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ENZYMATIC REMOVAL OF O^6-METHYLGUANINE FROM DNA BY MAMMALIAN CELL EXTRACTS

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SUMMARY Extracts from various rat tissues were incubated with [^{3}H]methylated DNA or chromatin in order to compare their abilities to catalyze the removal of labeled O^6-methylguanine from acid precipitable DNA. Liver extracts had the greatest activity. Kidney extracts had about 35% of the activity in liver and extracts from lung, colon, small intestine and brain were much less active. The enzyme responsible for this reaction does not appear to be an N-glycosidase because no labeled O^6-methylguanine could be detected in the supernatant fraction even though more than 50% of this base was lost from the DNA. The released radioactivity was present as methanol which is consistent with the possibility that the reaction may involve a demethylase action on either the DNA substrate or an oligonucleotide derived from it.

INTRODUCTION

Dimethylnitrosamine and a number of related carcinogens are thought to act by means of their conversion to reactive alkylating agents within the cell. This alkylating species reacts with cellular macromolecules at a number of sites (1,2). It has been suggested that the formation of O^6-alkylguanine in DNA may be a critical step in the induction of tumors by these agents and that the ability to remove this product from DNA may protect against carcinogenesis (2-5). Removal of O^6-alkylguanine from DNA has been observed *in vivo* following administration of labeled carcinogens (3-5). However, the enzymatic mechanism by which this removal occurs has not been characterized. Recently we observed that rat liver extracts catalyzed the loss of O^6-methylguanine when incubated with DNA isolated from the livers of rats treated with dimethylnitrosamine (6). In the present work the assay of this activity with different DNA substrates, the distribution of activity in various rat tissues and the nature of the material released from the methylated DNA has been investigated.

MATERIALS AND METHODS

The methylated DNA substrate used for most of the enzyme assays was prepared by incubation of calf thymus DNA (5 mg/ml) with 0.05 mM N-[^{3}H]methyl-N-nitro-

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sourea (1.055 Ci/mmole) in 0.04 M sodium phosphate buffer of pH 8.0 for 30 min at 37°. The DNA was then precipitated with 2 volumes of 2-ethoxyethanol, washed 6 times with 95% ethanol, once with ether and dried in vacuo for 2 days at 4°. DNA prepared in this way contained 3-8 pmoles of O⁶-methylguanine per µmole of the parent base depending on the preparation of labeled nitrosamide used. Labeled methylated DNA was also obtained by isolation of rat liver DNA (7) from livers of rats injected with 50 µg/kg body wt. of [^{3}H]dimethylnitrosamine (3.5 Ci/mmole) 20 min before death. This DNA contained 1-2 pmoles of O⁶methylguanine per µmole of the parent base. Chromatin was isolated from rat liver (8) and methylated by reaction with N-[^{3}H]methyl-N-nitrosourea in vitro as described above. Labeled chromatin was also obtained from livers of rats treated with 50 µg/kg body wt. of [^{3}H]dimethylnitrosamine 20 min before death. Tissue extracts containing enzymatic activity were prepared by homogenization in three volumes of 50 mM tris-HCl, pH 7.8, 1 mM-dithiothreitol, 0.1 mM disodium EDTA (Buffer A). Homogenization and all subsequent steps were performed at 4°. After centrifugation at 2,000 g for 5 min the supernatant was saved and the precipitate resuspended in buffer A and sonicated (6). Since activity was found both in the initial supernatant and the sonicated extract these were combined and the mixture centrifuged at 15,000 g for 30 min. The supernatant from this centrifugation was then precipitated by addition of $(NH_4)_2SO_4$ until 80% saturated. The resulting precipitate was collected by centrifugation, dissolved in buffer A and dialyzed overnight.

Activity was assayed by incubation of the labeled DNA or chromatin substrate with the extracts in the presence of 3 mM $MgCl_2$, 33 mM tris-HCl, pH 8 and 1 mM dithiothreitol. The total incubation volume was 6 ml. After incubation at 37° for one hour the reaction was stopped by the addition of ice-cold 1N perchloric acid to yield a final perchloric acid concentration of 0.25 N. The precipitated DNA and protein was collected by centrifugation and washed once with 0.25 N perchloric acid. The combined supernatants were neutralized by addition of 1 N KOH. The resulting precipitate was removed and the neutralized supernatant examined by column chromatography on Sephadex G10 (7). The content of methylated purines in the precipitated DNA was analyzed following release by dilute acid hydrolysis and separation by column chromatography on Sephadex G10 (7). The presence of radioactive methanol was determined by addition of 5 ml of carrier unlabeled methanol followed by distillation under reduced pressure. After collection of the first distillate at 50°, a further 5 ml of methanol was added to the distillation flask and a second distillate collected. This procedure was repeated one more time and an aliquot of the combined distillate counted in a toluene:Triton X100 (1:1) scintillation cocktail (9). The identity of the radioactivity as methanol was then confirmed by distillation of the methanol toluene binary azeotrope at 63-65° as described by O'Connor et al. (9). Recovery of authentic [^{14}C]methanol added to duplicate samples and subjected to this distillation procedure was between 75 and 80%.

RESULTS

Rat liver extracts were able to catalyze the loss of O⁶-methylguanine from acid precipitable DNA when incubated with methylated DNA or chromatin (Table 1). Incubation did not cause significant loss of 7-methylguanine or of guanine from the recovered DNA indicating that the loss of O⁶-methylguanine was specific and not due to non-specific nuclease activity. The liver extracts were able to bring about the loss of O⁶-methylguanine from purified rat liver

TABLE 1

LOSS OF O^6-METHYLGUANINE FROM VARIOUS METHYLATED DNA OR CHROMATIN PREPARATIONS ON INCUBATION WITH RAT LIVER EXTRACTS

SUBSTRATE	ENZYME	BASES PRESENT IN ACID-PRECIPITABLE DNA		
		GUANINE (μmoles)	7-METHYLGUANINE (pmoles)	O^6-METHYLGUANINE (pmoles)
DMN treated rat liver DNA	-	2.65	48	2.60
	+	2.79	48	1.51
NMU treated calf thymus DNA	-	2.42	124	12.7
	+	2.35	123	9.1
DMN treated chromatin	-	1.70	68	3.5
	+	1.61	63	2.3
NMU treated chromatin	-	1.58	35	3.3
	+	1.59	32	2.1

DMN, dimethylnitrosamine. NMU, N-methyl-N-nitrosourea. The DNA substrates were incubated for 60 min at 37° in the presence of 15 mg of liver protein or with no added protein as shown. Other experimental details are listed under methods.

DNA or rat liver chromatin which had been alkylated by reaction with [^{3}H]dimethylnitrosamine *in vivo*. However, chromatin or purified calf thymus DNA which had been methylated by reaction with labeled N-methyl-N-nitrosourea *in vitro* was also a substrate for the reaction. The most convenient substrate was calf thymus DNA alkylated by reaction with N-methyl-N-nitrosourea *in vitro* because this was the easiest to prepare and the extracts showed a maximum activity (measured as pmoles of O^6-methylguanine removed during a 1 hour incubation) with this substrate. Prolonged incubation of the substrate with high concentrations of liver protein led to loss of up to 80% of the O^6-methylguanine present.

When rat tissue extracts were incubated with this methylated DNA substrate the amount of O^6methylguanine lost from the DNA in a 60 minute in-

TABLE 2

COMPARISON OF ACTIVITIES OF RAT TISSUE EXTRACTS

IN CATALYZING REMOVAL OF O^6-METHYLGUANINE FROM DNA

TISSUE	ACTIVITY IN REMOVING O^6-METHYLGUANINE FROM DNA	
	(pmoles removed/ hr/gram wet wt.)	(fmoles removed/ hr/mg protein)
Liver	7.8	138
Kidney	2.9	61
Lung	0.4	13
Colon	0.6	19
Small Intestine	0.7	23
Brain	0.3	9

Activity was assayed using as substrate calf thymus DNA alkylated by reaction with labeled N-methyl-N-nitrosourea to an extent equal to 5.3 pmoles of O^6-methylguanine per μmole of guanine. Other details are given in the text and under methods.

cubation was proportional to the amount of protein added provided that not more than 60% of the O^6-methylguanine was removed. Comparisons of the activity extractable from various tissues were made under these conditions and the results are shown in Table 2. Rat liver had the greatest ability to catalyze this reaction; kidney extracts had about 35% of the activity in liver and extracts from various parts of the gastrointestinal tract, lung and brain were much less active ranging from 10% to less than 4% of the activity in liver. These results were expressed as activity from extracts equivalent to one gram wet weight of tissue but as shown in Table 2 even when expressed per mg of protein in the extracts used for assay liver was still by far the most active tissue and liver and kidney were much more active than the other tissues.

Radioactive O^6-methylguanine could not be detected in the supernatant fraction after incubation of [^{3}H]methylated DNA with rat liver protein such that more than half of the O^6-methylguanine was lost from the acid-pre-

TABLE 3

EFFECT OF UNLABELED O^6-METHYLGUANINE ON ENZYMATIC LOSS OF O^6-[^{3}H]-METHYLGUANINE FROM DNA

ADDITION	O^6-METHYLGUANINE PRESENT IN DNA (pmoles)	O^6-METHYLGUANINE PRESENT IN SUPERNATANT (pmoles)	[^{3}H]-METHANOL (pmoles)
None	8.3	< 0.1	0.2
Liver protein	2.7	< 0.1	4.4
Liver enzyme + 3 mM O^6-Methyl-guanine	3.5	< 0.1	4.3
Liver enzyme but not incubated	8.2	< 0.1	0.1

[^{3}H]-Methylated DNA was incubated with liver extracts (20 mg protein) for 2 hours and the amounts of labeled O^6-methylguanine remaining in acid-precipitable DNA and present in the perchloric acid supernatant and labeled methanol present in the supernatant were determined.

cipitable DNA (Table 3). This finding suggested that the removal was not catalyzed by the action of an N-glycosidase which would liberate the free base from the DNA leaving an apurinic site. However, it was possible that the crude enzyme preparation used contained an enzyme capable of further degradation of any O^6-methylguanine which was released. Therefore, a relatively high concentration (3 mM) of unlabeled O^6-methylguanine was added to the assay medium. This inhibited the reaction slightly but 4.8 pmoles of labeled O^6-methyl guanine were removed from the DNA by the liver enzyme and no radioactive O^6-methylguanine was found in the supernatant fraction even though more than 90% of the unlabeled O^6-methylguanine was recovered (based on the absorbance at 260 nm). Since any released labeled O^6-methylguanine should have been trapped in this way it appears that free O^6-methylguanine is not a product of the enzymatic reaction.

Another possible mechanism for the loss of O^6-methylguanine from DNA would involve the excision from the DNA of a small oligonucleotide containing

the methylated base. If this were the case treatment of the supernatant with 0.1N HCl at 70° for 30 minutes should have released free O^6-methylguanine but this was not found. Instead [^{3}H]methanol was present in the supernatant fraction after incubation of the [^{3}H]methylated DNA with the liver extracts.

The recovery of labeled methanol was about 75% and when this was taken into account there was a fair correlation between the amount of methanol released and the loss of O^6-methylguanine from DNA. However, it should be emphasized that the methylated DNA used as substrate in these experiments contains a number of methylated components and that it is possible that the methanol was derived from one of these products. It could not have arisen from 7-methylguanine which was not released by incubation with the enzyme or derived from 3-methyl- and 7-methyladenine which were removed from the DNA by the acid precipitation and recovered unchanged in the supernatant fraction. However, the content of methylphosphate triesters, O^2-methyl- and O^4-methylthymine was not determined in the present experiments and some of the released methanol could have been derived from these sources.

DISCUSSION

These results suggest that certain mammalian tissues contain an enzyme system capable of removing O^6-methylguanine from DNA which does not involve the action of an N-glycosidase similar to that reported to occur in *E. coli* (10). The findings raise the possibility that a demethylation of the O^6-methylguanine in DNA can take place. Such a reaction would be undetectable by conventional methods of assessing DNA repair synthesis such as measurement of single strand breaks or incorporation of radioactive nucleotides into repair patches (11). It is also possible that demethylation occurred after excision from the DNA of an oligonucleotide containing O^6-methylguanine. Demethylation could yield the parent deoxyguanosine or may be a more complex reaction resulting in other changes in the base. The observed activity could be due to a fortuitous reaction of an enzyme having some other cellular function not involved in DNA repair. Adenosine deaminase is known to be able to demethylate O^6-methylguanosine

(12) and will act at a reduced rate on adenosine in small oligonucleotides (13). The tissue distribution of enzyme activity which parallels relative rates of loss of O^6-methylguanine from DNA in these tissues in vivo (3-7) suggests that adenosine deaminase alone cannot account for the removal. Although the measured activity even in liver was quite low it is consistent with the rate of removal of O^6-methylguanine from DNA in vivo. A substantial fraction of the O^6-methylguanine produced by low doses of dimethylnitrosamine was removed in a few hours (6,7) but the maximal rate of removal calculated from such experiments is only about 10 pmoles/hour/gram of tissue. This is quite close to the observed activity with purified DNA as a substrate. The results of Table 1 suggest that the reaction with chromatin may be somewhat slower than with purified DNA as a substrate but the conditions used for the in vitro reaction may not be optimal. Activity promoting O^6-methylguanine removal from liver DNA may be inducible on prolonged exposure to dimethylnitrosamine (unpublished observation) although after large doses of the carcinogen removal is inhibited (5,14,15). The relationship between the present activity and the inducible repair system for the mutagenic damage produced by the methylating agent N-methyl-N'-nitro-N-nitrosoguanidine (16) is, therefore of particular interest.

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