Vol. 107, No. 2, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 30, 1982 Pages 546-553

NATURE OF THE CONSTITUTIVE AND INDUCED MAMMALIAN O^6-METHYLGUANINE DNA REPAIR ENZYME

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Received June 3, 1982

SUMMARY Removal of O^6-methylguanine from DNA had been shown to occur by transfer of the methyl group from the O^6-position of guanine to a cysteine residue in protein, forming the acid-stable product, S-methylcysteine. However, there is evidence that the reaction product is acid labile, methanol being produced. This apparent inconsistency was investigated. On incubation with cell and tissue extracts, O^6-methylguanine disappeared from DNA in proportion to the amount of S-methylcysteine formed, the reaction probably accounting for all the O^6-methylguanine removed. No evidence was obtained for acid-lability of the reaction product. The induced enzyme, like the constitutive enzyme, appears to be a transmethylase. Determination of S-methylcysteine provides a rapid simple method for estimation of repair activity in small samples of normal and pathological cells and tissues.

INTRODUCTION

Compelling evidence now supports the view that O^6-methylguanine is the major relevant lesion in DNA for mutagenesis and carcinogenesis induced by the potent SN1 class of alkylating agents (1). The rate of repair of this lesion apparently plays an important role in determining the extent of mutagenesis in cells (2) and of susceptibility to cancer induced by these agents (3). The nature of the repair process is therefore of much interest.

In E.coli (4) and in mouse liver (5) the methyl group is transferred from the O^6-position of guanine in DNA to a cysteine residue in protein, with the formation of S-methylcysteine. Evidence suggests that the reaction is stoichiometric, the 'enzyme' being consumed in the process (6,7,8). However, biphasic dose response effects and time curves suggest that more than one mechanism may be involved in the removal of O^6-methylguanine (6,8), possibly a rapid transmethylase reaction being followed by a slower glycosylase or demethylase. The biphasic effects apparently are not due to preferential

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repair of certain regions of chromatin (9). Another complication is the evidence that the product of the reaction is acid-labile (10,11,12), a volatile product, possibly methanol, being formed. This is inconsistent with the view that the product is S-methylcysteine (13).

Experiments were carried out in which the amount of O^6-methylguanine removed from DNA by extracts of rat liver and of Raji cells *in vitro* was compared with the amount of S-methylcysteine formed. The nature of the increased ability of liver of diethylnitrosamine-treated rats to remove O^6-methylguanine from DNA *in vivo* (14) was investigated by study of enzyme activity in liver extracts.

METHODS

Extraction of the repair enzyme. In previous studies, the O^6-methylguanine DNA repair enzyme was extracted from animal tissues by a method involving sonication of a homogenate (10). Enzyme extracts prepared by this method contain DNA, and this could affect the reactions being studied. Also, sonication could be a human hazard when applied to pathological human material. Detergents are known to contain peroxides which inactivate sulphydryl-dependent enzymes (15), and in fact extraction of rat liver with Nonidet gave low yields of the repair enzyme. Salt extraction was found to be a reliable reproducible technique. The method employed was similar to that used for extraction of other DNA repair enzymes from animal tissues, i.e. DNA polymerase (16) and 3-methyladenine glycosylase (17). Briefly, for rat liver, extraction with tris buffer 50 mM, pH 7.8, containing 0.1 mM EDTA and 1.0 mM dithiothreitol (3 ml/g liver) was followed by successive extractions with phosphate buffer 200 mM, pH 7.8. 80% ammonium sulphate precipitates of these extracts were redissolved in the tris buffer, and dialysed against tris buffer overnight. Raji cells were homogenised in the 200 mM phosphate/EDTA/DTT buffer containing tris buffer 50 mM, pH 7.8, (approximately 1 ml/50 x 10^6 cells), and dialysed against tris/EDTA/DTT buffer overnight. The tris and phosphate extracts, and the extracted pellets, were analysed for protein (18) and for DNA (19,20).

Assay of O^6-methylguanine DNA repair enzyme. DNA for use as substrate was methylated with [^{3}H-methyl]nitroso-methylurea, purchased from New England Nuclear, 1.6 Ci/mmol, essentially by the method of Pegg (10). The incubation medium contained methylated DNA, 1mg, containing approximately 2500 cpm in O^6-methylguanine, tris 50 mM, pH 7.9, dithiothreitol, 1.5 mM, EDTA, 0.05 mM, and enzyme extract, 0-0.9 ml. Enzyme preparations from Raji cells contained 2-3 mg protein/ml, tris extracts from rat liver contained 25-30 mg/ml, first phosphate extracts contained on an average 10 mg/ml. The mixtures were incubated at 37° for 2 hrs except where otherwise stated.

At the end of the incubation period the tubes were placed in ice and carrier DNA (6 mg) was added. DNA and protein were precipitated by bringing the mixture to 0.25 N with cold perchloric acid. DNA in the precipitate was depurinated by heating in 0.1 N HCl, 70°, 30 mins, (21) and the supernatant analysed for 7-methylguanine and O^6-methylguanine by analysis on Dowex-50, NH_4, 25 x 1 cms column, eluting with 0.3 M ammonium formate, pH 6.65, collecting 2.2 ml fractions. The protein precipitate was hydrolysed in 6 N HCl (13), and the hydrolysate analysed for S-methylcysteine by chromatography on Dowex-50, H^+, 25 x 1 cms column eluting with 1 N HCl, collecting

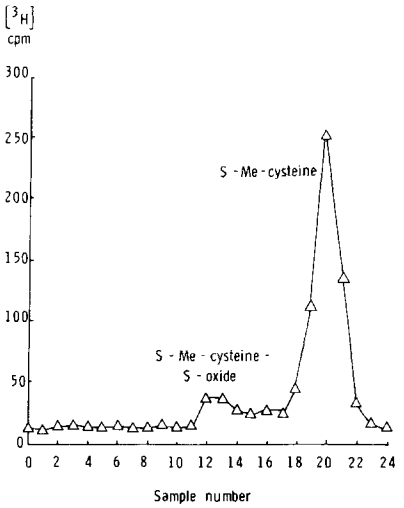

Fig. 1 Analysis on Dowex-50, H^+, of a 6 N HCl hydrolysate of the product of the reaction formed on incubation of an extract of rat liver for 2 hrs with DNA methylated with [^{3}H]nitrosomethylurea. Marker S-methylcysteine eluted in fractions no. 20-21.

4.2 ml fractions. Effluents from both types of column were assayed for tritium by liquid scintillation chromatography. Removal of O^6-methylguanine from DNA and formation of S-methylcysteine could thus be followed in the same experiment.

Tritiated material from the Dowex-50, H^+ column was further characterised by chromatography on AG-50, eluting with citrate buffer pH 3.2 (22), and by decending paper chromatography, using butanol:ethanol:water, 2:2:1, in each case the amino acids being located with Ninhydrin. Paper chromatograms were cut into small pieces, shaken in scintillation vials with 2 ml 0.1 N HCl, and radioactivity determined. S-methylcysteine-sulphoxide was prepared by treating the parent compound with hydrogen peroxide (23).

RESULTS AND DISCUSSION

Analysis of the acid hydrolysate of the reaction product showed that radioactivity co-elutes with reference S-methylcysteine (Fig. 1). The identity of the product was confirmed by re-analysis of the pooled fractions containing radioactivity by paper chromatography, by chromatography on AG/citrate columns, and by the shift in position of the tritium peak to the S-methylcysteine-sulphoxide location on Dowex-50, H^+, after treatment with hydrogen peroxide. The sulphoxide elutes faster than S-methylcysteine from Dowex-50, H^+ (Fig. 1).

Time curves showed that the formation of S-methylcysteine plateaued, the time necessary for reaching the maximum value depending on its

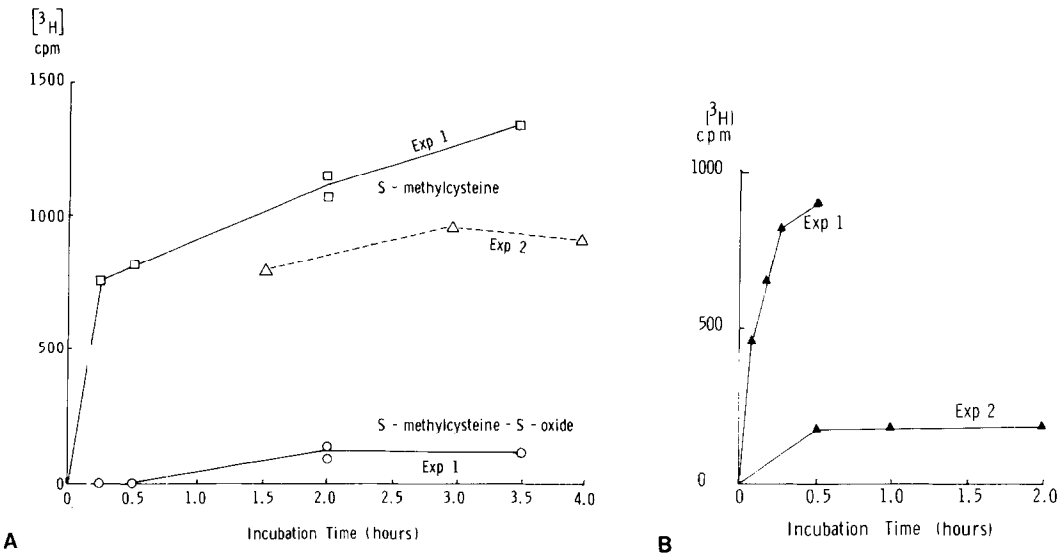

Fig. 2 Time curve for formation of S-methylcysteine and S-methylcysteine-sulphoxide formed on incubation of different preparations of rat liver (A) and of Raji cells (B) with methylated DNA.

magnitude (Fig. 2). At longer incubation times, a small amount of S-methylcysteine-sulphoxide was formed (Fig. 1 and Fig. 2). This compound is known to form spontaneously from S-methylcysteine in air (24). The enzyme concentration curve (Fig. 3) showed that the amount of S-methylcysteine formed to be proportional to the amount of enzyme added.

The repair enzyme was found in the tris and phosphate extracts of rat liver, when assayed by measuring either decrease in the O^6/N7-methylguanine ratio or by measuring the formation of S-methylcysteine (Table 1). Little enzyme was found on re-extraction of the pellet with phosphate buffer. Similarly, most activity in Raji cells was extracted on the first treatment with the tris/phosphate buffer (Table 1). The appearance of S-methylcysteine mirrored the disappearance of O^6-methylguanine when comparing different enzyme extracts, incubation times, or enzyme concentrations (Fig. 4).

When rats had been fed a diet containing diethylnitrosamine, 90 ppm, for 6 weeks, incubation of the same volume of enzyme extracts (0.9 ml) prepared in the same way as for normal animals removed all the O^6-methylguanine from the DNA substrate during the incubation period. The S-methylcysteine formed on incubation with a smaller volume (0.2 ml) of enzyme,

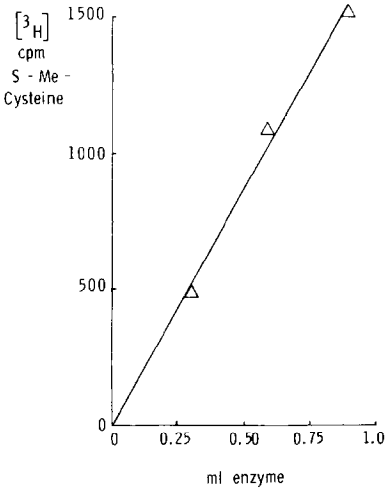

Fig. 3 Amount of S-methylcysteine formed in relation to the volume of extract of rat liver used in the incubation medium, calculated as total cpm in S-methylcysteine.

TABLE 1. REMOVAL OF O^6-METHYLGUANINE FROM DNA AND FORMATION OF S-METHYLCYSTEINE ON INCUBATION WITH ENZYME EXTRACTS

Sample	cpm S-Me-cys[b]	O^6-MG/N7-MG[a]	pmol S-Me-cys[d] per unit of tissue	pmol S-Me-cys[c] per g liver
Albumin	-	0.124	-	-
Normal rat liver				
Tris extr.	1295	0.042	1.76	4.89
1st PO_4	1524	0.012	0.97	2.69
2nd PO_4	375	0.088	0.20	0.55
			2.93	8.13
DEN-fed rat liver				
Tris extr.	3789		4.11	14.82
1st PO_4	4761		2.19	7.91
2nd PO_4	1908		0.58	2.10
3rd PO_4	1237		0.36	1.28
4th PO_4	517		0.10	0.35
			7.34	26.46
Raji cells (43 x 10^6)				
1st extr.	1364	0.044	2.80	
2nd extr.	129		0.27	
			3.07	

a O^6-MG/N7-MG, ratio of O^6-methylguanine/N7-methylguanine in DNA remaining at end of incubation period.

b cpm S-Me-cys are total cpm in the combined peaks of S-methylcysteine and S-methylcysteine-sulphoxide where present. With normal liver, 0.9 ml enzyme extract were used. With DEN-fed rat liver, 0.2 ml extract were incubated, and the cpm calculated to 0.9 ml. This calculation cannot be carried out for the O^6-MG/N7-MG ratios.

c pmol S-Me-cys formed per g liver was calculated from the specific radioactivity of the methyl group, the weight of liver extracted, the volume of the extracts, and the volumes used for assay.

d pmol S-Me-cys formed per unit of tissue gives the amount formed per μg DNA in the tissue sample used for extraction of the enzyme.

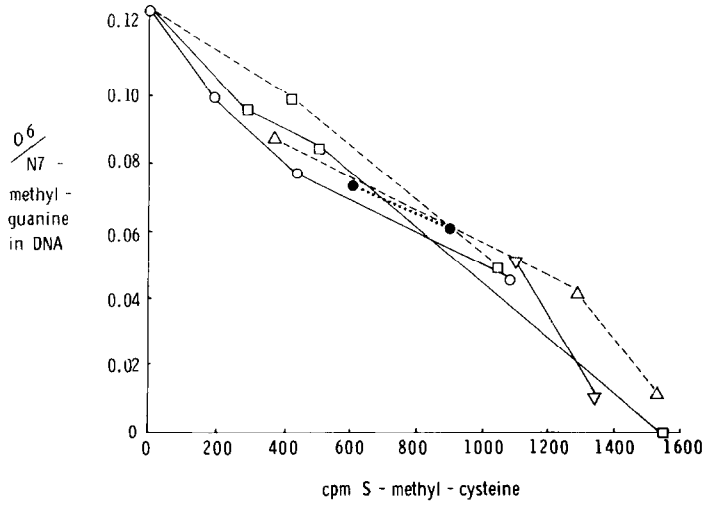

Fig. 4 Correlation between the amount of S-methylcysteine formed and O^6-methylguanine removed from DNA, expressed as the fall in the O^6-methylguanine to 7-methylguanine ratio, in experiments using different volumes of extracts from normal or DEN-fed rats. The graph is intended to show the correlation between S-methylcysteine formed and fall in ratio, rather than the results of any particular experiment. The ratio 0.124 was obtained when bovine serum albumin was used in place of an enzyme extract.

calculated to 0.9 ml for purposes of comparison, are shown in Table 1. The enzyme activity of each extract was higher than from normal animals, and repair enzyme occurred not only in the tris and first phosphate extracts but in the 3rd and 4th extracts also. The increase in the amount of O^6-methylguanine removed by the extracts from DEN-fed rats was thus paralleled by an increase in the amount of S-methylcysteine formed. The induction may be similar to the adaptive response to low levels of N-methyl-N'nitro-N-nitrosoguanidine which occurs in E.coli (25).

For comparison of enzyme activity in different extracts, the cpm S-methylcysteine as measured were converted into pmol S-methylcysteine formed per g liver, using the specific radioactivity of the nitrosomethylurea used for methylation of the DNA substrate, the volumes of tissue extracts in relation to the volumes used for assay, and the weight of tissue extracted. The result of approximately 8 pmol per g normal liver agrees reasonably well with the value of 10 pmol found by Pegg (10). DEN treatment increased repair enzyme activity approximately three times (Table 1).

To compare liver with other tissues, a more meaningful basis for comparison than tissue weight is μg DNA in the tissue sample from which the enzyme has been extracted. In this way, large variations in the amount of protein per cell between different cell types does not affect the result, which reflects instead the enzyme activity per cell nucleus. The DNA content of liver nuclei of adult rats of the colony used in these experiments (26) was similar to that of the Raji cells, approximately 9 μg/nucleus. On this basis, it is seen that the activities of normal rat liver and of Raji cells are very similar (Table 1).

The average recovery of the methyl group removed from O^6-methylguanine as S-methylcysteine in the case of the Raji cells was 98%. The value for rat liver was 80%. This lower value was probably due to the fact that a small amount of demethylation of DNA probably occurs during the perchloric acid treatment. This would result in disappearance of O^6-methylguanine from DNA without concurrent formation of S-methylcysteine, and while it would appear to reduce the extent of conversion of O^6-methylguanine to S-methylcysteine, it could be too small to affect appreciably the O^6/N7-methylguanine ratio. Artefactual demethylation of DNA by perchloric acid or by trichloracetic acid might also account for previous reports of the formation of a volatile reaction product (10,11,12). For technical reasons, exposure to perchloric acid and possible losses during experimental procedures would have been less with Raji cells than with rat liver experiments.

The data thus supports the view that removal of O^6-methylguanine from DNA occurs by transmethylation, the methyl group giving rise to acid-stable S-methylcysteine residues in protein. The induced enzyme, like the constitutive enzyme, appears to be a transmethylase. Acid hydrolysis of the reaction product followed by determination of the amount of S-methylcysteine formed provides a rapid simple method for assay of the O^6-methylguanine DNA transmethylase in small samples of normal and pathological cells and tissues.

ACKNOWLEDGEMENTS The authors would like to thank Dr Gilmour Harris for the supply of Raji cells and Dr P D Lawley for discussions.

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