The Molecular Basis for Biological Inactivation of Nucleic Acids

THE ACTION OF METHYLATING AGENTS ON THE RIBONUCLEIC ACID-CONTAINING BACTERIOPHAGE R17

By KENNETH V. SHOOTER, RUTH HOWSE, SUDHIKUMAR A. SHAH*
and PHILIP D. LAWLEY

*Chester Beatty Research Institute, Institute of Cancer Research, Royal Cancer Hospital,
Fulham Road, London SW3 6JB, U.K.*

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1. The inactivation of an RNA-containing bacteriophage after reaction with four methylating agents was studied. Measurements of the extent of methylation of the RNA and of the nature and amounts of the various reaction products were made. In experiments with dimethyl sulphate and methyl methanesulphonate inactivation can be quantitatively accounted for by methylation at two of the positions involved in hydrogen bonding: N-1 of adenine and N-3 of cytosine. In experiments with *N*-methyl-*N*-nitrosourea and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine methylation at N-1 of adenine and N-3 of cytosine accounts for only about one-half of the observed inactivation. Scission of the RNA chain during reaction accounts for a further 20% of the inactivation. To account for the remainder it seems necessary to postulate that formation of *O*⁶-methylguanine constitutes a lethal lesion. 2. Breaks in the RNA chain formed on reaction with the nitroso derivatives presumably result from methylation of the phosphate diester group followed by hydrolysis of the unstable triester thus formed.

A previous report from this laboratory described the use of the RNA-containing bacteriophage μ 2 in studies relating chemical reactions and biological effects of the alkylating agents, mustard gas and its monofunctional analogue, 2-chloroethyl 2-hydroxyethyl sulphide (Shooter *et al.*, 1971). It was clear that this system could usefully be applied to other alkylating mutagens and carcinogens, and for the present work typical methylating agents were selected.

The agents chosen may be classified into two groups. The first, represented by dimethyl sulphate and methyl methanesulphonate, are considered as reacting mainly through the S_N2 mechanism, and, in terms of biological action, can be classified as relatively weak carcinogens (Clapp *et al.*, 1968; Druckrey *et al.*, 1970; Frei, 1971; Swann & Magee, 1968). The second, represented by *N*-methyl-*N*-nitrosourea and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, are regarded as reacting through generation of the methyl diazonium ion, and may be classified as S_N1 agents. They are generally considered to be more potent carcinogens than the methanesulphonate or sulphate (Magee & Barnes, 1967; Sugimura *et al.*, 1970; Swann & Magee, 1968).

The differences in biological action of these two types of methylating agent have been ascribed in

* Present address: Cancer Research Unit, Royal Victoria Infirmary, Newcastle upon Tyne, U.K.

part to differences in their reactions with nucleic acids, in particular to the greater ability of the S_N1 agents to produce *O*⁶-methylguanine, a miscoding base in the Watson–Crick sense (Loveless, 1969). In general, the detailed chemistry of nucleic acid alkylation by methylating agents has advanced further than for homologous agents (reviewed by Lawley, 1972*a,b*).

Shooter *et al.* (1971) showed certain advantages of RNA-bacteriophage for studies of the molecular mechanisms involved in inactivation of nucleic acid templates. First, hydrolytic loss of 3- and 7-alkylpurines, at neutral pH and 37°C, did not occur from alkylated RNA, although this is an important feature in the action of alkylating agents on DNA-containing bacteriophages. Secondly, formation of phosphotriester groups in alkylated RNA, which is single-stranded in the bacteriophage used here, would be expected to be clearly revealed by fission of the macromolecule at neutral pH values and 37°C (Brown & Todd, 1955); whereas in DNA, because of alkylation-induced depurination, it might be difficult to assess the effects of phosphotriester formation [although recent work has shown that the DNA phosphotriesters are stable at neutral pH (Bannon & Verly, 1972)]. Thirdly, the RNA-bacteriophages are much more susceptible to inactivation by monofunctional alkylating agents than are DNA-bacteriophages containing double-stranded DNA

(Shooter *et al.*, 1971; cf. Lawley *et al.*, 1969). This enabled the deduction that formation of certain alkylation products in RNA were lethal events; in particular the sites involved were those in which the specific hydrogen-bonding to form nucleic acid duplexes, according to the Watson-Crick scheme, was blocked. It remains possible that these reactions are also lesions in double-stranded DNA, but that duplication of genetic information on the opposite strand masks their effect. Studies on inactivation of RNA may thus be relevant to corresponding effects on DNA, and may enable more detailed examination of precise effects on nucleic acid templates of the various reactions occurring.

Materials and Methods

Materials

[¹⁴C]Methyl methanesulphonate (50 mCi/mmol), di[¹⁴C]methyl sulphate (30 mCi/mmol) and di[³H]-methyl sulphate (15.4 Ci/mmol) were obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). Solutions of these compounds in ether were stored at -20°C. The ³H-labelled dimethyl sulphate was diluted with the unlabelled compound before use. *N*-[¹⁴C]Methyl-*N*-nitrosourea was synthesized from [¹⁴C]methylamine hydrochloride and potassium isocyanate by a modification of the procedure of Cox & Warne (1951), as described in detail by Lawley & Shah (1972). The final product (0.5 mCi/mmol) was stored as the dry solid in the dark. *N*-[¹⁴C]Methyl-*N'*-nitro-*N*-nitrosoguanidine was synthesized from [¹⁴C]methylamine hydrochloride and nitroguanidine by the method of McKay & Wright (1947) and McKay (1949). The product (0.34 mCi/mmol) was stored as a dry solid in the dark. With these two nitroso derivatives fresh solutions in ethanol (20 mg/ml) were made up before each experiment.

Preliminary experiments were performed with suspensions of bacteriophage μ 2 grown and isolated as described by Sargeant & Yeo (1966) and Shooter *et al.* (1971). Most of the work was done with suspensions of the closely related bacteriophage R17, grown and isolated by the method of Webster *et al.* (1967), but excluding the (NH₄)₂SO₄ precipitation, this step being replaced by the centrifugation stage described by Shooter *et al.* (1971). Inactivation of bacteriophage μ 2 by dimethyl sulphate and by *N*-methyl-*N*-nitrosourea was identical with that of bacteriophage R17, and only results with the latter will be reported in detail here.

The concentration of bacteriophage was estimated from E_{260} , an absorption of 8.5 in a cell of 1 cm path-length corresponding to 1.0 mg of bacteriophage/ml. When correcting to the molar scale of concentration, the molecular weight of the bacteriophage was taken as 3.6×10^6 , and of the single RNA molecule in each

bacteriophage as 1.1×10^6 (Gesteland & Boedtker, 1964). Assays of the number of infective particles or plaque-forming units in solutions were made by the agar-layer method (see Clowes & Hayes, 1968).

Assay of the extent of alkylation and inactivation of bacteriophage

For these studies suspensions containing 1–2 mg of bacteriophage/ml were used. In experiments with radioactively labelled methyl methanesulphonate and dimethyl sulphate, bacteriophage were suspended in 0.1 M-sodium acetate, pH 8.2; 5–50 μ l of a solution of the alkylating agent in ether was added to 1 ml of bacteriophage suspension at 37°C and dissolved by rapid stirring with a Rotamixer. The mixture was incubated for 1 h at 37°C and then samples (20–100 μ l) were taken for the assay of plaque-forming ability and for the assay of radioactivity to give the dose of alkylating agent. The remainder was chromatographed at 4°C on a column (35 cm $\times$ 2.5 cm) of Sephadex G-100 eluted with 0.02 M-sodium phosphate buffer, pH 7, to separate bacteriophage from unbound radioactive hydrolysis products. Fractions containing bacteriophage were pooled and the extent of reaction was determined from the u.v. absorption and the radioactivity. A portion of the pooled fractions from the Sephadex column was shaken with an equal volume of phenol reagent (phenol, 500 g; 8-hydroxyquinoline, 0.6 g; *m*-cresol, 50 ml; saturated with 0.02 M-sodium phosphate buffer, pH 7) and after centrifugation portions of the aqueous and phenol layers were assayed for radioactivity to determine the relative binding to RNA and to protein.

In experiments with *N*-methyl-*N*-nitrosourea, the bacteriophage were suspended in 0.1 M-Tris-HCl buffer pH 7.8, appropriate amounts of the nitroso derivative in ethanol (less than 0.1 vol. of the total reaction mixture) were added, and after rapid mixing the samples were incubated at 37°C for 30 min. Measurements of the rate of loss of absorption at 395 nm showed that, in the buffer system used, at least 95% of the nitroso derivative was hydrolysed in 30 min, and that even at an initial concentration of 2 mg/ml the final pH of the solution remained above 7.

Several methods were used in experiments with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. One group of experiments was performed with bacteriophage suspended in 0.2 M-Tris-HCl buffer, pH 9.0. Under these buffer conditions the half-life for the hydrolysis of the nitroso compound was 12.8 min. The nitroso compound in ethanol (less than 0.1 vol.) was added to the bacteriophage suspension and after rapid mixing was incubated for 1 h at 37°C. In a further group of experiments the bacteriophage was suspended in 0.02 M-sodium phosphate buffer, pH 7.0, and equimolar amounts of the nitroso compound and of *N*-acetylcysteine or of mercaptoethanol were added.

The activation of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine by thiols has been shown by Lawley & Thatcher (1970) to enhance markedly the rate of methylation of nucleic acids at this pH. Under the conditions used here, the half-life for the hydrolysis of the nitroso compound was 6min in the presence of *N*-acetylcysteine, and 4min in the presence of mercaptoethanol.

After the initial reaction with the nitroso compounds, the procedures used for isolation of methylated phage were as described above for methyl methanesulphonate and dimethyl sulphate except that a column (85cm×2.5cm) of Sephadex G-100 was used.

Analysis of the products of alkylation of bacteriophage RNA

The reaction conditions in these experiments were as described in the previous section except that much higher concentrations of bacteriophage (10mg/ml) were used. At the end of an incubation the bacteriophage were isolated by centrifugation at 100000g (r_{av} , 5.9cm) for 1h. The bacteriophage pellet was resuspended in buffer and shaken with phenol reagent. The aqueous layer was extracted twice more with phenol reagent and the RNA was precipitated with ethanol (2vol.) before being washed and redissolved in buffer. The precipitation and redissolving steps were repeated twice more. After three precipitations the RNA was washed with ethanol and ether and dried.

The methods for hydrolysis of the RNA and for chromatography of the products were described by Lawley & Shah (1972). In the present work part of each RNA sample was degraded enzymically to nucleosides and part was hydrolysed with acid, at 100°C, either with 1M-HCl to yield pyrimidine nucleotides and purine bases, or with HClO₄ to yield purine and pyrimidine bases. The latter techniques give the best estimates for 3- and 7-methyladenine and for

3- and 7-methylguanine, and the enzymic method has to be used to estimate *O*⁶-methylguanine as the nucleoside, because this product is unstable under the acid conditions for hydrolysis. The other products detected in this work, 1-methyladenine, 3-methyluracil and 3-methylcytosine, can be estimated after both methods of hydrolysis. Further details on the chromatographic techniques are given by Lawley & Shah (1972).

Physicochemical analysis of alkylated RNA

Since, with the bacteriophage used, a break in the single-stranded RNA molecule was expected to constitute a lethal lesion, it seemed necessary to determine whether or not the RNA chain was broken during the alkylation reactions. To test this possibility sedimentation experiments were performed with the RNA in a Beckman-Spinco analytical ultracentrifuge. In experiments with methyl methanesulphonate, dimethyl sulphate and *N*-methyl-*N*-nitrosourea, RNA was isolated from the bacteriophage at the end of the reaction by adding an equal volume of neutralized 8M-NaClO₄ and incubating at 37°C for 5min (Freifelder, 1965, 1966). After reaction with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine the samples of bacteriophage were dialysed against buffer to remove u.v.-absorbing reaction products before addition of the 8M-NaClO₄. After dilution with water to give an E_{260} of 2, the RNA solution (30μl) was banded on 2M-NaClO₄ (1.4ml) and centrifuged at 42000rev./min. Photographs were taken every 4min with u.v.-absorption optics. The photographs were traced by using a Joyce-Loebl recording microdensitometer. In the experiments with the nitroso derivatives, in addition to the main peak corresponding to unbroken RNA molecules, more slowly sedimenting u.v.-absorbing material corresponding to degraded RNA was observed (Fig. 1). For the analysis, areas under the peaks were determined with a planimeter. At least six photographs

Fig. 1. Band sedimentation profiles of bacteriophage R17-RNA

Sedimentation is from left to right. The meniscus is at the left, the peak corresponding to whole RNA molecules to the right and degraded RNA in the central area. A line has been drawn (----) to show the left-hand limit taken for the peak for whole molecules. In the four profiles for RNA from alkylated bacteriophage R17 the concentration of each reagent was chosen to decrease the survival of biological activity to 10–20%. Details of the measurements made are given in the text and the results of the calculations in Table 5. Control (a), after reaction with *N*-methyl-*N*-nitrosourea (b), *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (c), dimethyl sulphate (d) and methyl methanesulphonate (e).

were used to determine the total area corresponding to u.v.-absorbing material: this calculation served to position the baseline consistently. After this, the area under the main peak and that corresponding to degraded material were apportioned and the estimates made from at least four photographs were averaged.

A possible criticism of this technique to detect chain breakage is that breaks might occur in regions of the RNA chain that were within double-stranded, hydrogen-bonded, hairpin loops. Persistence of such ordered structures during isolation of the RNA might mask the effects of chain scission. To check this possibility, part of a solution of RNA after extraction with 8M-NaClO₄ and dilution, was treated at 75°C for 10 min in the presence of formaldehyde (7%, w/v). This process denatures the RNA, and reaction with formaldehyde prevents reformation of hydrogen-bonded regions, as shown by the absence of any decrease in the E_{260} when the solution is cooled (Freifelder & Davison, 1963; Boedtker, 1967). Results of a typical experiment with bacteriophage treated with *N*-methyl-*N*-nitrosourea are given in Table 1. The results showed that, although heating in formaldehyde did cause some further degradation of of both control and treated RNA species, the relative extent of degradation, after methylation of the RNA, expressed as survival of unbroken molecules, was not increased by denaturation in formaldehyde. Similar experiments with RNA from bacteriophage treated with methyl methanesulphonate, dimethyl sulphate or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine also failed to reveal the existence of 'hidden' breaks.

Results

The results for the analysis of the nature of the methylated products present in the RNA are summarized in Table 2. The distribution of products is

very similar to that observed in experiments on the methylation of isolated RNA (Lawley & Shah, 1972). With all four reagents the main product is 7-methylguanine. The main subsidiary products observed in experiments with methyl methanesulphonate and dimethyl sulphate were 3-methylcytosine and 1-methyladenine. Compared with these latter compounds the nitroso derivatives produced more 7-methylguanine but much less 3-methylcytosine and 1-methyladenine, though these latter two products together with *O*⁶-methylguanine were the main subsidiary products. Small amounts of other methylated bases were detected but the quantities involved were too small to contribute significantly to the observed extent of inactivation. Summation of the proportions of the various products fails to account for about 10% of the radioactivity eluted from the column. The apparent loss of material may be, in part, accounted for as cumulative errors, but it could include any methylated ribose or phosphate derivatives that had been formed. These latter two products, together with any methanol formed (e.g. by acid hydrolysis of *O*⁶-methylguanine), would be among the material eluting from the columns before the most weakly bound derivatives of the bases. No detailed analysis of the nature of the compounds in these early radioactivity-containing peaks has yet been made.

Dose-survival curves for the four compounds investigated are shown in Figs. 2(a) and 2(b). The lines drawn in these and subsequent figures connect the origin with the centroid of the results. These lines lie between the two lines calculated by regression analysis. The lower extent of inactivation observed with methyl methanesulphonate compared with dimethyl sulphate reflects the slower rate of hydrolysis of the sulphonate. The relationship between the extent of methylation and the degree of inactivation induced is shown in Figs. 3(a) and 3(b). In Fig. 3(b) no attempt has been made to deal

Table 1. *Effect of denaturation of RNA from bacteriophage R17 on the analysis of the number of breaks induced in the RNA molecules*

N-Methyl-*N*-nitrosourea (2.1 mg) was dissolved directly in a bacteriophage suspension in 0.1 M-Tris-HCl, pH 8.0 (1 ml containing 2.5 mg of bacteriophage), and incubated for 30 min at 37°C. Assay of a portion (0.1 ml) showed a survival of 0.058 relative to the control. A further portion (0.4 ml) was added to an equal volume of 8M-NaClO₄ and, after 5 min at 37°C, water (2 ml) was added. One-half of this sample was heated at 70°C for 5 min in the presence of formaldehyde (7%, w/w). Band sedimentation of the RNA was performed as described in the text and the area of the main peak as a fraction of the total area was determined. A control sample of bacteriophage was treated in the same way.

	Proportion of whole RNA molecules (area of main peak/total area)		Survival of unbroken molecules Treated/control
	Control	Treated	
Normal procedure	0.53	0.24	0.45
After heating with formaldehyde	0.35	0.19	0.54
Survival of unbroken molecules after heating with formaldehyde	0.66	0.79	

Table 2. Products in RNA from methylated bacteriophage R17

Suspensions of R17 were treated with the methylating agent, RNA was extracted, degraded and chromatographed as described in the text. The peaks corresponding to products formed in the reactions were identified by using appropriate markers and the relative amounts of each product were calculated from the radioactivity associated with the peak relative to the total amount of radioactivity applied to the column. The limits for the detection of minor products depended upon the shape of the eluted peak and on the total amount of radioactivity applied to the column. In general a peak could be detected with a significance level of 1% if the radioactivity was not less than 0.5% of the total. The extent of methylation of the RNA used in these analyses, measured as mol of methyl bound/mol of RNA with the four agents, was 1.4 for methyl methanesulphonate, 0.8 for dimethyl sulphate, 29 for *N*-methyl-*N*-nitroso-urea and 14 for *N*-methyl-*N'*-nitro-*N*-nitroso-guanidine.

Fraction of RNA methylation products				
	Methyl methanesulphonate	Dimethyl sulphate	<i>N</i> -Methyl- <i>N</i> -nitroso-urea	<i>N</i> -Methyl- <i>N'</i> -nitro- <i>N</i> -nitroso-guanidine
3-Methyluracil	0.004	—	—	—
3-Methylcytosine	0.07	0.10	0.015	0.01
7-Methylguanine	0.72	0.70	0.80	0.80
3-Methylguanine	—	—	0.01	—
<i>O</i> ⁶ -Methylguanine	0.002	0.002	0.04	0.03
1-Methyladenine	0.11	0.12	0.02	—
3-Methyladenine	0.015	—	—	—
Total	0.92	0.92	0.89	0.88

Fig. 2. Dose-survival curves

The relationship between dose of alkylating agent and survival of biological activity of R17 bacteriophage as measured by the plaque-forming assay is shown; (a) Δ , dimethyl sulphate; $\blacktriangle$, methyl methanesulphonate; (b) $\circ$, *N*-methyl-*N*-nitroso-urea; $\bullet$, *N*-methyl-*N'*-nitro-*N*-nitroso-guanidine. Details of methods are given in the text. Survival values are given on a logarithmic scale in all figures.

separately with the points for the two nitroso derivatives. Estimates of the mean lethal dose from Figs. 2(a) and 2(b) and from Figs. 3(a) and 3(b), the extent of methylation at the point where there is, on average, one lethal event for each bacteriophage particle, are given in Table 3. The standard errors of these latter estimates have been calculated from the regression of logarithm (survival) on dose (Davies,

1947). Note that although the mean lethal dose expressed as concentration of reagent varies by a factor of nearly 3, the extent of methylation required to induce one lethal event/bacteriophage does not differ greatly among the four compounds tested. The fraction of the methyl groups bound to RNA as a proportion of the total bound to the whole bacteriophage was determined, for each of the

reagents, by phenol extraction of the methylated bacteriophage. This fraction was very similar for methyl methanesulphonate (average 0.73, s.d. 0.04, six observations) and for the two nitroso derivatives (average 0.75, s.d. 0.02, two observations with each compound); with dimethyl sulphate a markedly smaller fraction was bound to the RNA (average

0.43, s.d. 0.03, six observations). From these data, the number of methyl groups bound to the protein moiety and to the RNA molecule of the bacteriophage at the mean lethal dose was calculated. As shown in Table 3, the number of methylations of the bacteriophage protein varied quite markedly (from 2.5 to 8) for the four reagents. There was much

Fig. 3. Binding-survival curves

The relationship between survival of biological activity and the extent of methylation of bacteriophage R17 is shown; symbols for (a) and (b) as in Fig. 2. Details of methods are given in the text.

Table 3. Mean lethal doses for methylated bacteriophage R17

The doses required to decrease survival to 0.37 (e^{-1}) are shown, together with the extents of methylation at the mean lethal dose, and the distribution of bound methyl groups between protein and RNA. For the nitroso compounds, estimates of the extents of methylation have been made from the combined data for both compounds, which did not differ significantly. The values in parentheses give s.e. of the estimates. From these data and those of Table 3 the number of mol of each methylated base present at the mean lethal dose has been calculated.

	Methyl methanesulphonate	Dimethyl sulphate	N-Methyl-N- nitroso-urea	N-Methyl-N'- nitro-N-nitroso- guanidine
Mean lethal dose (mg/ml)	0.33 (0.11)	0.13 (0.04)	0.25 (0.08)	0.25 (0.05)
At the mean lethal dose				
Mol of methyl/mol of phage	9 (1.8)	14 (3.6)		11 (1.4)
Bound to protein	2.5	8		3
Bound to RNA	6.5 (1.3)	6 (1.5)		8 (1.0)
From chromatographic analysis: mol of methyl- ated bases at the mean lethal dose				
3-Methyluracil	—	—	—	—
3-Methylcytosine	0.5	0.6	0.2	0.1
7-Methylguanine	4.7	4.2	6.4	6.4
3-Methylguanine	—	—	—	—
O ⁶ -Methylguanine	—	—	0.3	0.25
1-Methyladenine	0.7	0.7	0.2	0.25
3-Methyladenine	0.1	—	—	—
Methyl groups not accounted for	0.5	0.5	0.9	1.0

Table 4. *Degradation of the RNA molecule of bacteriophage R17*

Suspensions of the bacteriophage were treated with the reagents as described in the text, and samples were taken for assay of biological activity and to determine the fraction of whole RNA molecules remaining. These data were obtained from the experiments illustrated in Fig. 1.

Reagent	Fraction of unbroken RNA molecules (area of main peak/total area)	Fraction of unbroken molecules relative to control	Survival (plaque-forming units relative to control)
None	0.58	1.0	1.0
<i>N</i> -Methyl- <i>N</i> -nitroso-urea	0.33	0.57	0.1
<i>N</i> -Methyl- <i>N'</i> -nitro- <i>N</i> -nitrosoguanidine	0.40	0.69	0.1
Methyl methanesulphonate	0.57	0.98	0.01
Dimethyl sulphate	0.61	1.05	0.01

less difference between the reagents in the number of methyl groups bound to the RNA. Evidently inactivation requires the binding of more methyl groups after reaction with the nitroso derivatives than in reactions with dimethyl sulphate or methyl methanesulphonate, but as the two pairs of values differ by little more than 1 s.d. the significance of this difference is, statistically, not great.

Reaction with *N*-methyl-*N*-nitroso-urea and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine leads to degradation of the RNA molecule in the bacteriophage, whereas reaction with methyl methanesulphonate and dimethyl sulphate causes no significant degradation (see the Materials and Methods section). These observations are illustrated in Fig. 1. The results of the calculations based on these latter ultracentrifuge experiments (Table 4) show the differences in the amount of degradation observed with the two classes of reagent. The formation of the breaks in the RNA molecules after reaction with the nitroso compounds occurred during the time required for the hydrolysis of the compound. As is shown in Table 5 continued incubation after the first ½ h did not affect survival of biological activity, nor was any increase in the number of degraded molecules found. Further experiments were performed with the nitroso derivatives to determine the relationship between chain breakage and survival of biological activity. Within experimental error the results of these experiments with both compounds were identical (Fig. 4). This finding is consistent with the view that both nitroso compounds act through the same intermediate, i.e. most probably the methyl diazonium ion (Lawley & Thatcher, 1970). Breakage of the RNA chain is not a major factor leading to inactivation of the bacteriophage (Fig. 4). At the mean lethal dose, with survival decreased to 0.37 (e^{-1}) of the control, the fraction of RNA molecules remaining unbroken amounted to 0.83 (s.e. ± 0.03). In the reactions with methyl methanesulphonate and dimethyl sulphate the contribution of chain breaks to inactivation was negligible.

Table 5. *Stability of methylated RNA in bacteriophage R17*

N-Methyl-*N*-nitroso-urea (1 mg/ml) was added to a suspension of bacteriophage R17 in 0.1 M-Tris-HCl buffer, pH 8.0, and the mixture incubated at 37°C. At intervals a sample was taken for assay of biological activity and RNA was extracted from a further sample by the perchlorate method. The proportion of whole RNA molecules remaining relative to a control sample was determined by analytical ultracentrifugation as described in the Materials and Methods section.

Time at 37°C (h)	Survival of biological activity (fraction of the control value)	Fraction of molecules unbroken
0.5	0.11	0.60
1	0.10	0.62
2	0.12	0.57
4	0.16	0.60
7.5	0.13	0.58

Discussion

In a previous study of the inactivation of an RNA-containing bacteriophage, $\mu 2$, by alkylating agents (Shooter *et al.*, 1971) the results were consistent with the view that loss of biological activity resulted from the formation of specific lethal lesions and was not the cumulative effect of many lesions, all of which interfered only partially with transcription or translation. In particular, mono-alkylation of guanine in bacteriophage RNA to yield 7-alkylguanine did not appear to constitute a lethal lesion. On this basis the lethal lesions are to be found among the minor products of the methylation reactions. At the mean lethal dose, by definition, the sum of these lethal products should amount to one mol of alkyl/mol of RNA.

In the reactions involving methyl methanesulphonate and dimethyl sulphate the sum of the amounts of 3-methylcytosine and 1-methyladenine are 1.2 and 1.3 mol/mol of RNA respectively. These two products are thus present in sufficient amounts

Fig. 4. Correlation between survival of biological activity and the extent to which RNA molecules are degraded after reaction of bacteriophage R17 with methyl-nitroso compounds

The degradation of RNA was determined as shown in Fig. 1; $\circ$, *N*-methyl-*N*-nitrosourea; $\bullet$, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; methyl methanesulphonate and dimethyl sulphate did not degrade RNA over this range of survival.

to account for the inactivation observed. Note that, with each of these products, the methyl group blocks one of the specific hydrogen-bonding positions of the base in the Watson-Crick scheme for base-pairing in a nucleic acid duplex. Some support for the hypothesis that the formation of 3-methylcytosine and 1-methyladenine constitute lethal lesions comes from studies *in vitro* of the coding properties and template activities of synthetic polymers containing these modified bases. Ludlum & Wilhelm (1968) found that the presence of 3-methylcytosine residues in poly(C) decreased the template activity in the pyrophosphorylase system, and led to the incorporation of uracil into the newly synthesized polymer. Michelson & Pochon (1966) investigated the properties of poly-(1-methyladenine) and showed that it did not form a co-polymer with poly(U). In other studies, Ludlum *et al.* (1964) observed that the presence of 4% of 1-methyladenine in poly(A)-poly(U) mixtures and, even at this low frequency of substitution, markedly decreased the template efficiency for the synthesis of polylysine.

In the present experiments with *N*-methyl-*N*-nitrosourea and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, 3-methylcytosine and 1-methyladenine are among the minor products of the reactions but they are not present in sufficient amounts to account for the inactivation at the mean lethal dose, totalling only about 0.4 mol/mol of RNA. Physico-

chemical studies showed that reaction with these nitroso derivatives introduced breaks in the RNA chain. Fig. 4 showed that, at the mean lethal dose, breaks have been introduced in about 20% of the RNA molecules. In molar terms, the contribution of chain breaks to inactivation amounts to 0.2 breaks/mol of RNA. Even when this further contribution is added to the amounts of 3-methylcytosine and 1-methyladenine found, the total contribution to inactivation is only 0.6 of a lethal event/molecule of RNA. It would appear that to bring the sum of lethal events up to 1/molecule of RNA the formation of *O*⁶-methylguanine must be included as a lethal lesion. As the presence of the methyl group on the *O*⁶-position would block the specific hydrogen bonding of guanine to cytosine, it is quite possible that this methylated base would constitute a lethal lesion.

Loveless (1969) and Loveless & Hampton (1969) have suggested that, in a DNA-containing bacteriophage system (T4), miscoding may occur at a trinucleotide containing *O*⁶-methylguanine, i.e. that this product constitutes a mutagenic lesion. Support for this suggestion has come from recent work by Gerchman & Ludlum (1973) who have shown that when a copolymer of cytidylic acid and *O*⁶-methylguanylic acid was used as template in an RNA polymerase reaction *in vitro* misincorporation of uridylic acid into the product copolymer occurred. The evidence available is therefore apparently at variance with the present postulate that the occurrence of one *O*⁶-methylguanine moiety anywhere among the 3600 nucleotides of an R17-RNA molecule constitutes a lethal lesion *in vivo*. It should, however, be stressed that other lethal products may yet be identified in the 10–12% of radioactive material applied to the chromatographic columns that cannot be accounted for (Table 2). The work of Gerard *et al.* (1972), for example, suggests that methylation of the free 2'-OH group of the ribose moiety of purine nucleotides may inhibit template efficiency. No estimate has yet been made of the extent to which hydroxyl groups of the ribose are methylated in reactions of the bacteriophage with the alkylating agents. Also note that a lethal lesion in a single-stranded nucleic acid may not be lethal in a double-stranded DNA (Freifelder, 1965; Lawley *et al.*, 1969) because of the presence of an intact complementary chain.

The observation that reaction of the bacteriophage with the methyl-nitroso compounds leads to scission of the RNA molecule provides yet another difference in the mode of action of these methylating agents when compared with compounds such as dimethyl sulphate and methyl methanesulphonate. With regard to the action of alkylating agents on nucleic acids, Lawley (1972*a,b*) has suggested that changes in the spectrum of products may be related to changes in

alkylation mechanism. Dimethyl sulphate and methyl methanesulphonate react predominantly by the S_N2 mechanism, whereas both *N*-methyl-*N*-nitrosourea and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine can be regarded as S_N1 methylating agents generating the methylcarbonium ion. The S_N1 reagents would be expected to attack a different, and wider, spectrum of groups in nucleic acids than the S_N2 reagents. This is observed, because the S_N1 agents react with O-atom sites as well as with ring N atoms, as is exemplified by the formation of *O*⁶-methylguanine in RNA. The formation of chain breaks presumably results from alkylation of an O of the phosphodiester group followed by hydrolysis of the triester formed. The instability of ribonucleotide triesters at neutral pH has been shown by Brown *et al.* (1955).

Although the possibility and the effect of alkylation of the phosphate group of nucleic acids have been argued for many years, quantitative evidence for this reaction has been lacking until recently. Bannon & Verly (1972) have now shown that after reaction of DNA *in vitro* with methyl methanesulphonate or ethyl methanesulphonate, 1 and 15% respectively of the total alkylation derives from reaction with the phosphodiester group. These authors also showed that the triesters formed in reaction with DNA were stable for 90 min at 100°C at pH 7.0. Lawley (1973) has found from experiments on the reaction of DNA with *N*-methyl-*N*-nitrosourea that about 18% of the total alkylation products have properties consistent with those expected for the derivatives B-(pMe)-D where B and D are deoxyribonucleoside residues and (pMe) is a methylated phosphate group. These observations show, first, that triester formation can account for a considerable part of the total alkylation

and, secondly, that the extent of triester formation depends upon the nature of the alkylating agent.

In contrast with the stability of the triesters formed in DNA, triesters of ribonucleotides hydrolyse rapidly as was first shown by Brown *et al.* (1955). Ludlum (1969) showed that whereas the molecular weight of poly(A) was unaffected by reaction with methyl methanesulphonate, incubation of the polymer with ethyl methanesulphonate resulted in progressive and extensive degradation. These latter observations thus parallel those of Bannon & Verly (1972) in indicating a more extensive alkylation of the phosphate group by the ethylating agent. The finding that reaction with methyl methanesulphonate did not lead to degradation of poly(A) parallels the finding in the present work that this reagent does not cause any significant amount of chain breakage of the bacteriophage RNA.

Experiments with RNA or polyribonucleotides do not afford a measure of the total reaction with the phosphate groups. Hydrolysis of any triester formed can be envisaged (Brown & Todd, 1955) as resulting from the attack of the 2'-OH group of the ribose on the P=O bond to form an intermediate as shown in Scheme 1 followed by scission of either bond (i) or (ii). The former would lead to loss of the alkyl group and the latter to chain scission. There is also the possibility that ring opening after scission of bond (i) or (ii) may leave the P attached to the 2'-OH of the ribose. The physicochemical studies measure therefore only the extent to which triester formation occurs and is followed by the hydrolysis step involving chain scission. However, as the hydrolysis step is rapid, unless switching of the RNA chain from the 3'- to

Scheme 1. A suggested intermediate complex in the hydrolysis of phosphotriesters

Hydrolysis of a phosphotriester may lead to the formation of an unstable cyclic intermediate in which scission may first occur either at bond (i) with the loss of the methyl group; or at bond (ii) breaking the nucleic acid chain. In each case the subsequent ring-opening may result in the formation of either the 2'- or the 3'-phosphate.

the 2'-OH occurs to an appreciable extent, the chain scission will be the only part of the process that can contribute to inactivation. The present data show that in reaction with *N*-methyl-*N*-nitrosourea only about 3% of the total, measured, alkylation results from the formation of methyl phosphate group, which is followed by hydrolytic loss of part of the RNA chain. The amount of this reaction appears small compared with the value of 18% for the suggested proportion of methylation to yield phosphotriesters in DNA (Lawley, 1973). However, the comparison is difficult because no estimate can be made at present of the frequency of hydrolysis of a ribonucleotide triester to give a chain break, relative to the frequency of hydrolysis involving loss of the alkyl group.

It may be concluded from the present work that lethal lesions are induced by methylating agents when methylation occurs at one of the groups involved in the specific Watson-Crick base pairing. With the agents tested, the groups of this type most frequently involved are N-1 of adenine, N-3 of cytosine and O-6 of guanine. Methylation of phosphodiester groups followed by hydrolysis and scission of the RNA chain can occur to a measurable extent, but only when S_N1 -type reagents are used. Such chain breakage contributes considerably (20%) to the biological inactivation observed with *N*-methyl-*N*-nitrosourea or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

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