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Differences in the Patterns of Methylation in Rat Liver Ribosomal Ribonucleic Acid after Reaction *in vivo* with Methyl Methanesulphonate and *NN*-Dimethylnitrosamine

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1. rRNA was isolated from rat liver at short intervals after the intraperitoneal injection of [^{14}C]methyl methanesulphonate (50mg/kg) or *NN*-di[^{14}C]methylnitrosamine (2mg/kg). These doses were chosen to minimize the effects of toxicity. 2. The following methods of hydrolysis of [^{14}C]methylated rRNA were employed: enzymic digestion to nucleosides at pH8; alkaline hydrolysis and conversion into nucleosides; acid hydrolysis to bases. 3. The methylation products were analysed by chromatography on columns of Dowex-50 (H^+ form) and Dowex-50 (NH_4^+ form). 4. With both methylating agents the principal product of methylation was 7-methylguanine. Differences were obtained, however, in the molar proportions of the minor bases 3-methylcytosine, 1-methyladenine and 7-methyladenine. Methylation at the *O*-6 position of guanine was a significant feature of rRNA obtained from the *NN*-di[^{14}C]methylnitrosamine-treated animals but was not detected in rRNA after treatment with [^{14}C]methyl methanesulphonate.

Cellular RNA species of rat liver are significant targets for reaction with alkylating agents (Craddock & Magee, 1963; Lawley *et al.*, 1968; Whittle, 1969).

The emphasis of previous reports relating to liver carcinogenesis has been placed at the overall level of reaction of alkylating agents, and usually analyses have been restricted to the major reaction site, at the N-7 position of guanine in nucleic acids (see, e.g., Swann & Magee, 1968). However, the available evidence indicates that methylation of RNA at minor sites can be an important event. For example, in the RNA-containing organisms bacteriophage $\mu 2$ and tobacco mosaic virus, the presence of only one or two methylations at minor sites is sufficient to cause inactivation (Singer & Fraenkel-Conrat, 1969*b*; Shooter, 1971). Also, evidence for miscoding has been reported for methylated poly(C) in an RNA polymerase system *in vitro* (Ludlum & Wilhelm, 1968; Ludlum, 1970; Singer & Fraenkel-Conrat, 1970), but with methylated poly(C) in an amino acid-incorporating system *in vitro* Singer & Fraenkel-Conrat (1970) failed to find evidence of miscoding. It is essential, therefore, to gain detailed information on the patterns of methylation that might be expected for the reaction of these agents with cellular RNA species, since during the early stages of carcinogenesis the alkylation of certain cellular RNA species may well be important, especially those of the mRNA type where an altered base might introduce some imbalance into the regulatory control mechanisms of the cell.

In previous comparative studies with the potent liver carcinogen *NN*-dimethylnitrosamine and with methyl methanesulphonate, which is not known to be a carcinogen in rat liver, we examined liver rRNA under conditions in which the cell may be able to deal with these modifications to rRNA structure without detectable interference from degradative changes caused by the hepatotoxicity brought about by large dosages of these agents (Craig & O'Connor, 1971; McElhone *et al.*, 1971). With the low dosage employed it was evident that alkylated rRNA was intrinsically stable *in vivo* with respect to its biological half-life and to the content of 7-methylguanine.

In the present paper the overall patterns of methylation obtained with these two agents in liver rRNA are described and compared with results on the treatment of bacteriophage $\mu 2$ RNA and rabbit reticulocyte RNA with *N*-methyl-*N*-nitrosourea and dimethyl sulphate *in vitro* (Lawley & Shah, 1972).

When liver RNA isolated from rats 5h after administration of *NN*-di[^{14}C]methylnitrosamine was analysed by paper chromatography (Lawley *et al.*, 1968), the proportions of the principal minor

methylated bases, 1-methyladenine and 3-methylcytosine, in this RNA were less than those in RNA methylated *in vitro* with methyl methanesulphonate (Lawley & Brookes, 1963).

However, the analytical procedures used were of limited quantitative accuracy. Further, the presence of a minor product, O^6-methylguanine, which would have been destroyed by the procedures of acid hydrolysis used, has since been indicated. This follows from the work of Loveless (1969) on the methylation of deoxyguanosine by *N*-methyl-*N*-nitrosourea, and by the subsequent work of Lawley & Thatcher (1970) on methylation of nucleic acids in cultured mammalian cells by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. For both these nitroso compounds, and for *NN*-dimethylnitrosamine after metabolic oxidation (Heath, 1962), the common proximate reactive molecular species is expected to be the methyldiazonium ion.

Analytical procedures have recently been developed to improve the accuracy of quantitative determination of methylation products in RNA, including O^6-methylguanosine (Lawley & Shah, 1972), and these have been applied in the present work.

Materials and Methods

Materials

[^{14}C]Methyl methanesulphonate (46mCi/mmol) in diethyl ether solution and di[^{14}C]methylamine hydrochloride (20.6mCi/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Labelled methyl methanesulphonate was diluted with chemically pure unlabelled methyl methanesulphonate before removal of ether by vacuum evaporation at −78°C and was redissolved in 0.9% NaCl for injection (0.915mCi/mmol). *NN*-Di[^{14}C]methylnitrosamine was prepared from di[^{14}C]methylamine hydrochloride by the method of Dutton & Heath (1956). The di[^{14}C]methylamine hydrochloride was diluted with an appropriate amount of unlabelled material at the beginning of the reaction. The yield was 96% from the amine and the final specific radioactivity of the *NN*-di[^{14}C]methylnitrosamine was 2.28mCi/mmol.

Materials for column chromatography were obtained as follows. Dowex-50W (X4; 200–400 mesh and minus 400 mesh), Dowex-AG1 (X8; 200–400 mesh) were from Bio-Rad Laboratories, Richmond, Calif., U.S.A., and Sephadex G-100 was from Pharmacia (G.B.) Ltd., London W.5. U.K. Acrylamide was purchased from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K. Ribonuclease-free sucrose was from Schwarz–Mann, Orangeburg, N.Y., U.S.A., and rat liver tRNA was supplied by General Biochemicals, Chagrin Falls, Ohio, U.S.A. *Escherichia coli* alkaline phosphatase (type IIIS), crude snake venom (*Crotalus adamanteus*), venom phosphodiesterase (type II) and ribonuclease (type A) were obtained from Sigma (London) Chemical Co., London S.W.6, U.K. The crude snake venom was dissolved in 0.1M-tris–HCl (pH8.9) and incubated for 15min at 37°C before passage through a column of Sephadex G-100, [2cm × 20cm, as described for step 4 of Laskowski (1966)]. This process was used to remove ribonucleosides present in the incubated crude venom. The partially purified venom phosphodiesterase, containing 12% of the original protein, 64% of the phosphodiesterase activity and 95% of the 5'-nucleotidase activity, was stored at −15°C. Phosphodiesterase activity was assayed with bis-(*p*-nitrophenyl) phosphate, and 5'-nucleotidase was assayed with 5'-AMP (Laskowski, 1966).

The following marker compounds were supplied by Sigma (London) Chemical Co.: 7-methylguanine, 3-methylcytosine, 3-methyladenine, N^6-methyladenosine and 1-methyladenine; 7-methyladenine was obtained from Cyclo Chemicals, Los Angeles, Calif., U.S.A., and O^6-methylguanosine, 1-methyladenosine, 3-methyluracil and 3-methylcytidine were obtained as described by Lawley & Shah (1972).

Methods

Animal experiments. Animals were given single injections by the intraperitoneal route and were allowed access to food and water throughout these experiments. (*a*) Methyl methanesulphonate: male Wistar rats (230–250g) were injected between 10:30 and 11:00h and killed by decapitation without anaesthesia 4h later. (*b*) *NN*-Dimethylnitrosamine: male Wistar rats (210–220g) were injected at 11:00h and killed 3h or 5h later as described above.

Purification of rat liver rRNA. The liver was removed, rinsed in ice-cold NaCl (0.9%), weighed, frozen on solid CO_2 and stored at −15°C for subsequent extraction of rRNA. Liver was homogenized in 15vol. of 6% (w/v) 4-aminosalicylate containing 1% (w/v) NaCl and shaken with an equal volume of phenol–cresol mixture (500g of phenol, 70ml of *m*-cresol, 0.5g of 8-hydroxyquinoline and 55ml of water). The rRNA was prepared as described under procedure 2 of Kirby (1965). The yield of rRNA was 4mg/g of fresh liver. Samples of rRNA were checked for the absence of DNA, after digestion to the constituent nucleosides with snake-venom exonuclease and *Escherichia coli* alkaline phosphatase, by chromatography on a column (10cm × 1cm) of Dowex-1 (formate form) by using an exponential gradient (0.01M-NH_3 to 0.03M-ammonium formate, pH4.2). In this system, nucleosides are eluted in the order cytidine, adenosine, thymidine, uridine and guanosine.

Polyacrylamide-gel electrophoresis. Gels (0.6cm × 6.0cm; 2.6% with respect to acrylamide) were prepared in a buffer solution (pH7.6) containing 36mM-tris–HCl, 30mM-NaH_2PO_4 and 1mM-EDTA (disodium salt). The concentration of bisacrylamide was 5% of that of the acrylamide. The same buffer solution was used for electrophoresis, with the addition of sodium dodecyl sulphate (2g/l). Gels were pre-run at 8mA/gel for 1h, and, after the addition of the RNA sample in the same buffer containing 15% (w/v) ribonuclease-free sucrose, electrophoresis was carried out for 80min at 5mA/gel. Gels were monitored at 265nm by using a Joyce–Loebl u.v. scanner.

Hydrolysis of rRNA. For chromatography on columns of Dowex-50 (H^+ form) samples were hydrolysed with 72% (w/v) $HClO_4$ (50μl/mg) for 1h at 100°C and diluted to 2ml for application to the column. For chromatography on columns of Dowex-50 (NH_4^+ form) digests were prepared as follows. (*a*) With $HClO_4$ as described above but adjusted to pH8.9 with NH_3 before application to the column. (*b*) By incubation in 0.3M-NaOH (0.5ml) for 18h at 37°C; the solution was adjusted to pH8.9 with HCl and incubated for 6h with bacterial alkaline phosphatase (8.4 EC units), then the sample was diluted to 2ml and adjusted to pH8.9 before application to the column. (*c*) By incubation for 18h at 37°C with partially purified venom phosphodiesterase (0.22 EC unit) in the presence of 0.1M-tris–HCl (pH8.9)–2mM-$MgCl_2$ and bacterial alkaline phosphatase (8.4 EC units); the total volume was 2ml. (*d*) By treatment with ribonuclease (0.1mg) in water (1ml), the mixture was then adjusted to pH8 with 0.1M-tris–HCl buffer, pH8.9 (0.1ml), and venom phosphodiesterase (0.01 EC unit) and bacterial alkaline phosphatase (3 EC units) were added and the mixture was incubated for 16h at 37°C. These enzymic hydrolytic procedures were tested by chromatography of a portion of the digest either on Whatman no. 1 paper or on Polygram CEL 300 UV (Camlab, Cambridge, U.K.) developed with the solvent propan-2-ol–NH_3 (sp.gr. 0.88)–water (7:1:2, by vol.). Digestion was complete as judged from the appearance of the u.v.-absorbing bands under 254nm light and the absence of u.v.-absorbing material at the origin or in the position of nucleotides.

Column chromatography of hydrolysates of rRNA. (*a*) Samples of acid hydrolysates of rRNA (equivalent to 6–12mg of RNA) were applied to columns (28cm × 1cm) of Dowex-50 (H^+ form; 200–400 mesh), which were equilibrated with 0.75M-HCl (Figs. 2 and 3 and Table 1). Columns were developed at a flow rate of 15ml/h by using a convex gradient of 0.75–2.5M-HCl; the volume of the mixing vessel was 200ml. Fractions (3ml) were collected and their u.v.-absorption was monitored at 254nm with an LKB Uvicord photometer and measured accurately with a Unicam SP. 3000 spectrophotometer. Radioactivity was assayed as described below.

(*b*) Alkaline, $HClO_4$ or enzymic hydrolysates were applied to columns of Dowex-50 (NH_4^+ form) that had been equilibrated with 0.3M-ammonium formate (pH8.9). Enzymic digests made at pH8.9 and alkaline hydrolysates were applied to columns (200–400 mesh; 83cm × 1.5cm) which were developed at a flow rate of 15ml/h, first with the same solution and then with a solution of 1M-ammonium formate, pH8.9; fractions (8ml) were collected. Enzymic digests made at pH8.0 and $HClO_4$ hydrolysates were applied to columns (60cm × 1.5cm) of Dowex-50 (NH_4^+ form, minus 400 mesh) and eluted at a flow rate of 30ml/h. A Uvicord photometer was used to monitor the effluent at 254nm. The fraction volume was 6.4ml and radioactivity was assayed as described by Lawley & Shah (1972).

Paper chromatography. For the further identification of radioactive methylated bases, pooled fractions from the effluents of columns developed with HCl were evaporated to dryness by freeze-drying, dissolved in 0.1M-HCl with the appropriate marker substances and applied individually to chromatography paper (Whatman no. 1; H. Reeve Angel and Co., London E.C.4, U.K.). Papers were developed in two directions, first by descending chromatography in solvent (1) [butan-1-ol–NH_3 (sp.gr. 0.88)–water (85:2:12, by vol.)] and then by ascending chromatography in solvent (2) [methanol–conc. HCl–water (7:2:1, by vol.)]. Areas of the paper containing the appropriate markers (3-methylcytosine, 1-methyladenine and 7-methyladenine) and adjacent control areas were assayed for radioactivity as described below. For the separation of 7-methylguanine from 3-methyladenine ascending chromatography was used with solvent (2) and the entire paper was cut into 1 cm strips for assay of radioactivity.

Determination of radioactivity. Samples (2ml) from the Dowex-50 (H^+ form) column effluents were blended with 6ml of a scintillation mixture [2,5-diphenyloxazole (16g/l) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (0.4g/l) in A.R. toluene] mixed with 6ml of Triton X-100 immediately before counting. Samples (3ml) from the Dowex-50 (NH_4^+ form) column effluents were blended with 6ml of the scintillation mixture and 12ml of Triton X-100.

Paper strips were counted for radioactivity in 10ml of a dioxan scintillation mixture [naphthalene (125g) and 2,5-bis-(5-t-butylbenzoxazol-2-yl)thiophen (3g) dissolved in 1 litre of 1,4-dioxan] mixed with 1ml of 0.1M-HCl.

A background rate was determined for each vial and the efficiency of counting was determined by using an internal standard. Samples were assayed for radioactivity in a Tracerlab or a Packard liquid-scintillation spectrometer.

Results

Analysis of alkylated rRNA

Rat liver rRNA was prepared by a phenol procedure (Kirby, 1965) that selectively extracts rRNA and a small amount of rapidly labelled RNA. Samples of alkylated rRNA were examined by polyacrylamide disc-gel electrophoresis (Fig. 1). The preparation was shown to be essentially free of tRNA and comprised two major fractions that corresponded, on the basis of their mobility in 2.6% polyacrylamide gel, to the 28S and 18S components described by Loening (1967).

Samples of rRNA were analysed for their content of radioactively labelled methylated bases by the following column chromatographic procedures, essentially as described by Lawley & Shah (1972): (*a*) Dowex-50 (H^+ form) eluted with a convex gradient of HCl and (*b*) Dowex-50 (NH_4^+ form) eluted with ammonium formate. For chromatography under procedure (*a*) rRNA samples were hydrolysed in $HClO_4$ and for procedure (*b*) $HClO_4$, NaOH and enzymic procedures were used. Details of these procedures are given in the Materials and Methods section.

Methylation of rRNA in vivo

As previously described (McElhone *et al.*, 1971) injections of methyl methanesulphonate (50mg/kg) and *NN*-dimethylnitrosamine (2mg/kg) were given intraperitoneally. The samples of RNA that form the subject of the present paper have been prepared from animals killed at the time when methylation of RNA was about maximal, i.e. at 4h for methyl methanesulphonate and at 3 or 5h for *NN*-dimethylnitrosamine (Whittle, 1969; Craddock & Magee, 1963; McElhone *et al.*, 1971). In our experiments with *NN*-dimethylnitrosamine, methylation at 3h was 90% of the reaction observed at 5h. Methylation by methyl methanesulphonate was complete by 2h. At these early times, the incorporation of radioactivity by metabolic pathways was small and was detected only in the purine residues (Figs. 2 and 3).

After methyl methanesulphonate treatment the extent of the reaction for rRNA was 0.4μmol of [^{14}C]methyl groups/g of RNA, and 0.03% of the guanine residues were converted into 7-methylguanine. With *NN*-dimethylnitrosamine the corresponding values were 1.2μmol and 0.08% (McElhone *et al.*, 1971). For comparison, the reaction with DNA after treatment with methyl methanesulphonate yielded 0.4μmol of [^{14}C]methyl groups/g and 0.05% of the guanine residues were converted into 7-methylguanine. With *NN*-dimethylnitrosamine the corresponding values were 0.5μmol and 0.05%. The difference in the extent of methylation relative to the reaction with guanine for DNA was due to the different distribution of methylation products. In calculating the extent of reaction allowance has been made for the radioactivity incorporated into guanine and adenine for RNA, and into the purine bases and thymine for DNA and on the assumption that the specific radioactivity of [^{14}C]methyl groups remained unchanged.

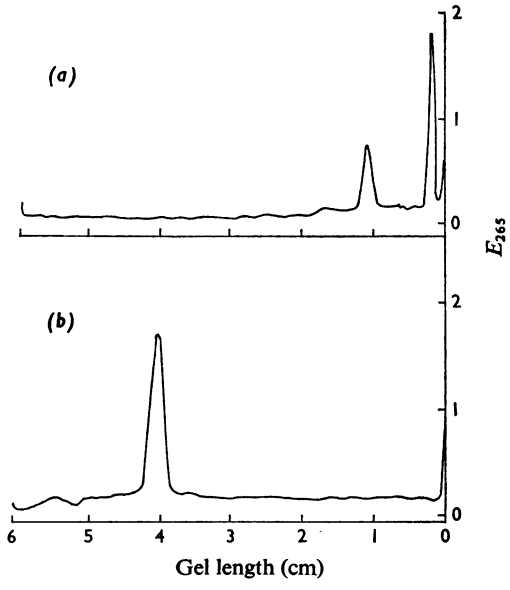

Fig. 1. *Polyacrylamide-gel electrophoresis of RNA*

The gel concentration was 2.6%. (*a*) Rat liver rRNA obtained from a treated animal killed 3h after an injection of *NN*-di[^{14}C]methylnitrosamine (2mg/kg); (*b*) marker tRNA. Details are given in the Materials and Methods section.

Patterns of methylation in rRNA

7-Methylguanine and 3-methyladenine. After methylation with methyl methanesulphonate and *NN*-dimethylnitrosamine, the principal reaction product was 7-methylguanine (Figs. 2 and 3 and Table 1). Since chromatography of acid hydrolysates on columns of Dowex-50 (H^+ form) failed to separate 7-methylguanine from 3-methyladenine the radioactive material eluted in this peak was examined by paper chromatography. It was found that in both cases this radioactive peak contained only a small amount of 3-methyladenine (Table 1). Approximately twice as much of this minor base was produced after treatment with methyl methanesulphonate as with *NN*-dimethylnitrosamine. This result was confirmed by chromatography of $HClO_4$ hydrolysates on columns of Dowex-50 (NH_4^+ form) (Table 1).

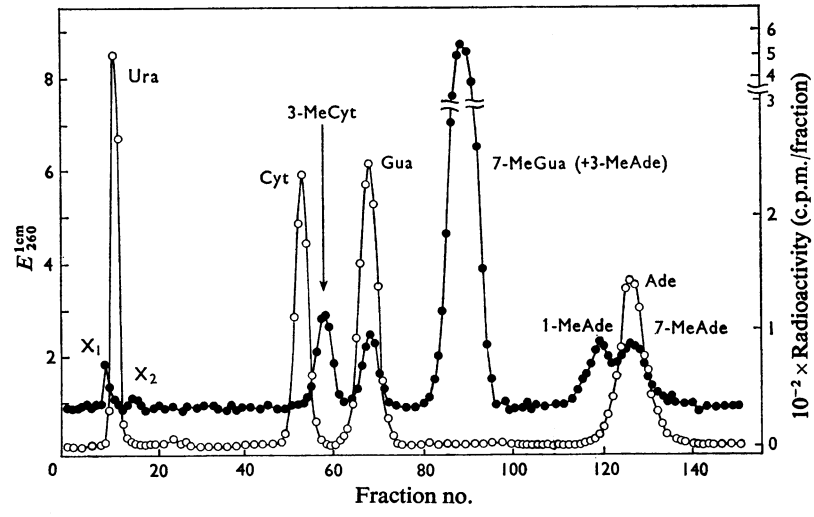

Fig. 2. *Ion-exchange chromatography on Dowex-50 (H^+ form) of an acid hydrolysate of rRNA obtained from the liver of a rat killed 4h after treatment with [^{14}C]methyl methanesulphonate (50mg/kg)*

Details of the chromatography are given in the text; o, E^{1cm}_{260} (some base-line points have been omitted); ●, radioactivity; positions of the bases are indicated as follows: Ura, uracil; Cyt, cytosine; 3-MeCyt, 3-methylcytosine; Gua, guanine; 7-MeGua, 7-methylguanine; 3-MeAde, 3-methyladenine; 1-MeAde, 1-methyladenine; Ade, adenine; 7-MeAde, 7-methyladenine; X_1 and X_2 are discussed in the text.

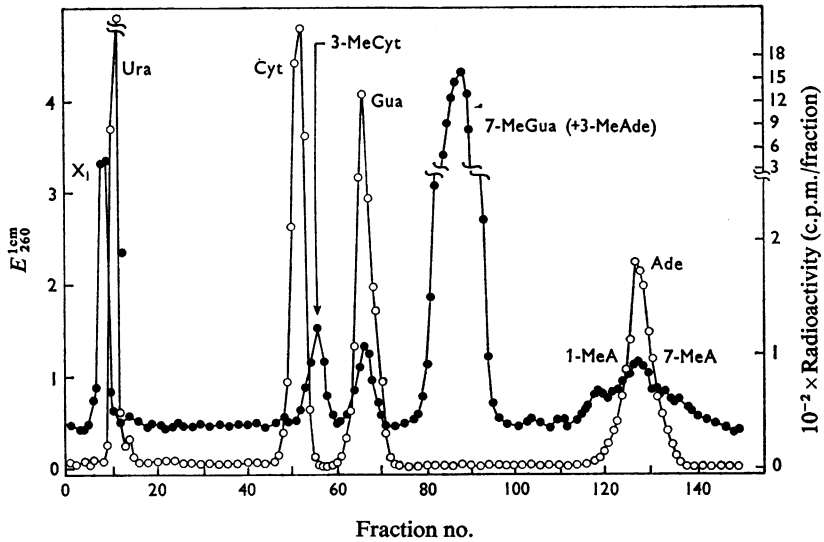

Fig. 3. *Ion-exchange chromatography on Dowex-50 (H^+ form) of an acid hydrolysate of rRNA obtained from the liver of a rat killed 3h after an injection of NN-di[^{14}C]methylnitrosamine (2mg/kg)*

Details and abbreviations are as given in Fig. 2.

Minor products analysed by Dowex-50 (H^+ form) chromatography. Column chromatography of acid hydrolysates revealed characteristic differences in the methylation of other minor bases. Methyl methanesulphonate treatment produced more 1-methyladenine and similarly, though to a lesser extent,

Table 1. *Products from the methylation of rat liver rRNA in vivo by [^{14}C]methyl methanesulphonate and NN-di[^{14}C]methylnitrosamine*

The molar proportions of the products are expressed as a percentage of the total radioactivity eluted from the columns after correction for the metabolic incorporation of radioactivity into guanine and adenine. The methods of column chromatography are denoted as *a*, Dowex-50 (H^+ form); *b*, Dowex-50 (NH_4^+ form); *c* indicates the use of paper chromatography to separate 3-methyladenine from 7-methylguanine after elution from a column of Dowex-50 (H^+ form). Precise details are given in the Materials and Methods section. ND, Not detected.

	[^{14}C]Methyl methanesulphonate						*NN*-Di[^{14}C]methylnitrosamine						
Time after injection (h)	4						3			5	3	5	
Method of hydrolysis	$HClO_4$	$HClO_4$	$HClO_4$	$HClO_4$	Phospho-diesterase (pH8.0)	Phospho-diesterase (pH8.9)	$HClO_4$	$HClO_4$	$HClO_4$	$HClO_4$	NaOH	Phospho-diesterase (pH8.0)	Phospho-diesterase (pH8.9)
Method of analysis	*a*	*a*	*a, c*	*b*	*b*	*b*	*a*	*a*	*a, c*	*b*	*b*	*b*	*b*
Products													
X_1	2.2	1.9					7.0	7.3					
X_2	0.4	0.5											
3-Methylcytosine	8.3	9.1					3.0	3.1					
1-Methyladenine	10.0	9.2					1.7	1.5					
7-Methyladenine	0.6	0.9					1.9	1.3					
7-Methylguanine	76.8*	78.4*	75.2				86.1*	87.0*	85.2				
3-Methyladenine			3.2	2.0					0.9	0.9			
O^6-Methylguanosine					<1.4	?Trace					3.5	3.7	3.7
1-Methyladenosine +N^6-methyladenosine					8.1†	6.0†					1.9‡	1.2†	1.5†
3-Methylcytidine					8.0	9.0					ND‡	2.2	2.1

* 7-Methylguanine and 3-methyladenine were eluted as a single peak from columns of Dowex-50 (H^+ form).

† Under alkaline conditions 1-methyladenosine undergoes slow rearrangement to yield N^6-methyladenosine; therefore the proportions of these two products have been summed.

‡ Under severe alkaline conditions N^6-methyladenosine was the only alkyladenosine product detected and 3-methylcytidine was completely degraded (Lawley & Brookes, 1963).

more 1-methylcytosine than was the case with *NN*-dimethylnitrosamine (Figs. 2 and 3 and Table 1). There was also a characteristic difference in the ratio of 1-methyladenine to 7-methyladenine after reaction with these agents. These two minor bases were present in approximately equal amounts, to the extent of 1–2%, after *NN*-dimethylnitrosamine treatment, but after methyl methanesulphonate treatment the molar proportion of 1-methyladenine was about 10 times that of 7-methyladenine, which again was of the order of 1% (Figs. 2 and 3 and Table 1).

Minor products analysed by Dowex-50 (NH_4^+ form) chromatography. Column chromatography of alkaline and enzymic digests on Dowex-50 (NH_4^+ form) was employed to confirm the results obtained for the molar proportions of 3-methylcytosine and 1-methyladenine (Table 1, and see footnotes about the degradation of these compounds under alkaline conditions) and also to estimate the amount of the acid-labile base O^6-methylguanine in these samples. With this system the molar proportions of the bases are estimated at the nucleoside level. After methyl methanesulphonate treatment methylation at the O-6 position of guanine, if present, was below the limit of detection (Fig. 4 and Table 1), whereas after reaction with *NN*-dimethylnitrosamine 3–4% of the total methylation products were present as O^6-methylguanosine (Fig. 5 and Table 1).

Other peaks of radioactivity. Two other peaks of radioactivity were observed and have not been positively identified. In the methyl methanesulphonate-treated series the elution profiles showed a small peak of radioactivity (X_2; Fig. 2 and Table 1) that was eluted immediately after uracil in the position of the 3-methyluracil marker. A small amount of radioactivity that was eluted in the position of peak X_2 after chromatography of hydrolysates of rRNA from the *NN*-dimethylnitrosamine-treated series was always associated with a small peak of u.v.-absorbing material (Fig. 3) and for this reason was not regarded as a putative methylated product.

The other peak, X_1, was present in the elution profiles after chromatography on Dowex-50 (H^+ form) of acid hydrolysates of rRNA after treatment with both agents (Figs. 2 and 3 and Table 1). The radioactivity was eluted with the void volume, indicating that the material was uncharged in acid solution. Since this material did not bind to the column, the radioactive material present after acid hydrolysis may not be the same after each type of treatment. However, there was significantly more radioactivity eluted with the void volume after chromatography of hydrolysates of rRNA obtained from the *NN*-dimethylnitrosamine-treated animals, although incorporation of the radioactivity into the purines from the C_1-compound pool was much higher after methyl methanesulphonate treatment (McElhone *et al.*, 1971). This difference in metabolic incorporation was presumably due to the greater dosage of methyl methanesulphonate (50mg/kg), which represented a

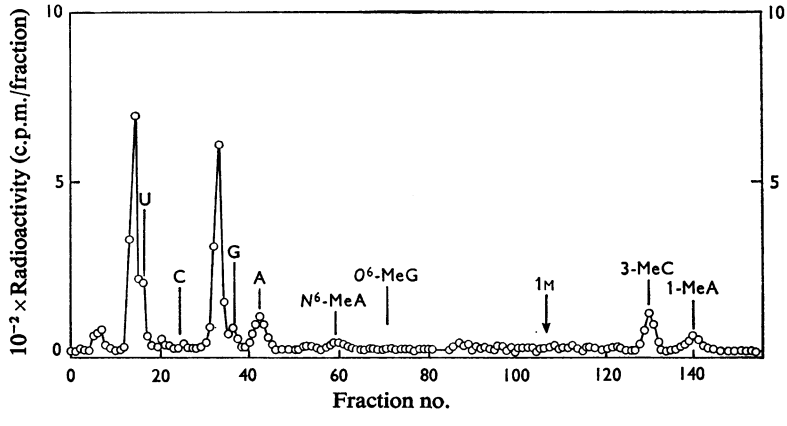

Fig. 4. *Ion-exchange chromatography on Dowex-50 (NH_4^+ form) of an enzymic hydrolysate (venom phosphodiesterase, pH 8.0) of rRNA obtained from the liver of a rat killed 4h after treatment with [^{14}C]methyl methanesulphonate (50mg/kg)*

Positions of the nucleosides are indicated as follows: U, uridine; C, cytidine; G, guanosine; A, adenosine; N^6-MeA, N^6-methyladenosine; O^6-MeG, O^6-methylguanosine; 3-MeC, 3-methylcytidine; 1-MeA, 1-methyladenosine; 1 M, indicates the change to 1 M-ammonium formate, pH 8.9. Details of the chromatography are given in the text.

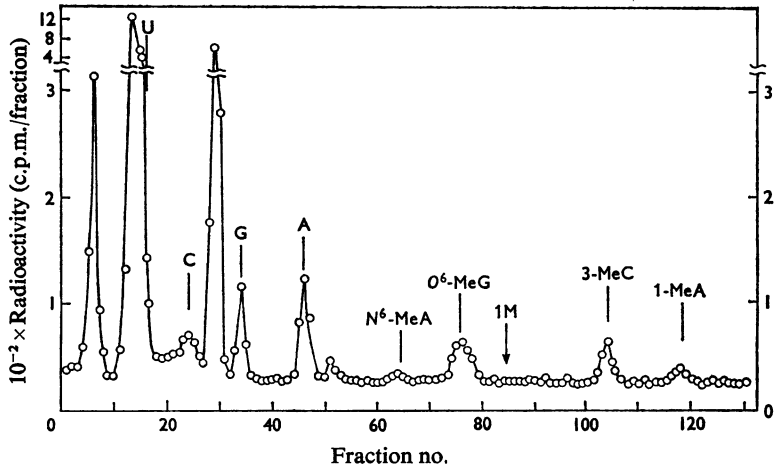

Fig. 5. *Ion-exchange chromatography on Dowex-50 (NH_4^+ form) of an enzymic hydrolysate (venom phosphodiesterase, pH 8.0) of rRNA obtained from the liver of a rat killed 5h after treatment with NN-[^{14}C]dimethylnitrosamine (2mg/kg)*

Details and abbreviations are as given in Fig. 4.

20-fold molar excess over the dose of *NN*-dimethylnitrosamine (2mg/kg) and suggests that the radioactivity in peak X_1 was not derived from the C_1-compound pool.

Discussion

The present paper is concerned with patterns of methylation present in samples of rRNA extracted from the livers of rats treated with low doses of methyl methanesulphonate and *NN*-dimethylnitrosamine. Under these conditions rRNA is stable for at least 14 days (McElhone *et al.*, 1971), and as the time of maximum methylation occurs at about 4h (Whittle, 1969; Craddock & Magee, 1963; McElhone *et al.*, 1971) after the administration of the methylating agent it is unlikely that any substantial degradative changes have taken place. Details of the methylated products described here should therefore be representative of the initial reaction within the cell.

This conclusion is supported when comparison is made with the results obtained by Lawley & Shah (1972) for the methylation *in vitro* of bacteriophage μ2 RNA and rabbit reticulocyte RNA with *N*-methyl-*N*-nitrosourea and dimethyl sulphate. These agents give essentially the same methylation patterns as reported here for rat liver rRNA, indicating that the nitroso compounds and the alkyl sulphonates react with cellular RNA species in a very characteristic way. Further, the comparison of these results emphasizes that these reagents can be distinguished on the basis of their reaction with nucleic acids (Lawley & Thatcher, 1970). *NN*-Dimethylnitrosamine and *N*-methyl-*N*-nitrosourea are envisaged to give the methyldiazonium ion as a reactive intermediate, and this in turn will methylate in part by the S_N1 mechanism (March, 1968). A significant feature after reaction with the nitroso compounds was methylation at the O-6 position of the guanine molecule (Fig. 5 and Table 1), whereas with the S_N2 compounds, methyl methanesulphonate and dimethyl sulphate, methylation at the O-6 position of guanine, if present, was below the limits of detection (Fig. 4 and Table 1). For both types of reagent 7-methylguanine was the major product, although the proportion was slightly larger with the S_N1 reagents (Table 1).

A further point emerges from the comparison of these results *in vitro* and *in vivo*, namely that the type of RNA involved in the reaction has little influence on the resultant sites of methylation, the principal determinant being the nature of the methylating agent employed. The RNA species that have been compared in this discussion are bacteriophage μ2 RNA, rabbit reticulocyte rRNA and tRNA *in vitro* (Lawley & Shah, 1972) and rat liver rRNA *in vivo*. The essentially similar patterns of methylation that are obtained suggest not only that conformation of the RNA plays at the most a minor role in the determination of these patterns, but that the accessibility of the RNA, as for instance protection of the molecule by the protein moieties of the ribosome, does not lead to a significant alteration of these patterns.

The conclusion relating to conformation would not necessarily have been expected, since previous results

have indicated that in some circumstances the secondary structure of the molecule apparently affected the methylation reaction. For example, hydrogen-bonding of reactive groups in polynucleotides can partially inhibit methylation (Ludlum, 1965; Pochon & Michelson, 1967), and the conformation of tobacco-mosaic-virus RNA was reported to influence its methylation, particularly where *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was concerned (Singer & Fraenkel-Conrat, 1969*a*,*b*). In DNA the N-3 atom of adenine is more reactive than in RNA, as shown by studies with methyl methanesulphonate (Lawley & Brookes, 1963) and with dimethyl sulphate (Lawley & Thatcher, 1970). The N-1 atom of adenine is more reactive with methyl methanesulphonate in heat-denatured DNA than in native DNA (Lawley & Brookes, 1963). However, in DNA, where the O-6 atom of guanine is involved in the base-pairing mechanism, approximately twice as much methylation occurs as in RNA (Lawley & Thatcher, 1970).

More extensive studies are required into the effects of secondary structure on the methylation process, but an important tentative conclusion to be drawn from the studies reported and discussed here is that these observed patterns of methylation might be expected in other as yet unexamined species of cellular RNA, and this may be relevant to considerations of molecular mechanisms for the biological effects of these agents.

After treatment with *NN*-dimethylnitrosamine, rat liver rRNA was methylated to a greater extent than the DNA from the same animals. An explanation for this could follow from the concept that the metabolism of *NN*-dimethylnitrosamine by the liver microsomal enzymes releases a short-lived active intermediate (Craddock & Magee, 1963) which would preferentially react with the microsomal RNA fraction when compared with DNA. The other nitroso compounds, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Lawley & Thatcher, 1970) and *N*-methyl-*N*-nitrosourea (Swann & Magee, 1968), also produced more methylation of cellular RNA than of DNA, probably by an intermediate agent identical with that for *NN*-dimethylnitrosamine, although release of this intermediate would not require enzymic action. Hydrolysis of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine to the active methylating intermediate is enhanced at neutral pH in the presence of thiols (Schulz & McCalla, 1969; Lawley & Thatcher, 1970; Wheeler & Bowdon, 1972) but this is not the case with *N*-methyl-*N*-nitrosourea (Wheeler & Bowdon, 1972). In animals or in cultured mammalian cells treated with either dimethyl sulphate or methyl methanesulphonate the extent of methylation of RNA and DNA was either similar or there was slightly more methylation of DNA (Swann & Magee, 1968; Lawley & Thatcher, 1970; and the present work). Although it seems reasonable to explain these differences on the basis of the liberation of short-lived intermediates with the S_N1 reagents, too little is known of their metabolism *in vivo* to be certain of this.

On column chromatography of hydrolysates of labelled RNA on Dowex-50 (H^+ form) an early radioactive peak was detected which preceded any of the bases, with little or no retention on the column. The relative amount of radioactivity in this peak was greater after *NN*-dimethylnitrosamine treatment than with methyl methanesulphonate. A peak of radioactivity which behaved similarly on Dowex-50 (H^+ form) was reported by Schoental (1967) after analysis of DNA treated with *N*-methyl-*N*-nitrosourethane. The fraction contained sugar moieties and it was suggested that it may contain methylated products. After reaction with *NN*-dimethylnitrosamine *in vivo* (Craddock, 1971) a similar peak was observed after analysis of RNA samples, and to a greater extent with DNA. In this case it was suggested that the material was an uncharged metabolite of formaldehyde. We have confirmed this difference between samples of RNA and DNA obtained from animals treated with *NN*-dimethylnitrosamine (M. J. Capps, P. J. O'Connor & A. W. Craig, unpublished work). However, if this material were derived from the C_1 pool more might be expected in the methyl methanesulphonate-treated series where the dosage is 20-fold higher on a molar basis than the dosage of *NN*-dimethylnitrosamine, and clearly this is not so.

It is also possible that some of this material may be derived from the alkylation of phosphomonoester or phosphodiester groups in RNA. This hypothesis is discussed by Lawley & Shah (1972), who found that reaction of *N*-methyl-*N*-nitrosourea with poly(U) yields as much as 60% of the products eluted early in the chromatographic profile of columns of Dowex-50 (H^+ form or NH_4^+ form), whereas the yield was about 8% after reaction with dimethyl sulphate.

In conclusion, it may be stated that the qualitative pattern of methylation of RNA *in vivo* or *in vitro* depends for the greater part on the methylating agent employed, rather than on the nature of the RNA involved in the reaction, and that there are consistent differences between the carcinogenic nitroso compounds and the other methylating agents examined. These differences appear to be best explained at present by the hypothesis that the common proximate methylating agent derived from the nitroso compounds is the methyldiazonium ion. To support this hypothesis further, the various products should now be examined in more detail, to determine whether the methyl group is introduced intact, as shown for 7-methylguanine by Lijinsky *et al.* (1968).

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