Molecular Mechanisms in Alkylation Mutagenesis

INDUCED REVERSION OF BACTERIOPHAGE T4rII AP72 BY ETHYL METHANESULPHONATE IN RELATION TO EXTENT AND MODE OF ETHYLATION OF PURINES IN BACTERIOPHAGE DEOXYRIBONUCLEIC ACID

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Survival and reversion to T4r⁺ of bacteriophage T4rII AP72 after treatment with ethyl methanesulphonate at 37° or 45°C were studied in relation to the extent and mode of alkylation of purines in DNA of ethylated bacteriophage. A single-burst technique was used for reversion assay. Survival was lower at 45°C than at 37°C at a given extent of ethylation of bacteriophage DNA, confirming that events subsequent to ethylation, probably depurinations, are the main cause of decreased survival. Reversion was positively correlated (approximately linearly except at low extents at 37°C) with ethylation of bacteriophage DNA, showing that ethylation itself causes mutation. Following the concept that reversion results from G-C → A-T transition at a single site (Krieg, 1963*a,b*) and the suggestion that O⁶-alkylation of guanine generates the miscoding base (Loveless, 1969), it was calculated that about one-third of induced O⁶-ethylguanines at this site would miscode to induce mutation.

Mutation induced in T-even bacteriophage by extracellular alkylation has been deduced to involve principally the Watson-Crick miscoding mechanism (Watson & Crick, 1953), in particular predominantly G-C → A-T transitions (reviewed by Krieg, 1963*a*; Lawley, 1966; Loveless, 1966; Drake, 1970). Inactivation of bacteriophage in relation to the extent and mode of alkylation of bacteriophage DNA has been studied quite extensively (Brookes & Lawley, 1963; reviewed by Verly, 1974), but, as noted previously (Lawley, 1966), mutagenesis has not so far been correlated in this fashion.

The most attractive hypothesis to account for induction of G-C → A-T transitions is that due to Loveless (1969), namely the induction in DNA by certain alkylating agents of the miscoding base O⁶-alkylguanine. This hypothesis received support from observations that alkylating agents such as methyl methanesulphonate are virtually non-mutagenic towards bacteriophage (Loveless, 1966) despite their ability to cause extensive methylation of bacteriophage DNA (Brookes & Lawley, 1963), whereas other agents, including ethyl methanesulphonate and *N*-methyl-*N*-nitrosourea, are powerful mutagens in this system (Loveless & Hampton, 1969). It now appears that, for alkylating agents in general, reactivity of the agents can be correlated with mutagenicity, in the sense that

those agents with greater S_N1 character (Lawley & Thatcher, 1970; reviewed by Lawley, 1972) or with lower Swain-Scott *s* factor (reviewed by Ehrenberg, 1971) are more potent mutagens. These reactivity factors in turn correlate positively with ability of alkylating agents to react relatively more extensively at O-atom sites in DNA, including the O-6 atom of guanine residues (Loveless, 1969; Lawley, 1972; Lawley *et al.*, 1975).

The hypothesis of Loveless (1969) implies that, at a single mutable site, induction of O⁶-alkylguanine should cause mutation at a high frequency, with possibly every replication of this base resulting in a miscoding with thymine instead of the normal partner of guanine, cytosine or a 5-substituted cytosine. To investigate this possibility in quantitative fashion, a bacteriophage mutant, T4rII AP72, was selected, which is known to be reverted to T4r⁺ at relatively high frequency, against a low background, after treatment with ethyl methanesulphonate (Krieg, 1963*b*), and was concluded to revert by G-C → A-T transition. The rate of reversion was correlated with the extent of ethylation of bacteriophage DNA at various sites in DNA purines, by using methods of bacteriophage growth and analysis of alkylated DNA previously developed in this laboratory (Brookes & Lawley, 1963; Lawley, 1972).

In some experiments it was decided to use the con-

ditions employed by Krieg (1963*b*), particularly with regard to bacteriophage survival after ethylation. However, with regard to assay of bacteriophage reversion it was thought desirable to modify Krieg's (1963*b*) method, which involved some growth of ethylated bacteriophage on a permissive host, and which was thought to make quantitative assay of reversion somewhat inaccurate. It appeared, however, from the work of Krieg (1963*b*) that some degree of DNA replication of ethylated bacteriophage was necessary to obtain maximal yield of revertants. Accordingly, it was decided to use a technique involving limited growth of ethylated bacteriophage on a permissive host, but in a stationary phase of growth, that is the single-burst technique, analogous to that described by Ellis & Delbrück (1939). It was found that treatment of the permissive host with KCN at appropriate concentration before adsorption of ethylated bacteriophage, followed by plating of adsorbed bacteriophage on the indicator strain with a large excess of indicator over permissive host cells, was satisfactory for this purpose.

Materials and Methods

Bacteriophage

T4rII AP72 bacteriophage and host bacterial strains were obtained from Dr. W. H. McClain, M.R.C., Molecular Biology Laboratory, Cambridge. The bacteriophage was normally prepared by using as host bacterium *Escherichia coli* BB; generally 2-litre cultures containing 2×10^8 bacteria/ml in the exponential phase of growth were infected with 10^9 bacteriophage/ml. After 4 h the cultures were lysed with chloroform (about 1%, v/v) and the bacteriophage was isolated by differential centrifugation (Brookes & Lawley, 1963); the yield was about 2×10^{15} bacteriophages as obtained resuspended in 0.28 M-sodium acetate (10 ml).

Bacteriophage was assayed by the agar-layer method on *E. coli* BB at a plating efficiency of more than 90% relative to the u.v. absorption of the bacteriophage suspension (taking a value for the molar extinction coefficient per mol of DNA phosphorus, ϵ_p , of DNA in bacteriophage of 7300).

Ethylation of bacteriophage

Ethyl methanesulphonate was obtained from Koch-Light Laboratories, Colnbrook, Bucks., U.K.; [$1\text{-}^{14}\text{C}$]ethyl methanesulphonate was from The Radiochemical Centre, Amersham, Bucks., U.K. (sp. radioactivity 5.3 mCi/mmol), or from Mallinckrodt Laboratories, St. Louis, Mo., U.S.A. (sp. radioactivity 0.36 mCi/mmol); and [3H]ethyl methanesulphonate was from New England Nuclear Corp., Boston, Mass., U.S.A. (sp. radioactivity 72 mCi/mmol).

In a typical experiment, to a bacteriophage suspension (10 ml in 0.28 M-sodium acetate) con-

taining 10^{14} bacteriophage at 37°C (or at 45°C) was added labelled ethyl methanesulphonate (320 mg; 2.58 mmol) of specific radioactivity, when using ^{14}C label after dilution with unlabelled ethyl methanesulphonate, 23 $\mu\text{Ci}/\text{mmol}$ (or with ^3H label, 292 $\mu\text{Ci}/\text{mmol}$). After 40 min at 45°C, or 90 min at 37°C, sodium thiosulphate was added with shaking, to give a concentration of 0.04 M after appropriate dilution of the suspension. The ethylated bacteriophage was isolated by centrifugation at 78 000g (30 000 rev./min) for 1 h in the Beckman model L ultracentrifuge (Ti30 rotor). The bacteriophage was then resuspended in 0.28 M-sodium acetate (10 ml) and protein was extracted twice into phenol reagent (9 ml) (Lawley & Thatcher, 1970). DNA (4 mg) was isolated from the aqueous layer by precipitation with ethanol (2 vol.), redissolved in 0.01 M-sodium acetate and reprecipitated.

Assay of bacteriophage and reversion

Survival of bacteriophage after ethylation was assayed at various times up to 100 min at 37°C or 120 min at 45°C after addition of ethyl methane-sulphonate. Portions (0.1 ml) of the suspension were diluted into 0.28 M-sodium acetate and assayed by the agar-layer method (cf. Brookes & Lawley, 1963) on *E. coli* BB. It was checked, by adding appropriate amounts of HCl to suspensions, that the pH decrease (7.2 to 5.5) due to hydrolysis of ethyl methane-sulphonate had no effect on survival. It was also checked that survival was the same whether direct plating, or treatment as for the single-burst technique, was used. Background reversion was also the same for both methods, showing that the use of the single-burst technique enhanced only the scoring of induced revertants.

Reversion of ethylated bacteriophage T4rII AP72 to T4r⁺ was assayed by a single-burst technique. A portion (1 ml) of the ethylated bacteriophage suspension ($2 \times 10^9/\text{ml}$) was diluted into nutrient broth (Oxoid no. 2) (9 ml) at 37°C containing *E. coli* BB in stationary phase (2×10^9 cells/ml). These cells were obtained by diluting an overnight culture of bacteria into nutrient broth containing KCN (0.02 M), and were used generally about 20 min after dilution. Adsorption of bacteriophage to the cells occurred during 10 min at 37°C. The suspension of cells with adsorbed bacteriophage was then diluted serially into nutrient broth (10 vol.) at 37°C and left for 10 min to permit initiation of bacteriophage DNA synthesis. Bacteriophage was then assayed by the agar-layer method by using *E. coli* K12(λ)-CA244 as indicator strain for T4r⁺ revertants.

Assay of radioactivity and analysis of ethylated bacteriophage DNA

Radioactivity of portions of bacteriophage suspensions or of column fractions was assayed by

liquid-scintillation counting with a detergent-based phosphor (Lawley & Shah, 1972).

Concentration of DNA was measured by u.v. absorption at 260nm, by using values of the molar extinction coefficient per mol of DNA phosphorus, ϵ_p , 6500 in aqueous buffer, or 8900 in acid hydrolysates.

Hydrolysis of DNA to yield purine bases was in 0.1M-HCl (about 2ml for column chromatography or 0.5ml for paper chromatography) at 70°C for about 20min (Lawley & Thatcher, 1970).

Three systems were used for column chromatography. Dowex AG50 (X8; minus 400 mesh; NH_4^+ form) was eluted with 0.3M-ammonium formate either at pH6.65 or pH4.3; in the former case, the column size was 25cm $\times$ 1.5cm; in the latter, 11cm $\times$ 1.5cm; 6.4ml fractions were collected. Dowex 50 (H^+ form) was also used, eluted with a gradient of 0.75–2.5M-HCl (600ml), the column size being 12cm $\times$ 1.5cm, and 3.2ml fractions were collected. For paper chromatography Whatman 3MM paper was used (downflow) with as solvent 2-methylpropan-2-ol-ethyl methyl ketone-conc. NH_3 (sp.gr. 0.88)–water (4:3:2:1, by vol.) (Maitra & Frei, 1974). The dried papers were cut into segments (4cm $\times$ 1cm) and counted for radioactivity in toluene-based phosphor (Lawley & Thatcher, 1970).

Chromatography on Dowex 50 (H^+ form) enabled determination of 7-ethyladenine, and 7-ethylguanine plus 3-ethyladenine (not separated), but O^6 -ethylguanine was de-ethylated in this system. The amount of DNA on the column was checked by summation of the u.v. absorption of fractions containing adenine and guanine.

Chromatography on Dowex 50 (NH_4^+ form) at pH6.65 enabled the determination of 3- and 7-ethyladenines, and 7- and O^6 -ethylguanines; at pH4.3, 3-ethyladenine and O^6 -ethylguanine were separated, but 7-ethyladenine and 7-ethylguanine were not separated.

Chromatography on paper enabled determination

of 7- and O^6 -ethylguanines, but 3- and 7-ethyladenines were not separated.

Positions of elution of the various ethylpurines were indicated by u.v. absorption of added markers (see Table 1). 7-Ethylguanine was obtained by the method of Brookes & Lawley (1961), O^6 -ethylguanine by the method of Balsiger & Montgomery (1960), 3- and 7-ethyladenines were from Dr. R. Denayer (see Denayer, 1962), and 1-ethyladenine and 3-ethylguanine (which were evidently too low in amount to be detected in this work) were as described by Lawley *et al.* (1975).

Results

Survival of bacteriophage at various times after treatment with ethyl methanesulphonate (0.258M) at 37°C or 45°C is shown in Fig. 1. In Fig. 1(a), the log of numbers surviving is plotted versus time, and, in Fig. 1(b), versus (time)², showing that, as found by Krieg (1963b), the log of numbers surviving decreases approximately linearly with the square of time at 45°C, but at both temperatures this relationship does not hold at high extents of survival.

Reversion of ethylated bacteriophage, measured as number of revertants per survivor, is shown in Figs. 2(a) and 2(b). As found by Krieg (1963b), at 45°C reversion increases linearly with time. For treatment at 37°C, there was some deviation from linearity (Fig. 2b), in that at shorter times reversion appeared to be somewhat lower than the linear relationship would predict. The present values obtained by the single-burst method are close to, but somewhat lower than, those reported by Krieg (1963b) by using growth after treatment in the permissive host, but are higher than those obtained by direct plating on the indicator strain.

Extent of ethylation of bacteriophage DNA after various treatments is shown in Table 2. Over the range of times used, which were short compared with the

Table 1. Positions of elution in column chromatography, or R_F values on paper, of ethylpurines

Details of chromatographic procedures are given in the Materials and Methods section.

Chromatographic support	Eluent	Fraction no. at peak of elution profile (254nm) or R_F value					
		1-Ethyl-Adenine	3-Ethyl-Guanine	7-Ethyl-adenine	3-Ethyl-adenine	7-Ethyl-guanine	adenine
Dowex 50 (H^+ form)	0.75–2.5M-HCl	116	61	—	97	121	97
Dowex 50 (NH_4^+ form)	0.3M-ammonium formate, pH4.3	52	19	—	118	52	46
Dowex 50 (NH_4^+ form)	0.3M-ammonium formate, pH6.65	27	27	125	92	32	36
Paper (Whatman 3MM)	2-methylpropan-2-ol-ethyl methyl ketone- NH_3 -water (4:3:2:1, by vol.)	0.36	0.14	—	0.72	0.66	0.36

Fig. 1. Survival of T4rII AP72 bacteriophage at various times after treatment with ethyl methanesulphonate (0.258 M)

(a) log of proportion surviving versus time; (b) log of proportion surviving versus (time)²; ●, at 37°C; ▲, at 45°C. For detail see the text.

Fig. 2. Reversion of bacteriophage T4rII AP72 to T4r+ by extracellular treatment with ethyl methanesulphonate (0.258 M) at 45°C or 37°C

Revertants were assayed by a single-burst technique as described in detail in the Materials and Methods section; survival is shown in Fig. 1. (a) Ethylation at 45°C; the straight line represents a rate of reversion ($\pm$ s.d.) of $(2.36 \pm 0.2) \times 10^{-6}$ (11 determinations) per min. (b) Ethylation at 37°C; the best straight line through the points was obtained by regression analysis and as shown did not pass through the origin.

Table 2. *Extent of ethylation of DNA in bacteriophage T4rII AP72 treated with isotopically-labelled methanesulphonate*

Details of methods are described in the text. Only selected values are given, referring mainly to treatment times of 40 min at 35°C and 90 min at 37°C. The mean $\pm$ s.d. from 16 experiments for number of ethylations per molecule of DNA (mol.wt. 108×10^6) for a treatment with the standard dose of 0.258 M-ethyl methanesulphonate for 1 min were, at 37°C, 9.2 ± 1.3 ethylations/min, and at 45°C, 18.4 ± 4.1 ethylations/min.

Label	Concn. of ethyl methanesulphonate (mM)	Time (min)	Sp. radioactivity of ethyl methanesulphonate (μ Ci/mmol)	Temperature (°C)	Extent of ethylation (μ mol of ethyl group/mmol of DNA P)
^3H	128	40	568	45	1.15
^{14}C	208	40	25	45	2.04
^3H	256	40	292	45	2.32
^{14}C	258	90	18	37	2.70
^{14}C	246	45	23	37	1.34
^{14}C	246	90	23	37	2.58

Extent of ethylation (μ mol/mmol of DNA P) Fig. 3. *Survival of bacteriophage T4rII AP72 in relation to the extent of ethylation of bacteriophage DNA*

The extent of ethylation was determined by using isotopically labelled ethyl methanesulphonate (0.258 M) under the same conditions as those used in experiments to measure survival (cf. Table 2); ●, 37°C; ▲, 45°C. (N.B. survival values at 45°C lower than 0.1 are omitted; points represent mean values at various times of exposure.)

half-life of hydrolysis of ethyl methanesulphonate [about 10.6 h at 37°C and 4.5 h at 45°C (Robertson, 1967)] ethylation was proportional to time. It was also checked that ethylation was proportional to concentration of ethyl methanesulphonate for a given time of treatment. Consistent results were obtained irrespective of the radioisotopic label used.

From the data on extent of ethylation, survival and reversion were correlated with ethylation of DNA (Figs. 3 and 4). This showed that a given extent of ethylation at 45°C decreased survival to a greater extent than at 37°C. In contrast, reversion correlated well with extent of ethylation, irrespective of temperature, and in linear fashion, except, as noted, for some divergence at lower extents at 37°C. The

Extent of ethylation of DNA (μ mol/mmol of DNA P)Fig. 4. *Reversion of bacteriophage T4rII AP72 in relation to extent of ethylation at either 37°C (●) or 45°C (▲)*

The abscissa denotes μ mol of ethyl groups/mmol of DNA P determined from data as in Table 2. From the proportion of *O*⁶-ethylguanine in ethylated DNA (see Table 3) 1 μ mol of ethyl group/mmol of DNA P corresponds to 122 ± 36 μ mol of *O*⁶-ethylguanine/mol of DNA guanine. The best straight line through the data corresponds to a reversion rate per survivor of $[44.5 \pm 2.7 \text{ (s.d.)}] \times 10^{-6}$ (21) per μ mol of ethyl group/mmol of DNA P, or $[0.36 \pm 0.11 \text{ (s.d.)}]$ (21) reversion per molecule of *O*⁶-ethylguanine induced by ethylation at a single guanine site.

combined values for the two temperatures when related with the extents of ethylation (as in Table 2) corresponded to the relation (revertants/ 10^6 survivors) = $[44.5 \pm 2.7 \text{ (s.d.)}] \times (\mu\text{mol of ethyl groups/mmol of DNA P})$.

The proportion of ethylated purines in DNA from ethyl methanesulphonate-treated bacteriophage are shown in Table 3. These were independent of the

Table 3. *Proportions of ethylated purines in DNA of bacteriophage T4rII AP72 after ethylation by [³H]- or [¹⁴C]-ethyl methanesulphonate*

For details see the text. n.d., not determined, but known from the reaction time (90 min at 45°C) to be about 800 mol/mol of DNA. The amounts of radioactivity recovered from the columns ranged from 1574 to 5754 d.p.m. for ¹⁴C and were greater than 7500 for ³H; efficiencies of counting were 61% for ¹⁴C and 42% for ³H. The values for proportions of the products refer to percentage of d.p.m. associated with u.v. absorption of the appropriate marker bases. The percentage of O⁶-ethylguanine from these data is 2.16±0.63 (s.d.).

Label	Temperature of ethylation (°C)	Mol of ethyl group/mol of DNA	Method for chromatography	Products of ethylation			
				3-Ethyladenine	7-Ethyladenine	7-Ethylguanine	O ⁶ -Ethylguanine
³ H	45	735	pH 6.65	9.6	0.7	55.5	2.2
³ H	45	737	HCl	—*	1.0	65.7*	—†
¹⁴ C	45	670	pH 6.65	7.7	1.1	54.9	3.0
¹⁴ C	45	826	pH 6.65	9.4	1.1	60.9	1.5
¹⁴ C	45	n.d.	pH 6.65	7.0	2.0	52.5	1.9
¹⁴ C	45	n.d.	pH 6.65	9.0	1.7	60.4	2.0
¹⁴ C	45	n.d.	pH 6.65	9.3	0.6	51.3	2.5
¹⁴ C	37	n.d.	Paper	10.8‡	—	66.0	1.6
¹⁴ C	37	n.d.	pH 4.3	9.4	—	64.8§	3.2
¹⁴ C	37	893	pH 6.65	9.2	2.4	59.1	1.5

* 3-Ethyladenine not separated from 7-ethylguanine.

† De-ethylated by acid chromatography.

‡ 7-Ethyladenine not separated from 3-ethyladenine.

§ 7-Ethyladenine not separated from 7-ethylguanine.

extent of ethylation, and were closely similar to those found for isolated DNA ethylated *in vitro* (Lawley *et al.*, 1975) except that 1-ethyladenine and 3-ethylguanine were too small in amount to be detected.

Discussion

The observations that survival of bacteriophage was lower at a given extent of ethylation at 45°C than at 37°C suggest that ethylation by itself cannot account entirely for inactivation of bacteriophage. However, the relationship proposed by Krieg (1963*b*) that the log of survival correlates linearly with (time)² holds only at lower extents of survival. It may therefore be concluded that survival depends in part on ethylation itself, and in part on a process subsequent to ethylation. The latter, as previously suggested (Brookes & Lawley, 1963; Verly, 1974) may well be depurination (or subsequent chain fission) of ethylated bacteriophage DNA.

In contrast, reversion correlates only with ethylation, and therefore subsequent reactions such as depurination are unlikely to contribute significantly to this effect. In confirmation of this view, no increase in reversion was found when ethylated bacteriophage (0.258 M-ethyl methanesulphonate at 37°C for 40 min) was incubated for up to 48 h at 37°C, although survival fell to 0.01 of control. These results parallel the findings of Strauss (1961), who reported that post-treatment incubation of ethylated

T4 bacteriophage did not increase the rate of induced forward mutation to T4*r*.

Several ethylation products were detected in bacteriophage DNA in the present work. It is at present not possible to define which of these products are promutagenic. However, Loveless (1969) has suggested that the mutagenic action of ethyl methanesulphonate, in contrast with the virtual non-mutagenicity of the analogue methyl methanesulphonate, can be ascribed to the ability of ethyl methanesulphonate to induce in DNA the base O⁶-ethylguanine. This product, on structural grounds, is expected to miscode by base-pairing with thymine, instead of with the normal partner of guanine, cytosine. According to Krieg (1963*b*), the reversion of bacteriophage T4rII AP72 can result only from a G-C → A-T transition at a specific site; therefore this concept could well account for the present observations.

It was therefore desirable to correlate the extent of ethylation of bacteriophage DNA at the O-6 position of guanine, as determined here, with the observed rate of reversion. From the data obtained, this correlation is presented in Fig. 4. These data were calculated on the basis that ethylation of DNA is a random process, in a bacteriophage genome of 3.15×10^5 DNA nucleotides (mol.wt. of DNA 108×10^6), 17.2% of which are deoxyguanylate residues (Freifelder, 1970; Thomas & MacHattie, 1967).

The calculations showed that the rate of reversion was about one-third [0.36 ± 0.11 (s.d.)] (21 deter-

minations)] of the rate of formation of O^6 -ethyl-guanine in DNA, whereas if every O^6 -ethylated guanine base at the mutant site caused reversion, this value should be unity.

Several explanations of this discrepancy could be suggested. The ethylations may not be, as assumed, at random, but this seems unlikely. The promutagenic base may not always miscode because of 'discrimination' by DNA polymerase (cf. Drake & Greening, 1970). Alternatively, bacterial enzymes may remove some ethylated purines from bacteriophage DNA as part of a 'repair' process. Removal of O^6 -methylguanine from DNA of *E. coli* after methylation has been observed (Lawley & Orr, 1970). The slight non-linearity of reversion against ethylation at 37°C, which appears from the present data to be more marked at low extents of ethylation, might be held to support this latter alternative. A further possibility is that all potential mutants were not scored; however, this possibility, which may be true for the direct plating method, seems unlikely for the single-burst technique used here.

It may be suggested that investigations with other alkylating mutagens, which are known (Lawley *et al.*, 1974) to give proportions of alkylation products in DNA, including O^6 -alkylguanines, different from those given by ethyl methanesulphonate will throw further light on the quantitative aspects of the hypothesis of Loveless (1969).

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