Biochem. J. (1967) **104**, 435

Effect of Administration of the Carcinogen Dimethylnitrosamine on Urinary 7-Methylguanine

By VALDA M. CRADDOCK and P. N. MAGEE
Toxicology Research Unit, Medical Research Council, Woodmansterne Road, Carshalton, Surrey

(*Received* 17 *November* 1966)

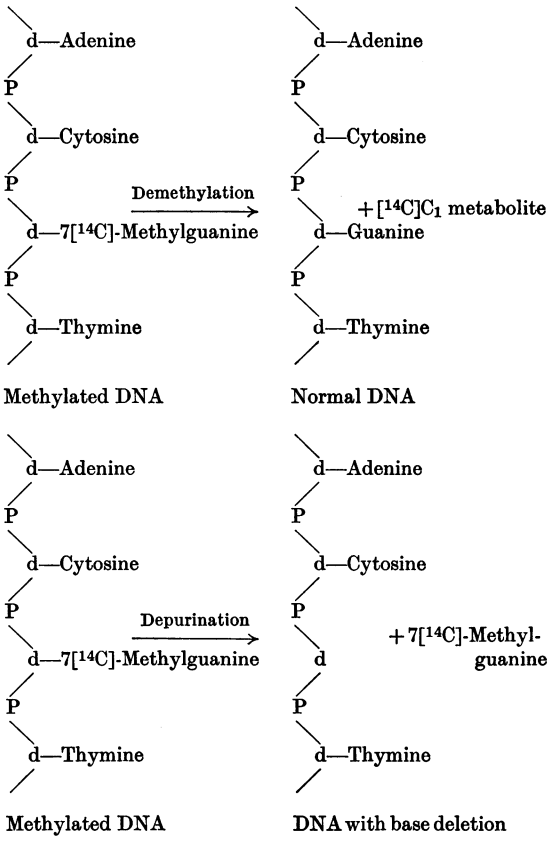
1. Evidence is presented for the excretion of 7-methylguanine in normal rat urine at a rate of approx. 65μg./day. Experiments with animals in which the nucleic acids had been prelabelled by treatment of the neonatal rats with [^{14}C]-formate gave evidence that the methylated base originated in the nucleic acids of the rat. 2. Injection of [^{14}C]dimethylnitrosamine leads to an increased excretion of 7-methylguanine, and the base becomes labelled in the methyl group. The disappearance of labelled 7-methylguanine formed in nucleic acids of rats treated with the carcinogen therefore does not take place by an *N*-demethylation reaction, but by liberation of the intact methylated base.

A single injection of dimethylnitrosamine brings about methylation of nucleic acids of liver and to a much smaller extent of kidney, the major part of the methylation being on the 7-position of guanine (Magee & Farber, 1962). The extent of methylation in each case reaches a maximum approx. 5hr. after injection, and then decreases, in the case of liver nucleic acids to very low levels within 2 days (Craddock & Magee, 1963; J. V. Frei & P. N. Magee, unpublished work). The main reason for carrying out the experiments now reported was to study the metabolic fate of the methylated DNA and RNA formed after administration of dimethylnitrosamine, as part of an assessment of the possible significance of methylation of nucleic acids in carcinogenesis. The mechanism of disappearance of the methyl group was studied. There are four conceivable reactions that could account for this: (1) enzymic *N*-demethylation of 7-methylguanine while still incorporated in the nucleic acid (Scheme 1, upper reaction); (2) splitting-out of the intact methylated base from the nucleic acid (Scheme 1, lower reaction); (3) excision of part of the nucleic acid chain, possibly by a so-called 'repair enzyme'; (4) breakdown of the entire nucleic acid molecule. In case (1), the methyl group would presumably enter the C_1 metabolic pool, and the original nucleic acid would be restored; in case (2), a nucleic acid would be produced containing a base deletion. The second type of reaction might be more likely to have biological significance. Depurination of 7-methylguanine is known to take place spontaneously from methylated DNA *in vitro* (Lawley & Brookes, 1963).

If free 7-methylguanine is formed by one of these reactions it would be expected to appear in the urine, since the methylated base, unlike guanine itself, is not rapidly deaminated in the rat, as it is not a substrate for guanase (Hitchings & Falco, 1944). 7-Methylguanine is partly converted into 8-hydroxy-7-methylguanine in man (Litwack & Weissmann, 1966), but there is evidence that this base is not formed in the rat (Mandel, Srinivasan & Borek, 1966). A first approach to the problem was therefore to look for 7-methylguanine in rat urine as a result of the administration of dimethylnitrosamine.

Normal urine from man (Krüger & Salomon, 1898*a*,*b*; Weissmann, Bromberg & Gutman, 1957) and the dog (Jackson & Entenman, 1957) is known to contain 7-methylguanine. Experiments were done to determine whether 7-methylguanine was present in normal urine of the rat. Its presence was established, and this was confirmed by work published while the present experiments were in progress (Mandel *et al.* 1966). The effect of administration of dimethylnitrosamine on the excretion of 7-methylguanine was then examined, and an increased excretion of the base was found to occur. An attempt was then made to find out the origin of the naturally occurring urinary 7-methylguanine, and of the increased amount excreted after dimethylnitrosamine treatment.

The 7-methylguanine in normal urine could conceivably have originated in the intestinal bacteria, as RNA of *Escherichia coli* is known to contain the methylated base (Dunn, 1962). Alternatively it could have originated in acid-soluble components of the tissues, or in nucleic acids. The RNA of pig liver (Dunn, 1963) and the RNA of rat liver (Villa-Trevino & Magee, 1966) are known to



Scheme 1. Possible mechanisms for the disappearance of the [14C]methyl group from methylated nucleic acids. P, phosphate; d, deoxyribose.

contain 7-methylguanine, and turnover of RNA appears to be a likely source of the urinary methylated base. To study this, a neonatal rat was given repeated injections of [14C]formate, and then kept for a period of 9 months. After this time, the nucleic acids of liver are known to be highly radioactive (Craddock & Magee, 1967). The urine of such a rat was examined for radioactive 7-methylguanine.

Preliminary accounts of this work have been published (Craddock & Magee, 1966; Magee, Craddock & Swann, 1966).

EXPERIMENTAL

Animals. Wistar albino rats derived from a 'specific-pathogen-free' colony of the Porton strain were maintained on M.R.C. diet 41B (Bruce & Parkes, 1956). The animals were approx. 200g. body wt. except where otherwise stated.

Materials. Sodium [14C]formate was purchased from The Radiochemical Centre (Amersham, Bucks.). Dimethylnitrosamine supplied by British Drug Houses Ltd. (Poole, Dorset) was redistilled before use. [14C]Dimethylnitrosamine was prepared by Dr D. F. Heath by the method of Dutton & Heath (1956). [3H]Dimethylnitrosamine was a gift from Dr W. Lijinsky (Division of Oncology, Chicago Medical School). We are indebted to Dr P. Lawley (Chester Beatty Research Institute) for specimens of 7-methylguanine and 1-methyladenine, and to Dr G. Hitchings (Wellcome Research Laboratories) for 1-methylhypoxanthine.

Animal experiments. Animals were housed in metabolism cages fitted with urine/faeces separators. The urine was frozen as collected.

To label the purines of nucleic acids in the intact animal, a litter of newborn rats was treated with sodium [14C]formate. Each neonatal rat was given a series of 11 injections during the first 22 days of life, the first nine being subcutaneous and the last two intraperitoneal. Each rat was given a total of 52·5μc of [14C]formate. The rats were maintained on a normal diet after weaning.

Treatment of urine. The purines were precipitated from urine essentially by the method of Jackson & Entenman (1957). The urine was adjusted to pH 2–3 with a few drops of conc. H_2SO_4 and centrifuged. An equal volume of M-$AgNO_3$ was added to the supernatant, and the mixture left for a short time to complete the precipitation. The precipitate was washed twice with 0·1 M-$AgNO_3$, and the purines were then released by addition of N-HCl, the mixture being stirred at intervals in the dark for 30min.

The purine preparations were fractionated on a Dowex-50 (X12) column (1 cm. × 10 cm.) with N-HCl and then either an exponential or a linear gradient to 4N-HCl. The E_{260} value was determined on each fraction of the effluent, and the u.v. spectra were recorded on a Unicam SP.800 automatic recording spectrophotometer where necessary.

Determination of radioactivity. Fractions of the effluents from column chromatography were evaporated to dryness in a stream of air. The residue was dissolved in 0·5ml. of methanolic M-Hyamine 10X and an addition was made of 10ml. of scintillator solution [0·1g. of 1,4-bis-(2-methyl-5-phenyloxazol-2-yl)benzene and 4g. of 2,5-diphenyloxazole in 1l. of toluene]. The samples were counted in a Packard Tri-Carb model 3000 liquid-scintillation counter, with internal standards to correct for quenching.

Paper chromatography. Fractions from column chromatography were concentrated by use of a rotary evaporator and examined by paper chromatography. The solvent systems used were propan-2-ol–aq. NH_3 (Markham & Smith, 1952), propan-2-ol–HCl (Wyatt, 1951), methanol–HCl (Lawley & Brookes, 1963) and 3-methylbutan-1-ol–aq. 5% (w/v) Na_2HPO_4 (Colburn, Richardson & Boutwell, 1965).

RESULTS

In an experiment in which five male rats were each given an injection of [14C]dimethylnitrosamine (30mg./kg. body wt., 200μc/rat) the urine was collected for 5hr. Labelled 7-methylguanine was found to be present at this short time-interval (Fig. 1). An experiment was carried out with a more quantitative urine collection than was possible during a short time-interval. A male rat was injected with [3H]dimethylnitrosamine (26mg./kg. body wt., 260μc/rat) and the urine collected for

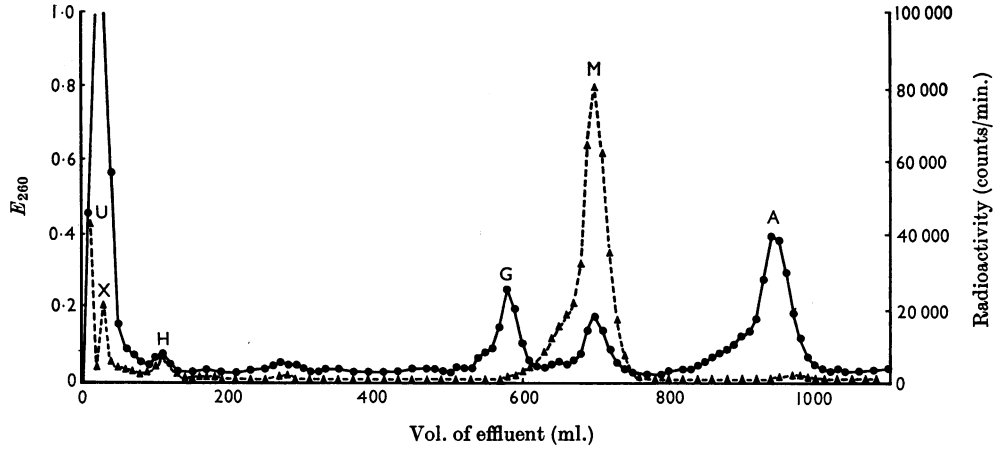


Fig. 1. Ion-exchange chromatography (Dowex 50, H^+ form) of the purine fraction of urine collected during the 5 hr. after the administration of [^{14}C]dimethylnitrosamine (30 mg./kg.). Carrier guanine, 7-methylguanine and adenine were added. Abbreviations: U, uric acid; X, xanthine; H, hypoxanthine; G, guanine; M, 7-methylguanine; A, adenine. ●, E_{260}; ▲, radioactivity (background not deducted).

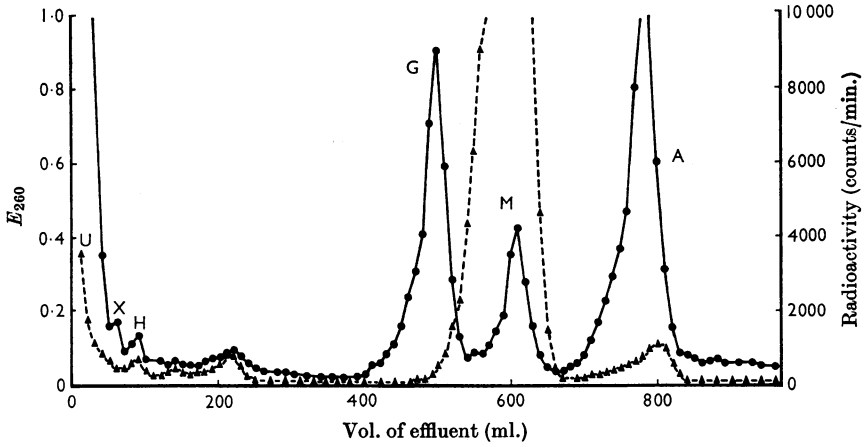


Fig. 2. Ion-exchange chromatography (Dowex 50, H^+ form) of purine fraction of urine collected during 24 hr. after the administration of [^{3}H]dimethylnitrosamine (26 mg./kg., 260 μc/rat). Carrier guanine, 7-methylguanine and adenine were added. Abbreviations are the same as in Fig. 1. ●, E_{260}; ▲, radioactivity (background not deducted).

24 hr. The results (Fig. 2) confirmed the previous experiment, in that labelled 7-methylguanine was present in the urine. In addition, a much smaller radioactive peak was found to move slightly slower than adenine and this is therefore presumably a more basic component.

To study the origin of the 7-methylguanine in urine, unlabelled dimethylnitrosamine (30 mg./kg.) was given to a 12-month-old rat that had been treated at birth with [^{14}C]formate, and the urine was collected frozen for a period of 2 days. A peak of radioactivity appeared in the 7-methylguanine position (Fig. 3), and the urinary excretion of 7-methylguanine increased about fourfold (Table 1). Since the extent of methylation by dimethylnitrosamine is highest in liver, it is reasonable to suppose that most of the increased excretion of 7-methylguanine derives from the liver. At the age of 12 months, most of the radioactivity in liver remaining from injection of tracer formate during the neonatal period is known to be in DNA, with possibly a smaller amount in RNA, and very little in

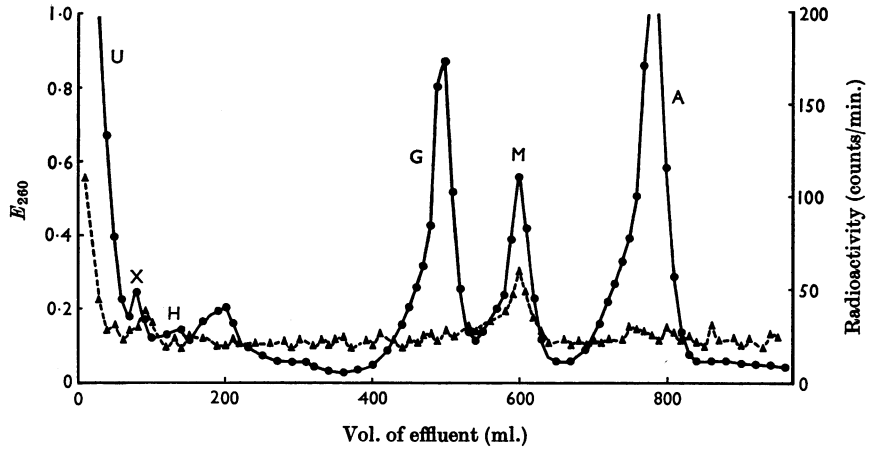


Fig. 3. Ion-exchange chromatography (Dowex 50, H^+ form) of purine fraction of urine collected during 2 days after the administration of unlabelled dimethylnitrosamine (30 mg./kg.) to a [^{14}C]formate-labelled rat. Carrier guanine and adenine were added. Abbreviations are the same as in Fig. 1. ●, E_{260}; ▲, radioactivity (background not deducted).

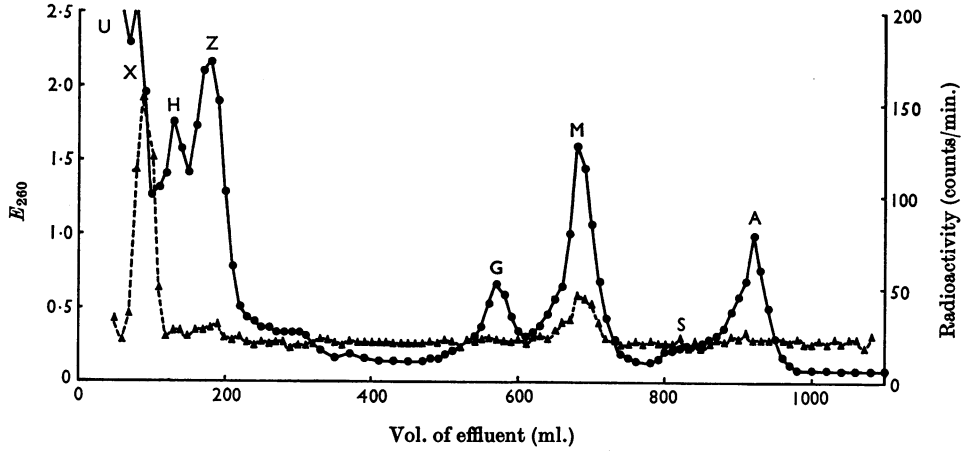


Fig. 4. Ion-exchange chromatography (Dowex 50, H^+ form) of purine fraction of urine of rat treated as a neonatal animal with [^{14}C]formate, with dimethylnitrosamine (50 p.p.m.) in the diet. Urine was collected over a 1-month period. No carriers were added. Abbreviations: S, 1-methyladenine; Z, contains 1-methylhypoxanthine; other abbreviations are the same as in Fig. 1. ●, E_{260}; ▲, radioactivity (background not deducted).

other cell components (Craddock & Magee, 1967). It is therefore probable that the radioactive 7-methylguanine (Fig. 3) appearing in response to dimethylnitrosamine arose from methylated nucleic acids rather than from smaller-molecular-weight guanine-containing compounds. Radioactivity in urinary 7-methylguanine in a 2-day volume of urine of a rat 12 months after treatment with [^{14}C]formate would not have been detectable in the absence of treatment with dimethylnitrosamine (see below). The result was confirmed when [^{3}H]dimethylnitrosamine was given to a [^{14}C]formate-labelled rat, because the ^{3}H and ^{14}C profiles both followed the E_{260} profile of 7-methylguanine. The tritium assays confirmed that another labelled compound is formed that moves more slowly than adenine.

The effect of prolonged administration of dimethylnitrosamine was studied in a rat in which the nucleic acids had been prelabelled by treatment at birth with [^{14}C]formate. The urine fractionation was carried out on a larger scale than previously, to increase the sensitivity of the test for radioactivity

Table 1. *Urinary excretion of 7-methylguanine*

The results were calculated from E_{260} profiles of 7-methylguanine obtained by column chromatography of purine fractions of urine.

Treatment of animals	No. and sex of rats	Duration of urine collection	7-Methylguanine excreted (μg./rat/day)	Body wt. (g.)
Normal	1 ♀	4 days	70	260
Normal	1 ♀	30 days	52	275
Normal	8 ♀	1 day	46	200
Normal	2 ♂	2 days	78	200
Normal	1 ♂	5 days	89	550
Dimethylnitrosamine in diet (50 p.p.m.)	1 ♀	28 days	67	280
Dimethylnitrosamine injected (mg./kg.)				
18·5	6 ♀	18 hr.	123	200
26	1 ♂	1 day	270	200
30	1 ♂	30 hr.	261	500
30	1 ♀	2 days	252	325

in the normally occurring 7-methylguanine peak, and also to give sufficient of the urinary purines for further investigations. The urine was collected frozen during the ninth month of life (437ml.). After collection of the normal urine, at the end of the ninth month, the rat was fed on a diet containing dimethylnitrosamine for a period of 2 months. The dietary concentration of dimethylnitrosamine used (50 p.p.m.) is known to be carcinogenic for liver. During the second month the urine was collected frozen (394ml.). Both samples were subjected to the usual fractionation procedure and column chromatography, in this case without the addition of carriers. Fig. 4 shows the purines of the urine of the animal that had been fed on a diet containing dimethylnitrosamine. The sample of normal urine gave an essentially similar picture. Peak M was examined by paper chromatography, and by its absorption spectra after elution from the chromatogram. The spectral characteristics at pH 2·1 ($\lambda_{max.}$ 250mμ, $\lambda_{min.}$ 228mμ), at pH 6·1 ($\lambda_{max.}$ 283mμ, $\lambda_{min.}$ 260mμ) and at pH 9·0 ($\lambda_{max.}$ 282mμ, $\lambda_{min.}$ 260mμ) were identical with that of authentic 7-methylguanine similarly treated. The same criteria suggested that peak S was 1-methyladenine; this agrees with the results obtained by Mandel *et al.* (1966). The results gave further evidence that peak G is guanine, peak A is adenine and peak Z contains 1-methylhypoxanthine. Peaks U, X, and H contain uric acid, xanthine and hypoxanthine respectively, but other material is present in the initial four peaks from the column.

The amount of 7-methylguanine excreted was calculated from the E_{260} profile and expressed as μg./rat/day. It was compared with the value for normal rats and for animals that had received an injection of dimethylnitrosamine. The results (Table 1) show that the rate of excretion during dietary treatment with the carcinogen lies within the normal range, whereas there is a considerable increase in excretion after injection of the much larger doses of dimethylnitrosamine.

DISCUSSION

The experiments described show that 7-methylguanine is present in normal rat urine, and by use of rats that had been treated as neonatal animals with [^{14}C]formate to prelabel the nucleic acids it was shown that the base originates at least in part in the nucleic acids. The increased excretion of 7-methylguanine after administration of dimethylnitrosamine shows that the 7-methylguanine formed in liver nucleic acids after treatment with the carcinogen is excreted in the urine. Further evidence for this is the fact that the urinary 7-methylguanine has a high specific radioactivity after administration of the radioactive carcinogen. This occurs at a time when the labelling of guanine in nucleic acids by normal C_1 metabolites is very low, showing that the label is in the methyl group rather than in the purine ring. Thus the disappearance of the labelled 7-methylguanine from liver DNA and RNA formed by administration of labelled dimethylnitrosamine is due to liberation of the intact free base, and not to an *N*-demethylation reaction.

The mechanism by which free 7-methylguanine is formed is not yet clear. As the base becomes labelled as soon as 5hr. after administration of the carcinogen, and also after injection of very much lower doses (V. M. Craddock & P. N. Magee, unpublished work), it is clear that gross cellular damage is not entirely responsible. There is evidence that liver RNA is rapidly broken down after

injection of dimethylnitrosamine (P. N. Magee, unpublished work); thus some or all of the labelled 7-methylguanine appearing in urine may arise from the catabolized RNA. However, preliminary experiments with rats that had been treated at birth with [^{14}C]formate, and so containing labelled liver DNA, with very little radioactivity in the RNA when used, suggest that some of the urinary 7-methylguanine may originate in DNA. Depurination of the methylated base is known to take place spontaneously *in vitro* (Lawley & Brookes, 1963), but disappearance of labelled 7-methylguanine from DNA occurs at a greater rate in the intact animal (Craddock & Magee, 1963). It is conceivable that either an enzyme catalysed excision or a limited breakdown of DNA is taking place.

Evidence for an increase in the urinary excretion of methylated purines in tumour-bearing rats and mice (Mandel *et al.* 1966) and in human subjects with leukaemia (Park, Holland & Jenkins, 1962) has been presented. No significant increase was found on feeding rats with dimethylnitrosamine at a dietary concentration of 50 p.p.m. This does not necessarily imply that nucleic acids are not methylated under these conditions, because the extent of methylation may be so small that the increase in urinary 7-methylguanine could be within the normal range. Experiments with thioacetamide and aflatoxin show that, if there is an increase in urinary excretion of 7-methylguanine when these carcinogens are given in the diet, it is not very great under the conditions used. It is therefore necessary to study the nucleic acids of the organs concerned, rather than the urine, to study possible changes in methylation during carcinogenesis.

The authors thank Mr R. Hunt for skilled technical assistance.

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