Biochem. J. (1974) **137**, 313–317
Printed in Great Britain

The Reaction of Alkylating Agents with Bacteriophage R17

BIOLOGICAL EFFECTS OF PHOSPHOTRIESTER FORMATION

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(*Received* 24 *August* 1973)

The extent of biological inactivation and of the degradation of the RNA after reaction of bacteriophage R17 with ethyl methanesulphonate, isopropyl methanesulphonate and *N*-ethyl-*N*-nitrosourea was studied. Formation of breaks in the RNA chain probably results from hydrolysis of phosphotriesters formed in the alkylation reactions. Near neutral pH the ethyl and isopropyl phosphotriesters are sufficiently stable for the kinetics of the hydrolysis reaction to be followed. Results indicate that the rate of hydrolysis increases rapidly as the pH is raised. The evidence shows that a phosphotriester group does not itself constitute a lethal lesion. The extent of phosphotriester formation by the different agents is discussed in terms of reaction mechanism.

Previous work has demonstrated that bacteriophage R17, which contains one single-stranded RNA molecule, can be used to determine which of a spectrum of reaction products constitute lethal lesions (Shooter *et al.*, 1974). Use has now been made of this model system to determine the biological effect of phosphotriester formation after reaction with different alkylating agents.

Bannon & Verly (1972) have provided direct evidence for phosphotriester formation in the reaction of ethyl methanesulphonate with isolated DNA and have demonstrated that the triester is stable. Lawley (1973) has studied the reaction of *N*-[^{14}C]-methyl-*N*-nitrosourea with isolated ^{32}P-labelled DNA and from chromatography of enzymic digests has found that 18% of the bound methyl groups occur in products which can be assigned the structure $dN_1p(Me)dN_2$ where dN_1 and dN_2 denote deoxyribonucleoside residues and p(Me) is a methylated phosphate group. Rhaese & Boetker (1973) have analysed the kinetics of mutation and biological inactivation induced by alkylation of DNA and from their results have concluded that phosphotriesters constitute lethal lesions in nucleic acids. In contrast with the stability of phosphotriesters in DNA such moieties in RNA would be unstable and hydrolyse readily (Brown *et al.*, 1955; Brown & Todd, 1955). The chain breaks produced in the RNA molecules of bacteriophage R17 after reaction with two methyl nitroso compounds have been attributed to the hydrolysis of methyl phosphotriesters (Shooter *et al.*, 1974). In reactions with these methylating agents, although the extent of triester formation only accounted for 3% of the methyl groups bound to the RNA, the contribution to biological inactivation amounted to 18%. The hydrolysis of the methyl phosphotriesters formed was found to be complete within the time-scale for the alkylation reaction.

In the present work some effects of treating bacteriophage R17 with two ethylating agents and one isopropylating reagent have been investigated. It has been assumed, following the arguments presented in a previous paper (Shooter *et al.*, 1974), that the formation of breaks in the RNA chain results from the hydrolysis of phosphotriesters. Formal proof of the presence of alkyl phosphate residues in the RNA requires a detailed analysis of the products of alkylation, and this work is not yet complete. Lawley (1972*a*,*b*) has suggested that *O*-alkylation occurs mainly in reactions of the S_N1-type mechanism. In a series of alkyl methanesulphonates, the extent of phosphotriester formation would thus be expected to increase in the sequence methyl, ethyl, isopropyl, in parallel with the increasing predominance of the S_N1-type mechanism. In addition, reaction with *N*-ethyl-*N*-nitrosourea should produce more ethyl phosphotriester than reaction with the ethyl methanesulphonate, since the former compound breaks down to give the ethyldiazonium ion or the ethyl carbonium ion as the proximal alkylating agent (Lawley & Thatcher, 1970).

Experimental

Bacteriophage R17 was grown, isolated and purified as described previously (Shooter *et al.*, 1971, 1974). Assays of the number of infective particles (plaque-forming units) in solutions were made by using the agar-layer method (Clowes & Hayes, 1968). For physicochemical studies RNA was isolated by the method of Freifelder (1965, 1966)

and the extent of degradation determined from band-sedimentation experiments in an analytical ultracentrifuge. Further details of these methods are given by Shooter *et al.* (1974).

Ethyl methanesulphonate was obtained from Eastman–Kodak Co. (Rochester, N.Y., U.S.A.) and isopropyl methanesulphonate from Koch–Light Laboratories Ltd. (Colnbrook, Bucks., U.K.). *N*-Ethyl-*N*-nitrosourea was synthesized by using the method of Werner (1919) for *N*-methyl-*N*-nitrosourea.

Measured volumes of ethyl methanesulphonate, corresponding to final concentrations of 1–11mg/ml, were added to suspensions of bacteriophage (1.7mg/ml) in 0.2M-sodium phosphate buffer, pH7.0, and assays for biological activity and for degradation of the RNA were performed after incubation for 24h at 37°C. From data given by Barnard & Robertson (1961) the half-time for the hydrolysis of the sulphonate under the conditions used was calculated as 11.5h.

Experiments with isopropyl methanesulphonate (final concentrations 2–11mg/ml) were performed with suspensions of the bacteriophage (1.7mg/ml) in 0.2M-phosphate, pH7.0, or 0.2M-Tris–HCl, pH8 or pH9. At the highest dose liberation of acid on hydrolysis lowered the pH of the solution by 0.7–1.1pH units. Observations on the rate of fall of pH were used to determine the approximate half-time for the hydrolysis reaction as 7min at pH7 and 8, and 6min at pH9. Measured volumes of the sulphonate were added to the suspensions of bacteriophage preincubated to a temperature of 37°C and the incubation was continued at this temperature for 1–24h before sampling for the assays of plaque-forming units and RNA chain breaks.

N-Ethyl-*N*-nitrosourea was dissolved in ethanol (40mg/ml) and measured volumes giving final concentrations of 1–8mg/ml were added to suspensions of the bacteriophage (1.7mg/ml) in 0.2M-phosphate buffer, pH7.0, incubated at 37°C. Measurements of the rate of decrease of the E_{396} gave a half-time for the decomposition of the nitroso derivative of 15min.

Results and Discussion

The relationship between loss of biological activity and degradation of RNA after reaction with ethyl methanesulphonate is shown in Fig. 1. From the graph the mean lethal dose (the concentration of reagent needed to decrease survival relative to control to e^{-1}) is 1.2mg/ml. At this dose the fraction of unbroken RNA chains relative to the control is 0.85. Since, by definition, there is one lethal event per bacteriophage at the mean lethal dose, the formation of chain breaks accounts for only a small fraction (0.15) of the inactivating events, the remaining fraction (0.85) resulting from lethal lesions formed, e.g., by alkylation of the base residues (cf. Shooter *et al.*, 1974).

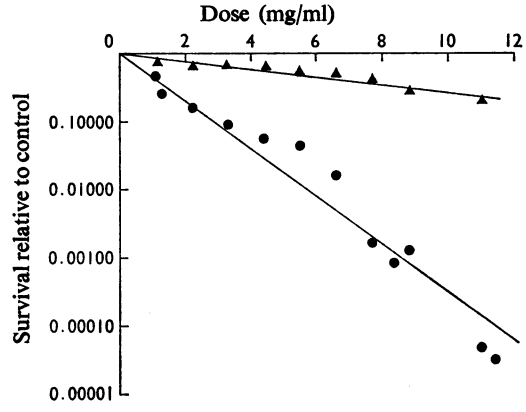

Fig. 1. *Reaction of bacteriophage R17 with ethyl methanesulphonate*

Bacteriophage R17 (1.7mg/ml) in 0.2M-sodium phosphate, pH7.0, was treated with ethyl methanesulphonate (1–11mg/ml) and the mixtures were incubated for 24h at 37°C. Assays were then made for survival of biological activity and for degradation of RNA. Data in each case are plotted as the ratio (treated/control). ●, Survival of infectivity; ▲, survival of unbroken RNA molecules.

In the experiments with *N*-ethyl-*N*-nitrosourea and isopropyl methanesulphonate the extent of inactivation of the bacteriophage increased if incubation was extended beyond 1h at 37°C even though at this stage hydrolysis was at least 95%. Measurements showed that, with time, the extent of inactivation observed reached a limiting value and then remained unchanged, relative to the control, for a further 48–72h.

In Figs. 2 and 3 the estimates of RNA chain breaks and survival of infectivity observed after 24h at 37°C are compared with results observed after 1h at 37°C. The data at 24h represent the limit for both changes. The increase in chain breaks and the decrease in survival are, in both cases, clearly too large to be accounted for by reaction with the 5% or less of reagent still present after 1h. Further experiments showed that, with all the compounds used in the present work, no loss of biological activity and no chain breaks occurred if the compounds were fully hydrolysed before addition of the bacteriophage. Experiments with other alkylating agents that do not induce breaks in the RNA chain, namely di-(2-chloroethyl) sulphide, 2-chloroethyl 2-hydroxyethyl sulphide (Shooter *et al.*, 1971), dimethyl sulphate and methyl methanesulphonate (Shooter *et al.*,

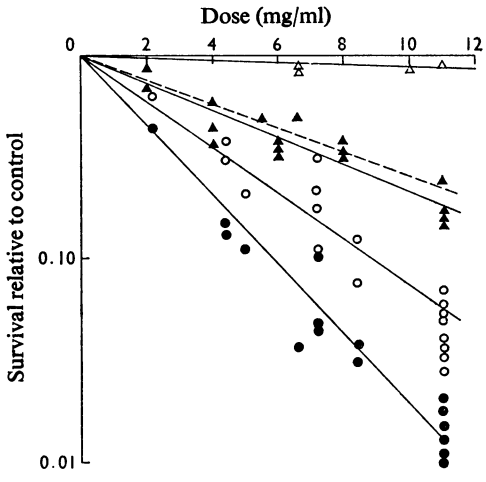

Fig. 2. *Reaction of bacteriophage R17 with isopropyl methanesulphonate*

Bacteriophage R17 (1.7mg/ml) in 0.2M-sodium phosphate, pH7.0, was treated with isopropyl methanesulphonate (2–11mg/ml) and incubated at 37°C. After 1h and 24h survival of biological activity and survival of unbroken RNA molecules were determined. Survival of infectivity relative to control after 1h (○) and 24h (●); survival of unbroken RNA molecules relative to control after 1h (△) and 24h (▲).

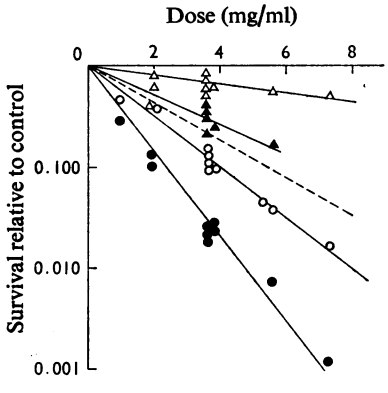

Fig. 3. *Reaction of bacteriophage R17 with N-ethyl-N-nitrosourea*

Bacteriophage R17 (1.7mg/ml) was treated with *N*-ethyl-*N*-nitrosourea (1–8mg/ml) in 0.2M-sodium phosphate, pH7.0, and incubated at 37°C. Measurements of biological infectivity and of the degradation of the RNA molecules were made after incubation for 1h and 24h. Survival of infectivity relative to control after 1h (○) and 24h (●); survival of unbroken RNA molecules after 1h (△) and 24h (▲).

1974), have shown that there is no delayed inactivating reaction once the hydrolysis of the reagent is complete and that alkylation of RNA bases does not lead to an instability reflected in loss of alkylated bases followed by chain scission. Breakage of the RNA chain appears to be produced solely by reagents that are capable of alkylating the phosphate groups to form unstable triesters. In previous work (Shooter *et al.*, 1974) it was found that methyl phosphotriesters produced by reaction with *N*-methyl-*N*-nitrosourea or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine were completely hydrolysed within the first hour of incubation. The present results suggest that phosphotriesters involving an ethyl or an isopropyl moiety may hydrolyse more slowly than the corresponding methyl derivatives and that the progressive increase in chain breaks and the decrease in survival with time reflect this slower rate of hydrolysis.

The increase in the number of lethal lesions on incubation between 1h and 24h can be determined from calculations of the ratio (survival of infectivity at 24h)/(survival of infectivity at 1h). Adding this increase to the lesions produced by chain breaks at 1h gives a dose-dependent plot shown by the dashed lines in Figs. 2 and 3. Statistical calculations show that the slopes of the dashed lines do not differ by more than 1.5 standard deviations from the slopes of the lines showing survival of unbroken RNA molecules at 24h. This suggests that with both compounds tested the further loss of infectivity if incubation is continued beyond the first hour can be attributed to the breakage of the RNA molecules.

Further support for the suggestion that, after the first hour, the decrease in survival of biological activity parallels the decrease in the number of unbroken RNA molecules can be obtained from a study of the kinetics of the two processes. The results of an experiment with *N*-ethyl-*N*-nitrosourea are shown in Fig. 4. The slopes of the two lines have been calculated by regression analysis to be $-0.040h^{-1}$ (S.E.M. ± 0.006) for survival of infectivity and $-0.040h^{-1}$ (S.E.M. ± 0.004) for chain breaks. The comparison indicates that within the errors of measurement both processes occur at the same speed.

The data shown in Fig. 4 give a half-time for the hydrolysis of the ethyl phosphotriesters of about 6h. Similar experiments on the reaction of isopropyl methanesulphonate with bacteriophage suspended in 0.2M-phosphate, pH7, gave a half-time for the hydrolysis of the phosphotriesters formed of 10–13h. Although this difference might reflect an increase in the stability of the phosphotriester as the complexity of the alkyl group increases it was also possible that the differences might merely result from the use of different pH in the experiments. In the experiment with the nitrosourea the pH remained at 7 throughout the experiment, whereas with isopropyl methane-

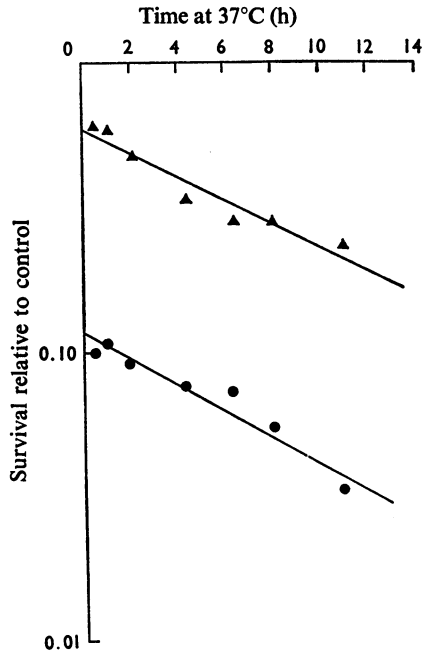

Fig. 4. *Comparison of the time-course for inactivation and for degradation of RNA*

N-Ethyl-*N*-nitrosourea (3.6mg/ml) was added to bacteriophage R17 (1.7mg/ml) suspended in 0.2M-sodium phosphate, pH7.0. Samples were taken at intervals and assayed for biological activity and for degradation of the RNA. ●, Survival of infectivity relative to control; ▲, survival of unbroken RNA molecules relative to control.

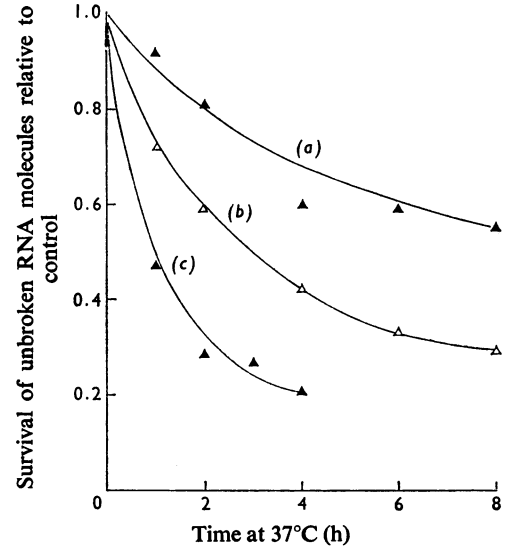

Fig. 5. *Effect of pH on the rate of degradation of RNA after reaction with isopropyl methanesulphonate*

Isopropyl methanesulphonate (11mg/ml) was added to suspensions of bacteriophage R17 (1.7mg/ml) suspended in 0.2M-sodium phosphate at pH7, or 0.2M-Tris–HCl, pH8 or 9, and incubated at 37°C. At intervals samples were taken and the extent of the degradation of the RNA determined as described in the Experimental section. (*a*), pH7; (*b*), pH8; (*c*), pH9.

sulphonate the pH fell to 6.3 after 1h. To test the effect of pH on the hydrolysis rate, isopropyl methanesulphonate (11mg/ml) was added to suspensions of bacteriophage (1.7mg/ml) in 0.2M-sodium phosphate, pH7, or in 0.2M-Tris–HCl, pH8 or pH9. At intervals, samples were taken and the extent of RNA degradation was determined. The final pH values of the solutions, initially 7, 8 and 9, measured after 2h were 6.3, 7.0 and 7.9 respectively. The results of these experiments are compared in Fig. 5 which shows that increasing the pH of the buffer used in the reaction accelerates the hydrolysis rate of the phosphotriester. The half-time for chain breaking decreases from 10–13h in the buffer starting at pH7 to 3h in pH8 buffer and about 1h in pH9 buffer. The fraction of RNA molecules remaining unbroken after each reaction mixture was incubated for 24h was 0.18 at pH7, 0.21 at pH8 and 0.21 at pH9. Changing the pH of the buffer thus accelerates the reactions but does not affect the magnitude of the total reaction. These results on the pH-dependence of the hydrolysis rate of the phosphotriesters suggest that this factor probably accounts for most of the differences in rate observed after ethylation or isopropylation and that effects caused by changes in the alkyl group are of secondary importance.

Studies of the products formed when RNA is treated with a variety of methylating agents (Lawley & Shah, 1972*a*) have led Lawley (1972*a*,*b*) to suggest that alkylation at oxygen sites occurs with reagents which react predominantly by the S_N1 mechanism. Examples of such reagents are *N*-methyl-*N*-nitrosourea and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, both of which alkylate O-6 of guanine as well as the oxygen of the backbone phosphate group. Reagents which react predominantly by the S_N2 mechanism, e.g. methyl methanesulphonate and dimethyl sulphate, alkylate O-6 of guanine very little if at all (Lawley & Shah, 1972*b*) and do not form detectable amounts of phosphotriester as judged by the degradation of RNA (Shooter *et al.*, 1974). The contribution of phosphotriester formation, and the consequent degradation of the RNA, to the inactivating effects of the various alkylating agents is summarized in Table 1. Taking the extent to which the RNA is degraded as a measure

Table 1. *Contribution of RNA chain breaks to the inactivation of bacteriophage R17*

For details see the text.

Reagent	Fraction of RNA chains broken at the mean lethal dose
Dimethyl sulphate	0.0
Methyl methanesulphonate	0.0
N-Methyl-*N*-nitrosourea	0.18
N-Methyl-*N'*-nitro-*N*-nitroso-guanidine	0.18
Ethyl methanesulphonate	0.15
N-Ethyl-*N*-nitrosourea	0.30
Isopropyl methanesulphonate	0.34

of the tendency to the S_N1 type of reaction, the data of Table 1 indicate that in the homologous series of the methanesulphonates, a predominantly S_N2 type reaction occurs with the methyl derivative, whereas the ethyl derivative shows some S_N1 character and the isopropyl derivative even more. This transition in the reaction mechanism in an homologous series parallels that predicted and illustrated by the work of Ingold and his colleagues on the reactions of alkyl halides (Ingold, 1970).

Studies of the mode of action of the nitroso derivatives (Lawley & Thatcher, 1970; Lawley, 1972*a*,*b*) have suggested that the reaction mechanism is predominantly S_N1 type, the proximal alkylating entity being the alkyldiazonium ion or the alkylcarbonium ion derived from this. The data of Table 1 reflect the transition from S_N2 to S_N1 mechanism going from methyl methanesulphonate to *N*-methyl-*N*-nitrosourea and also indicate the more predominantly S_N1 character of reactions involving *N*-ethyl-*N*-nitrosourea compared with ethyl methanesulphonate.

A point that may be stressed is that the hydrolysis of an alkyl phosphotriester may occur in two ways, either by loss of the terminal part of the RNA molecule giving a chain break, or simply by loss of the alkyl group leaving the RNA chain intact. The relative frequency with which these two pathways for hydrolysis are followed is not known.

The present work has shown that the loss of biological activity after the end of the initial reaction period parallels fairly closely, both quantitatively and kinetically, the increase in the number of breaks in the RNA molecules. Since, therefore, the formation of a break in the RNA chain introduces a new lethal lesion into the bacteriophage, it follows that the alkyl phosphotriester itself is not a lethal lesion.

This investigation was supported by grants to the Chester Beatty Research Institute, Institute of Cancer Research: Royal Cancer Hospital, from the Medical Research Council and the Cancer Research Campaign. We thank Dr. P. D. Lawley for helpful discussions during the course of this work, and Miss W. L. House for expert technical assistance.

References

Bannon, P. & Verly, W. (1972) *Eur. J. Biochem.* **31**, 103–111

Barnard, P. W. C. & Robertson, R. E. (1961) *Can. J. Chem.* **39**, 881–884

Brown, D. M. & Todd, A. R. (1955) *Annu. Rev. Biochem.* **24**, 311–338

Brown, D. M., Magrath, D. K. & Todd, A. R. (1955) *J. Chem. Soc. London* 4396–4401

Clowes, R. C. & Hayes, W. (1968) *Experiments in Microbial Genetics*, pp. 3–9, Blackwell Scientific Publications, Oxford

Freifelder, D. (1965) *Proc. Nat. Acad. Sci. U.S.* **54**, 128–134

Freifelder, D. (1966) *Virology* **28**, 742–750

Ingold, C. K. (1970) *Structure and Mechanism in Organic Chemistry*, pp. 421–555, G. Bell and Sons, Edinburgh

Lawley, P. D. (1972*a*) in *Jerusalem Symp. Quantum Chem. Biochem.* (Bergman, E. D. & Pullman, B., eds.), vol. 4, pp. 579–592, Academic Press Inc., New York

Lawley, P. D. (1972*b*) in *Topics in Chemical Carcinogenesis* (Nakahara, W., Takayama, S., Sugimura, T. & Odashima, S., eds.), pp. 237–258, University of Tokyo Press, Tokyo

Lawley, P. D. (1973) *Chem.-Biol. Interactions* **7**, 127–130

Lawley, P. D. & Shah, S. A. (1972*a*) *Biochem. J.* **128**, 117–132

Lawley, P. D. & Shah, S. A. (1972*b*) *Chem.-Biol. Interactions* **5**, 286–288

Lawley, P. D. & Thatcher, C. J. (1970) *Biochem. J.* **116**, 693–697

Rhaese, H.-J. & Boetker, N. K. (1973) *Eur. J. Biochem.* **32**, 166–172

Shooter, K. V., Edwards, P. A. & Lawley, P. D. (1971) *Biochem. J.* **125**, 829–840

Shooter, K. V., Howse, R., Shah, S. A. & Lawley, P. D. (1974) *Biochem. J.* **137**, 303–312

Werner, E. A. (1919) *J. Chem. Soc. London Trans.* **115**, 1093–1102