Methylation of Ribonucleic Acid by the Carcinogens Dimethyl Sulphate, *N*-Methyl-*N*-nitrosourea and *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine

COMPARISONS OF CHEMICAL ANALYSES AT THE NUCLEOSIDE AND BASE LEVELS

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1. The following methods for hydrolysis of methyl-¹⁴C-labelled RNA, and for chromatographic isolation and determination of the products, were investigated: enzymic digestion to nucleosides at pH 6 or 8; alkaline hydrolysis and conversion into nucleosides; hydrolysis by acid to pyrimidine nucleotides and purine bases, or completely to bases; chromatography on Dowex 50 (NH₄⁺ form) at pH 6 or 8.9, or on Dowex 50 (H⁺ form), or on Sephadex G-10. 2. The suitability of the various methods for determination of methylation products was assessed. The principal product, 7-methylguanosine, was unstable under the conditions used for determinations of nucleosides. 3- and 7-Methyladenine and 3- and 7-methylguanine are best determined as bases; 1-methyladenine and 3-methylcytosine can be isolated as either nucleosides or bases; *O*⁶-methylguanine is unstable under the acid hydrolysis conditions used and can be determined as the nucleoside; 3-methyluracil was detected, but may be derived from methylation of the ionized form of uracil. 3. Differences between the patterns of methylation of RNA and homopolyribonucleotides by the *N*-methyl-*N*-nitroso compounds and dimethyl sulphate were found: the nitroso compounds were able to methylate O-6 of guanine, were relatively more reactive at N-7 of adenine and probably at N-3 of guanine, but less reactive at N-1 of adenine, N-3 of cytosine and probably at N-3 of uridine. They probably reacted more with the ribose-phosphate chain, but no products from this were identified. 4. The possible influences of these differences on biological action of the methylating agents is discussed. Nitroso compounds may differ principally in their ability to induce miscoding in the Watson-Crick sense by reaction at O-6 of guanine. Both types of agent may induce miscoding to a lesser extent through methylation at N-3 of guanine; both can methylate N atoms, presumably preventing Watson-Crick hydrogen-bonding. *N*-Methyl-*N*-nitrosourea can degrade RNA, possibly through phosphotriester formation, but this mechanism is not proven.

Several carcinogens are known to methylate nucleic acids of target tissues *in vivo* (Magee & Farber, 1962; reviews, Magee & Barnes, 1967; Lawley, 1966).

Some recent interest in this field has concerned the nature and relative amounts of the various methylation products. Earlier studies (Lawley, 1966) showed little specificity in this respect between alkylating agents of various chemical types. This apparent lack of specificity became of particular importance in view of the apparent differences in biological action of certain methylating agents; for example, *N*-methyl-*N*-nitrosourea is a potent carcinogen (Druckrey *et al.*, 1965) and can induce mutations in bacteriophage T2 treated extracellularly (Loveless & Hampton, 1969). Methyl methanesulphonate and dimethyl sulphate, although often classified as relatively weak carcinogens, can induce sarcoma locally after subcutaneous injection in rats (Druckrey *et al.*, 1970). Methyl

methanesulphonate does not induce mutations in the T2 bacteriophage system quoted, although it can be a potent mutagen in other organisms (Loveless, 1966; Mallings & de Serres, 1969). Both types of carcinogen can methylate nucleic acids of potential target tissues *in vivo*, but for one site (kidney of the rat) studied in some detail extent of methylation of nucleic acids, as measured by the yield of the principal product, 7-methylguanine, did not correlate with induction of tumours. Methyl methanesulphonate gave more extensive methylation, but no tumours, whereas *N*-methyl-*N*-nitrosourea proved a potent carcinogen (Swann & Magee, 1968).

Among possible explanations of these differences between methylating agents it could be considered either that methylation of nucleic acids is irrelevant to carcinogenesis, or that certain distributions of methylation products in the nucleic acids are more

likely to favour tumour induction than others, and this factor depends on the nature of the carcinogen and on the target tissue concerned. One difference between the action of the two agents mentioned has already been proposed to be significant, i.e. that the supposedly more potent carcinogen *N*-methyl-*N*-nitrosourea can methylate deoxyguanosine at the O-6 position (Loveless, 1969), and therefore probably give O⁶-methylguanine residues in nucleic acids. The structure of these modified base residues is such as to permit anomalous base-pairing in the scheme for nucleic acid replication proposed by Watson & Crick (1953), and thus suggests an attractive hypothesis to account for induction of transition mutations. Moreover, it would follow that the same molecular mechanism was involved both in mutagenesis by the transition mechanism and in some types of carcinogenesis. Other minor sites of methylation in nucleic acids have been suggested to be involved in mutagenesis; for example, methylation of poly(C) at the N-3 position has been associated with alteration in template properties of the polynucleotide (Ludlum & Wilhelm, 1968), and methylation of phosphodiester groups in DNA has been suggested as a cause of mutation (Olson & Baird, 1969), but in neither case is a completely detailed molecular mechanism obvious.

Some evidence that certain potent methylating mutagens and carcinogens can give a distribution of products in nucleic acids different from that characteristic of the 'weaker' carcinogens has been obtained. *N*-Methyl-*N*'-nitro-*N*-nitrosoguanidine was compared with dimethyl sulphate, and found to give O⁶-methylguanine as a product additional to those given by the alkyl sulphate; the overall distribution of minor products in relation to the common major product, 7-methylguanine, also differed for both RNA and DNA with these two agents (Lawley & Thatcher, 1970).

The mechanism of reaction of alkylnitrosamides is very likely to resemble that of the alkylnitrosamidinium quoted, in that the reactive species generated will be the methyldiazonium ion (Lawley, 1968). The characteristic mode of reaction of these agents can then be ascribed either to this ion or to the methylcarbonium ion derived from it. The alkyl sulphate, on the other hand, would react by the S_N2 mechanism (Ingold, 1953) with the various centres of nucleic acids according to their relative nucleophilicities, the N-7 atom of guanine being the most reactive in this respect (Lawley, 1966).

Further studies in this field will clearly require sensitive methods for the detection and determination of the various methylation products in nucleic acids. From what was already known of the chemistry of these reactions (Lawley, 1966; Lawley & Thatcher, 1970), it seemed unlikely that any single simple procedure for analysis of methylated nucleic acids

would be satisfactory for all known products. As a particular instance, the usual procedures for analysis of methylated RNA involving acid hydrolysis would destroy O⁶-methylguanine. Several other problems remained unsolved; for example, methods for the separation of the relevant methyladenines and methylcytosines remained unsatisfactory. It was therefore thought desirable to re-examine some existing methods, which generally involved acid hydrolysis of nucleic acids, and to explore methods involving enzymic degradation of nucleic acids to nucleosides. RNA has been used in the studies reported here but clearly, with certain provisos, the application of the methods to DNA can be envisaged. Further, the methods would be expected to prove of value for studies of the action of agents other than the methylating agents used here.

It may be noted that considerable attention has been devoted to nucleoside-separation techniques, mainly with regard to biomethylation as opposed to chemical methylation. Among other methods (Hall, 1967) ion-exchange chromatography has been employed. Reichard & Estborn (1950) early reported the use of Dowex 50 (NH₄⁺ form) eluted at pH4.5; subsequently the use of this material was studied in more detail, e.g. by Uziel *et al.* (1968), with elution at pH4.7, and by Junowicz & Spencer (1969), at pH8.9. In view of the known acid lability of certain nucleic acid methylation products, for example 7-methylguanosine and O⁶-methylguanosine, it seemed likely that these techniques could be usefully complementary to the widely used methods of separating the bases obtained by acid hydrolysis of methylated RNA on Dowex 50 (H⁺ form) eluted with acid, or by paper chromatography (Brookes & Lawley, 1971). It was decided therefore to investigate the application of similar methods to nucleosides derived from enzymic digestion of chemically methylated RNA.

It was also decided to compare Dowex 50 (NH₄⁺ form) with the same material in the acid form as an ion-exchanger for the separation of bases obtained from methylated RNA. To remove ammonium formate used in the elution solvents, it was found convenient to rechromatograph column fractions on Sephadex G-10; this technique itself proved of some value for separation and identification of methylated bases and nucleosides, and thus worthy of inclusion here.

A preliminary report including results of some applications of these methods has been given (Lawley *et al.*, 1971).

Materials and Methods

Materials

Di[¹⁴C]methyl sulphate (58 mCi/mmol) and [¹⁴C]-methylamine hydrochloride (54 mCi/mmol) were

obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

N -[^{14}C]Methyl- N' -nitro- N -nitrosoguanidine was prepared as described by Lawley & Thatcher (1970). N -[^{14}C]Methyl- N -nitrosourea was prepared by a modification of the method of Cox & Warne (1951) (personal communication from Dr. J. E. Plant and Dr. J. J. Roberts of this Institute). In a typical example, [^{14}C]methylamine hydrochloride (0.5 mCi, 0.63 mg) was diluted with unlabelled material (67 mg) in water (0.68 ml), and potassium cyanate (200 mg) and a drop of H_2SO_4 (0.1 M) were added. The mixture was refluxed for 12 min, cooled in ice, and H_2SO_4 (2.75 M, 1.2 ml) was added; then NaNO_2 (167 mg) was added gradually over a period of 25 min. Some N -[^{14}C]methyl- N -nitrosourea precipitated, but it was found advantageous to extract the whole reaction mixture with ether (eight portions of 2 ml) to obtain maximal yields. The ether extract was dried over MgSO_4 in a desiccator in the dark for about 1 h. The ether solution was then decanted and evaporated in a desiccator, again in the dark, leaving a residue of crystals of N -[^{14}C]methyl- N -nitrosourea (63% yield of radioactivity; m.p. 123°C ; λ_{max} in ethanol, 395 nm, ϵ_{max} 96).

1-Methyladenosine, 1-methyladenine, 3-methyladenine, 7-methylguanosine and 7-methylguanine were obtained as described by Lawley & Thatcher (1970); 3-methylcytidine and 3-methylcytosine were prepared by the method of Brookes & Lawley (1962); 3-methyluridine by the method of Levene & Tipson (1934); 3-methyluracil by the method of D. J. Brown *et al.* (1955); 8-methylguanine by the method of Koppel & Robins (1958); and O^6 -methylguanosine by the method of Gerster *et al.* (1963). 7-Methyladenine was obtained from Fluka A.G., Buchs, Switzerland; N^6 -methyladenosine and N^6 -methyladenine from Sigma (London) Chemical Co., London S.W.6, U.K.; N^2 -methylguanosine and 1-methylguanosine from Cyclo Chemical Corp., Los Angeles, Calif., U.S.A.

Methylation of nucleotides

3-Methylcytidylic acid was prepared by the method of Griffin (1967). 7-Methylguanylic acid was also obtained by a procedure based on this general method for methylation of nucleotides. Guanosine 2'(3')-phosphate (sodium salt, 5 mmol) was treated with dimethyl sulphate (50 mmol) with stirring in water (25 ml), the pH being maintained at about 5 by addition of 0.3 M-ammonium formate, pH 8.9, until all the dimethyl sulphate had dissolved (about 4 h). The mixture was applied to a column (25 cm $\times$ 2.5 cm) of Dowex 1 (formate form) and eluted with water, 6 ml fractions being collected. Three major u.v.-absorbing peaks were found, in fractions 15,

18 and 21; their spectra were closely similar, with λ_{max} at pH 6.5 259 and 279 nm, changing irreversibly at pH 12 to 265 nm, i.e. characteristic of 7-methylguanylic acid. Their R_F values on Whatman 3MM paper, with satd. aq. $(\text{NH}_4)_2\text{SO}_4$ –0.1 M-sodium phosphate (pH 6.5)–propan-2-ol (79:19:2, by vol.) as solvent were also closely similar (0.65, 0.65 and 0.64 respectively). When treated with the enzymic mixture used to degrade RNA at pH 6, they again showed closely similar chromatographic properties, but each split into several components, as described in the Results and Discussion section. The products were also investigated by electrophoresis on a Pherograph 65 apparatus (Northern Media Supply Co., Hull, U.K.), by using Whatman 3MM paper, with aq. 2% (v/v) pyridine adjusted to pH 6.5 with acetic acid or 0.05 M-sodium citrate, pH 6.6, as solvent, and a voltage of about 25 V/cm, giving a current of 120 mA for 3 h. All three products moved 5 cm in the first system, and 7 cm in the second system, compared with guanosine 2'(3')-phosphate, 12 cm.

To obtain [methyl- ^{14}C]guanylic acid, di[^{14}C]methyl sulphate (20 μCi , 44 μg) was added to guanosine 2'(3')-phosphate disodium salt (100 mg) in 0.1 M-sodium citrate, pH 6 (1.2 ml), at 37°C . After 2 h the solution was cooled and extracted three times with ether (3 ml); the ether then contained 5×10^5 c.p.m. and the aqueous layer 3.5×10^7 c.p.m. The aqueous layer was then evaporated in a desiccator and was examined by chromatography after enzymic dephosphorylation as described for RNA digests.

α -D-Ribose 1-phosphate (4 mg) was similarly methylated with di[^{14}C]methyl sulphate (2 μCi) in 0.2 M-tris-HCl buffer (0.5 ml, pH 8). After extraction with ether, the solvent was removed by evaporation, leaving an oily residue.

Procedures with ribonucleic acid

Sources of RNA. Bacteriophage $\mu 2$ RNA was supplied by Dr. K. V. Shooter, of this Institute. RNA from rabbit reticulocytes was isolated as described by Kirby (1965) and tRNA was purified by the method of Delias & Staehelin (1966); reticulocytes were obtained by the method of Borsook *et al.* (1952).

Homopolynucleotides were obtained from Miles Laboratories, Maidenhead, Berks., U.K.

Methods for methylation of RNA. RNA (about 10 mg) dissolved in 0.2 M-tris-HCl buffer (2 ml) (generally at pH 8, but in some experiments, at pH 7) was treated with the ^{14}C -labelled reagents added in ethanol (N -methyl- N -nitrosourea) or ether (dimethyl sulphate) and, after reaction for 1 h at 37°C , methylated RNA was isolated by addition of 2.5 M-sodium acetate (0.1 vol.) and ethanol (2 vol.). The RNA was obtained by centrifugation, redissolved

in 0.25M-sodium acetate (2ml) and reprecipitated twice, and washed with ethanol and ether and dried. Methylation with *N*-[¹⁴C]methyl-*N'*-nitro-*N*-nitroso-guanidine was carried out as described by Lawley & Thatcher (1970).

Methods for degradation of RNA. Enzymes were from Sigma (London) Chemical Co. In the method denoted 'pH6', RNA (about 2mg, containing generally more than 20000 c.p.m.) was dissolved in 0.5M-sodium citrate, pH6 (0.5ml), and ribonuclease (0.05mg), spleen phosphodiesterase (0.05 unit) and wheat-germ acid phosphatase (0.25 unit) were added. The mixture was incubated at 37°C for 16h. The digest was then examined by t.l.c. on Polygram CEL 300 UV (Camlab Ltd., Cambridge, U.K.), with propan-2-ol-aq. NH₃ soln. (sp.gr. 0.88)-water (7:1:2, by vol.) as solvent. Digestion was complete, as judged from the appearance of u.v.-absorbing bands under light of 254nm, at the *R_F* values of the nucleosides adenosine (0.8), cytidine (0.7), guanosine (0.5), uridine (0.7, not separated from cytidine); no u.v.-absorbing material remained at the origin in the position of nucleotides or oligonucleotides.

This enzyme system was tested for its effect on various methylated nucleosides by incubating the nucleosides (solutions of *E*_{max}^{1cm}, about 3) under the conditions used for RNA digestion. One sample of spleen phosphodiesterase did cause partial degradation of *O*⁶-methylguanosine (as shown by a loss of characteristic u.v. absorption of about 50%). This action may be ascribed to deaminase activity in this type of enzyme preparation; adenylate deaminase has been reported to convert 6-methoxypurine riboside into hypoxanthine (Wolfenden & Kirsch, 1968). Spleen phosphodiesterase consistently deaminated adenosine under the conditions used here.

Alternative methods for degradation of RNA were therefore employed. In the method denoted 'pH8', RNA was treated with ribonuclease (0.1mg/ml) in water (1 ml) for 30min, the solution was then adjusted to pH8 with 0.1M-tris-HCl buffer (0.1 ml) and venom phosphodiesterase (0.03 unit) and bacterial alkaline phosphatase (3 units) were added. After 16h at 37°C, degradation of RNA to nucleosides was found to be complete. No deamination of marker nucleosides by this enzyme system was detected.

In the method denoted 'KOH', RNA was converted into nucleotides by hydrolysis in 1M-KOH (0.5ml) at 37°C for 16h, the cooled solution was neutralized with HClO₄ and the precipitate of KClO₄ was removed by centrifugation. The solution was adjusted to pH9 with 0.2M-tris-HCl buffer (0.125ml) and bacterial alkaline phosphatase (3 units) was added; after 2h at 37°C, degradation to nucleosides was complete.

RNA was degraded by acid, either by hydrolysis in 1M-HCl (0.5ml) for 1h at 100°C giving purine bases and pyrimidine nucleotides, or in HClO₄

(72%, w/v; 15μl/mg of RNA) for 1h at 100°C, giving purine and pyrimidine bases.

Before chromatography, appropriate marker compounds were added to the hydrolysates (about 200μg of each).

Methods for chromatography

Method 1. A column (60cm×1.5cm) of Dowex 50 AG 50W (X4; NH₄⁺ form; 400 mesh; Bio-Rad Laboratories, Richmond, Calif., U.S.A.) was eluted with a linear gradient of 1mM-2M-ammonium formate, pH6 (350ml of each), 6.4ml fractions being collected at a flow-rate of about 30ml/h, and u.v.-absorption at 254nm being monitored with a Uvicord photometer (LKB Instruments, S. Croydon, Surrey, U.K.).

Method 2. A column (8cm×1.5cm) of Dowex 50 (NH₄⁺ form) was eluted with 0.3M-ammonium formate, pH8.9 (6.4ml fractions); after *O*⁶-methylguanosine had been removed, at about fraction 100, elution was continued with 1M-ammonium formate, pH8.9, until 3-methylcytidine and 1-methyladenosine had been removed, after about 160 fractions. The column was then re-equilibrated with 0.3M-ammonium formate. In some experiments Dowex 50 (NH₄⁺ form) was eluted with 0.3M-ammonium formate, pH6.65, for about 110 fractions, and then with 1M-ammonium formate, pH6.65.

The positions of elution of various bases and nucleosides are given in Tables 1 and 2.

Method 3. Some acid hydrolysates were chromatographed on a Dowex 50 (H⁺ form) column (10cm×1.5cm) eluted with a linear gradient of 0.75-2.5M-HCl (300ml of each; 3.8ml fractions).

Method 4. Sephadex G-10 (Pharmacia Ltd., London W.5, U.K.) columns (85cm×1.5cm), eluted with 0.05M-ammonium formate, pH6.8, were also used for separation of bases and occasionally for rechromatography of nucleosides obtained from Dowex 50 columns.

Measurement of radioactivity

T.l.c. chromatograms were cut into segments (0.5cm) and counted for radioactivity in a Packard Tri-Carb (model 375 or 3375) scintillation counter, after moistening with 0.5ml of a toluene-based phosphor as described by Lawley & Thatcher (1970). Column fractions were assayed by adding a detergent-based phosphor (10ml) to give a gel in the scintillation vials. The phosphor contained Triton X-100 (Lennig Chemicals, Jarrow, U.K.; 4 litres), toluene (6 litres), 2,5-diphenyloxazole (70g) and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (3.5g). The efficiency of counting of ¹⁴C was about 65% for column fractions containing ammonium formate and about 57% for fractions containing HCl. A similar

Table 1. *Elution of purine and pyrimidine bases from column chromatograms*

The systems used were: Dowex AG 50W (X4; H⁺ form), 10 cm × 1.5 cm column, eluted with a linear gradient of 0.75–2.5M-HCl (300 ml of each); Dowex AG 50W (X4; NH₄⁺ form; 400 mesh), 85 cm × 1.5 cm column, eluted with 0.3M-ammonium formate, pH8.9, or 60 cm × 1.5 cm column eluted with 0.3M-ammonium formate, pH6.65 (700 ml), then with 1M-ammonium formate, pH6.65; Sephadex G-10, 85 cm × 1.5 cm column, eluted with 0.05M-ammonium formate, pH6.8. The positions of elution of various bases are given as their elution volumes expressed as percentages of those of adenine: the elution volume of adenine from Dowex 50 (H⁺ form) was about 160 ml; from Dowex 50 (NH₄⁺ form), at pH8.9, about 400 ml, and at pH6.65, about 500 ml; from Sephadex G-10, about 400 ml. The values refer to averages of several determinations, which generally did not differ by more than 3%.

Column material	...	Dowex 50 (H ⁺ form)		Dowex 50 (NH ₄ ⁺ form)		Dowex 50 (NH ₄ ⁺ form)		Sephadex G-10	
		Eluent ...		0.75–2.5M-HCl		0.3M-Ammonium formate, pH8.9		0.05M-Ammonium formate, pH6.65	
1-Methyladenine			92		85		290		38
3-Methyladenine			67		72		238		53
N ⁶ -Methyladenine			126		143		150		100
7-Methyladenine			107		109		98		52
Cytosine			39		56		57		33
3-Methylcytosine			45		90		285		26
5-Methylcytosine			48		74				34
Guanine			50		88		95		82
1-Methylguanine			62		105		110		66
N ⁷ -Methylguanine			60				124		75
3-Methylguanine			57		64		72		43
O ⁶ -Methylguanine					164		149		130
7-Methylguanine			67		95		107		61
8-Methylguanine			60		87		94		83
Uracil			7		32		33		34
3-Methyluracil			10		44		42		31

Table 2. *Elution of nucleosides from column chromatograms*

The systems used are as described in Table 1, and are given in detail in the Materials and Methods section. The positions of elution of various nucleosides are shown as percentages of those of adenosine; the elution volume for adenosine was about 300ml at pH8.9 and about 120ml at pH6.0.

Column material ... Eluent ...	Dowex 50 (NH ₄ ⁺ form) 0.3M-(then 1M)-Ammon- ium formate, pH8.9	Dowex 50 (NH ₄ ⁺ form) 1mM-2M-Ammonium formate, pH6.0
1-Methyladenosine	327	272
N ⁶ -Methyladenosine	135	120
Cytidine	49	
3-Methylcytidine	286	256
Guanosine	88	83
1-Methylguanosine	92	
N ² -Methylguanosine	126	
O ⁶ -Methylguanosine	160	122
7-Methylguanosine	65	323
7-Methylguanosine (main alkaline conversion products)	(12, 26, 33)	(28, 39)
Inosine	51	55
Uridine	33	28
3-Methyluridine	53	33

phosphor was used for counting radioactivity of HCl-containing column fractions by Whittle (1969).

Results and Discussion

Assessment of chromatographic procedures

The effectiveness of the methods for separation of the various products can be assessed from Tables 1 and 2. Typical examples of the application of some of these methods are shown in Figs. 1 and 2. It is convenient to discuss this aspect of the work by drawing attention to certain difficulties apparently inherent in procedures of this kind.

The major product given in RNA by all the methylating agents so far investigated is 7-methylguanosine. This nucleoside is known to be unstable to both acid and alkaline hydrolysis (Lawley & Wallick, 1957; Brookes & Lawley, 1961; Lawley & Brookes, 1963; Haines *et al.*, 1962; Kriek & Emmelot, 1963; Zoltewicz *et al.*, 1970). For this reason most analytical procedures for methylated RNA had used acid hydrolysis, and purines had been determined at the level of bases. The instability of O⁶-alkylguanines under the conditions used for RNA hydrolysis (Lawley & Thatcher, 1970; Lawley & Orr, 1970) stimulated further studies of enzymic hydrolyses of methylated RNA. As expected, during the digestion and subsequent chromatography, the principal product, 7-methylguanosine, split into several components.

As a model for the principal methylated nucleotide

in RNA, methylated guanosine 2'(3')-phosphate was prepared, both unlabelled and *methyl*-¹⁴C-labelled. When subjected to the conditions used for digestion and chromatography of RNA, several components other than 7-methylguanosine were obtained. One of these was eluted from the columns after 7-methylguanosine and was identified by its absorption spectra at three pH values, and by the fact that the authentic base was eluted at the same position as 7-methylguanine (Fig. 1).

Several components were eluted earlier than 7-methylguanosine (Fig. 1), and these had absorption spectra that were closely similar to each other, and were identical with those of the products resulting from alkaline conversion of 7-methylguanosine ($\lambda_{\max.}$ at pH12, 265nm; E_{280}/E_{260} 0.7; $\lambda_{\max.}$ at pH8.9, 272.5nm; E_{280}/E_{260} 1.4). The elution patterns of the *methyl*-¹⁴C-labelled products from guanylic acid, determined by the elution of radioactivity from the columns, were closely similar to those shown by the u.v.-absorption method.

These results agreed broadly with those reported by Kriek & Emmelot (1963), who used Dowex 50 (H⁺ form) in analogous experiments, but appeared to be somewhat at variance with the report by Haines *et al.* (1962) that the alkaline conversion product of 7-methylguanosine could be obtained as a chromatographically homogeneous material by treating this nucleoside with dilute aq. NH₃ soln. The procedure of Haines *et al.* (1962) was therefore repeated, by using aq. 2M-NH₃ soln. at 20°C for 2.5h for the conversion. The product did appear homo-

Fig. 1. Chromatography of 7-methylguanosine and derivatives from dephosphorylated 7-methylguanosine 2'(3')-phosphate, on Dowex 50 (NH_4^+ form) at pH8.9

(a) 7-Methylguanylic acid, obtained as described in the text, was treated with ribonuclease, spleen phosphodiesterase and wheat-germ acid phosphatase, at pH6 under the conditions used for digestion of RNA, and eluted from the column with 0.3M-ammonium formate, pH8.9 (see the Materials and Methods section for details). The u.v. absorption as obtained with the LKB Uvicord is shown. The products were identified by examination of the u.v.-absorption spectra of the fractions. PyNuc denotes the products from imidazole ring-fission of 7-methylguanosine; 7-MeGuo, 7-methylguanosine; 7-MeGua, 7-methylguanine. (b) 7-[^{14}C]Methylguanosine 2'(3')-phosphate was obtained as described in the text, and treated as in (a). The products were detected by scintillation counting of the fractions. (To show minor peaks, the major peaks are not drawn to scale.)

geneous on chromatography on Whatman 3MM paper, with propan-2-ol-water (7:3, v/v) as solvent (R_F 0.6). But when this material was chromatographed on Dowex 50 (NH_4^+ form) at pH8.9, it gave two principal peaks in fractions 13 and 16, both having the same absorption spectra (λ_{max} 272.5 nm at pH8.9). The immediate product from the action of 1 M-KOH

at 20°C on 7-methylguanosine was not homogeneous on paper chromatography; in the system as used for the ammonia-conversion product, two principal spots were observed at R_F 0.27 and 0.48. These spots were eluted into water and chromatographed on the column. They both split into the two main components previously found, as described above.

Fig. 2. Comparison of chromatograms of the products from methylation of bacteriophage $\mu 2$ RNA (a) by N -[^{14}C]-methyl- N -nitrosourea or (b) by $\text{di}[^{14}\text{C}]$ methyl sulphate

RNA was degraded to nucleosides enzymically at pH6, and chromatographed on Dowex 50 (NH_4^+ form) columns ($80\text{cm} \times 1.5\text{cm}$) at pH8.9 (6.4 ml fractions) as described in detail in the Materials and Methods section. The breaks in the abscissae indicate the switch of solvent for elution from 0.3M- to 1M-ammonium formate. For the proportions of radioactivity in the various products, see Table 3. The positions of nucleosides and added marker compounds, detected by u.v. absorption, are marked by arrows: Urd, uridine; 7-MeGuo, 7-methylguanosine; Guo, guanosine; Ado, adenosine; 3-MeUrd, 3-methyluridine; 7-MeGua, 7-methylguanine; N^6 -MeAdo, N^6 -methyladenosine; O^6 -MeGuo, O^6 -methylguanosine; 3-MeCyd, 3-methylcytidine; 1-MeAdo, 1-methyladenosine.

Evidently the products of alkaline hydrolysis of 7-methylguanosine are an equilibrium mixture; some degree of separation can be achieved by the procedures used here (see Fig. 1), but the nature of these products was not investigated further.

The conclusion reached was that the proportion of 7-methylguanosine in the methylated nucleosides derived from RNA could not be determined with precision. The reasons were that partial conversion into the pyrimidine derivatives described, by fission of the imidazole ring of the molecule, did not occur to a reproducible extent; the variety of products could not be characterized, nor were they separated from labelled material that was eluted early from the columns, and which, as discussed below, could originate from methylation of the sugar-phosphate moiety of RNA, or from methylated pyrimidines. When 7-methylguanosine was eluted from Dowex 50 (NH_4^+ form) at pH6 in the hope of decreasing the extent of alkaline conversion, it proved difficult to elute completely, and fairly concentrated ammonium formate was required.

It thus appears that 7-methylguanine in methylated

RNA is best determined as the free base, obtained by acid hydrolysis. It should be pointed out that this base is not separated from 3-methyladenine, another methylation product, by chromatography on Dowex 50 (H^+ form) eluted with HCl (although in methylated RNA the proportion of the latter was small). These bases can be separated on Dowex 50 (NH_4^+ form) (see Table 1).

Other known products of methylation of RNA that cannot at present be isolated at the nucleoside level, but can be detected as bases, include 3- and 7-methyladenine; the corresponding nucleosides are not known, but would be expected to be unstable in alkaline solutions. The nucleoside of the recently discovered methylation product 3-methylguanine (Lawley *et al.*, 1972) has also yet to be isolated.

1-Methyladenine and 3-methylcytosine were expected to be isolated satisfactorily as either bases or nucleosides. The latter, by virtue of their relatively high pK'_a values [8.8 and 8.3 have been reported for 1-methyladenosine (Martin & Reese, 1968; Macon & Wolfenden, 1968) and 8.9 for 3-methylcytidine (Brookes & Lawley, 1962)], are well separated from

other nucleosides on Dowex 50 (NH_4^+ form) at pH 8.9 (see Table 2). After hydrolysis of RNA in KOH, 3-methylcytidine is destroyed as expected (Brookes & Lawley, 1962) and 3-methyluridine is also unstable under these conditions (Shugar & Fox, 1952); 1-methyladenosine is converted into N^6 -methyladenosine (Brookes & Lawley, 1960; Macon & Wolfenden, 1968). It is thus of some importance to ensure separation of N^6 -methyladenosine and O^6 -methylguanosine, which have similar chromatographic properties, as do the corresponding bases (see Tables 1 and 2). Some conversion of 1-methyladenylic acid residues in RNA also occurs during the methylation reactions and during digestion and chromatography under mild alkaline conditions.

As previously reported (Lawley & Thatcher, 1970; Lawley & Orr, 1970), O^6 -methylguanine is unstable under conditions used for acid hydrolysis of RNA, the half-life in 1M-HCl at 100°C being about 12 min (Lawley & Orr, 1970), and this product cannot therefore be isolated as a base from methylated RNA. This procedure for acid hydrolysis also causes some conversion of 1-methyladenine (Brookes & Lawley, 1960; Whittle, 1969; Singer & Fraenkel-Conrat, 1969a) into a product found in the present work to be eluted from Dowex 50 (H^+ form) at the same position as 7-methyladenine. When 1-methyladenine (1 mg) was heated in 1M-HCl (0.5 ml) at 100°C for 1 h the u.v.-absorption spectrum showed a shift to longer wavelengths (λ_{max} , increasing from 258 to 265 nm); the products were chromatographed on Dowex 50 (H^+ form) eluted with HCl, and in addition to unchanged 1-methyladenine a u.v.-absorbing peak appeared at the position of 7-methyladenine; the extent of conversion of 1-methyladenine was about 30% as estimated from the loss of u.v. absorption. When 1-methyladenine was heated with HClO_4 (72%, w/v) at 100°C for 1 h, no change in u.v. absorption was found.

Evidently HClO_4 is preferable for acid hydrolysis of methylated RNA. It has the additional advantage that pyrimidine bases are liberated, and these are more easily characterized than are the corresponding nucleotides. 3-Methylcytidylic acid could be identified in HCl hydrolysates of methylated RNA, but an unidentified product was eluted together with this nucleotide on chromatography on Dowex 50 (H^+ form).

3-Methyluracil was best isolated by chromatography on Dowex 50 (NH_4^+ form), which effectively separated it from other unidentified material eluted early from the column.

Sephadex G-10 was useful for re-isolation of compounds obtained by elution from Dowex 50 (NH_4^+ form) columns, which could thus be freed from ammonium formate. This column also separated some groups of bases more effectively than did Dowex 50; as examples, 3- and 5-methylcytosine,

and the various methylguanines, which were eluted together from Dowex 50, could be re-chromatographed on Sephadex G-10 to give improved separations (see Table 1).

The conclusions therefore, in summary, are that no single procedure can enable the isolation and estimation of all the known products of methylation in RNA. O^6 -Methylguanine cannot be obtained in full yield, so that its determination must be carried out at a less-degraded level, in the present case at the level of the nucleoside. On the other hand, products that are unstable as nucleosides, such as 3-methyladenine, 7-methyladenine, 3-methylguanine and 7-methylguanine, require isolation and determination as bases. In view of the instability of 1-methyladenine to HCl hydrolysis, HClO_4 appears to be the hydrolytic procedure of choice; this also liberates pyrimidine bases, which can be readily isolated.

1-Methyladenine and 3-methylcytosine can be isolated either as nucleosides or bases; with the former some losses may occur during the procedures of digestion and chromatography, but except where strongly alkaline conditions have been used, these losses are not likely to be great.

For isolation and separation of nucleosides, digestion of RNA with venom phosphodiesterase and alkaline phosphatase followed by chromatography on Dowex 50 (NH_4^+ form) eluted at pH 8.9 was a satisfactory procedure.

For bases, chromatography on Dowex 50 (H^+ form) separated 1- and 7-methyladenine, but not 7-methylguanine and 3-methyladenine; chromatography on Dowex 50 (NH_4^+ form) eluted at pH 8.9 did not separate several groups of bases, e.g.: 3-methylguanine and 3-methyladenine, 1-methyladenine, 3-methylcytosine and 7-methylguanine, but chromatography on Dowex 50 (NH_4^+ form) eluted at pH 6.65 was especially effective for isolation of bases with basic pK_a' values in the region 5.5–7.5, i.e. 3-methyladenine (pK_a' about 6.0), 3-methylcytosine (7.4) and 1-methyladenine (7.2), which were eluted in the order stated, well after the other bases (see Table 1).

Sephadex G-10 did not separate 3- and 7-methyladenine, but otherwise was useful for base separations.

Applications of the methods to analysis of methylated RNA; differences in the modes of reaction of N-methyl-N-nitrosourea, N-methyl-N'-nitro-N-nitrosoguanidine and dimethyl sulphate

A comparison of the results obtained by some of the analytical procedures described in the previous section is given in Table 3, in which only values for products that were isolated in satisfactory fashion, as discussed, are quoted. The results show that the

Table 3. *Products from methylation of bacteriophage $\mu 2$ RNA by N -[^{14}C]methyl-N-nitrosoarea or by di[^{14}C]methyl sulphate and comparison of various analytical methods*

RNA (about 10 mg) dissolved in 0.2 M-tris-HCl buffer, pH 8 (2 ml), was treated with the reagents added in ethanol or ether (0.2 ml) for 1 h at 37°C; methylated RNA was then isolated as described in the text. It was degraded either enzymically or by acid hydrolysis. The methods used were: A ('pH 6 method'), ribonuclease, spleen phosphodiesterase and acid phosphatase; B ('KOH method'), KOH, then alkaline phosphatase; C, 1 M-HCl, 1 h, 100°C; D, HClO₄ (72% w/v), 1 h, 100°C. The products (from enzymic digests, nucleosides; from HCl hydrolysis, purine bases and pyrimidine nucleotides; from HClO₄ hydrolysis, purine and pyrimidine bases) were separated chromatographically by using Dowex AG 50W (X4, 400 mesh), at pH 6 (method 1) or pH 8.9 (method 2) (NH₄⁺ form eluted with ammonium formate; H⁺ form eluted with HCl, method 3), or by using Sephadex G-10 (method 4) eluted with 0.05 M-ammonium formate, pH 6.8. For details of these methods, see the text. The molar proportions of the products were determined as the percentage of total radioactivity eluted from the columns together with the u.v.-absorbing peaks of added marker compounds; only values for those compounds that were isolated by the method used are quoted, except for some values for 7-methylguanine, which eluted with 3-methyladenine (see the footnote ‡).

Reagent	Extent of methylation (mmol/mol of RNA P)	N-Methyl-N-nitrosoarea										Dimethyl sulphate					
		13	13	13	13	13	13	17	17	17	13	17	13	17	13	17	13
Method for hydrolysis	A	A	A	B	B	B	D	C	C	C	D	D	D	D	D	D	D
Method for chromatography	1	1	2	2	2	2	4	3	3	3	3	3	3	3	3	4	2
1-Methyladenine	2	3	2	†	†	†	†	3*	3*	3*	2	3	14	13	11		
3-Methyladenine								†	†	†	†	†	†	†	†		1
7-Methyladenine								3*	3*	3*	2	2			1	2	
3-Methylcytosine	1	2	1	†	†	†							12	11	13		
3-Methylcytidine							0.3										
3-Methylguanine							79	79†	82†	80†	76†						
7-Methylguanine																	
O ⁶ -Methylguanosine		4	4	3	3	3									54†	63†	0.4
3-Methyluracil																58	64
3-Methyluridine																	

* 1-Methyladenine was partially converted into a product eluted together with 7-methyladenine by HCl hydrolysis, and these values were corrected appropriately.

† 1-Methyladenine was converted into N⁶-methyladenosine, and 3-methylcytidine was destroyed, by treatment with KOH.

‡ 3-Methyladenine was eluted together with 7-methylguanine; since the proportion was small, no correction was made.

Fig. 3. Comparison of two methods for chromatography of methylated bases obtained from bacteriophage $\mu 2$ RNA after reaction with N -[^{14}C]methyl- N -nitrosourea

Chromatography was on (a) a Dowex 50 (NH_4^+ form) column ($80\text{cm} \times 1.5\text{cm}$) eluted with 0.3M -ammonium formate, pH 8.9 (6.4 ml fractions), or (b) a Dowex 50 (H^+ form) column ($10\text{cm} \times 1.5\text{cm}$) eluted with a gradient of 0.75 – 2.5M -HCl (3.8 ml fractions; total vol. 600 ml). RNA was hydrolysed with perchloric acid. For details see the Materials and Methods section. Ura, uracil; 3-MeUra, 3-methyluracil; Cyt, cytosine; 3-MeGua, 3-methylguanine; 3-MeAde, 3-methyladenine; Ade, adenine; 1-MeAde, 1-methyladenine; 3-MeCyt, 3-methylcytosine; 7-MeGua, 7-methylguanine; 7-MeAde, 7-methyladenine; N^6 -MeAde, N^6 -methyladenine (6-methylaminopurine). For the proportions of radioactivity in the various products, see Table 3.

level, whether nucleoside or base, at which analysis was carried out, is largely irrelevant to the results obtained, although in some cases the proportion of 3-methylcytidine recovered was rather less than that of the corresponding base. This may reflect some loss of the nucleoside during exposure to alkaline pH. The composite pattern that emerges is clear. The reagents N -methyl- N -nitrosourea and dimethyl sulphate give the same major product, 7-methylguanine, but differ significantly with regard to the distribution of the minor products of methylation in RNA (Tables 3, 4 and 5).

The principal minor products from dimethyl sulphate are those previously found for methyl methane-sulphonate, both *in vitro* (Lawley & Brookes, 1963) and *in vivo* (Whittle, 1969), namely 1-methyladenine and 3-methylcytosine. The principal minor product from N -methyl- N -nitrosourea or from N -methyl- N' -nitro- N -nitrosoguanidine (Table 6) is O^6 -methylguanine; this is not given in detectable amounts by dimethyl sulphate.

With regard to adenine derivatives, the nitroso compounds favour reaction at N-7 almost as much as at N-1. This is shown most clearly in the pattern

of methylation products from poly(A) (Table 5). Ludlum (1966) showed that the N-1 and N-3 atoms of adenine in poly(A) were methylated. Singer & Fraenkel-Conrat (1969a,b) had further found 7-methyladenine as a product of methylation of poly(A) and of tobacco mosaic virus RNA by dimethyl sulphate or N -methyl- N' -nitro- N -nitrosoguanidine, but the proportions of the products were rather different from those found here. The principal differences between the two sets of results for poly(A) are that Singer & Fraenkel-Conrat (1969a) did not find that a higher proportion of 7-methyladenine was given by N -methyl- N' -nitro- N -nitrosoguanidine, but they did find this for tobacco mosaic virus RNA. They also found that the proportions of various products varied with the solvent, but not with the extent of methylation.

Ludlum (1965) and Pochon & Michelson (1967) found that hydrogen-bonding of reactive groups in polyribonucleotides could partially inhibit their methylation, and conformation of tobacco-mosaic-virus RNA was found by Singer & Fraenkel-Conrat (1969a,b) to influence its methylation, particularly by N -methyl- N' -nitro- N -nitrosoguanidine.

Table 6. *Minor products from methylation of RNA by N-[¹⁴C]methyl-N'-nitro-N-nitrosoguanidine*

RNA was methylated in the presence of *N*-acetyl-cysteine at pH 7 (Lawley & Thatcher, 1970); bacteriophage $\mu 2$ RNA was degraded with venom phosphodiesterase and alkaline phosphatase and chromatographed on Dowex 50 (NH₄⁺ form) at pH 8.9; yeast RNA was hydrolysed with perchloric acid and chromatographed on Dowex 50 (H⁺ form) (see the text for details). Molar proportions of products are given as percentages of the total radioactivity eluted.

Extent of methylation (mmol/mol of P) ...	Bacteriophage $\mu 2$ RNA	Yeast RNA
	4	8
1-Methyladenine	2	2
7-Methyladenine		2
3-Methylcytosine	2	2
O ⁶ -Methylguanine	3	

In the present work, as shown by Tables 3 and 4, the type of RNA, whether from bacteriophage or from cellular rRNA or tRNA, has little influence on the patterns of methylation; certainly this influence is less than that of the type of reagent. The differences between the nitroso compounds and dimethyl sulphate were found consistently. The possibility that hydrogen-bonding or other conformational factors could influence the relative reactivities of the various methylated sites remains for further investigation. It should be noted in this connexion that, in DNA, in the native double-stranded form, the reactivity of the N-3 atom of adenine residues is much greater than in RNA or in poly(A). This conclusion applies both to dimethyl sulphate (Lawley & Thatcher, 1970) or methyl methanesulphonate (Lawley & Brookes, 1963) and to the nitroso compounds (Lawley & Thatcher, 1970), but towards the last-named type of reagent the relative reactivity is decreased to about one-half.

With regard to methylation of cytosine, 3-methylcytosine is the product identified in the present work, as in previous studies of polynucleotide methylations (Lawley & Brookes, 1963; Brimacombe *et al.*, 1965; Bollack *et al.*, 1965; Ludlum, 1966, 1970; Singer & Fraenkel-Conrat, 1969*a,b*). The lower reactivity of N-3 of cytosine in RNA towards the nitroso compounds as compared with that towards dimethyl sulphate or methyl methanesulphonate (Lawley & Brookes, 1963) is the significant difference between the types of reagent. Whether *O*-methylated cytosine can be formed is not known; the corresponding nucleoside is not available, and the product would presumably be unstable to acid hydrolysis.

With regard to methylation of guanine, the present results with RNA extend those previously found with

DNA (Lawley & Thatcher, 1970), showing that the nitroso compounds can methylate at the O-6 atom of this base. The relative extent of this methylation in RNA is about half that in DNA. This finding is contrary to expectations based on the concept that involvement of the O-6 atom in hydrogen-bonding of the Watson-Crick type would decrease its reactivity, as found with N-1 of adenine. The possibility that the O-6 atom of guanine in RNA is hydrogen-bonded more strongly to water, if not to cytosine, might explain this behaviour. Here studies of the guanosine residue in other polymers of known different conformations might be of interest. In poly(G) the proportion of O-6 methylation is similar to that in RNA, i.e. about 3%.

The recently detected minor product 3-methylguanine (Lawley *et al.*, 1972) is given by both the nitroso compounds and the sulphonate esters, but apparently rather more by the former. The small proportions found (around 1%) make this comparison tentative at present. Other conceivable guanine products have not been positively identified in methylated poly(G) or RNA, but have been found in other guanine methylations. Litwack & Weissmann (1966) have reported that *N*²-methylguanine and 1-methylguanine are among the products from the action of excess of methyl chloride on guanine itself in alkali. Hoffmann & Müller (1969) claimed that diazomethane could methylate guanosine to a small extent at the C-8 position, but experimental details were not given. From the present results, it seems unlikely that these products, if present at all, could exceed about 0.5% of total methylations.

Uracil, as previously reported (Lawley & Brookes, 1963; Bollack *et al.*, 1965), is not a reactive base in RNA at neutral pH values, although methylation at N-3 can be achieved in alkaline media (Kriek & Emmelot, 1963; Pochon & Michelson, 1967). In the present work, to enhance the rate of methylation by *N*-methyl-*N*-nitrosourea, RNA was methylated at the slightly alkaline pH of 8, and dimethyl sulphate was for comparison used also at this pH, although its rate of reaction is not affected much, if at all, by pH in this range.

Somewhat surprisingly, the results for methylation of poly(U) showed that dimethyl sulphate had relatively more affinity for reaction at the N-3 atom of the uridine moiety (Table 5). The sample of poly(U) used contained some guanylic acid residues, as evidenced by the production of 7-methylguanine. Dimethyl sulphate at pH 8 gave the highest proportion of 3-methyluracil in the methylation products (87%), and at pH 7 it gave a lower proportion (63%), reflecting probably the lower extent of acidic ionization of poly(U), and indicating that the ionized uracil residue is probably the predominant reactive form. *N*-Methyl-*N*-nitrosourea at either pH gave lower proportions of 3-methyluracil, 22% at pH 8 and 11%

at pH7. Clearly more intensive studies of uracil-containing polymers will be required to define the relative reactivities of the neutral and ionized forms of the uracil residue, but the indications at present are that the N-3 atom of uracil has no specific affinity for methylation by *N*-methyl-*N*-nitrosourea. It remains possible, however, that the latter may methylate at O-4 of uridine moieties, since Wong & Fuchs (1971) have found that 1-methyluracil will react at this position with diazomethane in the ether-methanol solvent system, to about one-tenth the extent of reaction at N-3.

These findings are of some interest with respect to the report by Loveless & Hampton (1969) that thymidine in DNA is methylated at N-3 by *N*-methyl-*N*-nitrosourea, but it was notable that this product was found in a reaction mixture that could have been made transiently alkaline; *O*-methylation was apparently not detected.

In RNA, N-3 of uracil is feebly reactive at pH8 (Tables 3 and 4) and it is uncertain whether this reactivity can be ascribed to the ionized form of this base.

As with cytosine, it remains for further investigation therefore whether *O*-methylation of uridine residues can occur in RNA.

The question now arises whether the residual radioactivity in the hydrolysates of methyl-¹⁴C-labelled RNA that cannot be accounted for as known methylated nucleosides or bases is derived from methylation of the sugar-phosphate chain of the polymer.

First, it should be noted that this proportion is generally less for dimethyl sulphate as methylating agent than for the nitroso compounds. This is seen most clearly with methylated poly(U), where the basic moiety of the polymer is weakly reactive. When poly(U) methylated with di[¹⁴C]methyl sulphate at pH8 was degraded with venom phosphodiesterase, 8% of the radioactivity was eluted from Dowex 50 (NH₄⁺ form) before 3-methyluridine. For poly(U) methylated with *N*-[¹⁴C]methyl-*N*-nitrosourea this proportion was about 60%. Hydrolyses with HClO₄ gave analogous results.

The nature of the material that is eluted from the columns before the most weakly retained nucleoside or base has yet to be ascertained. When α -D-ribose 1-phosphate was treated with di[¹⁴C]methyl sulphate and the resulting labelled material, after the various hydrolytic procedures, was chromatographed in the systems used for RNA analysis, the products were eluted in the earlier fractions as expected. The labelled methylating agents, after hydrolysis in the buffer used for RNA methylations, were also eluted in this region.

The most likely source of non-base methylated material from dimethyl sulphate is the terminal phosphate group. Abell *et al.* (1965) have shown

alkylation of this group in poly(U) by mustard gas. In agreement with their findings that this agent did not degrade the polymer, Shooter *et al.* (1971) showed that bacteriophage μ 2 RNA remained intact after extensive alkylation by the mustard. It appears to be well established that RNA and various polyribonucleotides can be methylated extensively by dimethyl sulphate without degradation (Brimacombe *et al.*, 1965; Smith *et al.*, 1967), but several studies have shown that diazomethane can degrade RNA (Kriek & Emmelot, 1963) or polynucleotides (Holy & Scheit, 1967). More recently, the actions in this respect of dimethyl sulphate and *N*-methyl-*N*-nitrosourea on bacteriophage μ 2 have been compared; the latter, but not the former, caused degradation of the RNA of the bacteriophage during the methylation process (Lawley *et al.*, 1971; Shooter, 1971; personal communication from Dr. K. V. Shooter).

The present results could be consistent with the hypothesis that *N*-methyl-*N*-nitrosourea, which is now known to methylate *O*-atoms in guanine of RNA, could also methylate the phosphodiester group, and thus cause degradation of RNA (Brown & Todd, 1953; D. M. Brown *et al.*, 1955; Kriek & Emmelot, 1963; Holy & Scheit, 1967). This hypothesis could also be extended to cover other alkylating agents that are thought to react in part through the S_N1 mechanism, such as ethyl methanesulphonate, which has been shown to degrade poly(A) (Ludlum, 1969). The S_N2 reagents, such as mustard gas and dimethyl sulphate, may be unable to alkylate to appreciable extents at O atoms other than those of the more nucleophilic terminal phosphomonoester groups. Clearly, analytical chemical procedures for positive identification of phosphotriester groups are still required to test this hypothesis further.

The general conclusion from the results so far obtained can be summarized therefore as showing that the modes of reaction of the *N*-methyl-*N*-nitroso compounds on the one hand, and the methyl sulphonates on the other, do influence their respective modes of reaction with RNA and polyribonucleotides. The salient feature is the ability of the nitroso compounds to methylate the weakly nucleophilic O atoms of guanine residues. The results could also be consistent with ability of this group of compounds to methylate the O atoms of the ribose-phosphate chain, but this has yet to be proved.

There are also significant differences between the patterns of methylation of ring N atoms in the RNA bases. The nitroso compounds give a higher proportion of the major product 7-methylguanine and show a greater tendency to methylate N-7 of adenine, but less tendency to methylate N-1 of adenine and N-3 of cytosine in RNA. Whether the two types of methylating agent differ in their abilities to methylate the weakly reactive N-3 atoms of guanine and uridine

is less clear at present, but the tentative conclusion is that the nitroso compounds have relatively more affinity for N-3 of guanine and less for N-3 of uracil; but this latter question is complicated by the indication that the ionized form of uracil is more reactive than the neutral form.

With respect to the possible significance of these differences for methylations *in vivo*, correlations must remain tentative until it is well established that these differences are in fact maintained for reactions *in vivo*.

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