The Action of Mono- and Di-functional Sulphur Mustards on the Ribonucleic Acid-Containing Bacteriophage $\mu 2$

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Bacteriophage $\mu 2$ is inactivated by both mono- and di-functional sulphur mustards at relatively low extents of alkylation. No degradation of alkylated RNA was detected. Cross-linking of RNA to protein was observed with the difunctional agent, but this reaction was only a minor contribution to the inactivation. Analyses of the reaction products in bacteriophage RNA showed that, at the mean lethal doses, more than one mono-alkylation of guanine had occurred but the sum total of other types of RNA alkylation was close to a single event. The results therefore suggest that inactivation results from the mono-alkylation of adenine or cytosine. In experiments with the difunctional agent cross-linking of RNA bases or of RNA to protein also prevented replication, the existence of these reactions accounting for the greater sensitivity of the bacteriophage to this agent.

Many cytotoxic, mutagenic and carcinogenic chemicals are capable of reacting with cellular material directly as alkylating agents; and many others are converted by metabolism (chiefly oxidative) into reactive molecular species of the alkylating type. There is now little doubt that nucleic acids can be significant cellular targets for such agents (Lawley, 1966a,b; Dipple, Lawley & Brookes, 1968).

Considerable attention has been devoted to the reaction with DNA, and bacteriophages containing DNA were shown to be valuable as model systems (Loveless, 1966). Rather fewer investigations of RNA bacteriophages have been reported, although the studies by Fraenkel-Conrat (1961) with tobacco-mosaic virus clearly showed the value of such viruses in studies of the mode of action of chemical agents (cf. Singer & Fraenkel-Conrat, 1969).

In bacterial cells (Venitt, Brookes & Lawley, 1968) and in liver of rats (Mizrahi & De Vries, 1965; Villa-Trevino, 1967) inhibition of protein synthesis by alkylating agents has been associated with alkylation of mRNA. However, in the bacterial system translation was less affected by alkylation than was transcription, and both processes were more difficult to inhibit than was cell division.

The crucial cellular targets of chemical carcino-

gens are not yet clearly defined, although the concept that some essential template macromolecule should be irreversibly modified in the process of tumour initiation is difficult to avoid (Lawley, 1966a; Dipple *et al.* 1968). The processes in carcinogenesis termed 'promotion', which certain experimental approaches have suggested to be qualitatively different from those of tumour initiation by reason of their reversible nature, also remain ill-defined in molecular terms.

Whether cellular RNA could be involved as a target in tumour promotion is not known; however, it remains possible that some RNA, perhaps of the mRNA type, could be a significant receptor of carcinogens in the initiation stage. It is conceivable that destruction of the template functions of certain cellular RNA molecules by carcinogens could lead to permanent imbalance in the organization of animal tissues, possibly of neoplastic potential; alternatively, certain 'normal' cellular RNA molecules could be converted by a process of chemical mutagenesis into potentially oncogenic molecules, analogous to those of tumour viruses.

For these reasons, therefore, it appears that studies of the action of chemical carcinogens on biologically active RNA may be of potential significance in the field of carcinogenesis. The main difficulty with this concept is that even the largest RNA molecules are relatively small targets compared with cellular DNA; but, whereas there is

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evidence for repair of lesions in DNA, this may not be possible for RNA.

Clearly, the systems available at present for practicable study appear to be remote from those thought to exist in mammalian cells. The difficulties presented by the study of RNA function in the complex milieu of the cell are well known, and the concept of transfer of non-viral RNA between cells remains controversial. It therefore appeared necessary to turn to better-defined systems and to establish quantitative relationships between extents of chemical modification of RNA in such systems and biological effects; the subsequent return to cellular studies might then be more fruitful.

The RNA bacteriophages seemed useful from this point of view, and the action of certain simple chemical agents on these systems was therefore selected for study. The reasonably well characterized $\mu 2$ bacteriophage of *Escherichia coli* (Dettori, Maccacaro & Piccinin, 1961; Matthews, 1968) was available in quantities suitable for investigations of this type (Isenberg, 1963; Sargeant & Yeo, 1966). The inactivation of this bacteriophage by radiation and by nitrous acid has been studied (Turri, Maccacaro & Dettori, 1964). For chemical agents in the present work we chose the sulphur mustards, since mono- and di-functional agents of equally high chemical reactivity were available isotopically labelled at high specific radioactivity, and these have been extensively investigated in previous work (Brookes & Lawley, 1960; Lawley, Lethbridge, Edwards & Shooter, 1969). Mustard gas is now established to be a carcinogen for humans (Wada, Miyaniishi, Nishimoto, Kambe & Miller, 1968).

Several points of interest arose from previous studies on the chemical inactivation of RNA. First, Yamamoto & Naito (1965) had found that, for inactivation of bacteriophages containing single-stranded DNA or RNA, the difunctional nitrogen mustard di-(2-chloroethyl)methylamine (HN 2) was effective, but the monofunctional analogue was either much less effective or ineffective. This finding was surprising in view of reports that mono- and di-functional alkylating agents were of approximately equal effectiveness in this type of test (Fraenkel-Conrat, 1961; Loveless, 1966). However, the extents of alkylation of bacteriophage nucleic acids were not determined by Yamamoto & Naito (1965), so that their results were not readily interpreted in detail.

Secondly, conflicting evidence had been obtained about the part played by degradation of alkylated RNA in its inactivation. The evidence for sulphur mustards was that degradation of RNA did not occur; for example, tobacco-mosaic virus or its infectious RNA was not degraded by doses of mustard sufficient to cause inactivation (Fraenkel-Conrat, 1961). Inactivation of poly(U) as template

for polypeptide synthesis *in vitro* was shown to result from alkylation of terminal phosphomonoester groups, but not of chain phosphodiester groups (Abell, Rosini & Ramseur, 1965). The latter process would be expected to cause fission of polyribonucleotides at neutral pH (Brown, Magrath & Todd, 1955); although in DNA, which lacks the 2'-hydroxyl group, such instability of phosphotriester groups would not necessarily result (Brown & Todd, 1955). Ludlum (1967, 1969) reported that di-(2-chloroethyl)methylamine and ethyl methanesulphonate did degrade polyribonucleotides at neutral pH; Kriek & Emmelot (1963) and Holy & Scheit (1966) found degradation of RNA or oligonucleotides respectively after extensive alkylation *in vitro* by diazomethane. The action of dimethylnitrosamine *in vivo* on polyribosomes of rat liver was interpreted by Mizrahi & DeVries (1965) as showing degradation of mRNA; whereas from analogous experiments Villa-Trevino (1967) concluded that the inhibition of protein synthesis could be caused by methylation of mRNA, but not necessarily by its degradation. Brimacombe, Griffin, Haines, Haslam & Reese (1965) found that a small extent of degradation of polyribonucleotides accompanied extensive methylation by diazomethane *in vitro*, but pointed out that the neutral molecule of this agent could act as a basic catalyst. Griffin, Haines & Reese (1967) found no evidence for attack of a large excess of diazomethane on the phosphodiester linkage in diribonucleotides such as UpA, and did not confirm the contrary claim from similar work of Holy & Scheit (1966).

On the basis of their reactions with nucleic acids, alkylating agents appear to be divided into two categories (Ludlum, 1969; Lawley & Thatcher, 1970): those that react almost exclusively with basic ring nitrogen atoms [perhaps analogous to the classical S_N2 reagents of Ingold (1953)]; and those that can additionally react with oxygen atoms, perhaps reacting in part through the S_N1 mechanism. The latter may include alkylnitrosamides, which can react with the extranuclear 6-oxygen atom of guanine nucleosides (Loveless, 1969) and of guanine residues in nucleic acids under *in vivo* conditions (Lawley & Thatcher, 1970). Whether reaction with phosphodiester groups occurs is not yet conclusively established, although the evidence of Ludlum (1969) for degradation of ethylated polynucleotides is particularly suggestive in this respect.

It was therefore expected that both sulphur mustards would inactivate RNA bacteriophage, but that probably this would be caused by alkylation rather than degradation of RNA. The action of these mustards could then serve as a basis for comparison with the effects of other agents, which might differ in this respect.

MATERIALS AND METHODS

Materials

Di-(2-chloroethyl) [^{35}S]sulphide (mustard gas) and 2-chloroethyl 2-hydroxyethyl [^{35}S]sulphide (half mustard) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Methods for the use and assay of these mustards have been described by Lawley *et al.* (1969).

Homopolyribonucleotides were obtained from Miles-Seravac Ltd., Maidenhead, Berks., U.K.

Bacteriophage $\mu 2$, grown as described by Sargeant & Yeo (1966), was obtained from the Microbiological Research Establishment, Porton, Wilts., U.K., as an ammonium sulphate precipitate of the bacterial lysate. Purification was by the method of Dr H. Isenberg (personal communication), as follows. The precipitate was resuspended in 0.1 M-sodium acetate-10 mM-magnesium acetate-1 mM-disodium EDTA and centrifuged at 7000g for 10 min to remove insoluble debris. To 80 g of the supernatant was added 45 g of CsCl and the solution was centrifuged at 22500 rev./min in an SW25 rotor of a Beckman-Spinco model L ultracentrifuge for 48 h. The band containing the bacteriophage was removed by pipetting and dialysed against five changes of suspension medium.

Bacteriophage concentration was estimated from the E_{260} of solutions. In a cell of 1 cm path-length an absorption of 1.0 is observed with a solution containing 8.5 mg of $\mu 2$ bacteriophage/ml. When converting into a molar scale of concentration the molecular weight of the bacteriophage has been taken as 3.6×10^6 and that of the RNA molecule within the bacteriophage as 1.1×10^6 (Gesteland & Boedtker, 1964). Assays of the number of viable bacteriophage in solutions were made using the agar-layer method. After suitable dilution samples of bacteriophage solutions were mixed with 2.5 ml of soft agar containing 10^9 *E. coli* strain K12 D10 and spread on a nutrient containing a layer of hard agar in a petri dish. Dishes were then incubated overnight at 43°C in air saturated with chloroform. The presence of a viable bacteriophage particle in the test solution showed as a clear circle in the translucent layer of bacteria. Counts of the number of clear circles or plaques, corrected for dilution, give the number of viable bacteriophage or plaque-forming units in the original solution. Further discussion and details of the assay method may be found in Clowes & Hayes (1968).

Most of the work reported here refers to preparations of bacteriophage with a plating efficiency (ratio of viable bacteriophage to total number of bacteriophage) of 4–7%. Later, freshly grown bacteriophage was isolated from bacterial lysates by centrifugation instead of precipitation with ammonium sulphate and these preparations had a plating efficiency of at least 30%.

Assay of extent of alkylation of bacteriophage

In a typical experiment ^{35}S -labelled mustard gas in ether (5–25 μl) was added to a bacteriophage suspension (0.5 ml; 1–2 mg/ml) at 37°C, and dissolved by rapid stirring with a Rotamixer. After 30 min, samples (10 μl) were taken for assay of radioactivity of the suspension medium, and the remainder was chromatographed at

4°C on a column (20 cm $\times$ 1.5 cm) of Sephadex G-100 eluted with the suspension medium (flow rate 10 ml/h; 2 ml fractions). Bacteriophage was eluted in fractions 15–18 and hydrolysed unbound mustard in fractions 30–50, with a clear separation of radioactivity into these two peaks shown by the presence of only background counts in the intervening fractions. The fractions containing bacteriophage were pooled; the extent of reaction was determined from the u.v. absorption and the radioactivity, and the number of plaque-forming units was assayed after appropriate dilutions. The relative extents of alkylation of RNA and proteins in bacteriophage were determined by two methods. 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 suspension medium). After centrifugation, portions of the phenol and aqueous layers were assayed for radioactivity. Alternatively, with a more concentrated suspension of bacteriophage (about 5 mg/ml), the alkylated bacteriophage was collected by centrifugation and resuspended three times; the suspension was then extracted with phenol reagent. The aqueous layer was shaken with two more portions of phenol and the RNA was precipitated by addition of ethanol (2 vol.). The first phenol layer was twice washed with buffer and protein was then precipitated with methanol (10 vol.). RNA was redissolved in 2% (w/v) sodium acetate-1 mM-disodium EDTA, pH 7, then reprecipitated and washed with ethanol and ether. The protein was washed with methanol and ether. The radioactivity and concentration of RNA was assayed in aqueous solutions (ϵ_p 9300 at 260 nm). The protein was weighed and dissolved in 10% (w/v) tetraethylammonium hydroxide for assay of radioactivity. Estimates of the relative binding to RNA and protein obtained by the two procedures were in excellent agreement.

To determine the relative binding of mustard to coat protein and A protein of the bacteriophage the two were separated by electrophoresis in polyacrylamide gel (Vinuela, Algrananti & Ochoa, 1967; Fromageot & Zinder, 1968). After reacting the bacteriophage with ^{35}S -labelled half mustard the proteins were isolated by phenol extraction as described above and dissolved in 0.5 M-urea-1% (w/v) sodium dodecyl sulphate-20 mM-sodium phosphate buffer, pH 7. A sample (0.2 ml) of the solution was applied to a 7.5% polyacrylamide gel and subjected to electrophoresis at 50 V, 6 mA for 4 h. The distribution of protein within the gel was determined with an automatic scanning densitometer (Vitatron, Dieren, Holland; 280 nm filter). For the determination of radioactivity gels were cut into 2 mm slices, each slice was heated in 0.3 ml of 10% (w/v) trichloroacetic acid for 10 min at 100°C and the radioactivity of 0.2 ml samples was counted.

Separation of reaction products in RNA or polyribonucleotides

Acid hydrolysis. RNA (about 0.5 mg) isolated after reaction with ^{35}S -labelled mustard, and containing more than 20000 c.p.m., was hydrolysed in 1 M-HCl (0.1 ml) for 0.75 h at 100°C and the hydrolysate was chromatographed on a strip of Whatman 3 MM paper (3 cm wide) with methanol-conc. HCl-water (7:2:1, by vol.) as solvent (upflow). The dried and neutralized paper was examined

under u.v. light (254nm) and either scanned with a windowless gas-flow counter (Packard model 385) or was cut into 1cm segments, the radioactivities of which were counted in scintillation bottles containing 0.5ml of phosphor [toluene, 1 litre; 2,5-diphenyloxazole, 5g; 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene, 0.05g]. An alternative solvent used for chromatography (3-methylbutan-1-ol saturated with 0.3M-disodium hydrogen phosphate, both phases present) gave essentially similar results.

Marker substances for chromatography were 7-alkyl-guanine, prepared as described by Brookes & Lawley (1960), and 1-alkyladenine and 3-alkyladenine, prepared by reaction of half mustard with deoxyadenylic acid and adenine respectively, and isolated chromatographically. The u.v. properties used for identification of the adenine derivatives are in Table 1.

In some cases ^{35}S -labelled RNA after acid hydrolysis was chromatographed on a column (8cm $\times$ 0.7cm) of the cation-exchange resin Dowex 50 AG (H^+ form), eluted with 1M-HCl (3ml fractions). In a typical experiment with RNA treated with half mustard, the peak fractions containing the various components of the mixture were as follows: uridylic acid, 2; cytidylic acid, 4; guanine, 17; adenine, 61; ^{35}S -labelled products were found in the region of pyrimidine nucleotides (4% of total ^{35}S recovered) together with marker 7-alkylguanine (peak fraction 44, 86% of ^{35}S); and eluted after adenine, with 1.5M-HCl as solvent, alkylated adenines (5% of ^{35}S). An unidentified peak containing 4% of ^{35}S was eluted at fraction 13. This method was also used to investigate

the presence of alkylated RNA in protein extracted from alkylated bacteriophage.

Degradation of RNA to nucleosides. RNA (about 1mg) was dissolved in water (0.5ml), and 1M-tris-HCl buffer, pH 8 (0.1ml), was added together with venom phosphodiesterase (Calbiochem, Los Angeles, Calif., U.S.A.) (380 units/ml; 0.1ml) and bacterial alkaline phosphatase (280 units/ml; 0.01ml). The mixture was incubated for 18h at 37°C and a portion (0.01ml) was examined by chromatography on thin-layer cellulose plates with propan-2-ol-aq. NH_3 (sp.gr. 0.88)-water (7:1:2, by vol.) as solvent. The digest was evaporated to dryness in a desiccator and the residue was dissolved in methanol (5ml at 46°C for about 30 min). The resultant solution of nucleosides was clarified by centrifugation and applied to a column (70cm $\times$ 3cm) of Sephadex LH-20 eluted with methanol (10ml fractions). The unchanged nucleosides of RNA were eluted in three peaks; the first peak contained cytidine (shoulder at fraction 34) and uridine (fraction 37); guanosine (fraction 44) and adenosine (fraction 50) were separated. The positions of elution of alkylated nucleosides derived from homopolyribonucleotides after reaction with half mustard were: alkylated cytidine, 33; alkylated adenosine, 42; a minor peak, 58. Polyguanylic acid was not degraded by the enzymes, and mustard gas-treated polynucleotides were not completely degraded. Alkylated guanosine from alkylated RNA was not separated from alkylated cytosine (peak fraction 34). The method was therefore useful primarily for the isolation of alkylated adenosines from other products.

Degradation of RNA to nucleotides. RNA (about 0.5mg)

Table 1. *Properties of 2-hydroxyethylthioethylated adenine and adenylic acid compared with those of methyl analogues*

In the table below 'alkyl' refers to 2-hydroxyethylthioethyl. Solvents for paper chromatography were as follows: (1) methanol-conc. HCl-water (7:2:1, by vol.); (2) 3-methylbutan-1-ol saturated with 0.3M-sodium phosphate, pH 7, both phases present in the chromatography trough; (3) saturated $(\text{NH}_4)_2\text{SO}_4$ -propan-2-ol-0.1M-sodium phosphate buffer, pH 7 (79:2:19, by vol.).

Compound	pH	λ_{max} (nm)	E_{280}/E_{260} ratio	pK'_a	R_F		
					Solvent (1)	Solvent (2)	Solvent (3)
1-Alkyladenine	2	261.5	0.42	7.2 10 approx. 6	0.48	0.73	—
	8.9	271	1.03				
	11.9	271	0.95				
3-Alkyladenine	1	274.5	1.25	approx. 6	0.74	0.84	—
	12	274	1.64				
1-Alkyladenylic acid	7	260.5	0.40	—	—	0.83	—
	12*	260.5	0.43				
N^6 -Alkyladenylic acid	7	268	0.71	—	—	—	0.20
	12	268	0.71				
1-Methyladenine	2	259	0.23	7.2 11.0	0.48	—	—
	13	270	0.85				
3-Methyladenine	2	274	1.26	6.0	0.55	—	—
	13	273	1.48				
1-Methyladenylic acid	7	258.5	0.26	—	—	—	—
	12*	259	0.39				
N^6 -Methyladenylic acid	7	266	0.66	—	—	—	—
	12	266	0.66				

* Initial values; the compound is ultimately converted in alkali into N^6 -alkyladenylic acid.

was dissolved in 1 M-KOH (0.2 ml) at 37°C overnight, and the resulting hydrolysate, containing nucleoside 2'- and 3'-phosphates, was chromatographed on Whatman 3MM paper with, as solvent, saturated $(\text{NH}_4)_2\text{SO}_4$ -propan-2-ol-0.1 M- Na_2HPO_4 (79:2:19, by vol.; downflow). The R_F values of ^{35}S -labelled nucleotides from reaction with half mustard were as follows: from poly(A), 0.16-0.20; from poly(C), 0.91; from poly(G), 0.71 and 0.79 with a small peak at 0.36; from poly(U), no peak of radioactivity. When ^{35}S -labelled mustard gas was used essentially the same peaks were found, but a 'tail' of radioactivity to lower R_F values was found. With ^{35}S -labelled half mustard the peaks corresponding to those observed with labelled poly(A) and poly(G) were found and at the position of alkylated cytidylic acid a shoulder of the main peak was observed. The alkylated material from poly(G) is presumably derived from opening of the imidazole ring of 7-alkylguanylic acid in alkali. The R_F of alkyladenylic acid was identical with that of the alkaline conversion product of 1-alkyladenylic acid. The proportion of the alkylation products from RNA treated with ^{35}S -labelled mustard gas in this latter zone was 9-10%, and with the half mustard was 5-6%.

Sedimentation experiments

A Beckman-Spinco analytical ultracentrifuge fitted with u.v.-absorption optics was used. RNA was isolated from samples of bacteriophage (approx. 1 mg/ml) by adding an equal volume of neutralized 8 M- HClO_4 , then incubating the mixture for 5 min at 37°C and then diluting it with a further 8 vol. of water (Freifelder, 1966). Boundary-sedimentation experiments were made at 42040 rev./min. From the photographs obtained, distribution curves of sedimentation coefficients were calculated in the manner outlined by Shooter & Butler (1956). Sedimentation experiments were also used to check the purity of the preparations of the bacteriophage after banding in CsCl .

Effect of pretreatment with formaldehyde on the reaction of bacteriophage $\mu 2$ RNA with mustard gas

The RNA (1 mg/ml in 0.1 M-sodium acetate-1 mM-magnesium acetate) was added to formaldehyde in the same medium [2 vol.; to give concentrations of 1-13% (w/v) formaldehyde]. The solutions were then heated at 95°C for 15 min and cooled at 0°C. ^{35}S -labelled mustard gas was added (100 $\mu\text{g}/\text{ml}$), and after being kept at 37°C for 30 min the RNA was isolated and analysed by acid hydrolysis and chromatography on paper.

RESULTS

The extent of reaction of the two mustards with whole $\mu 2$ bacteriophage was proportional to the dose (Fig. 1). The binding of mustard gas (0.27 mol of mustard/mol of bacteriophage $\mu 2$ with a unit dose of 1 μg of mustard/ml) was slightly greater than that for half mustard (0.21 mol/mol of bacteriophage $\mu 2$ per unit dose of 1 $\mu\text{g}/\text{ml}$). The effect of mustards on the plaque-forming ability of the bacteriophage is shown in Fig. 2. Evidently

Fig. 1. Extent of reaction of bacteriophage $\mu 2$ with sulphur mustard (●) and half mustard (○). Experimental details are given in the text.

the half mustard is less toxic than mustard gas at a given extent of binding. At the dose required to decrease survival to 37%, i.e. a dose corresponding to one lethal event for each bacteriophage present, the extent of reaction of mustard gas was 5 mol of mustard/mol of bacteriophage $\mu 2$ and that of half mustard was 11 mol/mol of bacteriophage $\mu 2$.

Separation of bacteriophage protein and RNA showed that the fraction of bound half mustard combined with RNA was 0.91 (s.d. ± 0.02 , 14 assays). For mustard gas the corresponding fraction was smaller (0.74-0.80), and decreased somewhat as the extent of binding increased (statistical analysis of 18 experiments showed this trend to be significant). A possible explanation for the apparently greater extent of reaction of mustard gas with bacteriophage protein is that cross-linking of bacteriophage RNA to protein had occurred and that RNA cross-linked to protein was carried into the phenol phase.

Experiments were therefore done to investigate the presence of alkylated RNA in the denatured proteins extracted from [^{35}S]sulphur mustard-treated bacteriophage by phenol. The dried protein (40-50 mg) was weighed and hydrolysed in 1 M-hydrochloric acid for 1 h at 100°C; this procedure solubilized 80-90% of the radioactivity. The diluted hydrolysate (0.1 M-hydrochloric acid) was then applied to a column of Dowex 50 (H^+ form) after addition of marker 7-alkylguanine, and eluted with 0.1 M-hydrochloric acid (3 ml fractions). After 9 fractions had been collected the eluent was changed to 1 M-hydrochloric acid, and 7-alkylguanine was subsequently eluted in fractions 14-47. With half mustard-treated bacteriophage all the radioactivity was found in the first two fractions from the column. With mustard gas-treated

bacteriophage part of the radioactivity was found in the 7-alkylguanine fractions and, in addition, u.v. absorption caused by RNA guanine and adenine was detected (Fig. 3). The specific radioactivity of the RNA carried into the phenol layer could thus be calculated and compared with that of the bulk of RNA isolated in the aqueous layer after

phenol extraction (Table 2). The amount of RNA associated with protein could also be calculated from the amount of guanine observed in the hydrolysate and was found to be 0.02–0.03 mol of RNA/mol of mustard gas bound to the bacteriophage. The small RNA-containing bacteriophages have been shown to contain one molecule of protein (the A protein) per bacteriophage, this being essential for the process of infection (Lodish, Horiuchi & Zinder, 1965; Argetsinger & Gussin, 1966). Such a unique molecule could be a vital target of a cytotoxic agent. As shown in Fig. 4, however, the greater part of the mustard is bound to coat protein rather than to the A protein.

With alkylated DNA bacteriophages marked effects of post-treatment incubation in diminishing survival and extent of alkylation were found (Loveless & Stock, 1959; Brookes & Lawley, 1963; Lawley *et al.* 1969). Corresponding investigations of alkylated $\mu 2$ bacteriophage were therefore done. As shown in Tables 3 and 4 prolonged further incubation of mustard-treated bacteriophage had no effect on survival and no significant loss of alkyl groups occurred.

It remained possible that degradation of bacteriophage $\mu 2$ RNA had accompanied its alkylation. A suspension of bacteriophage $\mu 2$ was divided into three parts, one being treated with mustard gas and one with half mustard to give extents of reaction of 41 and 56 mol of mustard/mol of bacteriophage $\mu 2$ respectively. After the samples had been maintained at 37°C for 30 min, RNA was liberated by the method of Freifelder (1966) and the integral distribution curve of sedimentation coefficients was determined for each sample. As shown in Fig. 5 no significant differences could be detected.

It therefore appeared that the only reactions of mustards with RNA that were significant lesions

Fig. 2. Inactivation of bacteriophage $\mu 2$ by mustard gas (●) and half mustard (○). Experimental details are given in the text.

Fig. 3. RNA cross-linked to protein after reaction of bacteriophage $\mu 2$ with ^{35}S -labelled mustard gas. Protein was isolated by extraction with phenol, hydrolysed in 1M-HCl for 1 h at 100°C and chromatographed on Dowex 50 (H^+ form) (3 ml fractions). —, E_{260} (the three main peaks are from the left guanine, 7-alkylguanine added as marker and adenine); ----, radioactivity.

Table 2. Binding of ^{35}S -labelled mustard gas to RNA and protein of bacteriophage $\mu 2$

Samples of the bacteriophage suspension were treated with concentrations of mustard gas of 4.5, 9.0 and 63 $\mu\text{g}/\text{ml}$ for 30 min at 37°C and were then extracted with phenol as described in detail in the text.

Extent of binding (mol of mustard/mol of bacteriophage $\mu 2$)	Sp. radioactivity of RNA ($\mu\text{Ci}/\text{g}$)		
	From aqueous layer	From protein in phenol layer	Fraction of RNA bound to protein
1.15	8.4	7.2	0.03
2.3	15	17	0.07
17	125	110	0.35

Fig. 4. Binding of mustard to the proteins of bacteriophage $\mu 2$. Bacteriophage $\mu 2$ was treated with ^{35}S -labelled half mustard, and the proteins were isolated by phenol extraction, dissolved in 0.5M-urea-1% (w/v) sodium dodecyl sulphate-20 mM-sodium phosphate buffer, pH7, and separated by electrophoresis in 7.5% polyacrylamide gel. Experimental details are given in the text. —, E_{260} ; ·····, radioactivity. The main peak corresponds to coat protein and the minor peak to the A protein.

should be sought among the alkylation products themselves. For chemical analyses alkylated RNA was degraded to yield bases, nucleosides or nucleotides, and comparisons at each level were made with alkylated homopolyribonucleotides. Typical radiochromatograms are shown in Fig. 6. The salient feature of reactions of the bacteriophage with both mustards was the predominance of 7-alkylguanine among the products. The principal minor product in experiments with half mustard (Table 5) was a derivative of adenine. Although this product had chromatographic properties identical with those of 1-alkyladenine, the possibility that some 7-alkyl-

adenine was formed cannot be ruled out. It is known that the N-7 position of adenine is reactive in nucleotides and in DNA (Lawley & Brookes, 1964), and also in RNA (Singer & Fraenkel-Conrat, 1969). The 7-alkyladenine derivative produced by reaction with the mustards has yet to be isolated and characterized, but by analogy with 7-methyladenine it is expected to have R_F values close to those of 1-alkyladenine (Lawley & Brookes, 1964). A minor product from adenine had R_F values identical with those of 3-alkyladenine. A product from cytidylic acid had properties consistent with those of 3-alkyletydylidic acid, at present the sole established site of alkylation for this nucleotide (Brookes & Lawley, 1962). Small proportions (generally less than 1%) of radioactivity on chromatograms of hydrolysed alkylated RNA ran near the solvent front; this proportion was greater for the polyribonucleotides. Abell *et al.* (1965) found that the terminal phosphomonoester groups of poly(U) reacted with mustard gas, but no evidence was found for reaction with phosphodiester groups in the macromolecular chain. Since in the present work no degradation of RNA was found after extensive reaction it is probable that alkylation of phosphodiester groups does not occur. The traces of mustard residues found near the solvent front presumably originate from alkylation of terminal phosphate groups, from partial decomposition of alkylated bases or from hydrolysed mustard incompletely removed from RNA by the preparative procedures.

Differences were found between the mono- and di-functional mustards in their mode of reaction with the bacteriophage, the difunctional mustard yielding with RNA an additional product that had chromatographic properties identical with those of di(guanin-7-ylethyl) sulphide. The possibility that other cross-linked derivatives are formed cannot be eliminated. Poly(A) and poly(C) (Fig. 6) gave additional products with mustard gas, which were probably cross-linked derivatives. But in reactions with bacteriophage the amounts of such products formed would be far too small to be detected by the

Table 3. *Effect of post-treatment incubation on plaque-forming ability of alkylated bacteriophage $\mu 2$*

The extents of reaction were, for half mustard, 56 mol/mol of bacteriophage and for mustard gas, 41 mol/mol of bacteriophage $\mu 2$.

Time, at 37°C (h)	10 ⁻⁹ × Plaque-forming ability (plaque-forming units/ml)		
	Control	Half mustard	Mustard gas
0.5	160	1.3	0.16
24	170	1.5	0.18
48	150	1.3	0.14
89	150	1.4	0.16
161	23	0.24	0.14

Table 4. *Effect of post-treatment incubation on the extent of alkylation of bacteriophage $\mu 2$*

Two samples of bacteriophage suspension were treated with mustard gas (140 μ g/ml) at 37°C for 30 min. Then each sample was divided into two portions, one being kept at 37°C and the other at 4°C. After various times the extent of alkylation was measured by Sephadex chromatography as described in detail in the text.

Time after treatment (h)		Observed extent of alkylation (mol of mustard/mol of bacteriophage $\mu 2$)			
Experiment ...	Temperature ...	A		B	
		4°C	37°C	4°C	37°C
0.5		34.8	38.0	35.8	37.0
24		36.4	34.8	34.0	37.2
48		32.7	33.5	38.6	40.0
72		—	—	36.2	37.7
144		34.0	30.9	—	—

Fig. 5. Distribution of sedimentation coefficients of RNA liberated from bacteriophage $\mu 2$ after incubation with or without sulphur mustards. The time of treatment before release of RNA was 30 min at 37°C, and the extents of alkylation were, for half mustard, 56 mol/mol of bacteriophage $\mu 2$, and, for mustard gas, 41 mol/mol of bacteriophage $\mu 2$. O, Control; Δ , half mustard; $\bullet$, mustard gas.

analytical methods used so far. The contribution of such products to the total biological effect would be negligibly small. The difunctional mustard

gave consistently, and somewhat unexpectedly, a small peak which has so far not been positively identified, with R_F close to that of 1-alkyladenine.

Bacteriophage RNA has some secondary structure, as reflected by its hypochromicity relative to the sum total of u.v. absorption of the constituent nucleotides ($\epsilon_{(P)}260$ in 0.15M-sodium chloride-15mM-sodium citrate, pH 7.0: bacteriophage, 8450; RNA, 7900; at pH 13: bacteriophage, 10500; RNA, 9800). We therefore investigated whether the observed guanine-guanine linkage was dependent on this secondary structure. Pretreatment of bacteriophage RNA with formaldehyde and heat destroyed the hypochromicity of RNA. But, when this treated RNA was subsequently alkylated with mustard gas, the proportion of diguanine derivatives remained the same as for 'native' RNA (Table 6).

In general, the RNA reacted with alkylating agents in the same way in whole bacteriophage as when treated in isolation *in vitro*.

DISCUSSION

The principal points of interest to emerge are, first, the comparatively low extent of alkylation of bacteriophage $\mu 2$ by either mustard that suffices to inactivate plaque-forming ability, and, secondly, the relatively small, but significant, difference between effectiveness of alkylation by the mono- and di-functional mustards.

Fig. 6. Radiochromatograms of acid hydrolysates of alkylated homopolyribonucleotides or of RNA from alkylated bacteriophage $\mu 2$. Abscissae indicate distance along paper (origin at the left); ordinates indicate ^{35}S radioactivity. At least 40000 c.p.m. was applied, corresponding to extents of alkylation of about 1 mmol/mol of RNA P. The solvent was methanol-conc. HCl-water (7:2:1, by vol.) and the distance run was about 30 cm. (a) Half mustard-poly(G); (b) half mustard-poly(A); (c) half mustard-poly(C); (d) mustard gas-poly(A); (e) mustard gas-poly(A); (f) mustard gas-poly(G); (g) half mustard-RNA; (h) mustard gas-RNA.

Table 5. Analyses of bacteriophage $\mu 2$ RNA alkylated by sulphur mustards

The R_f values relative to that of adenine (0.37) for products (I-V) were 0.05, 0.95, 1.4, 1.9 and 2.6 respectively. Marker substances with R_f values identical with those of the various products were: (I), di(guanin-7-ylethyl) sulphide; (II), 7-alkylguanine; (III), 1-alkyladenine; (IV), 3-alkyladenine; (V), 3-alkylcytidylic acid. As noted in the text, the as yet unknown compound 7-alkyladenine may have an R_f approximately equal to that of 1-alkyladenine ('alkyl' = 2-hydroxyethylthioethyl). The percentages of the various products were estimated from the amounts of ^{35}S radioactivity in the appropriate regions of the chromatograms.

(a) Paper chromatography of HCl hydrolysates; solvent: methanol-conc. HCl-water (7:2:1, by vol.).

Material treated	Reagent	Extent of alkylation (mol of mustard/mol of RNA)	% of different products of alkylation				
			(I)	(II)	(III)	(IV)	(V)
Bacteriophage $\mu 2$	Half mustard	43	—	91	6	1	2
		52	—	90	6	1	3
		82	—	88	6	2	2
Bacteriophage $\mu 2$ RNA	Half mustard	64	—	93	5	1	1
Bacteriophage $\mu 2$	Mustard gas	12	10	74	13	2	2
		17	12	73	12	3	—
		21	11	73	12	3	1
Bacteriophage $\mu 2$ RNA	Mustard gas	108	12	74	11	1	2

(b) Sephadex LH-20 chromatography of nucleosides.

Material treated	Reagent	Extent of alkylation (mol of mustard/mol of RNA)	Proportion of total radioactivity in principal alkylated adenosine (peak fraction 42)
Bacteriophage $\mu 2$	Half mustard	52	6
Bacteriophage $\mu 2$	Mustard gas	12	13

Table 6. *Effect of eliminating hydrogen-bonded double-stranded regions of RNA on the cross-linking of guanine moieties by di-(2-chloroethyl)sulphide*

Bacteriophage $\mu 2$ RNA was heated in the presence of various amounts of formaldehyde (95°C for 15 min). Then 100 μg of ^{35}S -labelled mustard gas was added/ml and the reaction mixture kept for 30 min at 37°C. The RNA was precipitated, washed, hydrolysed with 1M-HCl and chromatographed on paper. Distribution of radioactive products was determined by using a chromatogram scanner. The two columns of results refer to two separate experiments.

Concn. of formaldehyde (%, w/v)	% of di(guanin-7-ylethyl)sulphide in reaction products	
1	14	13
2	9	13
3	14	12
4	10	12
5	14	15

The first point reflects the higher susceptibility to lesions in the nucleic acid molecule of bacteriophage containing a single-stranded nucleic acid compared with those containing double-stranded DNA. Earlier examples of such differential susceptibility have been provided by the work of Fiers & Sinsheimer (1962a) who have shown that one break in the chain of the circular DNA molecule of ϕX174 is sufficient to inactivate this bacteriophage whereas with bacteriophage T7 at least ten single-strand breaks induced by X-rays are required for inactivation (Freifelder, 1965). The evidence of Fiers & Sinsheimer (1962b) on the thermal inactivation of ϕX174 suggests that the loss of probably only one purine base may be sufficient to inactivate whereas with bacteriophage T7 at least ten purine bases must be lost before inactivation results (Lawley *et al.* 1969; Brakier & Verly, 1970). It may also be noted that bacteriophages T2, T4 and T7 are not inactivated by at least ten times as many mono-alkylations as are required to inactivate bacteriophage $\mu 2$ (Brookes & Lawley, 1963; Lawley *et al.* 1969; Brakier & Verly, 1970).

The present observation that the mono- and di-alkylating agents do not differ much in the efficiency with which they inactivate bacteriophage $\mu 2$ is in marked contrast with the results obtained by Yamamoto & Naito (1965) but would be consistent with the observations by both Loveless (1966) and Fraenkel-Conrat (1961). The comment of these two latter authors that there was little difference between mono- and di-functional agents was, in each case, based on too few results to show significant differences at the level noted in the present work. No explanation for the lack of effect of the monofunctional agent by Yamamoto & Naito (1965) can be given, though it is notable that these authors provide no evidence of the extent of alkylation occurring under the conditions of their experiments.

The difference between the mono- and di-

functional agents appears to arise from differences in their mode of reaction with bacteriophage RNA. It seems reasonable to assume that the difunctional alkylation of RNA is as effective in preventing replication as is the difunctional alkylation of DNA. From the results obtained with formaldehyde-treated RNA, it would appear that the diguaninyl derivative can be formed readily, as with DNA, by linking neighbouring guanine residues rather than across 'loops' of double-stranded regions of the RNA. It is, however, clear in the present work that the inactivation of bacteriophage $\mu 2$ on reaction with mustard gas cannot be entirely attributed to the difunctional alkylation, since at the mean-lethal extent of reaction only 0.5 mol of diguaninyl derivative/mol of bacteriophage is present. Cross-linking of the RNA to protein presumably results in inactivation, but the contribution of this type of reaction is small. Other alkylation reactions must therefore contribute to the inactivation.

A possible explanation of the results can be obtained if it is assumed that mono-alkylated guanine either is not an effective lesion or is at most a very weak one. Inspection of the results of Table 5 shows that, if the alkylation of guanine is ignored, then, in the reaction with half mustard, the sum of all other types of alkylation product amount to 1 lesion/RNA molecule at the mean lethal dose. In the experiments with mustard gas, adding all alkylated derivatives of RNA other than the 7-guaninyl derivative again gives about 1 lesion/RNA molecule at the mean lethal dose.

Surprisingly, however, because of the low extents of alkylation of bacteriophage protein the observed 'single-hit' doses also correspond to a single alkylation in protein. It is possible that the 'single hit' in protein could affect a sensitive target, causing failure of the bacteriophage to attach to the host bacterium in the manner appropriate for infection. Analysis of the alkylated protein has shown that the greater part of the reaction is with coat protein. Since each bacteriophage contains 180 identical

molecules of coat protein it is unlikely that one alkyl group on any one of these molecules would result in inactivation.

Therefore it seems more probable that certain types of alkylation of RNA, notably at N-3 of cytosine or at N-1 of adenine, or guanine-guanine cross-linking, are responsible for the inactivation of replicative function. It may be noted that N-1 of adenine and N-3 of cytosine are involved in hydrogen-bonding of the Watson-Crick type whereas the N-7 atom of guanine is remote from the hydrogen-bonding sites. Further, it is known that 7-alkylation of guanine in polyribonucleotides does not cause mispairing of this base *in vitro* to a significant extent (Wilhelm & Ludlum, 1966; Ludlum, 1970), but methylated poly(C) has been shown to behave in anomalous fashion as a template *in vitro* after alkylation (Ludlum & Wilhelm, 1968). Singer & Fraenkel-Conrat (1969) concluded that methylation of cytosine in tobacco-mosaic-virus RNA could cause mutations, but that methylation of guanine was not likely to do so. The relative susceptibilities of the two template functions of RNA, for protein synthesis and for RNA synthesis, cannot be judged from the present work, but this question now invites attention.

The relatively high sensitivity of RNA to alkylation indicated by this interpretation of the results would presumably point to equally high probabilities of inactivation of single-stranded RNA within cells. Clearly mRNA would constitute a likely target of this type. The results are therefore consistent with previous interpretations of the inhibition of cellular protein synthesis by alkylating agents, namely that a relatively small number of alkylations in mRNA can inhibit its translation (Venitt *et al.* 1968).

The problem remains whether this action of alkylating agents could be significant in the overall effects of such agents *in vivo*. As mentioned above, individual RNA molecules are small targets relative to cellular DNA, itself known to be a sensitive target. The contribution of inactivation of RNA to the general cytotoxic action of alkylating agents must therefore be small, despite the inherent sensitivity of individual RNA molecules, and clearly could not account for the marked difference between mono- and di-functional agents in this respect.

Nevertheless the possibility remains that, after repair of lesions in cellular DNA, certain RNA molecules could remain with alkylation damage. The part played by such entities in processes of mutagenesis and carcinogenesis remains for further investigation.

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