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Formation and Loss of Alkylated Purines from DNA of Hamster Liver after Administration of Dimethylnitrosamine[1]

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ABSTRACT

The methylation of hamster liver DNA was studied as a function of dose of dimethylnitrosamine. 7-Methylguanine levels were proportional to a dose over the range of 10 μg/kg to 25 mg/kg when measured 5 to 24 hr after treatment. This product was lost from the DNA at a rate greater than expected from spontaneous depurination at neutral pH, suggesting that enzyme-catalyzed excision takes place. O^6-Methylguanine levels were not proportional to doses over this range but were much lower than expected (based on 7-methylguanine levels) when measured 5 to 24 hr after doses of dimethylnitrosamine below 0.5 mg/kg. It is suggested that this result may be due to the presence of an enzyme capable of removing O^6-methylguanine from DNA efficiently, provided the level of methylation was low. The presence of such an enzyme in hamster liver extracts was demonstrated by incubation with methylated DNA. The extracts brought about a significant decrease in the content of O^6-methylguanine present in acid-precipitable DNA. However, when doses of dimethylnitrosamine above 0.5 mg/kg were used, removal of O^6-methylguanine occurred much more slowly, and the capacity of hamster liver to carry out removal of O^6-methylguanine from DNA *in vivo* was considerably lower than that of rat liver. The possible relevance of these findings to the relative susceptibility of these species to liver cancer induction by single doses of dimethylnitrosamine is discussed.

INTRODUCTION

N-Nitroso compounds are potent carcinogens in many species including the hamster (4, 8). The chemically simplest representatives of these carcinogens, dimethylnitrosamine and *N*-methyl-*N*-nitrosourea, lead to methylation of cellular components, and there is evidence that the methylation of DNA may be responsible for their carcinogenic action (6, 8, 9, 16). Studies on rats have implicated the formation and persistence of O^6-alkylguanine in DNA as a critical factor in tumor production by these agents and their ethyl analogs (1, 3, 11, 16–19). It appears that rat liver and kidney contain an enzyme system capable of removing this product from DNA which may protect against carcinogenesis (18, 19). However, the Syrian golden hamster liver, which is more sensitive to the carcinogenic potential of single doses of dimethylnitrosamine than is the rat liver (4, 20), appeared to lack the potential to remove this product from its DNA after high doses (25 mg/kg) of dimethylnitrosamine (12). In the present study, the formation and persistence of alkylation products in DNA of hamster liver has been studied after various doses of dimethylnitrosamine. It was found that removal of O^6-methylguanine did take place after small doses of dimethylnitrosamine which produced lower extents of alkylation but that the enzymic system responsible for this reaction was much less effective after higher doses. Evidence supporting an earlier suggestion that 7-methylguanine is metabolically removed from hamster liver DNA (12) was also obtained in the present study. The possible significance of these repair mechanisms in carcinogenesis is discussed.

MATERIALS AND METHODS

Materials. [^{3}H]Dimethylnitrosamine (2.9 Ci/mmol) and [^{14}C]dimethylnitrosamine (3.2 to 26 mCi/mmol) were purchased from New England Nuclear, Boston, Mass., and diluted with unlabeled redistilled dimethylnitrosamine (Aldrich Chemical Co., Milwaukee, Wis., or Merck-Schuchardt, Munich, Federal Republic of Germany) to the required specific activity before use. Other biochemicals were products of the Sigma Chemical Co., St. Louis, Mo. except for O^6-methylguanine, which was synthesized by a published procedure (17).

Animals. Male Syrian golden hamsters were purchased from the Charles River Breeding Laboratories, Wilmington, Mass., or from Animalerie des Essertines, Rochetaillée, France. They were maintained under controlled diurnal lighting conditions (12 hr light, 12 hr dark commencing at 7 a.m.) and were allowed free access to food pellets and water. Dimethylnitrosamine was administered by i.p. injection of a solution in 0.9% (w/v) NaCl solution at about 10 a.m. The specific activity of the labeled dimethylnitrosamine administered was varied to ensure that sufficient radioactivity was present in the O^6-methylguanine fractions. (The specific activity used for doses of 10 μg/kg was 2900 mCi/mmol, and for 25 mg/kg it was 3.2 mCi/mmol.) For doses of 1 mg/kg or below, [^{3}H]dimethylnitrosamine was used; for higher doses, [^{14}C]dimethylnitrosamine was used. Previous experiments in rats have shown that similar extents of alkylation of DNA were obtained in direct comparisons of the ^{3}H- and ^{14}C-labeled dimethylnitrosamine preparations (19). Organs for the isolation of DNA were removed rapidly, frozen in liquid N_2, and stored at −30°.

Preparation and Assay of Enzymic Activity Removing O^6-Methylguanine from DNA. Hamster livers were homogenized in 2 volumes of 50 mM Tris-HCl-1 mM dithiothreitol-

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0.1 mM disodium EDTA, pH 7.5, and an ammonium sulfate-precipitated protein fraction was prepared as described by Pegg and Hui (19). These extracts were incubated with ^{3}H-methylated DNA [prepared from the livers of rats that had been given injections of [^{3}H]dimethylnitrosamine (0.01 mg/kg) approximately 10 min before death] in a medium containing 3.3 mM $MgCl_2$, 1.5 mM dithiothreitol, 75 mM Tris-HCl (pH 8), about 4 mg of DNA, and 15 mg of protein. After 60 min incubation at 37°, the reaction was halted by the addition of an equal volume of 0.5 N perchloric acid. The precipitate was collected by centrifugation, it was washed in 0.25 N perchloric acid, and the methylated purine content was determined as described below.

Isolation and Analysis of DNA. DNA was isolated by extraction with phenol as previously described (11, 17) and stored at −30°. The DNA was hydrolyzed to yield free purine bases by heating at 70° for 30 min in 0.1 N HCl. After neutralization and addition of unlabeled markers of methylated purines, the mixture was chromatographed on Sephadex G-10. The radioactivity present in the fractions corresponding to methylated bases was determined, and the amounts of adenine and guanine present in the DNA hydrolysate was determined from the absorbance of the fractions containing these bases. From these measurements, the amount of methylated bases present was calculated as µmol/mol of the parent base. Fuller details of this analytical procedure are given in recent publications (11, 17, 19).

RESULTS

The amount of 7-methylguanine and O^6-methylguanine present in hamster liver DNA 5 to 7 and 24 hr after injections of dimethylnitrosamine, varying between 0.01 and 25 mg/kg, are shown in Table 1. Within the limits of experimental error, the 7-methylguanine values were directly proportional to the dosage of dimethylnitrosamine over this range. There was a substantial decrease in 7-methylguanine levels between 5 and 24 hr. The amount of this decrease (about 30%) was not greatly affected by the dose of dimethylnitrosamine, and thence the starting level of 7-methylguanine. These results are in good agreement with the observations of Margison *et al.* (12), in which the levels of 7-methylguanine at various times after administration of dimethylnitrosamine (25 mg/kg) were measured. However, this dose causes extensive necrosis of the liver and the death of highly alkylated cells, which might contribute to the fall in 7-methylguanine levels. This is unlikely to be the case after the much lower doses of the carcinogen examined in the present experiments which, therefore, suggest that 7-methylguanine may be excised from hamster DNA. A time course of the persistence of 7-methylguanine in hamster liver DNA after dimethylnitrosamine (2.5 mg/kg) is shown in Chart 1. It can be seen that by 96 hr only 20% of the amount detected at 7 hr was present. This loss cannot be accounted for by necrosis and is substantially faster than expected from the rate of spontaneous hydrolysis of 7-methylguanine in DNA at neutral pH (2, 7, 10, 16).

O^6-Methylguanine levels in hamster liver DNA after dimethylnitrosamine (2.5 mg/kg) was administered are also shown in Chart 1. O^6-Methylguanine was lost from the DNA slightly more slowly that 7-methylguanine so that 27% of the amount present initially remained by 96 hr. However, as indicated in Table 1, the O^6-methylguanine levels present in DNA 5 to 24 hr after injection of dimethylnitrosamine were not directly proportional to the dose over the range tested but showed a striking change when the dose of dimethylnitrosamine was increased above 0.25 mg/kg. Five hr after a dose of 0.25 mg/kg, the O^6-methylguanine level in the DNA was 1.3 µmol/mol guanine, whereas, after doubling the dose to 0.5 mg/kg the O^6-methylguanine level was almost 10-fold greater at 12 µmol/mol guanine. This difference was also found when methylation was measured 24 hr after administration of the dimethylnitrosamine. It can also be seen from Table 1 that O^6-methylguanine levels in DNA

Table 1

Methylated guanine derivatives present in hamster liver DNA after injection of various doses of dimethylnitrosamine

At the times shown after i.p. injection of labeled dimethylnitrosamine, the hamsters were killed and the content of methylated guanine derivatives in the hepatic DNA was measured.

Dose of dimethylnitrosamine (mg/kg)	Time (hr)	Methylated guanine content of DNA: 7-Methylguanine (µmol/mol of guanine)	Methylated guanine content of DNA: O^6-Methylguanine (µmol/mol of guanine)
0.01	5	2	0.02
0.10	5	16	0.33
0.25	5	42	1.3
0.50	5	94	12
0.75	5	146	19
1.00	5	245	21
2.50	5	594	77
2.50	7	638	70
5.0	5	835	104
10.0	5	2130	258
20.0	5	3995	471
25.0	7	4210	546
0.01	24	1.8	0.01
0.10	24	10	0.18
0.25	24	26	0.7
0.50	24	58	10
0.75	24	83	19
1.0	24	141	19
2.5	24	366	60
5.0	24	632	114
10.0	24	1567	267
15.0	24	2315	493
20.0	24	2308	464
25.0	24	2902	620

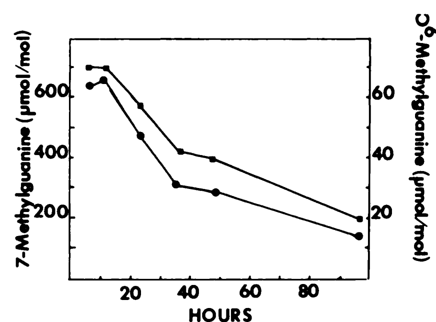

Chart 1. Time course of loss of 7-methylguanine (●) and of O^6-methyl guanine (■) from hamster liver DNA after injection of [^{14}C]dimethylnitrosamine (2.5 mg/kg; 29.6 mCi/mmol).

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declined to only a small extent (and in some cases not at all) over the period from 5 to 24 hr after administration of doses of greater than 0.5 mg/kg. However, at lower doses, about 50% of the O^6-methylguanine present at 5 hr was lost by 24 hr (Table 1).

These results might be explained by the presence of an enzymic excision mechanism which rapidly removes a significant proportion of the O^6-methylguanine from DNA after the DNA has been alkylated to a low extent but is either saturated or inhibited after higher doses of dimethylnitrosamine, leading to a greater extent of alkylation. Direct evidence for such an enzyme system is shown in Table 2. In this experiment, protein fractions isolated from hamster liver were incubated with alkylated DNA which was labeled at high specific activity in the added methyl groups. After a 60-min incubation at 37°, the DNA present was precipitated and analyzed to determine the content of methylated guanines remaining after exposure to the hamster liver enzymes. As shown in Table 2, the extract prepared from control hamster liver led to a significant decline in the content of O^6-methylguanine present in the acid-precipitable DNA, whereas little 7-methylguanine or guanine was lost. This result shows that an enzyme that is specific for the removal of O^6-methylguanine rather than a nonspecific nuclease is responsible for the loss of O^6-methylguanine from the acid-precipitable DNA. When similar extracts were prepared from the livers of hamsters which had been pretreated 24 hr before death with dimethylnitrosamine (20 mg/kg), the activity could not be detected (Table 2). Therefore, it appears that hamster liver cells do have the ability to remove O^6-methylguanine from DNA but that the capacity to carry out this reaction is small, and a significant decrease in O^6-methylguanine levels within 24 hr is observed only after doses below 0.5 mg/kg. This conclusion is supported by the observed ratios of O^6-methylguanine to 7-methylguanine in the DNA. A number of studies have shown that the relative reactivities of the O^6-methylguanine and 7-methylguanine sites in guanine of DNA under physiological conditions is such that this ratio is about 0.12 (6, 12, 16, 19). Five hr after administration of doses of dimethylnitrosamine greater than 0.25 mg/kg, this ratio was observed in the present experiments (Chart 2); but at doses of 0.01 to 0.25 mg/kg, much lower ratios of 0.01 to 0.03 were found. This can be explained by the rapid loss of up to 90% of the O^6-methylguanine during the 5-hr period. 7-Methylguanine excision over this short period is too small to make a significant difference to the ratio. However, at 24 hr after doses of greater than 0.25 mg/kg, the ratio had increased to about 0.2 because the rate of loss of 7-methylguanine exceeded that of O^6-methylguanine (Chart 2).

These results show interesting contrasts to results of similar experiments in rats. Data for the O^6-methylguanine to 7-methylguanine ratios in rat liver DNA after administration of a similar range of doses of dimethylnitrosamine are also shown in Chart 2. These ratios were calculated from the levels reported recently (17, 19). There are 2 major differences between the results in hamsters and those in rats: (*a*) in the rat O^6-methylguanine was also lost much more rapidly than was 7-methylguanine, resulting in ratios less than 0.1, but this occurred to a substantial extent at doses up to and including 2.5 mg/kg, an order of magnitude greater than the equivalent point in the hamster; (*b*) even at doses of 20 mg/kg, O^6-methylguanine was lost at least as rapidly as 7-methylguanine in the rat and, therefore, the ratio of O^6-methylguanine to 7-methylguanine did not rise above 0.12.

Table 2

Removal of O^6-methylguanine from alkylated DNA on incubation with hamster liver extracts in vitro

Extracts were prepared from control hamster livers and from hamsters treated 24 hr before death with dimethylnitrosamine (20 mg/kg). These extracts (15 mg of protein) were then incubated for 60 min at 37° with ^3H-methylated DNA, and the content of methylated bases remaining in the acid-precipitable DNA was determined as described under "Materials and Methods."

		Content of acid-precipitable DNA		
Extract added	Incubation time (min)	Guanine (μmol)	7-Methylguanine (pmol)	O^6-Methylguanine (fmol)
None	60	3.43	2.68	352
Control	0	3.30	2.70	367
Control	60	3.15	2.60	69
Pretreated with dimethylnitrosamine (20 mg/kg)	60	3.23	2.50	342

DISCUSSION

The loss of 7-methylguanine from DNA observed in the present experiments (Table 1; Fig. 1) and reported previously by Margison *et al.* (12) suggests that enzymatic excision of this purine takes place in the hamster. The rate

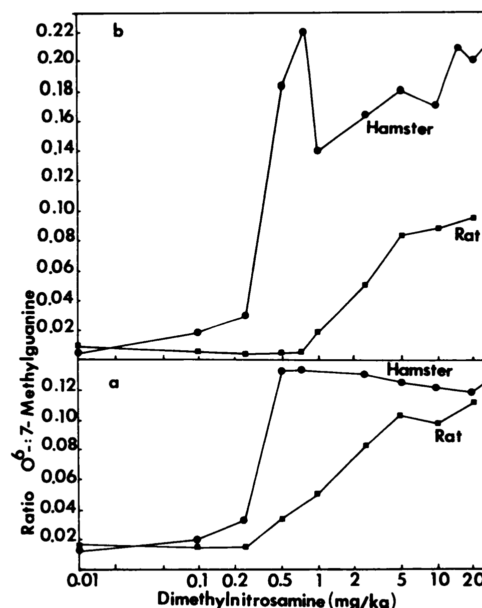

Chart 2. Ratio of O^6-methylguanine to 7-methylguanine in hamster (●) and rat liver (■) 4 or 5 hr (*a*) and 24 hr (*b*) after administration of dimethylnitrosamine. Values for the rat were calculated from the data given in Pegg and Hui (19) for alkylation 4 or 24 hr after administration of dimethylnitrosamine. Values for the hamster were calculated from results for alkylation 5 and 24 hr after dimethylnitrosamine treatment in Table 1.

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of loss was considerably faster than that which would be expected from the rate of spontaneous depurination at neutral pH. The exact pH and ionic strength of the nuclear environment could alter this rate significantly, and these are not known precisely. However, it appears unlikely that these factors could account for the discrepancy, and the rate of loss of 7-methylguanine from hamster liver DNA was much faster than that from rat liver DNA after the same extent of alkylation (*cf.* Table 1, Chart 1, and Refs. 10, 16, and 17). It is improbable that the rat and hamster nuclei differ in their ionic composition to an extent sufficient to bring about this difference if loss of 7-methylguanine were mediated solely by chemical depurination. In part, the apparent loss of 7-methylguanine from DNA after doses of dimethylnitrosamine as high as 25 mg/kg may be due to necrosis and death of highly alkylated cells, but little cell death occurs within the first 24 hr and after the much lower doses of dimethylnitrosamine examined in the present study. At present, there is no information on the nature of the enzymic reaction responsible for the loss of 7-methylguanine from DNA, but it must be distinct from that catalyzing removal of O^6-methylguanine, since the isolated cell extracts which carried out this latter reaction did not excise 7-methylguanine (see Table 2). The fact that these liver extracts did not appear to carry out this reaction is not surprising, since the data of Table 1 suggest that *in vivo* only about 30 to 40% of the 7-methylguanine is lost in 24 hr. This would suggest that the reaction is much slower than that responsible for the loss of small amounts of O^6-methylguanine, and a significant effect would not be expected during the 1 hr of reaction *in vitro*.

The results presented in the present paper are consistent with the hypothesis that cells in the hamster liver do possess the ability to remove O^6-methylguanine from their DNA but that substantial removal occurs only when the initial extent of methylation is low. Little decline in O^6-methylguanine levels was observed in 24 hr after doses of dimethylnitrosamine greater than 0.25 mg/kg in the present study (Table 1), and in a previous work (11) significant decline was not observed until 36 hr after 25 mg/kg, which produces widespread necrosis and regenerative cell growth at this time. Although the more detailed time course of Chart 1 shows that O^6-methylguanine was lost from hamster DNA after a dose of 2.5 mg/kg, the loss was quite slow, with 27% of the initial input still being present 4 days after administration of the carcinogen. These results differ from the situation in rat liver after administration of methylating carcinogens, for although O^6-methylguanine removal from rat liver DNA is much more efficient after low doses of dimethylnitrosamine or *N*-methyl-*N*-nitrosourea, removal does still occur after higher doses (5, 14, 17, 18). For example, after 2.5- or 20-mg/kg doses of dimethylnitrosamine to rats, O^6-methylguanine levels by 96 hr were substantially less than 10% of the input (14).

The enzyme activity capable of removing O^6-methylguanine from DNA *in vitro* which we have detected in hamster liver extracts (Table 2) is also present in rat liver (19) and is also undetectable in rat liver extracts after high doses of dimethylnitrosamine (18). Therefore, it appears that the rat liver must possess a second system for the excision of O^6-methylguanine from DNA, which is less efficient but has a greater capacity to act on DNA after higher extents of alkylation. This activity seems to be much less in the hamster liver. It is possible that the reduced activity of this system contributes towards the greater sensitivity of the hamster liver to tumor induction by single doses of dimethylnitrosamine. In rats (with the rare exception of the NZR/Gd strain), liver tumors were not produced by treatment of normal adults with single doses (20 to 30 mg/kg) of this carcinogen (4, 7, 15), whereas a 30% incidence was observed on treating hamsters with a single 25-mg/kg dose of dimethylnitrosamine (4, 12, 18). Tumors are produced in rats by prolonged feeding of a diet containing quite low levels of dimethylnitrosamine, and a dose-response curve is available, but comparable results for repeated doses in hamsters have not been published (4).

The mechanism underlying the loss of activity catalyzing removal of O^6-methylguanine from DNA *in vitro* when rat or hamster liver extracts are prepared from animals pretreated with dimethylnitrosamine (20 mg/kg) is not yet clear. As discussed (18), it could be due to direct inhibition of the enzyme or to the change in state of the enzyme in response to the high level of alkylation, leading to the activity becoming resistant to the extraction procedure used. Although hamster and rat liver extracts appear to be similar in this respect, the present experiments emphasize the probability that different species may vary in their abilities to repair alkylation damage to their DNA. It appears that hamster liver cells are able to remove 7-methylguanine from their DNA, whereas in rat liver this reaction occurs more slowly at a rate which cannot at present be definitely distinguished from that of spontaneous depurination (10, 16). There is also evidence favoring enzymic excision of 7-methylguanine from DNA in the mouse (13). Rat liver cells appear to be more active in removing O^6-methylguanine from their DNA than are hamster liver cells. These differences in the rate of removal of 7-methylguanine and of O^6-methylguanine both contribute to the striking species difference in the relative proportions of these products found after various doses of dimethylnitrosamine (Chart 2). It should be stressed that, although removal of O^6-methylguanine is strongly dose dependent, the initial degree of methylation of hepatic DNA in rats and hamsters after the same dose of dimethylnitrosamine is approximately the same (Table 1; Refs. 12, 17, and 19). Therefore, differences in the ability to remove methylation products from the DNA rather than in ability to activate the carcinogen are responsible for the differences in the observed amounts of methylated bases.

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