6-MeG in DNA is consumed during the repair process. In the untreated control culture, which was kept at the same cell density as the experimental culture, the DNA repair enzyme activities remained constant throughout the experimentation period (data not shown).

In E coli and rat liver, an increased capacity of O^6-MeG removal was observed after pretreatment with low concentrations of methylating agent [22–24]. We have not succeeded in demonstrating a similar adaption in HeLa cells after repeated additions of low doses. In these experiments HeLa cell cultures were treated every 24 hr with MNNG at concentrations of 0.01 μg/ml, 0.04 μg/ml, 0.1 μg/ml, or 1 μg/ml. Cells were collected at 6–12 hr intervals and cell extracts were prepared. We never observed an increase in the activity of O^6-MeG–cysteine-methyl-transferase activity. In fact, a 30–50% decrease in activity was seen during the "recovery period" after treatment with a single dose of MNNG (0.1 μg/ml). Cell growth was depressed by about 90% for the first 24 hr after a single dose of MNNG (0.1 μg/ml), but subsequently recovered almost completely. Repeated additions of MNNG of the same concentrations resulted in complete growth arrest and subsequent cell death.

DISCUSSION

In the present study we show that cell extracts from animal cells contain enzymatic activities which remove the three major alkylation products, (7-MeG, 3-

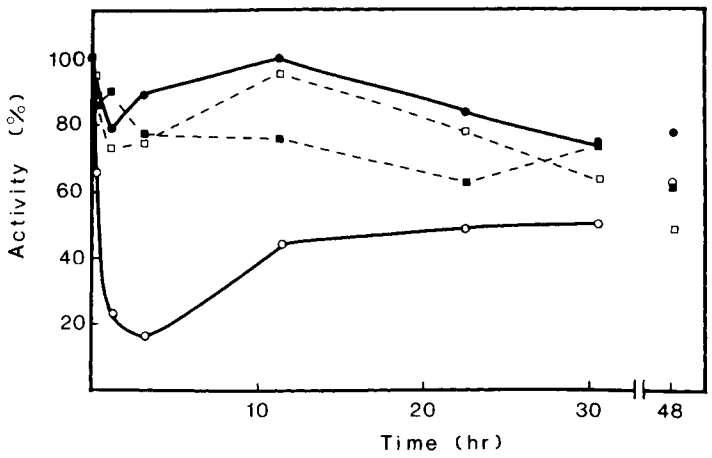

Fig. 5. Effect of pretreatment of HeLa cells with MNNG on DNA repair capacity of cell extracts. HeLa S_3 cells in the logarithmic growth phase (2×10^5 cells/ml) were treated with MNNG (0.1 μg/ml final concentration). Cells (about 2×10^8 cells) were harvested immediately before and at various times after addition of MNNG. Repair of O^6-MeG residues was assayed by the specific assay for methyl transfer (—○—). Release of radioactive 3-MeA (—●—) or, 7-MeG (--■--) from DNA was measured after separation by paper chromatography as described. Uracil-DNA-glycosylase activity (--□--) was measured as described earlier [13]. The activity is given as % of the activity in the extract from untreated cells and is corrected for the slight variation in protein content in the extracts. 100% activity was: 0.76 pmol/mg protein (O^6-MeG–cysteine-methyl-transferase); 0.72 nmol/mg protein/min (uracil-DNA-glycosylase); 1.1 pmol/mg protein/60 min (3-MeA–DNA-glycosylase); and 0.67 pmol/mg protein/60 min (7-MeG–DNA-glycosylase).

MeA, and O^6-MeG) resulting from treatment of DNA with methyl-nitroso-urea, although 7-MeG–DNA-glycosylase activity was not detected in human liver. 3-MeA–DNA-glycosylase was originally discovered in E coli [25] and has subsequently been reported in several animal cell lines [26,27] and rodent liver [28]. 7-MeG–DNA-glycosylase was recently reported in bacteria [29], human lymphoblasts [26], and rodent liver [28], but is apparently absent in some animal cell lines [27]. Possibly 7-MeG–DNA-glycosylase is not essential, since the *N*-7 modified base is also released relatively rapidly by nonenzymatic hydrolyses [5]. The significance of the methylation of *N*-3 of adenine and *N*-7 of guanine is not clear, although such lesions may be "cytotoxic" [30]. Baker et al. [31] have shown that different alkylation lesions are responsible for mutagenic and cytotoxic effects, but the lesions responsible for these effects were not identified. However, there is strong evidence suggesting that alkylation at the O^6-atom is a promutagenic and procarcinogenic event [2–4]. This alkylation alters the base pairing properties of guanine and, unless repaired before the next round of DNA replication, may lead to a GC → AT transition [32].

The mechanism for repair of O^6-alkyl-guanine in DNA is strikingly similar in E coli and human cells. The methyl group is transferred to a cysteine residue of an acceptor protein which is small: 16,000 daltons in E coli [6] and 23,000–24,000 in human cells (this work). Furthermore, the system appears to be consumed during the reaction [20,21, and this study]. In E coli, the system clearly is inducible [22,23] and this also seems to be the case in rat liver [24]. No evidence for a similar induction was observed for Raji cells [21] and HeLa cells [31 and this work], although evidence for an adaptive response after MNNG treatment was recently reported in HeLa S_3 cells [33]. The reason for this apparent contradiction cannot presently be explained, but may be due to biologic differences even between closely related cell lines grown in different laboratories. However, more data is needed to make any firm conclusion for animal cells. Other aspects of O^6-MeG repair remain unclear. It is not known whether the methyl group is transferred to the acceptor protein by an enzyme or whether the acceptor protein itself is a "suicide enzyme." Furthermore, it is not known whether the methylated acceptor protein is degraded or in some way demethylated and reutilized. It is also unclear whether more than one system for O^6-MeG removal exists. The properties of the most thoroughly studied DNA glycosylases are also fairly similar in bacteria and animal cells [5]. This suggests that the DNA repair enzymes may be highly conserved. Harris et al. [34] have shown that human lymphocytes vary considerably in their capacity to remove O^6-MeG from DNA. We observed a significant variation in the activity of O^6-MeG–cysteine-methyl-transferase activity in extracts of human liver. Whether this variation has a genetic basis or reflects variation in expression due to different environmental factors ("life-style") is not known, but should be worth a more thorough study.

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