Stability of Deoxyribonucleic Acid Methylated in the Intact Animal by Administration of Dimethylnitrosamine

RATE OF BREAKDOWN *IN VIVO* AND *IN VITRO* AT DIFFERENT DOSAGES

By VALDA M. CRADDOCK

Toxicology Research Unit, Medical Research Council Laboratories, Carshalton, Surrey

(Received 17 September 1968)

1. The effect of administration of various dosages of dimethylnitrosamine on the extent of methylation of liver and kidney nucleic acids in the intact rat was studied. Methylation of liver nucleic acids was linearly related to the dosage, but decreasing the dose produced relatively less lowering of the extent of alkylation of kidney nucleic acids. 2. The rates of disappearance of 7-methylguanine from DNA during the 2 days after administration of dimethylnitrosamine in the intact animal and on incubation under simulated physiological conditions *in vitro* were compared. At a high dosage this rate was greater *in vivo* than *in vitro*. At a low dosage the small difference between the two rates was not thought to be sufficient evidence for existence of a specific enzymic excision of the abnormal base.

After a single injection of the carcinogen dimethylnitrosamine, liver and kidney nucleic acids of rats become methylated, chiefly on the 7-position of guanine, the extent of methylation reaching a maximum at about 5 hr. after the injection and then decreasing (Craddock & Magee, 1963). As the intact base 7-methylguanine is found in increased concentration in the urine after injection of dimethylnitrosamine, the disappearance of 7-methylguanine from RNA and probably from DNA is due to excision of the methylated base or to breakdown of the nucleic acid molecule, rather than to *N*-demethylation (Craddock & Magee, 1967). The disappearance of 7-methylguanine from DNA of liver was very rapid during the first 2 days after the injection of dimethylnitrosamine. Although alkylation leads to spontaneous depurination of the alkylated guanine, the rate of loss was more rapid than would be expected to occur by uncatalysed hydrolysis of methylated DNA (Lawley & Brookes, 1963). In the experiments *in vivo*, DNA methylated by treatment of the intact animal with [¹⁴C]-dimethylnitrosamine had lost the 7-methylguanine to an extent of 50% in about 17 hr., whereas DNA from T4 phage or salmon sperm, methylated with [¹⁴C]methyl methanesulphonate and incubated at pH 7 at 37°, had lost 50% of its radioactivity by 6½ days. The increased rate of breakdown in the intact animal could have been due to necrosis and loss of those cells in liver that had become most highly methylated, or alternatively an enzyme-catalysed excision of 7-methylguanine could have been responsible. An enzyme that catalyses the differential destruction of methylated DNA has

been shown to occur in *Bacillus subtilis* (Strauss, 1962), and a DNA repair mechanism has been shown to operate in mammalian cells treated with mustard gas (Crathorn & Roberts, 1966; Roberts, Crathorn & Brent, 1968) and with ultraviolet light (Rasmussen & Painter, 1964). There is evidence that the same enzyme is responsible for recognizing different types of damage to DNA. Therefore it seemed possible that an enzyme in mammalian cells might specifically degrade methylated DNA. The existence of such a 'repair' enzyme could have far-reaching consequences: differences in the response of cells to toxic and carcinogenic chemicals could be due to differences in their ability to repair damaged DNA. There is evidence that a defective repair mechanism in skin fibroblasts occurs in xeroderma pigmentosum, and this may explain why patients with this disease often develop fatal skin cancers (Cleaver, 1968).

To study whether the loss of 7-methylguanine from DNA in rat liver was being catalysed by a specific enzyme mechanism, the rate of disappearance was measured by killing rats at intervals after a very low dosage of dimethylnitrosamine that was known not to cause frank necrosis in liver. This rate was compared with the rate of spontaneous chemical degradation under simulated physiological conditions *in vitro*.

To determine an appropriate low dosage of dimethylnitrosamine for use in these experiments, it was necessary first to study the effect of dosage on extent of methylation of nucleic acids. The dose-response relationships for liver and kidney DNA and RNA were of interest for several additional

reasons. It seems possible that the acute toxic effect of dimethylnitrosamine is due to methylation of some species of RNA (Villa-Treviño, 1967), and it was therefore of interest to determine whether the biological effectiveness of dimethylnitrosamine bears any quantitative relationship to the extent of alkylation of RNA. With reference to the carcinogenicity of dimethylnitrosamine, a single injection, or oral administration at a high dosage for a short time, produces tumours in rat kidney, whereas oral administration at a lower dosage for a longer time is carcinogenic for liver (Magee & Barnes, 1959, 1962). A comparison of the dose-response relationships for methylation of kidney and liver DNA and RNA might help to evaluate the significance of these reactions in carcinogenesis.

The dose-response relationships for alkylation of nucleic acids were studied at a constant time of 5 hr. after administration of the carcinogen at each dosage. The rate of disappearance of 7-methylguanine during the succeeding 2 days was then measured both *in vivo*, by killing rats at various intervals after injection of dimethylnitrosamine and isolating the nucleic acids for analysis, and *in vitro*, by incubating the DNA isolated at 5 hr. after injection for various times before reprecipitation and analysis.

7-Methylguanine had been shown to be excreted in the urine as a result of administration of high dosages (18.5–30.0 mg./kg.) of [^{14}C]dimethylnitrosamine, where the complication of liver necrosis had to be considered (Craddock & Magee, 1967). To determine whether the methylated base formed by action of the carcinogen on nucleic acids was excreted in the urine even at very low dosages, the urine collected after a dosage of 1 mg. of dimethylnitrosamine/kg. was analysed.

MATERIALS AND METHODS

Animals

Wistar albino rats of the Porton strain were maintained on MRC diet 41B (Bruce & Parkes, 1956). Males weighing 200 g. were used.

Materials

Cetyltrimethylammonium bromide (Cetab) was purchased from British Drug Houses Ltd., Poole, Dorset.

Dimethylnitrosamine was purchased from British Drug Houses Ltd. and was redistilled before use.

Two batches of [^{14}C]dimethylnitrosamine were used. One was prepared by Dr D. F. Heath by the method of Dutton & Heath (1956). The other was prepared by Dr A. R. Mattocks from [^{14}C]dimethylamine hydrochloride, purchased from The Radiochemical Centre, Amersham, Bucks., by the method of Dutton & Heath (1956). To remove any possible amines present in the product, the distillate of [^{14}C]dimethyl-

nitrosamine was acidified to pH 6 with dilute H_2SO_4 , saturated with NaCl and redistilled. The yield was 95% from the amine. Both preparations were diluted with unlabelled dimethylnitrosamine to give the specific radioactivity required in each experiment.

Animal experiments

Dose-response curve. Six rats were starved overnight and then given dimethylnitrosamine by intraperitoneal injection, of appropriate dosage and specific radioactivity, as described below. The urine was frozen as passed. After 5 hr. the animals were killed by a blow, and the livers and kidneys were removed and frozen immediately in liquid N_2 . DNA and RNA were prepared by a modification of the method of Kirby (1962) as described by Craddock, Villa-Treviño & Magee (1968). In some cases a known weight of normal rat kidneys was added to the experimental kidneys to facilitate the nucleic acid preparations.

The purine fraction of urine was analysed as described by Craddock & Magee (1967).

Stability of DNA. (a) After 26 mg. of dimethylnitrosamine/kg. Six male rats were starved overnight and then given an intraperitoneal injection of 26 mg. of [^{14}C]dimethylnitrosamine (0.31 $\mu\text{C}/\text{mg.}$)/kg. body wt. Four of the animals were killed after 5 hr., the livers removed and placed in liquid N_2 , and nucleic acids isolated as described above. Half of the liver DNA was hydrolysed and analysed as described below. The remaining half was incubated for 43 hr. before reprecipitation, hydrolysis and analysis. The remaining two rats were killed 2 days after the injection of dimethylnitrosamine, i.e. 43 hr. after the first group of animals, and liver DNA was isolated, hydrolysed and analysed. The effect of 43 hr. *in vivo* and 43 hr. *in vitro* could then be compared.

(b) After 1 mg. of dimethylnitrosamine/kg. Four male rats were starved overnight and then given an intraperitoneal injection of 1 mg. of [^{14}C]dimethylnitrosamine (33.20 $\mu\text{C}/\text{mg.}$)/kg. body wt. After 5 hr. the animals were killed and liver nucleic acids isolated. A 30 mg. sample of liver DNA was hydrolysed and analysed without further treatment. A 15 mg. sample was incubated for 43 hr. before reprecipitation, hydrolysis and analysis. Two male rats, treated as described above, were killed 24 hr. after the initial injection and a further two animals were killed 48 hr. after the injection, and the liver DNA was isolated, hydrolysed and analysed.

(c) After 2 mg. of dimethylnitrosamine/kg. Twelve male rats were starved overnight and then given an intraperitoneal injection of 2 mg. of [^{14}C]dimethylnitrosamine (5.86 $\mu\text{C}/\text{mg.}$)/kg. body wt. Eight animals were killed after 5 hr. The remaining animals were given food until the night before they were killed; two animals were killed 24 hr. after the initial injection, and the remaining two were killed 48 hr. after injection. Liver DNA was isolated from each group of animals. The preparations from the animals killed at 24 and 48 hr. were hydrolysed and analysed without further treatment. Of the DNA from the animals killed at 5 hr., 23 mg. was hydrolysed and analysed as prepared, 23 mg. was incubated at pH 6.9 for 19 hr. and a further 23 mg. was incubated for 43 hr. before reprecipitation, hydrolysis and analysis. A further sample was incubated at pH 7.4 for 19 hr. before analysis.

Treatment of nucleic acids

Incubation procedure. The incubation was carried out in a dialysis bag, as 7-methylguanine split from DNA may otherwise remain bound to the macromolecule. Alkylated guanine residues split from DNA have been shown to be released during dialysis and to pass into the diffuse (Lawley & Wallick, 1957). The buffer used was 0.02 M-sodium phosphate, pH 6.9, containing NaN_3 (0.1 M). The DNA was dissolved in 10 ml. of buffer, poured into a dialysis tube and dialysed against 100 ml. of buffer, with shaking, at 39° for the appropriate time. At 1 hr. before the end of the incubation time the bag was placed in 100 ml. of fresh buffer solution. The dialysis bag was rinsed and emptied, and an equal volume of 1% (w/v) Cetab added. The DNA precipitate was converted into the sodium salt by placing it in 2% (w/v) sodium acetate in 75% (v/v) ethanol, and was then dried by treatment with ethanol, ethanol-ether and ether.

Analysis of nucleic acids. Samples of nucleic acids were hydrolysed in 1 N-HCl at 100° for 1 hr., and were analysed by column chromatography on a column (0.5 cm. $\times$ 10 cm.) of Dowex 50 (H⁺ form), with as eluent at first 1 N-HCl then a 1-3 N-HCl exponential gradient, the rate of flow being 25 ml./hr. The E_{260} was measured on each fraction of the effluent, and radioactivity was determined as described by Craddock & Magee (1967). The amount of 7-methylguanine could be calculated from the total radioactivity of the 7-methylguanine peak, assuming that the methyl group had the same specific radioactivity as the methyl group in the dimethylnitrosamine injected. The amount of guanine could be calculated from the total E_{260} of the guanine peak. Thus the percentage conversion of guanine into 7-methylguanine could be calculated. No correction was made for the small amount of naturally occurring 7-methylguanine in RNA.

RESULTS AND DISCUSSION

There is evidence that dimethylnitrosamine is metabolized by an *N*-demethylating enzyme to give an unstable alkylating intermediate, which then spontaneously methylates certain cell constituents (Magee & Barnes, 1967). The extent of methylation of liver nucleic acids is linearly related to the dosage of dimethylnitrosamine (Fig. 1). As no plateau was reached, it is probable that the enzyme responsible for *N*-demethylation is very active in liver and was not saturated at high dosages. In contrast, the decrease in methylation of kidney nucleic acids that occurred on lowering the dimethylnitrosamine dosage from 26 mg./kg. to 15 mg./kg. was much smaller. The less active *N*-demethylase in kidney may therefore be nearly saturated with substrate even at 15 mg. of dimethylnitrosamine/kg. This may explain why the minimum amount of dimethylnitrosamine needed to produce kidney tumours is rather critical: it has been found (Riopelle & Jasmin, 1963) that 0.5 mg. given to rats twice daily for 6 days produced kidney tumours in 100% of the survivors, whereas 0.2 mg. given twice daily for 30 days produced no kidney tumours. At the lower

Fig. 1. Effect of dosage of dimethylnitrosamine on the extent of methylation of liver and kidney DNA and RNA, measured 5 hr. after injection. ○—○, Liver RNA; △—△, liver DNA; ○—○, kidney RNA; △—△, kidney DNA.

dosage effectively all the nitrosamine may be metabolized by liver, whereas at a slightly higher dosage sufficient nitrosamine may reach the kidney to saturate the *N*-demethylase and produce almost maximum possible methylation of nucleic acids.

The extent of methylation of liver DNA relative to that of liver RNA is approximately constant over the dosage range studied. This indicates that, even at very low dosages, the alkylating intermediate produced by metabolism of dimethylnitrosamine in the microsomes does in fact reach the nucleus and does not all react with microsomal RNA and protein. Thus the fact that oral administration of dimethylnitrosamine at low dosages produces liver tumours (Terracini, Magee & Barnes, 1967) does not exclude reaction with DNA from being one of the relevant reactions in carcinogenesis.

Evidence has already been given to suggest that, after a high dosage of dimethylnitrosamine, the disappearance of 7-methylguanine from the nucleic acids in the intact animal is due to excision of the methylated base (Craddock & Magee, 1967). In the present experiments analysis of urine showed that after a low dosage of 1 mg./kg. most of the radioactivity in the purine fraction of urine was in 7-methylguanine. Thus even after a dosage of the carcinogen that would not produce more than minimal detectable damage to the liver, if any, the 7-methylguanine formed in nucleic acids is excreted as the intact base in urine.

The results of the experiment studying the stability of methylated DNA *in vivo* and *in vitro*

Fig. 2. Stability of 7-methylguanine in rat liver DNA, methylated by administration of 26 mg. of [^{14}C]dimethylnitrosamine ($0.31 \mu\text{C}/\text{mg.}/\text{kg.}$). (a) DNA isolated 5 hr. after injection of dimethylnitrosamine; (b) DNA from (a) incubated for 43 hr. at pH 6.9 at 37° ; (c) DNA isolated 48 hr. after injection of dimethylnitrosamine. The DNA was hydrolysed in 1 N-HCl at 100° for 1 hr. and chromatographed on Dowex 50 (H^+ form) with a 1-3 N-HCl exponential gradient. T, Pyrimidine deoxynucleotides; G, guanine; A, adenine; M, 7-methylguanine. $\circ$, E_{280} ; Δ , radioactivity. Extents of methylation of DNA, calculated from radioactivity in the 7-methylguanine peak and total E_{280} in the guanine peak and expressed as percentage methylation of guanine residues, are (a) 0.81%, (b) 0.53% and (c) 0.14%.

after a high dosage of [^{14}C]dimethylnitrosamine (26 mg./kg.) are shown in Fig. 2. The reaction leading to methylation of nucleic acids begins to take place immediately after injection of dimethylnitrosamine, and 7-[^{14}C]methylguanine is known to appear in nucleic acids as soon as $\frac{1}{2}$ hr. after treatment. At the same time a normal C_1 metabolite is formed from the dimethylnitrosamine; this takes part in the normal C_1 metabolism of the cell, and is thus incorporated into guanine, adenine and the methyl group of thymine during biosynthesis. Therefore radioactivity appears first in 7-methylguanine and later in the normal bases. In Fig. 2(a), with the DNA from animals killed at 5 hr., most of the radioactivity is in 7-methylguanine (0.81% of the guanine residues methylated), with only a small amount in the normal bases as a result of biosynthesis of DNA during this period. In Fig. 2(b), with DNA from animals killed at 5 hr. incubated for 43 hr., the radioactivity in the normal bases remains but the amount of 7-methylguanine is decreased (0.53% of the guanine residues methylated) owing to excision of the methylated base. In Fig. 2(c), with DNA from rats killed 43 hr. after those in the experiment shown in Fig. 2(a), there is a greater amount of radioactivity in the normal bases owing to synthesis of DNA during the 43 hr. period. The amount of 7-methylguanine is greatly decreased (0.14% of the guanine residues methylated). The rate of disappearance of 7-methylguanine *in vivo* is thus much greater than during incubation at pH 6.9 *in vitro*. This result could be due to dilution of the DNA containing 7-[^{14}C]methylguanine, by necrosis of the most highly methylated cells and regeneration, with synthesis of DNA labelled in the normal bases only.

The experiments were therefore repeated with lower dosages of dimethylnitrosamine that were insufficient to cause frank necrosis of liver. At 7.5 mg./kg., 6 hr. after treatment, there is some loss of cytoplasmic basophilia, but no necrosis becomes apparent for 48 hr. (Shank, 1968). Dosages of 2 mg./kg. and 1 mg./kg. caused no histologically visible necrosis. When animals were given 2 mg. of [^{14}C]dimethylnitrosamine/kg. and killed at 5 hr., the pattern of labelling of DNA (Fig. 3a) was similar to that from animals given 26 mg./kg. (Fig. 2a). However, in animals killed 43 hr. later (Fig. 3c) there was very much less incorporation of radioactivity into the normal bases than at 26 mg./kg. (Fig. 2c), suggesting that necrosis and regeneration were minimal or absent, but loss of 7-methylguanine had occurred. DNA that had been incubated for 43 hr. showed a similar disappearance of 7-methylguanine (Fig. 3b). Similar results were obtained with a 1 mg./kg. dosage of dimethylnitrosamine.

The relative rates of disappearance of 7-methyl-

Fig. 3. Stability of 7-methylguanine in rat liver DNA, methylated by administration of 2mg. of [^{14}C]dimethylnitrosamine ($5.86 \mu\text{C}/\text{mg.}/\text{kg.}$). Details of (a), (b) and (c) and experimental details are as given in Fig. 2. The extents of methylation of the DNA samples are (a) 0.050%, (b) 0.041% and (c) 0.025%.

guanine from DNA *in vivo* and *in vitro* at different dosages are shown in Fig. 4, the scale for the extent of methylation of guanine of DNA being 20 times larger for the higher dosage. At 26mg./kg. the extent of methylation of guanine has fallen to 17% of the 5hr. value at 2 days after the injection, whereas at 2mg./kg. and 1mg./kg. the

Fig. 4. Rates of disappearance of 7-methylguanine from DNA *in vivo* and on incubation after different dosages of [^{14}C]dimethylnitrosamine: (a) 26mg./kg.; (b) 2mg./kg.; (c) 1mg./kg. Δ , During incubation at pH 6.9 at 37° for the appropriate time; $\circ$, in the intact animal, killed at the times as shown. The values in parentheses are the amounts of 7-methylguanine remaining in the DNA, expressed as percentages of the value at 5 hr. after injection of dimethylnitrosamine.

corresponding values are 50% and 60% respectively. The lower value at 26mg./kg. is probably due to liver necrosis, the value at 2mg./kg. may partly represent destruction of the most highly methylated cells and at 1mg./kg. the complications of cell death may have been avoided.

The rates of depurination of 7-methylguanine from DNA *in vitro* at the extents of methylation resulting from administration of 2mg./kg. and 1mg./kg. dosages of dimethylnitrosamine are very similar, i.e. the amounts of 7-methylguanine remaining in the DNA after 43hr. dialysis are 81% and 80% respectively. This agrees with the results of Lawley & Brookes (1963) with DNA methylated with [^{14}C]methyl methanesulphonate; after incubation for 43hr. approx. 80% of the radioactivity remained bound to the DNA. The corresponding

value after 26 mg. of dimethylnitrosamine/kg. is 65%. Thus it may be that 7-methylguanine occurring at certain sites in the DNA molecule is depurinated more rapidly than at other sites. In any case, at a high dosage of dimethylnitrosamine, the rate of disappearance of 7-methylguanine from DNA is much greater in the intact animal than *in vitro* (17% remaining after 43 hr. compared with 65% *in vitro*), but the difference is much less apparent at the lower dosage (60% remaining compared with 80% *in vitro*).

The question thus arises as to whether the difference in rate of loss of 7-methylguanine from DNA *in vivo* and *in vitro* is sufficient to suggest that enzymic excision is occurring in the intact animal. The DNA is obviously not in an identical condition *in vivo* and *in vitro*. The pH of the medium may be different, although it was found that the rate of loss of 7-methylguanine from DNA dialysed at pH 7.4 was not appreciably different from that occurring at pH 6.9. As the depurination is a hydrolytic reaction, catalysis by cations present in the cell nucleus, including the histones, may be sufficient to account for the greater rate of excision *in vivo*.

It therefore does not seem necessary to postulate the existence of a specific enzyme to account for the loss of 7-methylguanine from DNA alkylated in the liver after treatment with dimethylnitrosamine, although it is conceivable that some type of 'repair' enzyme is present, but that it is inhibited by the carcinogen, or that it can be induced by administration of the carcinogen. An enzyme exists in certain bacteria that can specifically degrade DNA methylated by treatment with methyl methane-sulphonate (Strauss, 1962). It is possible that DNA methylated by treatment of bacteria or of rats with methyl methane-sulphonate has a different pattern of methylation from DNA methylated by treatment of rats with dimethylnitrosamine, so that the latter may not be susceptible to hydrolysis by the same enzyme. A study of the effect of the bacterial enzyme on DNA from rats treated with dimethylnitrosamine might therefore be worth while.

In systems where repair of DNA has been shown to take place, excision of the abnormal base is followed by repair replication. Therefore another approach that may be used to study the possibility of repair in the intact animal would be to determine whether a very low dosage of carcinogen leads to an increased incorporation of labelled precursors into DNA.

The author thanks Mr J. A. E. Jarvis and Mr R. Hunt for skilled technical assistance, Dr R. C. Shank for the values of the extent of methylation of nucleic acids after a 7.5 mg./kg. dosage of dimethylnitrosamine and Professor P. N. Magee and Dr J. J. Roberts for reading the manuscript.

REFERENCES

- Bruce, H. M. & Parkes, A. S. (1956). *J. Anim. Tech. Ass.* **7**, 54.
 Cleaver, J. E. (1968). *Nature, Lond.*, **218**, 652.
 Craddock, V. M. & Magee, P. N. (1963). *Biochem. J.* **89**, 32.
 Craddock, V. M. & Magee, P. N. (1967). *Biochem. J.* **104**, 435.
 Craddock, V. M., Villa-Treviño, S. & Magee, P. N. (1968). *Biochem. J.* **107**, 179.
 Crathorn, A. R. & Roberts, J. J. (1966). *Nature, Lond.*, **211**, 150.
 Dutton, A. & Heath, D. F. (1956). *J. chem. Soc.* p. 1892.
 Kirby, K. S. (1962). *Biochim. biophys. Acta*, **55**, 545.
 Lawley, P. D. & Brookes, P. (1963). *Biochem. J.* **89**, 127.
 Lawley, P. D. & Wallick, C. A. (1957). *Chem. & Ind.* p. 633.
 Magee, P. N. & Barnes, J. M. (1959). *Acta Un. int. Cancr.* **15**, 188.
 Magee, P. N. & Barnes, J. M. (1962). *J. Path. Bact.* **84**, 19.
 Magee, P. N. & Barnes, J. M. (1967). *Advanc. Cancer Res.* **10**, 163.
 Rasmussen, R. E. & Painter, R. B. (1964). *Nature, Lond.*, **203**, 1360.
 Riopelle, J. L. & Jasmin, G. (1963). *Rev. Canad. Biol.* **22**, 365.
 Roberts, J. J., Crathorn, A. R. & Brent, T. P. (1968). *Nature, Lond.*, **218**, 970.
 Shank, R. C. (1968). *Biochem. J.* **108**, 625.
 Strauss, B. S. (1962). *Proc. nat. Acad. Sci., Wash.*, **48**, 1670.
 Terracini, B., Magee, P. N. & Barnes, J. M. (1967). *Brit. J. Cancer*, **21**, 559.
 Villa-Treviño, S. (1967). *Biochem. J.* **105**, 625.