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Formation and Subsequent Removal of O^6-Methylguanine from Deoxyribonucleic Acid in Rat Liver and Kidney after Small Doses of Dimethylnitrosamine

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1. The amounts of 7-methylguanine and O^6-methylguanine present in the DNA of liver and kidney of rats 4h and 24h after administration of low doses of dimethylnitrosamine were measured. 2. O^6-Methylguanine was rapidly removed from liver DNA so that less than 15% of the expected amount (on the basis of 7-methylguanine found) was present within 4h after doses of 0.25mg/kg body wt. or less. Within 24h of administration of dimethylnitrosamine at doses of 1mg/kg or below, more than 85% of the expected amount of O^6-methylguanine was removed. Removal was most efficient (defined in terms of the percentage of the O^6-methylguanine formed that was subsequently lost within 24h) after doses of 0.25–0.5mg/kg body wt. At doses greater or less than this the removal was less efficient, even though the absolute amount of O^6-methylguanine lost during 24h increased with the dose of dimethylnitrosamine over the entire range of doses from 0.001 to 20mg/kg body wt. 3. Alkylation of kidney DNA after intraperitoneal injections of 1–50μg of dimethylnitrosamine/kg body wt. occurred at about one-tenth the extent of alkylation of liver DNA. Removal of O^6-methylguanine from the DNA also took place in the kidney, but was slower than in the liver. 4. After oral administration of these doses of dimethylnitrosamine, the alkylation of kidney DNA was much less than after intraperitoneal administration and represented only 1–2% of that found in the liver. 5. Alkylation of liver and kidney DNA was readily detectable when measured 24h after the final injection in rats that received daily injections of 1μg of [^{3}H]dimethylnitrosamine/kg for 2 or 3 weeks. After 3 weeks, O^6-methylguanine contents in the liver DNA were about 1% of the 7-methylguanine contents. The amount of 7-methylguanine in the liver DNA was 10 times that in the kidney DNA, but liver O^6-methylguanine contents were only twice those in the kidney. 6. Extracts able to catalyse the removal of O^6-methylguanine from alkylated DNA *in vitro* were isolated from liver and kidney. These extracts did not lead to the loss of 7-methylguanine from DNA. 7. The possible relevance of the formation and removal of O^6-methylguanine in DNA to the risk of tumour induction by exposure to low concentrations of dimethylnitrosamine is discussed.

Dimethylnitrosamine is a potent carcinogen in many species. It is well established that the compound exerts its carcinogenic effects by means of its metabolic conversion into a reactive methylating agent (Magee & Barnes, 1967; Druckrey *et al.*, 1967; Magee *et al.*, 1976). It has been suggested that the critical reaction of the methylating agent in inducing cancer might be the methylation of DNA forming O^6-methylguanine (Loveless, 1969; Lawley, 1974, 1976; Magee *et al.*, 1976; Pegg, 1977*a*). The ability of cells to remove O^6-methylguanine from their DNA may provide a protective mechanism against carcinogenesis by dialkylnitrosamines and *N*-alkyl-*N*-nitrosamides (Goth & Rajewsky, 1974; Margison & Kleihues, 1975; Nicoll *et al.*, 1975; Pegg, 1977*a*). In both rat kidney and liver this removal process was much more efficient after low doses of these carcinogens (Nicoll *et al.*, 1975; Kleihues & Margison, 1976; Pegg, 1977*b*). In these experiments, the smallest dose of dimethylnitrosamine that could be tested was 0.25mg/kg body wt. because of the low specific radioactivity of the available ^{14}C-labelled carcinogen (Pegg, 1977*b*). This dose given daily in the diet corresponds approximately to that found to give a low incidence of liver tumours in rats (Terracini *et al.*, 1967). Lower doses than this have not been tested for carcinogenicity in experimental animals, and, because of the large numbers of animals needed to test doses of carcinogens that might produce tumour incidences of a few per cent or less, such tests are extremely expensive. It was therefore decided to obtain further information on the enzymic system responsible for

the loss of O^6-methylguanine from DNA and to determine to what extent the small amounts of O^6-methylguanine produced by very low doses of dimethylnitrosamine could be removed. Such small doses may be comparable with those to which humans are exposed. Nitrosamines including dimethylnitrosamine have been found in the environment and in certain foods, and can be formed in the stomach by the reaction of nitrite and amines (Lijinsky & Epstein, 1970; Fiddler, 1975; Scanlan, 1975; Mirvish, 1975; Archer & Wishnok, 1977; Fine *et al.*, 1976). Dimethylnitrosamine has been detected in the blood of humans after a meal of bacon and spinach (Fine *et al.*, 1977).

In the present study [^{3}H]dimethylnitrosamine was used to measure the degree of alkylation of DNA after doses as low as 1 μg/kg body wt. It was found that even though most of the O^6-methylguanine was removed very rapidly from liver DNA after these low doses, this product was still readily detectable in the DNA 24h after administration of the carcinogen. In addition, a cell-free extract capable of removing O^6-methylguanine from DNA has been prepared from rat liver and kidney, and some properties of this system are described. The significance of these results in the possible induction of tumours by low exposures to dimethylnitrosamine is discussed.

Methods and Materials

Chemicals

[^{14}C]Dimethylnitrosamine (sp. radioactivity 5.185 mCi/mmol) and [^{3}H]dimethylnitrosamine (sp. radioactivity 2.96Ci/mmol) were purchased from New England Nuclear Corp., Boston, MA, U.S.A., and diluted to the required specific radioactivity by addition of redistilled, unlabelled dimethylnitrosamine obtained from Aldrich Chemical Co., Milwaukee, WI, U.S.A. Sephadex G-10 was purchased from Pharmacia, Uppsala, Sweden. O^6-Methylguanine was synthesized by the method of Balsiger & Montgomery (1960). All other biochemical reagents were products of Sigma Chemical Co., St. Louis, MO, U.S.A.

Animals

Female Sprague–Dawley strain rats (200–250g body wt.) purchased from Charles River Breeding Laboratories, Wilmington, MA, U.S.A., were maintained on a 12h light/12h dark cycle and fed *ad libitum* on Purina Rat Chow. Dimethylnitrosamine was administered at about 10:00h by intraperitoneal injection of a solution in 0.9% NaCl or by oral administration of a solution in water. The concentration of the nitrosamine was adjusted so that the volume injected was 0.3–0.7ml and that given orally was 2–3ml.

Preparation and analysis of DNA

This was carried out essentially as described previously (Pegg, 1977*b*). Briefly, tissue samples were removed rapidly after death and frozen in liquid N_2. The frozen tissues were stored at −70°C and the DNA then isolated by extraction with phenol (Margison & Kleihues, 1975; Pegg, 1977*b*). The dried DNA was stored at −20°C. DNA was hydrolysed in 0.1M-HCl at 70°C for 30min to release free purine bases (Lawley & Thatcher, 1970). Authentic marker-methylated bases (3-methyladenine, 7-methylguanine and O^6-methylguanine) were added to the hydrolysate and the mixture was chromatographed on a column (70cm×1.6cm) of Sephadex G-10 eluted with 0.5M-ammonium formate, pH6.8 (Lawley & Shah, 1972). Fractions (5ml) were collected and the amounts of guanine and adenine present determined by measurement of the A_{260} in the appropriate fractions. The radioactivity present in each fraction was determined after the addition of 10ml of Formula-947 LSC Cocktail (New England Nuclear, Boston, MA, U.S.A.). The counting efficiency in a Beckman LS-350 liquid-scintillation counter was determined by use of an external standard and was around 65% for ^{14}C and 24% for ^{3}H. The amounts of the methylated bases present in the DNA sample were calculated as μmol of the methylated base per mol of the normal purine. Sufficient DNA was used to ensure that at least 200c.p.m. above background were present in the O^6-methylguanine peak. In a few cases this was not possible and values based on measurement of smaller amounts of radioactivity than this are indicated in the text.

Preparation and assay of enzyme fraction removing O^6-methylguanine from DNA

The tissue was removed from the rat as rapidly as possible and homogenized in 3 vol. of ice-cold 50mM-Tris/HCl (pH7.8)/1mM-dithiothreitol/0.1mM-disodium EDTA. The extract was centrifuged at 10000*g* for 5min at 4°C and the supernatant kept. An additional volume of the homogenizing buffer equal to that of the original tissue was then added to the pellet and the suspension sonicated with a model W-225R ultrasonic cell disrupter (Heat Systems–Ultrasonics, Plainview, NY, U.S.A.) at 80–100W and a 50% duty cycle for three periods of 30s each separated by 1min intervals. During sonication the extract was surrounded by an ice/water bath to keep the temperature as near to 0°C as possible. The sonicated extract was then added to that obtained from the initial centrifugation and the mixture centrifuged at 15000*g* for 30min at 4°C. The supernatant from this centrifugation was then made 80% saturated by addition of solid $(NH_4)_2SO_4$. After stirring for 30min, the precipitated protein was dissolved in as small a volume as possible of the homogenizing

buffer and dialysed overnight against 2 litres of the same solution. The dialysed extract was then used as a source of enzyme. It could be stored frozen at −20°C for up to 4 weeks without significant loss of activity.

Enzyme activity was assayed by the incubation in a total volume of 6ml of a solution containing up to 20mg of protein from the extracts prepared as described above, 1.5mM-dithiothreitol, 3.3mM-$MgCl_2$, 75mM-Tris/HCl, pH8.0, and 6–8mg of substrate DNA (see below). After incubation at 37°C the reaction was stopped by the addition of 6ml of cold 0.5M-$HClO_4$. The precipitate was collected by centrifugation at 3000*g* for 10min at 4°C and washed twice by resuspension in 0.25M-$HClO_4$ and subsequently centrifuged. After washing, the pellet was suspended in 10ml of 0.1M-HCl and incubated at 70°C for 30min. The residual pellet was removed by centrifugation at 3000*g* for 10min and the supernatant analysed for the presence of purines by chromatography on Sephadex G-10 as described above. In all experiments, a control incubation omitting the protein extract was used to measure the amounts of methylated purines lost from the substrate DNA incubated in the absence of enzymes. This value, which was very small, was subtracted from that released when the protein extract was added. The DNA used as substrate in these experiments was prepared from the livers of rats injected with 10μg of [^{3}H]dimethylnitrosamine (sp. radioactivity 2.96Ci/mmol)/kg body wt. 10min before death. The DNA was isolated as described above and stored dry at −20°C until required.

Results

The major product of the reaction of dimethylnitrosamine and DNA is 7-methylguanine, and the formation of this product can be used as a measure of the degree to which the carcinogen is converted into the methylating species within the cell (Swann & Magee, 1968; Craddock, 1969; Swann & McLean,

Table 1. *Amounts of 7-methylguanine and O^6-methylguanine present in liver DNA 4 and 24h after administration of dimethylnitrosamine*

Rats were injected intraperitoneally with either [^{3}H]dimethylnitrosamine or [^{14}C]dimethylnitrosamine at about 10:00h and killed 4 or 24h later. DNA was then isolated from the livers and the amounts of 7-methylguanine and O^6-methylguanine present were determined as described in the Methods and Materials section. The values for doses of dimethylnitrosamine of 0.25mg/kg body wt. or less were obtained by using [^{3}H]dimethylnitrosamine and those of more than 1 mg/kg body wt. were taken from previously published data in which [^{14}C]dimethylnitrosamine was used (Pegg, 1977*b*). The values for doses of 0.5 and 1.0mg/kg body wt. were measured with both [^{14}C]- and [^{3}H]-dimethylnitrosamine and the results shown are the mean of these measurements. All values shown are the mean of at least three estimations involving separate rats, which agreed within 10%. The expected value for O^6-methylguanine was taken as 0.115 × the measured value for 7-methylguanine. The actual O^6-methylguanine found was expressed as a percentage of this value.

Dose (mg/kg body wt.)	Time (h)	Methylated base (mol/10^6mol of guanine)		Percentage of expected value for O^6-methylguanine
		7-Methylguanine	O^6-Methylguanine	
0.001	4	0.36	0.005	12
0.01	4	4.1	0.067	14
0.025	4	14.5	0.22	13
0.05	4	20.6	0.28	12
0.25	4	85	1.0	10
0.5	4	160	5.5	30
1.0	4	292	14.3	43
2.5	4	694	57	72
5.0	4	1510	155	89
10.0	4	3280	322	85
20.0	4	5920	652	96
0.001	24	0.31	0.004	10
0.01	24	3.6	0.034	7
0.025	24	10.5	0.092	6
0.05	24	18.8	0.120	5
0.25	24	74	0.31	3
0.5	24	124	0.60	3
1.0	24	240	4.3	13
2.5	24	579	29	37
5.0	24	1180	99	57
10.0	24	2660	233	62
20.0	24	4610	447	66

1971; Montesano & Magee, 1974). Within 4h the alkylation of nucleic acids in rat liver is complete even after doses of dimethylnitrosamine as high as 20mg/kg body wt. (Heath, 1962; Swann & Magee, 1968; Pegg, 1977*b*). The amounts of 7-methylguanine and O^6-methylguanine present in hepatic DNA after administration of doses of dimethylnitrosamine ranging from 0.001 to 20mg/kg body wt. are shown in Table 1. The values for doses of greater than 1mg/kg body wt. are taken from a previous publication utilizing [^{14}C]dimethylnitrosamine (Pegg, 1977*b*). The data for lower doses were obtained by using [^{3}H]dimethylnitrosamine. The use of this material introduces two possible sources of error. The nominal specific radioactivity of the [^{3}H]dimethylnitrosamine may change on storage owing to ^{3}H exchange with the solvent, and there is the possibility of a significant isotope effect in the metabolism of the nitrosamine that generates the alkylating species. An isotope effect in the metabolism of fully deuterated dimethylnitrosamine has been observed (Dagani & Archer, 1976). However, in the experiments shown in Table 1 the production of 7-methylguanine at 4h was approximately proportional to dose over the entire range of dimethylnitrosamine used, which included both [^{14}C]- and [^{3}H]-dimethylnitrosamine. Also, when the observations at doses of 0.5 and 1.0mg/kg body wt. were made with both [^{14}C]- and [^{3}H]-dimethylnitrosamine there was no significant difference between the calculated amounts of alkylated guanines. Therefore the [^{3}H]dimethylnitrosamine was satisfactory for the measurement of the degree of alkylation of DNA.

O^6-Methylguanine was readily detected in DNA of rats treated with low doses of dimethylnitrosamine and could be measured even 24h after doses of only 1μg/kg body wt. However, as previously reported (Pegg, 1977*b*), after doses of less than 2.5mg/kg body wt. the amounts of O^6-methylguanine found were substantially less than might be expected from the amounts of 7-methylguanine. The methylating species derived from dimethylnitrosamine would be expected to react with DNA to produce 7-methylguanine and O^6-methylguanine in the relative proportions of about 9:1 (Lawley, 1974, 1976; Pegg & Nicoll, 1976; Pegg, 1977*a*). This value should not change with different amounts of the alkylating agent over the range in question (see below and Table 2). Therefore the predicted amount of O^6-methylguanine was calculated from an expected ratio of O^6-methylguanine/7-methylguanine of 0.115 and the amount of 7-methylguanine present at 4h, which was assumed to be the maximum amount present. The percentage of the calculated O^6-methylguanine value that was actually found is shown in Table 1. Within 4h more than 85% of the expected O^6-methylguanine had been lost after all doses of 0.25mg/kg body wt. or less, but there was little difference in the percentage of this base remaining when doses from 0.001 to 0.25mg/kg body wt. were compared. Within 24h more than 85% of the O^6-methylguanine expected had been lost after doses of 1.0mg/kg body wt. or less. However, the efficiency of removal, defined as the percentage loss of O^6-methylguanine over the first 24h, showed a peak for doses of 0.25–0.5mg/kg body wt. and was less than this for doses both higher and lower. One possible explanation for this could be that the enzymic system for removal of O^6-methylguanine is limited by substrate availability at the low concentrations of O^6-methylguanine present after very low doses of dimethylnitrosamine. It is also possible that O^6-methylguanine at certain sites within the chromosome is less susceptible to the removal process.

Although the efficiency of removal of O^6-methylguanine decreases with doses of the nitrosamine greater than 0.5mg/kg body wt. it should be noted that the reaction does not appear to be saturated even at the highest dose tested. Fig. 1 shows the relationship between dose of dimethylnitrosamine and amount of O^6-methylguanine formed and subsequently lost in a 24h period. There is an increase in the amount of O^6-methylguanine lost over the entire dose range.

All of the calculations discussed above are based

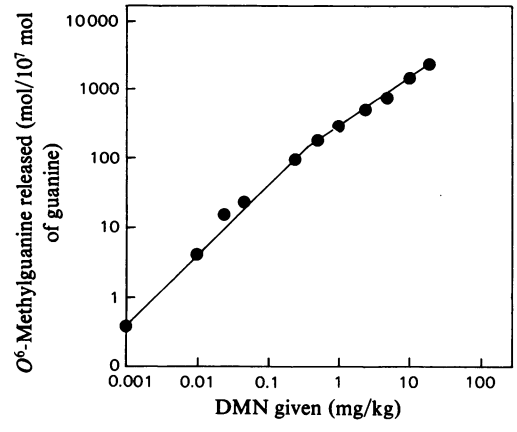

Fig. 1. *Loss of O^6-methylguanine from hepatic DNA over a 24h period as a function of dose of dimethylnitrosamine*

The amount of O^6-methylguanine expected to be present in liver DNA after administration of dimethylnitrosamine (DMN) was calculated as described in Table 1 and the amount lost in 24h determined by subtraction of that found in the DNA at 24h. This value, expressed as mol of O^6-methylguanine removed per 10^7mol of guanine in DNA, was then plotted as a function of the dose of dimethylnitrosamine.

on the assumption that at all doses of the nitrosamine the ratio of alkylation of guanine in DNA at the O^6- and 7- positions is 0.115. This value was taken because it was the mean of studies in our laboratory in which alkylation was measured after the reaction of double-stranded DNA with various concentrations of *N*-methyl-*N*-nitrosourea *in vitro* or of rat liver DNA with large doses of dimethylnitrosamine *in vivo* (Pegg & Nicoll, 1976; Pegg, 1977*a*,*b*). It is in good agreement with those obtained by others, which range from 0.10 to 0.125 (Lawley, 1974; Kleihues & Margison, 1976; Margison *et al.*, 1976), but there was no direct experimental evidence showing that the same ratio is found after alkylation with small amounts of dimethylnitrosamine. Since this concept is critical to the interpretation of Table 1, two experiments were carried out to prove that the same ratio of alkylation occurs after low doses of dimethylnitrosamine as after high doses (Table 2). In the first experiment rats were given a single dose of 20mg of unlabelled dimethylnitrosamine/kg body wt. to produce unlabelled methylated bases in the DNA that would minimize the effects of the enzymes catalysing removal of these products. Then 12h later, when all the unlabelled nitrosamine had been metabolized so that it was undetectable in the blood, an injection of 10μg of [^{3}H]dimethylnitrosamine/kg body wt. was given. The amounts of labelled O^6-methylguanine and 7-methylguanine present in the DNA 4h later are shown in Table 2 and the ratio was 0.120. This ratio is in close agreement to that found by Kleihues & Margison (1976), who gave a tracer dose of *N*-[^{3}H]methyl-*N*-nitrosourea after a large dose of dimethylnitrosamine. In the second experiment, untreated rats were given 10μg of [^{3}H]dimethylnitrosamine/kg body wt. by intravenous injection and the rats killed 10min later. A substantial portion of this dose was metabolized within this short period, as indicated by the production of 7-methylguanine (Table 2), which was only slightly less than that found 4h after injection (Table 1). The amount of O^6-methylguanine found 10min after injection was much greater than that found 4h later and the ratio of O^6- to 7-methylguanine was 0.083. The difference between this and the expected ratio probably indicates that removal of O^6-methylguanine occurs even over the 10min period.

The alkylation of kidney DNA by low doses (1–50μg/kg body wt.) of [^{3}H]dimethylnitrosamine is shown in Table 3. Dimethylnitrosamine is metabolized in the kidneys of normal rats at a rate about one-eighth to one-tenth that in the liver (Swann & Magee, 1968; Swann & McLean, 1971). As expected from this, after intraperitoneal injection of the carcinogen, alkylation of kidney DNA forming 7-methylguanine occurred to about one-tenth the extent of alkylation in the liver. O^6-Methylguanine was present in the kidney DNA at slightly less than the expected amount 4h after injection after doses of 1 and 10μg/kg body wt. and at only 40 and 32% that expected after doses of 25 and 50μg/kg body wt. (Table 3). By 24h, the O^6-methylguanine concentrations after the latter doses had fallen to 25 and 15% respectively (results not shown). These results suggest that enzymic removal of O^6-methylguanine occurred in the kidney after these low doses of dimethylnitrosamine, but was less efficient than in the liver. (This could be related to the low extent of alkylation limiting the activity of the removal system as discussed above.)

When the same low doses of [^{3}H]dimethylnitrosamine were administered orally rather than by intraperitoneal injection the alkylation of kidney DNA was much decreased, although it could still be detected. The alkylation of kidney DNA decreased to 1–2% of the alkylation of liver DNA (Table 3). This finding confirms the report of Diaz Gomez *et al.* (1977), who found that after low oral doses of dimethylnitrosamine, alkylation of kidney DNA occurred to a much smaller extent than expected from studies with higher doses or where the compound was given by injection. These results are probably explained by the liver's ability to metabolize almost completely low concentrations of dimethyl-

Table 2. *Alkylation of guanine in hepatic DNA after administration of* 10μg *of* [^{3}*H*]*dimethylnitrosamine/kg body wt.*
One group of rats was given [^{3}H]dimethylnitrosamine (10μg/kg body wt.; sp. radioactivity 2.96Ci/mmol) by intravenous administration 10min before death. A second group was given the same dose of [^{3}H]dimethylnitrosamine by intraperitoneal injection 12h after a dose of 20mg of unlabelled dimethylnitrosamine/kg body wt. and killed 4h later. DNA was then isolated from the livers and the amount of radioactive methylated guanine derivatives present determined as described in the Methods and Materials section.

Pretreatment	Time after [^{3}H]dimethylnitrosamine administration	[^{3}H]Methylated bases (mol/10^{6}mol of guanine)		Ratio
		7-Methylguanine	O^6-Methylguanine	O^6- to 7-methylguanine
Dimethylnitrosamine (20 mg/kg body wt.)	4h	3.94	0.47	0.12
None	10min	3.32	0.27	0.083

Table 3. *Alkylation of kidney DNA after intraperitoneal or oral administration of dimethylnitrosamine*
The dose of [^{3}H]dimethylnitrosamine shown was administered to rats either by intraperitoneal injection or orally: 4h later, the rats were killed and the 7-methylguanine and O^6-methylguanine present determined as described in the Methods and Materials section. The values for alkylation of kidney DNA at the O^6-position of guanine for all oral doses and for the intraperitoneal doses below 0.025mg/kg body wt. were based on c.p.m. above background in the O^6-methylguanine peak of more than 40 but less than 200. The ratio of 7-methylguanine production in the kidney DNA to that produced in the liver DNA in the same animals is also shown. The alkylation of liver DNA was not statistically significantly different when oral and intraperitoneal injection were compared.

Dose (μg/kg body wt.) and route of administration	Methylated base (mol/10^6mol of guanine)		Ratio of 7-methylguanine produced in liver DNA to that in kidney DNA
	7-Methylguanine	O^6-Methylguanine	
1 intraperitoneal	0.05	0.007	7.2
10 intraperitoneal	0.46	0.04	8.9
25 intraperitoneal	1.29	0.06	11.2
50 intraperitoneal	2.44	0.08	8.4
1 oral	0.013	<0.002	45
10 oral	0.06	0.005	119
50 oral	0.43	0.04	55

Table 4. *Alkylated guanine derivatives present in hepatic DNA after daily injections of 1 μg of dimethylnitrosamine/kg body wt.*
Rats were given [^{3}H]dimethylnitrosamine (1μg/kg body wt.; sp. radioactivity 2.96Ci/mmol) by intraperitoneal injection daily at 10:00h. They were killed 24h after the final injection and the methylated guanines present in the DNA determined as described in the Methods and Materials section.

Organ	Number of daily injections	Methylated bases (mol/10^6mol of guanine)	
		7-Methylguanine	O^6-Methylguanine
Liver	14	0.84	0.008
Liver	20	1.02	0.013
Kidney	14	0.101	0.006
Kidney	20	0.109	0.007

nitrosamine in the portal blood after oral administration, thus preventing it from reaching the kidney (Diaz Gomez *et al.*, 1977).

The amounts of alkylated guanine derivatives present in rat liver and kidney after repeated daily injections of 1μg of [^{3}H]dimethylnitrosamine/kg body wt. are shown in Table 4. Measurements were made 24h after the last injection. At 2 weeks after the start of treatment the amount of 7-methylguanine present in the liver DNA was about 3 times and the amount of O^6-methylguanine was about twice that present 24h after a single dose. The contents were increased only slightly after 3 weeks of injections, suggesting that a balance between synthesis from the daily dose of dimethylnitrosamine and removal had been reached, although treatment over a much longer period of time would be necessary to establish this conclusively. The O^6-methylguanine content in liver DNA represented about 1% of the 7-methylguanine present and in kidney DNA about 6%. This indicates that over the longer period the O^6-methylguanine is removed more completely in the liver than in the kidney. As a result of this, although the amount of 7-methylguanine found in the liver DNA was 10 times that found in the kidney DNA, there was only a 2-fold difference in the O^6-methylguanine amounts.

These results described above and previous studies (reviewed by Pegg, 1977*a*) suggest that an enzymic system for the removal of O^6-methylguanine is present in liver and kidney cells. We have been able to demonstrate the presence of such activity in cell-free extracts. Activity was assayed by measuring the loss of labelled O^6-methylguanine from DNA isolated from the livers of rats treated 10min before death with 10μg of [^{3}H]dimethylnitrosamine/kg body wt. It was necessary to use such DNA as a substrate because the extracts had only a very limited capacity to remove O^6-methylguanine. Since we were able to measure only the alkylated bases present in the remaining DNA rather than those liberated, it was essential to have a substrate that could be substantially altered by this limited activity. The activity was measured by incubating the DNA with the extract and then precipitating the DNA and determining the methylated base content. Fig. 2 shows the effects of incubating such DNA with an extract prepared from rat liver. Incubation with the extract led to a time-dependent loss of O^6-methylguanine from the DNA that was much greater than the small losses of 7-methylguanine and guanine. The loss of guanine represents the degree to which the DNA was degraded to fragments soluble in cold 0.25M-$HClO_4$, and this occurred to only an insignificant extent. The small loss of 7-methylguanine can be accounted for by this small decline of acid-insoluble

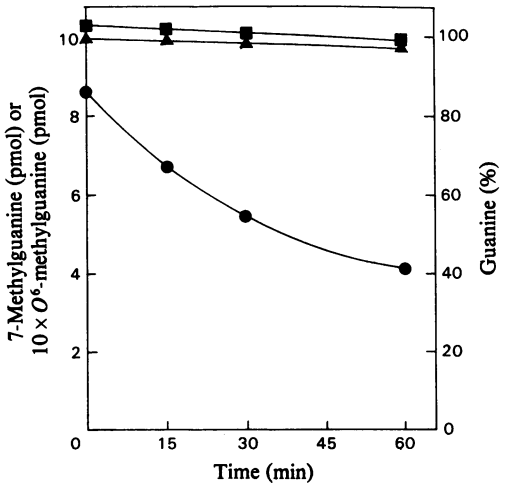

Fig. 2. *Removal of O^6-methylguanine from alkylated DNA incubated in vitro for different times with rat liver extracts*

Rat liver extract was incubated at 37°C with [^{3}H]-alkylated DNA, and at the times shown the reaction was halted and the content of alkylated bases and of guanine in the DNA precipitated by cold 0.25M-$HClO_4$ determined as described in the Methods and Materials section. The amounts of guanine (▲), 7-methylguanine (■), and O^6-methylguanine (●) present in the precipitated DNA are shown.

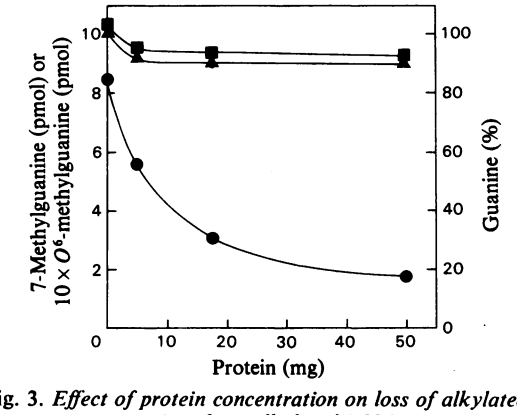

Fig. 3. *Effect of protein concentration on loss of alkylated guanines from alkylated DNA*

[^{3}H]Alkylated DNA was incubated with the amount of liver extract containing the protein shown for 2h at 37°C. The amounts of guanine (▲), 7-methylguanine (■) and O^6-methylguanine (●) present in the DNA precipitated by cold 0.25M-$HClO_4$ were determined as described in Fig. 2.

DNA, but this cannot account for the decline in O^6-methylguanine. Further, as shown in Fig. 3, the loss of O^6-methylguanine from the DNA was increased greatly by increasing the protein added, although it was not directly proportional to the protein concentration. Less than 2% of the O^6-methylguanine present in the DNA was rendered acid soluble on incubation for 2h at 37°C in the absence of the protein extract. Therefore an enzymic process specific for the removal of O^6-methylguanine and inactive on 7-methylguanine must be present in these extracts.

We were able to detect activity in both the post-nuclear-supernatant fractions from liver extracts and in sonicated extracts from the nuclear pellet. In the present work these extracts were combined, but it is not yet known whether these activities are indicative of the presence of two distinct enzymes. The activity could be fractionated by the addition of $(NH_4)_2SO_4$, but for unknown reasons, much greater non-specific deoxyribonuclease activity was present in the separate $(NH_4)_2SO_4$ fractions than in the 0–80%-satd.-$(NH_4)_2SO_4$ fraction used in the present experiments. Further purification by chromatography on DEAE-cellulose was needed to obtain active extracts that did not substantially degrade the substrate.

Extracts from liver, kidney and brain were prepared in an identical manner and their ability to remove O^6-methylguanine from DNA was compared in assays containing 15mg of protein incubated at 37°C for 2h. The brain extracts were completely inactive (<2% loss of O^6-methylguanine), the liver extracts had the greatest activity (83% lost), and the kidney extracts had definite activity, but were less active than the liver (32% lost). In this respect, therefore, the activity of these extracts resembles the abilities of these tissues to catalyse this reaction *in vivo* (Goth & Rajewsky, 1974; Margison & Kleihues, 1975; Pegg & Nicoll, 1976).

Discussion

There is substantial, but not overwhelming, support for the hypothesis that tumour induction by dimethylnitrosamine is related to the production and subsequent persistence in DNA of methylated nucleosides, which are likely to miscode (Lawley, 1974; Magee *et al.*, 1975, 1976; Pegg, 1977*a*). At least nine methylated bases and methyl phosphate triesters are produced by the reaction of dimethylnitrosamine with DNA. Although other methylations on oxygen atoms should not be ruled out and deserve more detailed investigation (Singer, 1975, 1976), O^6-methylguanine may be the most important product in tumour induction (Loveless, 1969; Goth & Rajewsky, 1974; Lawley, 1974; Craddock, 1975*a*,*b*, 1976; Margison & Kleihues, 1975; Nicoll *et al.*, 1975; Pegg, 1977*a*). If this hypothesis is correct the efficiency of removal of O^6-methylguanine may be critical in protecting against tumour induction.

The present studies confirm and extend earlier

reports showing that rat liver and kidney are capable of removing O^6-methylguanine from DNA (Craddock, 1973; O'Connor *et al.*, 1973; Goth & Rajewsky, 1974; Margison & Kleihues, 1975; Nicoll *et al.*, 1975; Pegg, 1977*a*,*b*). O^6-Methylguanine is stable in DNA (Lawley & Thatcher, 1970) and the loss *in vivo* that occurs at a much faster rate than the loss of 7-methylguanine must indicate an enzyme-catalysed reaction. 7-Methylguanine is lost from DNA *in vitro* by a spontaneous depurination that has a half-time of about 6 days at physiological pH (Craddock, 1969; Margison *et al.*, 1973; Lawley, 1974). Loss of 7-methylguanine from rat liver DNA *in vivo* occurs somewhat more rapidly than this. Half-times of 1–4 days with the higher values for greater extents of alkylation have been reported (Craddock, 1969; Margison *et al.*, 1973; Nicoll *et al.*, 1975; Pegg, 1977*a*,*b*). At such doses, the possibility of loss by cell necrosis may contribute to the observed rate and owing to the uncertainty of the exact pH and ionic strength of the nuclear environment it is still unclear whether there is an enzymic removal of 7-methylguanine in the rat. [More convincing evidence exists for the active excision from DNA of 7-methylguanine in the mouse (Nemoto & Takayama, 1974) and hamster liver (Margison *et al.*, 1976)]. Irrespective of the mechanism, loss of 7-methylguanine from the DNA is sufficiently slow in our experiments (compare data at 4 and 24h in Table 1) for the loss during the first 4h to be negligible in calculating the expected O^6-methylguanine values.

Although after low doses of the nitrosamine removal of O^6-methylguanine was very efficient, it was found that at very low extents of alkylation removal was slowed, perhaps by the low substrate concentration. It appears that extents of alkylation of the order of one O^6-methylguanine residue per 10^6 guanine residues may persist in DNA for longer than might be expected if the time taken for the extent to fall from 1 in 10^5 to 1 in 10^6 is considered. Therefore, although the O^6-methylguanine-removal system may protect against carcinogenesis by dimethylnitrosamine and may be responsible for lowering the carcinogenic risk from daily doses of between 5 and 0.5mg/kg body wt. by more than the simple factor between the doses (Pegg, 1977*a*,*b*), its existence does not imply that there is necessarily a threshold dose at which no tumours at all would be expected. Even after doses of dimethylnitrosamine as low as 1μg/kg body wt. we were able to detect O^6-methylguanine in DNA 24h after a single dose (Table 1) or a series of doses (Table 4). In this respect, our data differ from the report of Margison *et al.* (1977), who claimed that O^6-methylguanine could not be found in the DNA from livers of rats given daily doses of 2mg/kg body wt. dimethylnitrosamine 5 days a week for periods from 2 to 24 weeks. This discrepancy is likely to be due to the difference in sensitivity in the methods used. At present, there is no way of determining the extent below which O^6-methylguanine presence in DNA would not provide a hazard, particularly since the alkylation produced by dimethylnitrosamine may not be uniform within all the cells of the liver (Magee *et al.*, 1975; Pegg, 1977*a*). It must also be considered that if the carcinogen is administered daily the amount of alkylated products in the DNA will show daily variations, even after a steady-state concentration is reached where the amount of alkylation caused by the daily dose balances that lost during the day. For example, Tables 1 and 4 would suggest that on day 20 after administration of 1μg of dimethylnitrosamine/kg body wt. the alkylation of hepatic DNA would vary from 1.4 to 1.0 mol of 7-methylguanine and 0.05 to 0.01 mol of O^6-methylguanine per 10^6 mol of guanine.

After 2 weeks of daily injection of 1μg of dimethylnitrosamine/kg body wt. the amount of O^6-methylguanine present in the kidney DNA 24h after the last injection was somewhat more than half that present in the liver DNA. Since the ability to convert the nitrosamine into the alkylating species is almost an order of magnitude greater in the liver, this finding suggests that O^6-methylguanine is removed more efficiently in the liver. It also raises the question as to why only liver tumours and not renal tumours are produced by prolonged feeding of low doses of dimethylnitrosamine (Druckrey *et al.*, 1967; Magee & Barnes, 1967; Terracini *et al.*, 1967) if the formation of O^6-methylguanine is important in this induction. The answer to this important question may be provided by the finding that there is little reaction with the kidney after low oral doses of dimethylnitrosamine (Diaz Gomez *et al.*, 1977). This result is confirmed by our data in Table 3. Although with the high specific radioactivity of our [^{3}H]dimethylnitrosamine we were able to measure reaction with the kidney, even with doses below 40μg/kg body wt., at which Diaz Gomez *et al.* (1977) found no detectable alkylation, the amount of dimethylnitrosamine reacting with the kidney DNA was much less when the compound was given orally than when it was injected intraperitoneally. As suggested by Diaz Gomez *et al.* (1977), low doses of dimethylnitrosamine absorbed into the portal blood may therefore be almost completely metabolized by the liver and not reach other organs. Since the liver appears to be less sensitive than other organs to the carcinogenic action of dimethylnitrosamine (perhaps because of the high activity of the enzymes removing O^6-methylguanine from DNA) this may provide a protective mechanism against carcinogenesis. Dimethylnitrosamine is absorbed only slowly from the rat stomach, but very rapidly from the upper part of the small intestine (Heading *et al.*, 1974; Hashimoto *et al.*, 1976). The efficiency of clearance of the nitrosamine from the portal blood by the liver may therefore depend on the

rate of gastric emptying as well as the dose of dimethylnitrosamine given.

The ability of the rat liver to metabolize a substantial proportion of a small dose of dimethylnitrosamine very rapidly is shown both by these experiments and by the results of Table 2. It is known that human liver can metabolize dimethylnitrosamine, and although the kinetics of the reaction were not studied in detail, human liver slices were only slightly less active than rat liver slices (Montesano & Magee, 1974). Therefore the finding in human blood of concentrations of dimethylnitrosamine as high as 770ng/litre shortly after a meal (Fine *et al.*, 1977) may indicate exposure to significantly higher amounts.

The relative activities of extracts from liver, kidney and brain in catalysing removal of O^6-methylguanine from DNA *in vivo* suggest that the reaction that we are studying may be responsible for O^6-methylguanine removal *in vivo* after low extents of alkylation. The availability of this system *in vitro* should make possible the determination of its relationship to the known pathways for DNA repair in mammalian cells (Regan & Setlow, 1974; Cleaver, 1975; Grossman *et al.*, 1975). One of these pathways involves the breaking by an endonuclease of a phosphodiester bond near to the damaged site and removal of a number of residues including the altered nucleoside, followed by subsequent resynthesis and joining by a ligase. Endonucleases that attack DNA at apurinic sites (Verly & Paquette, 1973; Ljungquist *et al.*, 1974; Brent, 1975; Teebor & Duker, 1975; Linsley *et al.*, 1977; Kuebler & Goldthwait, 1977) and at sites damaged by u.v. irradiation or reaction with acetylaminofluorene (van Lancker & Tomora, 1974; Bacchetti & Benne, 1975; Brent, 1976; Teebor *et al.*, 1977) have been isolated from mammalian cells, but none of these have been reported to act on sites occupied by methylated nucleosides. O^6-Alkylguanine is excised from the DNA of *Escherichia coli in vivo* (Lawley & Orr, 1970; Lawley & Warren, 1975), and an enzyme that releases O^6-methylguanine or 3-methyladenine as free bases from alkylated DNA has been isolated from *E. coli* (Kirtikar & Goldthwait, 1974). This preparation also contained endonuclease activity, but whether the same enzyme is responsible for both the *N*-glycosidase activity releasing the methyl purines and for the strand breakage is controversial (Kirtikar *et al.*, 1976). Lindahl (1976) and Laval (1977) have provided evidence that they are separate enzymes. It is possible that O^6-methylguanine is removed from DNA in our experiments by the action of a similar *N*-glycosidase, and the apurinic site left behind is repaired by the action of the endonucleases and other repair enzymes described above. We have not been able to test this hypothesis by determining the chemical form in which the labelled O^6-methyl group is lost from the DNA. Mammalian cell extracts contain an enzyme capable of de-alkylating O^6-methyl- or O^6-ethylguanine (Miller *et al.*, 1973) and corresponding ribo- and deoxyribo-nucleosides (K. Rogers & A. E. Pegg, unpublished work). This activity contaminates our enzyme and complicates identification of the initial product. We cannot yet rule out the possibility that such de-alkylation may occur directly on O^6-methylguanine present in the DNA.

It is possible that liver cells have more than one mechanism for the removal of O^6-methylguanine and repair of DNA after alkylation by dimethylnitrosamine. The slow but extensive loss of this purine after high doses of dimethylnitrosamine (Fig. 1) seems unlikely to be achieved by the enzyme assayed *in vitro*, which had a very limited capacity. Fibroblasts from patients suffering from xeroderma pigmentosum were found to excise O^6-alkylguanine more slowly than controls, but some removal did still occur (Goth-Goldstein, 1977). Cells from patients suffering from xeroderma pigmentosum of the same complementation group are known to be defective in apurinic endonuclease activity (Kuhnlein *et al.*, 1976). Efficient DNA repair in eukaryotes may require the combined actions of a number of proteins, not all of which are yet characterized (Mortelmans *et al.*, 1976). In the present experiments, the removal of O^6-methylguanine from liver DNA commenced very rapidly after its formation. This suggests that enzyme induction may not be required for the DNA repair, but the induction of a repair system analogous to the bacterial 'SOS repair' (Witkin, 1976) or that described by Samson & Cairns (1977) cannot be ruled out at later times or after prolonged exposure to the nitrosamine.

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