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Nitrosamine-Induced Carcinogenesis

THE ALKYLATION OF NUCLEIC ACIDS OF THE RAT BY *N*-METHYL-*N*-NITROSOUREA, DIMETHYLNITROSAMINE, DIMETHYL SULPHATE AND METHYL METHANESULPHONATE

BY P. F. SWANN* AND P. N. MAGEE*
Medical Research Council Toxicology Research Unit, Carshalton, Surrey

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1. *N*[^{14}C]-Methyl-*N*-nitrosourea, [^{14}C]dimethylnitrosamine, [^{14}C]dimethyl sulphate and [^{14}C]methyl methanesulphonate were injected into rats, and nucleic acids were isolated from several organs after various time-intervals. Radioactivity was detected in DNA and RNA, partly in major base components and partly as the methylated base, 7-methylguanine. 2. No 7-methylguanine was detected in liver DNA from normal untreated rats. 3. The specific radioactivity of 7-methylguanine isolated from DNA prepared from rats treated with [^{14}C]dimethylnitrosamine was virtually the same as that of the dimethylnitrosamine injected. 4. The degree of methylation of RNA and DNA produced in various organs by each compound was determined, and expressed as a percentage of guanine residues converted into 7-methylguanine. With dimethylnitrosamine both nucleic acids were considerably more highly methylated in the liver (RNA, about 1% of guanine residues methylated; DNA, about 0·6% of guanine residues methylated) than in the other organs. Kidney nucleic acids were methylated to about one-tenth of the extent of those in the liver, lung showed slightly lower values and the other organs only very low values. *N*-Methyl-*N*-nitrosourea methylated nucleic acids to about the same extent in all the organs studied, the amount being about the same as that in the kidney after treatment with dimethylnitrosamine. In each case the RNA was more highly methylated than the DNA. Methyl methanesulphonate methylated the nucleic acids in several organs to about the same extent as *N*-methyl-*N*-nitrosourea, but the DNA was more highly methylated than the RNA. Dimethyl sulphate, even in toxic doses, gave considerably less methylation than *N*-methyl-*N*-nitrosourea in all the organs studied, the greatest methylation being in the brain. 5. The rate of removal of 7-methylguanine from DNA of kidneys from rats treated with dimethylnitrosamine was compared with the rate after treatment of rats with methyl methanesulphonate. No striking difference was found. 6. The results are discussed in connexion with the organ distribution of tumours induced by the compounds under study and in relation to the possible importance of alkylation of cellular components for the induction of cancer.

The remarkable carcinogenic properties of a number of *N*-nitroso compounds have been surveyed in reviews by Druckrey *et al.* (1967) and Magee & Barnes (1967). The most simple, dimethylnitrosamine, is both an acute hepatotoxin and a powerful carcinogen producing a high incidence of tumours of the liver, kidney and lung in rats. A single dose sufficient without further treatment to produce kidney tumours in the rat is completely metabolized within 5–6hr. During this short time a chemical reaction must have occurred sufficient in nature and intensity to start the growth of tumours in the kidney. The compound is converted *in vivo* into an active methylating agent that can react with proteins forming methylated histidine residues (Magee & Hultin, 1962) and with nucleic acids forming mainly 7-methylguanine (Magee & Farber, 1962; Lawley, Brookes, Magee, Craddock & Swann, 1968), and it is now known that other carcinogenic *N*-nitroso compounds also produce alkylation of guanine at N-7 (Magee & Lee, 1963; Swann, Craddock & Magee, 1965; Lee & Lijinsky, 1966). Since this discovery there has been speculation as to the role that alkylation of cellular components might play in the acute toxicity and carcinogenic potency of the nitrosamines. From metabolic

* Present address: Courtauld Institute of Biochemistry, The Middlesex Hospital Medical School, London, W. 1.

studies Heath (1962) concluded that alkylation of some cellular component, rather than any other possible chemical reaction, is responsible for the acute toxicity of these compounds, and it has since been suggested that the early inhibition of hepatic protein synthesis in acute dimethylnitrosamine poisoning may be caused by methylation of messenger RNA (Mizrahi & Emmelot, 1964; Villa-Treviño, 1967; Shank, 1968).

The biochemical lesion initiating the carcinogenic process is, however, still unknown. There is considerable circumstantial evidence that the nitrosamines are not themselves carcinogenic, but that an active carcinogen is produced in their breakdown, since in general only those nitroso compounds that break down spontaneously (e.g. *N*-methyl-*N*-nitrosourea) or that seem likely to be broken down enzymically (e.g. dimethylnitrosamine) are carcinogenic (Druckrey *et al.* 1967). Though alkylation may not be the only chemical reaction occurring as a result of this breakdown, we have taken as a working hypothesis that alkylation of some cellular constituent is responsible for the carcinogenic action of the nitrosamines.

N-7 of guanine is the most reactive site in nucleic acids towards alkylation *in vitro*, so that 7-methylguanine is the major methylated base produced by the incubation of nucleic acids or nucleosides with the methylating agents dimethyl sulphate (Lawley, 1957; Reiner & Zamenhof, 1957), methyl methanesulphonate (Brookes & Lawley, 1961) and diazomethane (Haines, Reese & Todd, 1962). Dimethyl sulphate and *N*-methyl-*N*-nitrosourea (Swann *et al.* 1965) and methyl methanesulphonate (Swann, 1967), as well as dimethylnitrosamine (Magee & Farber, 1962), if injected lead to the formation of 7-methylguanine in the nucleic acids of the intact rat. In the experiments described below we investigated the relation between the carcinogenic potency of a single dose of dimethylnitrosamine, *N*-methyl-*N*-nitrosourea, dimethyl sulphate and methyl methanesulphonate and the amount of methylation of nucleic acids that the same dose of each produces in different organs of the treated rat.

Preliminary reports of part of this work have appeared (Swann *et al.* 1965; Swann, 1967; Magee, Craddock & Swann, 1967).

MATERIALS AND METHODS

Animals. Wistar albino rats of the Porton strain were maintained on M.R.C. diet 41B (Bruce & Parkes, 1956).

Chemicals. Dimethylnitrosamine was obtained from British Drug Houses Ltd. (Poole, Dorset) and purified by distillation (b.p. 151°). Methyl methanesulphonate was obtained from Aldrich Chemical Co. (Milwaukee, Wis., U.S.A.) and redistilled (b.p. 83–84°/6·5 mm. Hg). *N*-Methyl-*N*-nitrosourea was prepared by the method of Werner (1919). *N*-Methylurea (0·06 mole) was dissolved in water (40 ml.) with $NaNO_2$ (0·06 mole), and 1·5 M-H_2SO_4 was added until the solution reached pH 3–4. During the addition of the acid the solution frothed and a mass of crystals rose to the surface. These were filtered on a sintered-glass disk and washed four times with ice-cold water. The crystals were dissolved in warm ethanol–water (1:1, v/v) containing a little acetic acid, and recrystallized by cooling. The crystals were washed with ethanol, ethanol–diethyl ether (1:1, v/v) and diethyl ether. The pale-yellow plates had m.p. 122–124° (decomp.) (decrepitation began at about 110°). A strong warning should be given that *N*-methyl-*N*-nitrosourea is among the most potent and versatile of known carcinogens. It has produced tumours in many organs of the rat after a single dose (Druckrey, Steinhoff, Preussmann & Ivancovic, 1964) and it produces skin tumours by topical application (Graffi, Hoffmann & Schütt, 1967). Utensils may be decontaminated by immersion in NaOH solution.

Radioactive compounds. *N*[^{14}C]-Methylurea and [^{14}C]-dimethyl sulphate (specific radioactivity 1·42 mc/m-mole) were purchased from New England Nuclear Corp. (Boston, Mass., U.S.A.). [^{14}C]Methyl iodide was purchased from The Radiochemical Centre (Amersham, Bucks.). [^{14}C]-Dimethylnitrosamine was prepared by Dr D. F. Heath by a previously published method (Dutton & Heath, 1956). The radioactive material (4·8 mc/m-mole; 3·06 mg./ml. of water) was diluted with unlabelled dimethylnitrosamine before use. *N*[^{14}C]-Methyl-*N*-nitrosourea (specific radioactivity 40 μc/m-mole) was prepared by Dr D. F. Heath by the method described for the unlabelled material. A solution in sodium citrate buffer, pH 6·5, was prepared by Dr Heath from the crystalline material. This was given to the animals within 45 min. of preparation. [^{14}C]Dimethyl sulphate was diluted with redistilled commercial dimethyl sulphate before use. 7[^{14}C]-Methylguanine (specific radioactivity 30 mc/m-mole) synthesized by the reaction of [^{14}C]methyl iodide and guanosine (Jones & Robbins, 1963) was prepared by Dr A. R. Mattocks. It was purified by ion-exchange chromatography on Dowex 50 (H^+ form). Two samples of [^{14}C]methyl methanesulphonate were used. The first, a gift from Dr P. Brookes and Dr P. D. Lawley, was prepared by the reaction of [^{14}C]methyl iodide in ethereal solution with an excess of silver methanesulphonate. The material (29·7 mc/m-mole) was supplied in ethereal solution. The second sample (0·286 mc/m-mole) was prepared in a vacuum line by the same reaction, but no solvents were used. The radiochemical homogeneity of this sample was checked by gas–liquid chromatography on a Perkin–Elmer model 800 gas chromatograph (Perkin–Elmer Corp., Norwalk, Conn., U.S.A.). The effluent gas stream was split: part was fed to a flame ionization detector and the remainder was led through CuO at 700° to oxidize the organic material. The $^{14}CO_2$ produced was passed through the dry anthracene-packed cell of a Packard model 317 flow detector (Karmen, McCaffrey & Bowman, 1962). The chromatography conditions were: column (6 ft. × 0·25 in.) packed with either 10% Apiezon L on Chromosorb P or silicone gum rubber on Chromosorb W; column temperature 100°; carrier gas N_2 (24 ml./min.). All the radioactivity was associated with the methyl methanesulphonate and there was no detectable [^{14}C]methyl iodide or [^{14}C]methanol.

Measurement of radioactivity. All radioactivity measurements were made in scintillation counters (Packard Instru-

ment Co., La Grange, Ill., U.S.A.) by conventional methods. Corrections from counts/min. to disintegrations/min. were normally made by the addition of standard [^{14}C]toluene (Packard Instrument Co.), but where the solutions were highly radioactive the method of pulse-height shift was used (Baillie, 1960). Two scintillation solutions were used: 0·6% 2,5-diphenyloxazole in toluene was used for samples that did not contain water, and 1% 2,5-diphenyloxazole, 0·05% 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene and 8% naphthalene dissolved in dioxan–Cellosolve–toluene (3:3:1, by vol.) (Bruno & Christian, 1961) was used for aqueous samples.

Conduct of animal experiments. (*a*) General procedure. The animals were allowed access to water, but they were not given food for 20hr. before they were killed by bleeding from the abdominal aorta after anaesthesia with sodium pentobarbital. The organs were removed and frozen in liquid N_2.

(*b*) Dimethyl sulphate. A 10μl. sample of [^{14}C]dimethyl sulphate (13·6mg.; 12·5μc) was mixed with 0·5ml. of 0·1M-citrate buffer, pH7·4, and injected into the tail vein of each of six male rats (each 150–180g.), which were killed 4hr. later.

(*c*) Methyl methanesulphonate. In the first and second experiments 2ml. of [^{14}C]methyl methanesulphonate solution in diethyl ether (270μc and 1mg./ml.) from the stock made by Dr Brookes and Dr Lawley was mixed with carrier methyl methanesulphonate (0·153ml., i.e. 198mg., in 5·5ml. of 0·9% NaCl). The ether was blown off in a gentle stream of air. In both experiments 0·5ml. of this solution (18mg., 120mg./kg. body wt.) was injected into the tail vein of each of ten male rats (each 150g.), which were killed 16hr. later. In the first experiment three rats died during the 16hr. and two rats were moribund. These two and the other five were killed. In the second experiment all the rats (ten males) survived. In the third experiment, [^{14}C]methyl methanesulphonate (0·10ml., i.e. 129mg.; 350μc) from the sample synthesized in the vacuum line was pipetted into 0·9% NaCl (3·5ml.). A 0·5ml. sample was given to each of two groups of seven male rats (each 150g.), which were killed 4hr. and 16hr. later. In the second and third experiments samples of the plasma of each rat were taken at the time they were killed. The radioactivity of these was determined to ensure that each rat had received a similar dose.

(*d*) *N*-Methyl-*N*-nitrosourea. *N*-Methyl-*N*-nitrosourea (7·8mg./ml.; 3·35μc/ml.) was dissolved in sodium citrate buffer, pH6·5. A 1ml. sample was given either by a metal cannula into the stomach (nine females) or intravenously (ten males) to each rat (80–100g.). They were killed 4hr. later.

(*e*) Dimethylnitrosamine. Six male rats were each given [^{14}C]dimethylnitrosamine (27mg./kg. body wt.; 182μc) by intraperitoneal injection. The rats were killed 5hr. later.

Preparation of nucleic acids. Both RNA and DNA were prepared from the same sample of tissue by the method of Kidson, Kirby & Ralph (1963), except that phenol–*m*-cresol–8-hydroxyquinoline–water (100:14:0·1:11, by wt.) was used in place of 90% phenol. The DNA was freed of RNA by ribonuclease treatment, and carbohydrates were removed from both RNA and DNA by the methoxyethanol–phosphate procedure (Kirby, 1956). The nucleic acids were finally precipitated by the addition of an equal volume of 1% (w/v) cetyltrimethylammonium bromide. The cetyltrimethylammonium salt was washed with water and converted into the sodium salt by treatment with 2% (w/v) sodium acetate in 70% (v/v) ethanol. The sodium salt of the nucleic acid was then washed with ethanol, ethanol–diethyl ether (1:1, v/v) and diethyl ether and dried *in vacuo*.

Determination of the proportion of guanine residues methylated. The nucleic acids were hydrolysed in 1N-HCl and chromatographed as described by Magee & Farber (1962). In all cases there was a radioactive peak eluted from Dowex 50 between guanine and adenine (Fig. 1). This peak was previously identified as 7-methylguanine when found after dimethylnitrosamine (Magee & Farber, 1962) and methyl methanesulphonate (Swann, 1967) poisoning. The amount of 7-methylguanine was calculated from the amount of radioactivity in the peak of 7-methylguanine, assuming that the specific radioactivity was the same as that of the methyl group of the injected methylating agent (see below). The amount of guanine was calculated from the extinction of the peak of guanine by taking ϵ_{260} in acid to be 8000.

Specific radioactivity of the 7-methylguanine found in DNA of rat liver after a single dose of dimethylnitrosamine. The [^{14}C]dimethylnitrosamine used in this experiment was purified by distillation from 3N-NaOH (Dutton & Heath, 1956). The distillate was adjusted to pH5–6 by the addition of a single drop of dil. H_2SO_4, saturated with NaCl and redistilled. The amount of dimethylnitrosamine was determined polarographically (Heath & Jarvis, 1955) and the radioactivity measured. This [^{14}C]dimethylnitrosamine was diluted with redistilled carrier dimethylnitrosamine and given by intraperitoneal injection in 0·9% NaCl to 20 female rats (each 200g.). The specific radioactivity of the injected solution was 23·63μc/m-mole. The rats were anaesthetized 5hr. after injection, the livers removed and RNA and DNA prepared from them as described above. The DNA (196mg.) was hydrolysed in 20ml. of 1N-HCl by heating it at 100° for 1hr. The hydrolysate was chromatographed on a Dowex 50W (X12; H^+ form) column (22cm.×1·75cm.) with a 0·25N–3N-HCl gradient, and the effluent was collected in fractions (10ml.). $E^{1cm.}_{260}$ of each fraction was measured, and the radioactivity in 0·1ml. from each fraction was determined. The fractions containing the major part of the 7-methylguanine were pooled and evaporated to dryness. The residue was taken up in water and applied in a 15cm. streak to Whatman 3MM paper and chromatographed with the propan-2-ol–aq. NH_3 system of Markham & Smith (1952). After the paper had been dried the position of 7-methylguanine was discovered by the use of an ultraviolet lamp, and the paper was cut into strips perpendicular to the direction of flow. Each strip was then eluted with water (approx. 5ml.). To each sample 1ml. of 0·33M-potassium phosphate buffer, pH7, was added and the spectrum was recorded on a recording spectrophotometer (SP.800; Unicam Instruments, Cambridge). The blank was taken as the adjacent strip, on the side nearer to the origin. The amount of 7-methylguanine was determined assuming ϵ_{247} 5890, ϵ_{260} 3890 and ϵ_{282} 7240 (Haines *et al.* 1962). The method was checked by passing synthetic 7[^{14}C]-methylguanine of known specific radioactivity (a gift from Dr A. R. Mattocks) through the procedure.

Test for the amount of 7-methylguanine in normal

DNA. The method used above to measure the specific radioactivity of 7-methylguanine was used to search for 7-methylguanine in normal rat liver DNA. DNA was prepared from the livers of 35 female rats (each 200g.). To this DNA (386mg.), 7[^{14}C]-methylguanine was added (0·22μc, 1·17μg.) as tracer, and 7-methylguanine was isolated from the acid hydrolysate by column and paper chromatography in the manner described in the preceding section.

Elimination of 7-methylguanine from rat kidney DNA after a single dose of dimethylnitrosamine or methyl methanesulphonate. A group of 35 female rats (each 140g.) were each given [^{14}C]methyl methanesulphonate (120mg./kg. body wt. in 0·5ml. of 0·9% NaCl) by intravenous injection. Four rats died overnight. Four rats were killed at 4, 8, 12 and 19hr. Five were killed at 48 and 72hr. DNA was prepared from the pooled kidneys of the rats killed at each time-interval by phenol extraction. Forty female rats (150g.) were each given [^{14}C]dimethylnitrosamine (30mg./kg. body wt. in 1ml. of 0·9% NaCl) by intraperitoneal injection. Five rats were killed under Nembutal anaesthesia at 4, 11, 19, 24, 41, 48, 72 and 96hr., and DNA was prepared from the pooled kidneys of the rats killed at each time-interval. In both experiments the rats were allowed free access to water and M.R.C. diet 41B throughout. The proportion of guanine residues methylated was determined as above.

RESULTS

After administration of all the alkylating agents the only methylated base found in the nucleic acid hydrolysates appeared as a radioactive peak eluted from Dowex 50 between guanine and adenine (Fig. 1). This peak had previously been identified as 7-methylguanine when found after dimethylnitrosamine (Magee & Farber, 1962) and methyl methanesulphonate (Swann, 1967) poisoning. Small amounts of other methylated bases had been found after methyl methanesulphonate had been allowed to react with RNA and DNA *in vitro* (Lawley & Brookes, 1963), and in RNA and DNA of rat liver after an injection of dimethylnitrosamine *in vivo* (Lawley *et al.* 1968), but no search was made for these. The amount of 7-methylguanine was determined from measurement of radioactivity by assuming that the specific radioactivity of the 7-methylguanine was the same as that of the methyl groups in the methylating agent. This assumption was tested, but only for dimethylnitrosamine. After isolation and purification of the 7-methylguanine by column and paper chromatography as described in the Materials and Methods section, the amount was determined by measuring the extinction of the buffered solution, pH7, at 247mμ, 260mμ and 282mμ. The method was checked by adding 7[^{14}C]-methylguanine of known specific radioactivity to liver DNA from normal untreated rats. The amount of 7[^{14}C]-methylguanine added was similar to the amount expected to be in a similar weight of liver DNA from dimethylnitrosamine-treated rats. The 7[^{14}C]-methylguanine added to the normal rat DNA was recovered with a specific radioactivity 94% of the starting material when measured at 247mμ, 90·4%

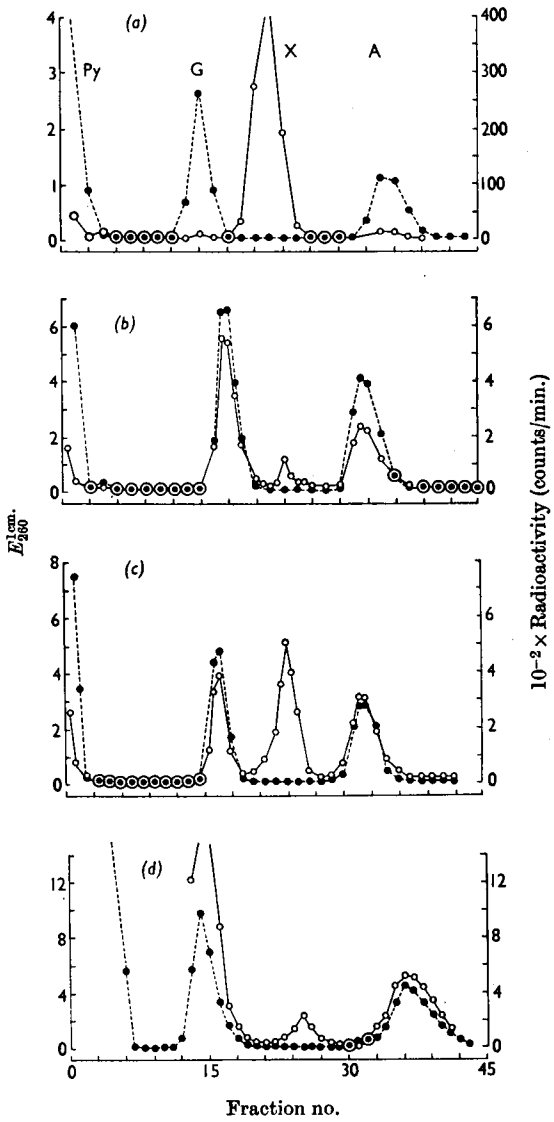

Fig. 1. Ion-exchange chromatography of liver RNA from rats given dimethylnitrosamine (*a*), dimethyl sulphate (*b*), *N*-methyl-*N*-nitrosourea (*c*) and methyl methanesulphonate (*d*). The isolated nucleic acids were hydrolysed in 1N-HCl at 100° for 1hr. and chromatographed on Dowex 50 (X12; H^+ form) with exponential 1N→4N-HCl gradient elution. ●, E_{260}; ○, radioactivity. Py, pyrimidine nucleotides; G, guanine; A, adenine. The radioactive peak X was shown to be 7-methylguanine when found after administration of dimethylnitrosamine (Magee & Farber, 1962) and methyl methanesulphonate (Swann, 1967). Fractions (10ml.) were collected.

Table 1. *Lifetime in the rat of single doses of dimethylnitrosamine, dimethyl sulphate, methyl methanesulphonate and* N-*methyl*-N-*nitrosourea*

Compound	Dose (mg./kg. body wt.)	Route	Approx. lifetime	Reference
Dimethylnitrosamine	30	Intraperitoneal	5–6hr.	Heath (1962)
Dimethyl sulphate	50	Intravenous	<5min.	Swann (1968)
Methyl methanesulphonate	100	Intravenous	1½hr.	Swann (1968)
N-Methyl-*N*-nitrosourea	90	Intravenous	15min.	Swann (1968)
N-Methyl-*N*-nitrosourea	90	Intragastric	2–3hr.	Swann (1968)

when measured at 260mμ and 97·0% when measured at 282mμ. The 7[^{14}C]-methylguanine isolated from the liver DNA of the dimethylnitrosamine-treated rats had a specific radioactivity 106% of that of the methyl groups in the injected [^{14}C]dimethylnitrosamine when measured at 247mμ, 98% when measured at 260mμ and 102% when measured at 282mμ.

7-Methylguanine is known to be present in small amounts in pig liver RNA (Dunn, 1963) and there was incorporation of radioactivity from [*Me*-^{14}C]-methionine into 7-methylguanine in RNA from normal rat liver. However, no absorption peak in the ultraviolet due to 7-methylguanine was seen in hydrolysates of DNA (Shank & Magee, 1967) and there was no incorporation of radioactivity from [*Me*-^{14}C]methionine into 7-methylguanine in rat liver DNA, and it was inferred that no 7-methylguanine was present (Craddock, Villa-Treviño & Magee, 1968). A similar method of isolating 7-methylguanine by a sequence of column and paper chromatography was used to search for 7-methylguanine in the DNA (386mg.) of livers from 35 male rats. A trace of 7[^{14}C]-methylguanine was added to the DNA; the DNA was hydrolysed and the 7-methylguanine reisolated. About 65% of the radioactivity added to the hydrolysate was recovered by elution from a single strip of chromatography paper, but there was no discernible spectrum of 7-methylguanine in this sample. At 282mμ an extinction of 0·1, which is probably the minimum for certain detection, is equivalent to 7·55μg. of 7-methylguanine. Allowing for the 35% either lost during the isolation or present in adjacent strips of the paper, the original DNA sample (386mg.) could not have contained more than 11μg. of 7-methylguanine.

The 7-methylguanine produced by alkylating agents did not remain permanently in the nucleic acids of the rat *in vivo*, but was metabolically unstable and was rapidly removed from both RNA and DNA (Craddock & Magee, 1963). The greatest methylation produced by dimethylnitrosamine was reached at a time just after the major part of the compound had broken down; as soon as formation of the active metabolite ceased the amount of 7-methylguanine fell to less than half the maximum within 24hr. Thus to find the maximum methylation of the nucleic acids it was necessary to know the approximate lifetimes of the doses of the alkylating agent *in vivo*. These were measured as described in the following paper (Swann, 1968); they are given in Table 1.

The amount of methylation produced by each methylating agent is given in Table 2. The most noteworthy results were obtained in the kidney and brain. Dimethylnitrosamine, *N*-methyl-*N*-nitrosourea and methyl methanesulphonate each produced a similar amount of methylation of kidney nucleic acids, and *N*-methyl-*N*-nitrosourea and methyl methanesulphonate each produced a similar amount of methylation of the DNA of the brain (Fig. 2).

It is possible that variations in the rate of removal of 7-methylguanine might influence the pathological response to methylation. For this reason the rate of disappearance of 7-methylguanine from the DNA of the kidney after treatment of rats with methyl methanesulphonate and dimethylnitrosamine was measured (Fig. 3). The amount of methylation of kidney DNA produced by methyl methanesulphonate was initially higher than that produced by dimethylnitrosamine, but the value fell more rapidly, so that after 20hr. they were similar and thereafter decreased at about the same rate.

As part of this investigation, tests for carcinogenic activity were carried out with dimethyl sulphate given by various routes (B. Terracini & P. N. Magee, unpublished work): a single intragastric dose of *N*-methyl-*N*-nitrosourea (D. D. Leaver, P. F. Swann & P. N. Magee, unpublished work) and a single intravenous dose of methyl methanesulphonate. The results of these and other previous investigations (Magee & Barnes, 1959; Druckrey *et al.* 1964) are given in Table 3.

DISCUSSION

These experiments were in two parts; in this paper we report the methylation of guanine produced in the nucleic acids of organs of the rat by administration of two *N*-methylnitrosamines and

Table 2. *Methylation of nucleic acids of the rat by dimethylnitrosamine, dimethyl sulphate,* N-*methyl*-N-*nitrosourea and methyl methanesulphonate*

The amount of 7-methylguanine is expressed as a percentage of the total guanine. The method of calculation is given in the Materials and Methods section. i.v., Intravenous; i.p., intraperitoneal.

Compound	Dose (mg./kg.) and route	No. and sex of rats	Time after dose (hr.)	7-Methylguanine (%) Liver DNA	Liver RNA	Kidney DNA	Kidney RNA	Lung DNA	Lung RNA	Small intestine DNA	Small intestine RNA	Large intestine DNA	Large intestine RNA	Stomach DNA	Stomach RNA	Testis DNA	Testis RNA	Brain DNA
Dimethylnitrosamine	27 i.p.	6 Males	5	0·87	1·16	0·106	0·16	0·058	0·065	0·0019	0·0039					~0·0007	~0·0006	
Dimethyl sulphate	80 i.v.	6 Males	4	0·008	0·007	0·014	0·01	0·052	0·03									0·046
N-Methyl-*N*-nitrosourea	90 Oral	9 Females	4	0·12	0·17	0·075	0·11							0·15	0·25			
N-Methyl-*N*-nitrosourea	90 i.v.	10 Males	4	0·12	0·15	0·11	0·13	0·095	0·12	0·072	0·125	0·078	0·11					0·085
Methyl methanesulphonate	120 i.v.	7 Males	16	0·09	0·053	0·096	0·051	0·09								0·05	0·025	0·105
	120 i.v.	10 Males*				0·09		0·11	0·068									
		7 Males†		0·107		0·11												
Methyl methanesulphonate	120 i.v.	7 Males	4	0·205	0·11	0·23	0·10											

* Expt. 2. † Expt. 3.

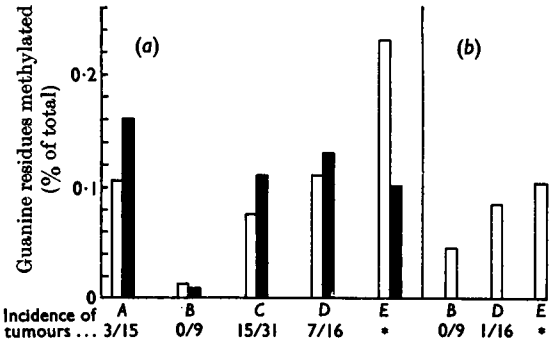

Fig. 2. Comparison between the amount of 7-methylguanine produced by (*A*) dimethylnitrosamine, (*B*) dimethyl sulphate, (*C*) *N*-methyl-*N*-nitrosourea (oral administration), (*D*) *N*-methyl-*N*-nitrosourea (intravenous administration) and (*E*) methyl methanesulphonate, in the DNA (□) and RNA (■) of the kidney (*a*) and brain (*b*) of the rat, and the incidence of tumours produced by a single dose similar to that used for the determination of methylation. The conditions are as in Table 1. *, See Table 3.

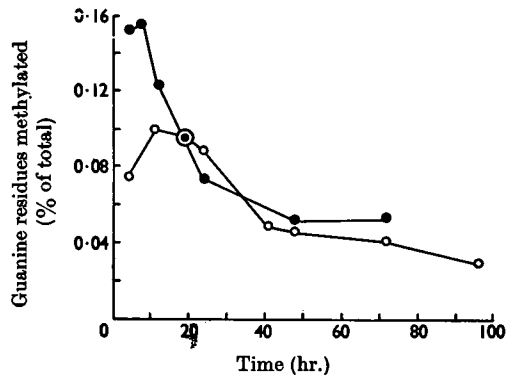

Fig. 3. Loss of 7-methylguanine from rat kidney *in vivo* after a single dose of dimethylnitrosamine (○) or methyl methanesulphonate (●). The dimethylnitrosamine was given by intraperitoneal injection, the methyl methanesulphonate by intravenous injection. For methyl methanesulphonate four rats were killed at 4, 8, 12 and 19 hr. and five killed at 48 and 72 hr. For dimethylnitrosamine five rats were killed at each time. The results are on pooled samples of DNA at each time.

two methylating agents; and in parallel with this we carried out tests for carcinogenic activity of each compound, where these had not previously been published. The purpose of these experiments was to discover whether there is a correlation between the ability of these compounds to produce 7-methylguanine in nucleic acids and their potency as carcinogens. The results (Fig. 1 and Table 2) show

Table 3. *Carcinogenic activity of compounds producing 7-methylguanine in nucleic acids* in vivo

The results in this Table are not an exhaustive report of all the carcinogenesis tests that have been carried out on these compounds, but are those where the dose of the compound used in the test for carcinogenesis was most similar to that used for the determination of the amount of methylation. i.v., Intravenous; i.p., intraperitoneal.

Compound	Dose (mg./kg.) and route	No. of rats	Tumours	Reference
N-Methyl-*N*-nitrosourea	70–100 (i.v.)	16	Squamous stomach (14), glandular stomach (1), kidney (7), large intestine (3), small intestine (2), jaw (2), brain (1), lung (1)	Druckrey *et al.* (1964)
	90 (oral)	31	Squamous stomach (24)*, kidney (15), large intestine (16), small intestine (6), jaw (2), skin (8)	D. D. Leaver, P. F. Swann & P. N. Magee (unpublished work)
Dimethylnitrosamine	30 (i.p.)	15	Kidney (3)	Magee & Barnes (1959)
Dimethyl sulphate	75–150 (i.v.)	9	None	B. Terracini & P. N. Magee (unpublished work)
Methyl methanesulphonate	120 (i.v.)	16	None	P. F. Swann & P. N. Magee (unpublished work)
	96 (i.v.)	20	Brain (1), nerve (1)	
	72 (i.v.)	20	Brain (2), spinal cord (1)	

* Nine of these were greater than 3mm. diam. The remaining were multiple pinhead hyperplastic nodules.

that administration of every one of the four compounds produces 7-methylguanine in the nucleic acids of the rat.

Differences in distribution of alkylation reflect the differing chemical reactivities of the compounds. *N*-Methyl-*N*-nitrosourea is unstable and reactive, it probably does not require enzymic activation in its breakdown, and alkylation therefore occurs in all the tissues penetrated by the compound. Dimethylnitrosamine is more stable and a high degree of methylation occurs only in those tissues, such as the liver and kidney, that have high activities of the enzyme systems required for its metabolism. If the data for the nitroso compounds are considered alone, there is considerable correlation between the amounts of alkylation of nucleic acids in a tissue and the distribution of tumours (Tables 2 and 3). The tumours induced by the nitrosamines occur in those organs in which there is a large amount of methylation of nucleic acids, and they have not been reported in organs in which the amount of alkylation is very low; for example, dimethylnitrosamine does not produce tumours in the small intestine or testes. Alkylation of the nucleic acids by nitrosamines is the result of the production of a reactive intermediate during the breakdown of the nitrosamine, and is therefore probably an index of the amount of breakdown occurring in the tissue. A similar pattern of results would have been expected if the amount of breakdown in each tissue had been measured by other means, or if another reaction, for example the the alkylation of protein, had been measured. The results therefore support the conclusion of Druckrey *et al.* (1967) that nitrosamine-induced carcinogenesis occurs as a result of a reaction that requires the breakdown of the nitrosamines, but they do not show that alkylation of nucleic acids or any other molecule is the cause of the tumours.

Though Druckrey *et al.* (1967) showed that tumours may be produced in virtually all organs of the rat by one or other nitrosamine, their work suggests that organs differ in susceptibility to the carcinogenic activity of these compounds, and therefore quantitative comparisons can only reasonably be made between the incidence of tumours and the alkylation in the same organs after each different compound. Dimethylnitrosamine given as a single intraperitoneal injection produces a significant incidence of kidney tumours (Magee & Barnes, 1959) and a single intravenous dose (90mg./kg. body wt.) of *N*-methyl-*N*-nitrosourea induces tumours in a wide variety of organs, including the kidney (Druckrey *et al.* 1964) (Table 3). Measurement on the nucleic acids of various organs showed that the degree of methylation achieved by a single intragastric dose of *N*-methyl-*N*-nitrosourea was similar to that which followed the intravenous dose. In particular, the amounts of methylation of kidney nucleic acids were similar, and similar to those found after a single renal carcinogenic dose of dimethylnitrosamine. Repeated oral dosing with *N*-methyl-*N*-nitrosourea produced only stomach tumours (Druckrey, Preussmann, Schmähl & Müller, 1961), but, if there was a connexion between the amount of methylation of nucleic acids and the induction of tumours, this large single intragastric dose of *N*-methyl-*N*-nitrosourea should produce

tumours in many organs of the rat, including the kidney. This was tested: a high incidence of kidney and other tumours was produced (D. D. Leaver, P. F. Swann & P. N. Magee, unpublished work) (Table 3).

If the carcinogenic activity of the nitroso compounds is related to alkylation then simple alkylating agents such as dimethyl sulphate and methyl methanesulphonate might also be expected to have carcinogenic activity if they produced similar amounts of methylation *in vivo*. A single intravenous dose of dimethyl sulphate of approximately the median lethal amount produced widespread methylation, particularly in the lung and brain (Table 2), but the methylation was much less than that found after the nitrosamines had been given. It would therefore be consistent if this single dose were not carcinogenic. Druckrey, Preussmann, Nashed & Ivancovic (1966) produced sarcomas by repeated subcutaneous injections of dimethyl sulphate, but tests in which the compound was given by other routes showed only very weak carcinogenic activity (B. Terracini & P. N. Magee, unpublished work).

Methyl methanesulphonate reacted with nucleic acids *in vivo* to a much greater degree than dimethyl sulphate. The extent of methylation of rat kidney DNA was greater than that produced by the dose of either *N*-methyl-*N*-nitrosourea or dimethylnitrosamine that induced a high incidence of kidney tumours (Fig. 2). It is noteworthy that methyl methanesulphonate methylated DNA to a greater degree than RNA, whereas the nitrosamines produced relatively higher methylation of RNA than DNA. Thus, though the methylation of kidney DNA was higher than with either of the nitrosamines, the methylation of RNA was lower. The methylation of brain DNA by methyl methanesulphonate was also higher than that produced by the single dose of *N*-methyl-*N*-nitrosourea that will produce a small but significant incidence of brain tumours. If the carcinogenic activity of the nitrosamines were linked to the alkylation of DNA, methyl methanesulphonate should also be carcinogenic in the kidney and brain. Three brain tumours, a tumour of a nerve and another of the spinal cord were found in rats given a single dose of methyl methanesulphonate (Table 3). None of these occurred in the rats given the largest dose, but it seems reasonable to attribute them to the effect of the compound, since no brain tumours were found in the control animals. Spontaneous brain tumours are uncommon in the rat (Snell, 1965) and only one has previously been found in the untreated rats of our colony. The production of these tumours of the nervous system is consistent with the hypothesis that alkylation of DNA and carcinogenesis are causally related. If this were so, a high incidence of kidney tumours would also be expected, since the methylation of kidney DNA is higher after methyl methanesulphonate than after either of the nitroso compounds (Fig. 2), but no tumour of the kidney was observed. The methylation of RNA of the kidney by methyl methanesulphonate is lower than with either nitroso compound but the difference is small and it is difficult to assess its significance.

The lack of a correlation between the amount of alkylation of DNA and the production of tumours could be interpreted in two ways: either that alkylation is responsible for the carcinogenic action of the nitrosamines, but alkylation of something other than DNA; or that alkylation plays no part and some entirely different reaction is involved. Some alkylating agents are indifferent carcinogens (Brookes & Lawley, 1964), but the production of brain tumours by methyl methanesulphonate, the production of kidney tumours by three doses of ethyl methanesulphonate in the mouse (Alexander & Connell, 1963) and the rat (P. F. Swann & P. N. Magee, unpublished work), and the carcinogenic properties of some lactones (Walpole, 1958; Dickens & Jones, 1961) suggest that alkylation can be sufficient stimulus to induce cancer. It is possible that the changes involved in carcinogenesis are analogous to those taking place in cellular differentiation where there is a change, not in DNA, but in the expression of genetic information. In this case the crucial site of interaction might be not with DNA, but with protein, RNA or some other cellular constituent (Pitot & Heidelberger, 1963). These ideas were invoked by Axel, Weinstein & Farber (1967) to explain the carcinogenic activity of ethionine, which, they claim, does not interact with DNA. Our results might also suggest that the alkylation of DNA is not the cause of the tumours produced by these nitroso compounds and that alkylation of some other cellular constituent is responsible, but for several reasons the results must be treated with caution.

It has been suggested that the excision of 7-methylguanine from the nucleic acids of nitrosamine-treated animals might represent a process similar to the repair of radiation damage that occurs in some bacteria (Craddock & Magee, 1965), and it is possible that the difference in the pathological response of the kidney to the action of the nitroso compounds and of methyl methanesulphonate might be caused by interference with this process. However, the excision of 7-methylguanine from kidney DNA followed a similar course whether the methylation had been effected with dimethylnitrosamine or methyl methanesulphonate (Fig. 2). It is possible that methyl methanesulphonate differs sufficiently in its chemical structure from the active methylating agent produced from the nitroso compounds that some crucial site in DNA that is

attacked by the nitroso compounds is not attacked by methyl methanesulphonate. Unfortunately the exact nature of the active methylating intermediate produced from the nitroso compounds is not known (Heath, 1962), but there is considerable similarity between the products of the reaction of methyl methanesulphonate on DNA *in vitro* and those of dimethylnitrosamine on DNA *in vivo* (Lawley *et al.* 1968). It is also possible that some cells are more highly methylated than others, with the tumours arising from these highly methylated cells, and that the distribution of methylation after dosage with methyl methanesulphonate is sufficiently different from that after dosage with the nitroso compounds to account for the observed differences in carcinogenic action.

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