Action of Ethyl and Methyl Methane Sulfonates on DNA Injection and Genetic Recombination in T7 Bacteriophage

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After treatment with methyl or ethyl methane sulfonate, T7 amber mutants display a reduced capacity for recombination. Moreover, alkylation reduces recombination frequency involving markers on the right-hand side of the genetic map more than it reduces recombination frequency involving markers on the left-hand side. We interpret this to mean that alkylation can stop DNA injection at any point along the DNA molecule, and that T7 phage injects its DNA in a unique fashion starting from the end carrying the genes for early proteins.

The immediate inactivation of bacteriophage T7 by monofunctional alkylating agents appears to be due to several causes. About half of the phage inactivation can be attributed to single-strand breaks or apurinic sites in the DNA (20). Terminal phosphate and base alkylation may be toxic, but its quantitative importance is not known. Alkylation also prevents phages from fully injecting their DNA into the host cells (9). Three models can be proposed to account for this injection defect: (i) all-or-none model, a particular fraction of alkylated phages is unable to inject any of its DNA; (ii) partial injection model, certain alkylated phages inject only a part of their DNA; (iii) a combination of (i) and (ii). The lethal effects of a given defect in DNA injection are obviously greatest in the case of the partial injection model.

To choose among these alternatives, we have carried out genetic recombination experiments. T7 phage is reported to inject its DNA in a unique direction, starting from the end containing the genes coding for the early proteins (13). Thus, partial injection of DNA by alkylated phages should give a decrease in recombination frequency going from those genes injected first towards those injected last. In contrast, injection according to the all-or-none model should produce no such gradient in recombination frequency.

MATERIALS AND METHODS

Chemicals. Ethyl methane sulfonate (EMS) and methyl methane sulfonate (MMS) were Eastman Organics products.

Bacteria. *Escherichia coli* B_{s-1} (*rec⁻*, UV^s, T1^r, Su$^-$), the nonpermissive host, and *E. coli* 011′ (*thy⁻*,

Su$^+$), the permissive host, were obtained from W. C. Summers.

Bacteriophages. T7 amber mutants, all from the collection of F. W. Studier (17), were am64 (gene 2), am208 (gene 4), am37 (gene 11), am9 (gene 16), am10 (gene 19). The various mutants used in these crosses are indicated on the T7 genetic map (Fig. 1); the amber mutation in each case is located close to the beginning of the affected gene (17). Phages were grown and assayed according to the techniques of Studier; they were stored as nonpurified lysates at 4 C (17). For crosses, helper phages were used directly in this form, whereas the phages to be alkylated were always purified (5).

Alkylation of T7 phage. Purified phage (5) at a concentration of 10^{11}/ml was alkylated for 2 h at 37 C in phosphate buffer (0.4 M Na_2HPO_4, pH 7.2) containing varying concentrations of MMS or EMS. The phage inactivation was measured immediately at the end of the reaction; the remaining phage suspension was dialyzed against cold 1.0 M NaCl–0.01 M $MgCl_2$–0.01 M Tris, pH 7.8, overnight. Controls were treated identically except for the absence of alkylating agent. These phage suspensions were used immediately at the end of the dialysis period to avoid any possible degradation of the alkylated phage.

Genetic recombination. The experimental protocol for crosses was adapted from that of Pao and Speyer (13). *E. coli* 011′(Su$^+$) in Thomas and Abelson medium (19) plus 40 µg of thymine per ml, at 10^8 cells/ml, was infected at a multiplicity of 10 phages per cell with the alkylated T7 amber mutant and at a multiplicity of 30 phages per cell with a different amber mutant (helper phage). Optimal recombination frequencies are observed at these multiplicities (17). After 7 min at 37 C, the culture was centrifuged and the infected cells were diluted 500-fold into T broth (17); incubation continued 100 min at 37 C, and lysis was completed by the addition of chloroform. Total progeny phage were assayed on 011′ (Su$^+$) cells and wild-type recombinants were assayed on B_{s-1} (Su$^-$) cells. The frequency of wild-type recombinants was used as a measure of recom-

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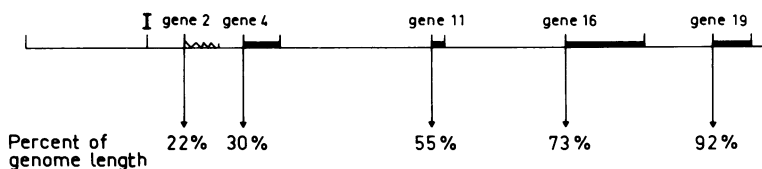
Fig. 1. *Location of pertinent amber mutants on the T7 genetic map. The map gives the position of the beginning of the genes in which the amber mutations of strains used here are located. The heavy lines correspond to the lengths of the T7 genes concerned as calculated from their known protein products; the jagged line indicates that the size of the gene 2 product is unknown (18). The initiation site for DNA replication is indicated by I (4). The simplified genetic map is adapted from Golomb and Chamberlin (6) and summarizes data from Studier (18), Simon and Studier (16) and Hyman et al. (8).*

bination frequency. Each cross was carried out in duplicate. Results are expressed as percentage of normal recombination: $RF_{alkylated}/RF_{control} \times 100$, where $RF_{alkylated}$ is the average (duplicate samples) recombination frequency observed in a cross involving an alkylated phage and a helper phage; $RF_{control}$ is the average (duplicate samples) recombination frequency observed when the alkylated phage was replaced by the nonalkylated control phage (normal recombination). Measurement of the number of wild-type revertants in each of the amber mutants (including the alkylated phages) indicated that the reversion frequency was negligible compared to the observed recombination frequencies.

Each cross with an alkylated phage was accompanied by the corresponding control cross, since recombination frequencies for a given cross were found to vary significantly from one experiment to another. (Studier [17] has noted this same phenomenon.) In contrast, the experimental error, for the average of duplicate samples treated the same day, was ±10%. Thus, for each different preparation of alkylated T7 phage, the appropriate control crosses were repeated. The average values of the frequency of wild-type recombinants in these control crosses were: with untreated am37 (gene 11) control, 5.0, 4.6, 3.8, and 3.6% for am64 (gene 2), am208 (gene 4), am9 (gene 16), and am10 (gene 19), respectively; and with untreated am208 (gene 4) control, 3.4, 2.3, 9.4, and 10.9% for am64 (gene 2), am37 (gene 11), am9 (gene 16), and am10 (gene 19), respectively.

Analysis of genotype. For several crosses, the genotypes of the recombinants among the progeny phage were determined by method I of Doermann and Boehner (3) developed for phage T4. For each cross, 100 plaques produced on *E. coli* 011′ (Su⁺) were analyzed. Material from each plaque was transferred by toothpick to four kinds of petri dishes containing, in the upper layer of soft agar, *E. coli* 011′ (Su⁺), *E. coli* B$_{s-1}$ (Su⁻), *E. coli* B$_{s-1}$ (Su⁻) with one of the parent amber mutants, or *E. coli* B$_{s-1}$ (Su⁻) with the other parent amber mutant. Phage genotypes were identified by the following criteria: wild-type recombinants give plaques on the four kinds of petri dishes; double amber mutants give plaques only on petri dishes containing *E. coli* 011′ (Su⁺).

Burst size. Burst size was determined by the method of Kerr and Sadowski (10).

Kinetics of DNA synthesis. Measurement of the incorporation of [methyl-³H]thymidine into acid-precipitable material was based on the method described by Studier (17). For these experiments, only purified phages (5) were used. Infected cell suspensions were prepared exactly as indicated above for adsorption in genetic recombination experiments. At appropriate times, a 2-ml sample of an infected culture was added to 0.1 ml of water containing 10 µCi of [methyl-³H]thymidine (6.7 Ci/mmol) and was then incubated for 30 s at 37 C. The reaction was stopped by addition of 2 ml of ice-cold 10% trichloroacetic acid containing 100 µg of unlabeled thymidine per ml, and the tubes were placed on ice for at least 5 min. The precipitated material was retained on Whatman GF/C glass fiber filter disks (24-mm diameter) and was washed three times with 5 ml of ice-cold 5% trichloroacetic acid. The filter disks were then rinsed with 95% ethanol, thoroughly dried, and placed in glass vials, after which a scintillation mixture {4 g of PPO [2,5 diphenyloxazole] and 0.1 g of dimethyl POPOP [1,4-bis-(2-[4-methyl-5-phenyloxazoyl])benzene] in 1 liter of toluene} was added, and the radioactivity was measured in a Packard Tri-Carb spectrometer.

RESULTS

Defect in DNA injection. Using radioactive wild-type T7 phage, Karska-Wysocki et al. (9) have shown that alkylation prevents a certain percentage of DNA from entering the host cell (Table 1). For similar levels of phage inactivation, EMS and MMS cause about the same defect in DNA injection.

Genetic recombination. Experiments were first carried out using T7 am37 treated with MMS; the mutation here is in a centrally located gene, number 11. The results of these experiments are shown in Table 2. After alkylation, there is a decrease in phage survival, S/S_0, which represents the ratio of plaque-forming units in the alkylated phage sample to plaque-forming units in the control sample as measured on the permissive host. Survival levels are similar to those published previously (20). For a given cross, the percentage of normal recombination decreases as the alkylation dose increases. In addition, at a given dose, the

TABLE 1. Inhibition of DNA injection after treatment of wild-type T7 phage with alkylating agents[a]

Alkylating agent	Concn (M)	S/S_0[b] (%)	DNA not injected (%)
EMS	0.20	8	12
	0.74	0.4	26
MMS	0.02	0.4	23

[a] A detailed presentation of these results is published elsewhere (9).

[b] S/S_0 represents the ratio of plaque-forming units in the alkylated phage sample to plaque-forming units in the control sample.

TABLE 2. Recombination between MMS-alkylated T7 phage am37 (gene 11) and various other amber mutants

Concn (M) (MMS)	$S/S_0 \times$ 100	Percentage of normal recombination			
		Gene 2	Gene 4	Gene 16	Gene 19
0.001	80	66	65	54	36
0.0025	47		52		18
0.004	30	49		21	11
0.006	12	42	23	12	8
0.01	4		8		1

percentage of normal recombination depends on the location of the mutation in the helper phage. These results are shown graphically in Fig. 2. After alkylation, recombination with the sites on the left of the genetic map has a higher probability than recombination with those on the right side of the map. Pao and Speyer (13) observed a similar gradient after X-irradiation of T7 phage.

To show that these results are not unique to alkylated T7 am37, we repeated the same type of experiment with T7 am208 (gene 4) as alkylated phage (Table 3). As observed for the gene 11 mutant, recombination of T7 am208 with T7 am9 (gene 16) and T7 am10 (gene 19) was greatly diminished by alkylation in a fashion related to dose. However recombination with am64 (gene 2) and with am37 (gene 11) was stimulated by moderate doses of MMS. At a dose of 0.001 M MMS, the average percentage of normal recombination was 125% for am64 (gene 2); at a dose of 0.006 M MMS, the average percentage of normal recombination was 110% for am64 (gene 2) and 155% for am37 (gene 11). Only at a dose of 0.016 M MMS were recombination frequencies diminished by alkylation for all genes. In the three cases tested, the percentage of normal recombination was greater for the gene 11 helper phage than for the gene 2 helper phage. The results for the three series of

crosses which include the four different helper phages are illustrated in Fig. 3.

Several crosses were carried out with T7 amber mutants alkylated by EMS (Table 4). Despite known differences in the mechanism of alkylation by EMS and MMS (14), the results of these crosses are qualitatively similar to those obtained with MMS. In all cases, recombination with helper phage T7 am10 (gene 19) is markedly decreased. For phage T7 am208 (gene 4), alkylation stimulates recombination with
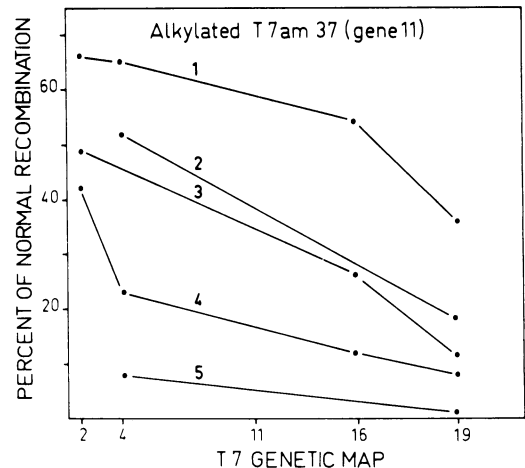

FIG. 2. Frequency of recombination between alkylated T7 am37 (gene 11) and other T7 amber mutants (identified by gene number on genetic map) expressed as percentage of normal recombination. For each experimental point, two control crosses and two crosses with alkylated phage were carried out; percentage of normal recombination was calculated as explained in Materials and Methods. All crosses for a given curve were made on the same day with the same preparations of alkylated or control phage. Different curves correspond to different MMS concentrations (see Table 2): 1, 0.001 M; 2, 0.0025 M; 3, 0.004 M; 4, 0.006 M; 5, 0.01 M.

TABLE 3. Recombination between MMS-alkylated T7 phage am208 (gene 4) and various other amber mutants

Concn (M) (MMS)	$S/S_0 \times$ 100	Percentage of normal recombination			
		Gene 2 (am64)	Gene 11 (am37)	Gene 16 (am9)	Gene 19 (am10)
0.001	65	130			56
0.006	10	110			6
	8.5	130			10
	7.3	87	180	37	14
	6.8	120	130	15	6
0.016	0.96	11	22	4	2

helper phages $am64$ (gene 2) and $am37$ (gene 11). There is, however, a quantitative difference between the two alkylating agents. At a similar survival level (at similar phage survival levels, EMS and MMS introduce about the same number of alkyl groups into T7 phage DNA [20]), EMS treatment of phage T7 $am37$ appears to inhibit recombination more than MMS treatment (compare Tables 2 and 4). At
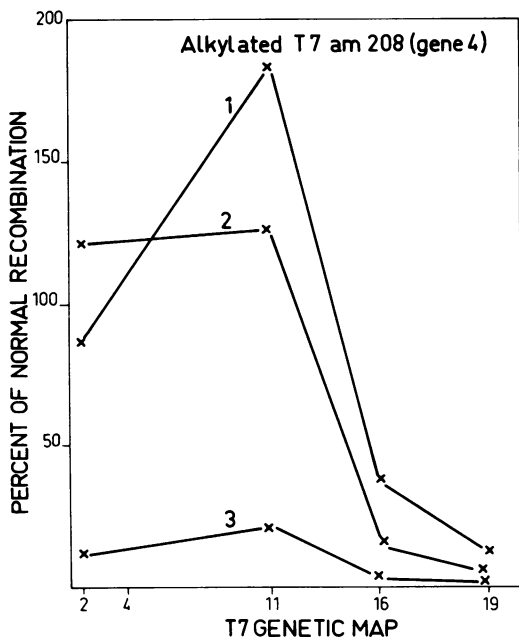

FIG. 3. *Frequency of recombination between alkylated T7 am208 (gene 4) and other T7 amber mutants (identified by gene number on genetic map) expressed as percentage of normal recombination. For each experimental point, two control crosses and two crosses with alkylated phage were carried out; percentage of normal recombination was calculated as explained in Materials and Methods. All crosses for a given curve were made on the same day with the same preparations of alkylated or control phage. Different curves correspond to different MMS concentrations (see Table 3): 1 and 2, 0.006 M; 3, 0.016 M.*

$S/S_0 \times 100 = 80\%$ (MMS) or 76% (EMS) this difference is relatively small. But at $S/S_0 \times 100 = 12\%$ (MMS) or 9.6% (EMS), the difference is clearly visible; for $am64$ (gene 2) the percentage of normal recombination is 42% (MMS) compared to 3.4% (EMS); for $am10$ (gene 19) 8% (MMS) compared to 0% (EMS).

Analysis of genotype. Thus far, the frequency of wild-type progeny phages has been taken as a measure of recombination frequency. It was thus necessary to check that alkylation did not cause the production of unequal numbers of wild-type recombinants and double amber mutants. This verification appeared particularly important for the cross between alkylated $am37$ (gene 11) and helper $am10$ (gene 19), where we observed a large reduction in recombination frequency as compared to the control cross.

We thus have analyzed the genotypes of recombinant progeny phages resulting from two crosses: $am37 \times am208$; $am37 \times am10$. The results (Table 5) show that the frequencies of wild-type and double mutant genotypes are approximately the same for a given cross; alkylation of $am37$ resulted in a similar decrease in the frequencies of both types of recombinants.

Burst size. Burst sizes, measured in the permissive host, were similar for all crosses and also for the single parental-type infections run as controls; they varied from 13 to 25 phages per bacterium. No differences were detected after alkylation of the phages. The burst sizes observed are slightly lower than those reported by Hausmann and Gomez (7) for T7 amber mutants. They were, however, much lower than those observed by Studier (17).

Kinetics of DNA synthesis. We have compared the kinetics of DNA synthesis in permissive host cells after infection by various alkylated and nonalkylated phages, to see if alkylation causes any major change. This point was of particular interest since the same phage-specific enzymes are involved in recombination and replication (10). Incorporation of [³H]-thymidine into acid-precipitable material dur-

TABLE 4. *Recombination between EMS-alkylated T7 phage am37 (gene 11) or am208 (gene 4) and various other amber mutants*

Alkylated mutant phage	Concn (M) (EMS)	$S/S_0 \times 100$	Percentage of normal recombination				
			Gene 2 ($am64$)	Gene 4 ($am208$)	Gene 11 ($am37$)	Gene 16 ($am9$)	Gene 19 ($am10$)
$am37$ (gene 11)	0.04	76	54	47		50	30
	0.2	9.6	3.4	2.5		0.95	0
$am208$ (gene 4)	0.06	46	150		190	79	29

TABLE 5. *Frequency of recombinant genotypes found among progeny phages*

Cross	Genotype	Frequency of genotype (%)	
		Cross with control phage	Cross with alkylated phage[a]
am37 × am208 (gene 11 × gene 4)	Wild type	4	2
	Double mutant	6	4
am37 × am10 (gene 11 × gene 19)	Wild type	6	2
	Double mutant	4	1

[a] Alkylation of phage am37 (gene 11) was carried out at a concentration of 0.0025 M MMS.

ing a 30-s pulse is taken as a measure of the rate of DNA synthesis.

Figure 4 presents the pulse-labeling curves for phages am37, am208, and am10, alkylated (0.0025 M MMS) or nonalkylated, as well as those for two crosses involving am37 as alkylated phage. The curve (Fig. 4A) for phage am37 is similar to those normally observed for wild-type T7 phage (17); host DNA synthesis stops within 5 to 10 min after infection; the synthesis of T7 DNA starts at this time and reaches a maximum rate between 15 and 20 min after infection (18). Alkylation (Fig. 4B) causes a shift in the time scale of the curve but no major change in rate. The curves for am208 (Fig. 4C,D) resemble the curve obtained by Studier (17) for gene 4 amber mutants under non-
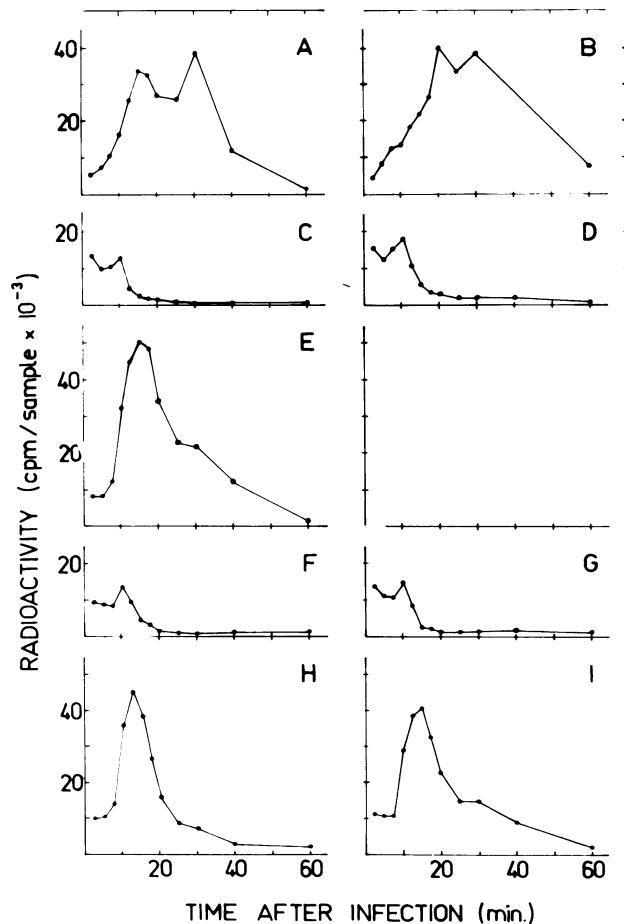

FIG. 4. *Patterns of DNA synthesis after infection of permissive host E. coli 011' by various T7 amber mutants. Infected cell suspensions were prepared and treated as indicated in the text. The right-hand curves relate to alkylated (0.0025 M MMS) phage, whereas the left-hand curves relate to the corresponding controls. A and B, am37 (gene 11); C and D, am208 (gene 4); E, am10 (gene 19); F and G, am37 × am208 (in G, only am37 is alkylated); H and I, am37 × am10 (in I, only am37 is alkylated).*

permissive conditions; this undoubtedly reflects, in our case, the imperfect suppression of the nonsense mutation in gene 4, one of the genes responsible for DNA synthesis (17). For phage am 10 (Fig. 4E), the curve is similar to those observed for wild-type phage. For the two crosses, we see no difference between the curves for alkylated am37 and the control phage. We thus conclude that changes in recombination frequencies observed after alkylation of phage cannot be explained by major differences in the kinetics of DNA synthesis.

DISCUSSION

Comparing alkylated T7 am37 (mutation in gene 11) with nonalkylated controls, we have found that alkylation decreases the frequencies of recombination with other T7 amber mutants in a way related to their positions on the T7 genetic map. Recombination frequencies were lowest for those genes located to the right of the map (Fig. 1). Pao and Speyer (13) reported a similar gradient in recombination frequencies for T7 phage treated with X-rays, but not for T7 phages treated with UV radiation. Krisch (11) (also D. M. Green, quoted in references 11 and 13) has likewise observed a gradient for T7 phage labeled with radioactive phosphorus (^{32}P) and stored at 4 C.

Pao and Speyer (13) and Krisch (11) interpret such a gradient in recombination frequencies as indicating that a lesion in the phage DNA prevents transfer of markers distal to that lesion. We feel that this is the most probable interpretation of our experimental results. However, these results may also depend on other causes such as alteration of DNA synthesis and effect of alkylation on recombination. We can eliminate an effect on burst size since it is clear that, in our experimental conditions, alkylation does not change it.

For a given amber mutant, the rate of DNA synthesis is about the same whether the phage is alkylated or not. This makes unlikely the hypothesis that the alkylating agent introduces into DNA lesions that act to block replication. However, if this were the case, segments of DNA located between initiation sites for replication and those lesions would exist in more copies and rescue of the genetic markers localized in these segments would thus be favored. This interpretation was given by Doermann (2) to explain the UV stimulation of recombination with phage T4 (21). For T7 phage, the initiation site for replication lies at 17% from the left end of T7 DNA (Fig. 1), i.e., to the left of all the amber mutations in the phages used here; interruption of DNA synthesis might thus explain the observed gradient in recombination frequency. It seems, however, unlikely that a lesion caused by MMS or EMS in T7 DNA might interrupt replication since only one out of eight apurinic sites causes phage inactivation (1, 12), and since more than 100 alkylated sites are created per inactivation hit at the end of the treatment with a monofunctional alkylating agent (20). We thus consider it improbable that an interference with replication could be the sole cause of the observed gradient in recombination frequencies. We do not have any direct evidence, however, to show that it makes no contribution whatsoever to the observed gradient.

Alkylation affects recombination. Tables 2 and 4 show numerous instances where the decrease in recombination frequency is greater than the decrease in survival. For example, after MMS treatment of T7 am37 (see Table 2), the percentage of normal recombination for gene 19 is always lower than S/S_0. If we assume that the ability of a phage to inject its entire DNA molecule is a necessary condition for survival, then this observation simply means that the decrease in recombination frequency that we observe is not due uniquely to a defect in DNA injection. Alkylation of the DNA that is injected is clearly affecting recombination.

The most likely interpretation of our experimental results is that alkylation can stop the injection process at any point. Since the injection of T7 DNA is believed to be unidirectional (13), partial injection of alkylated DNA should give rise to a gradient in recombination frequencies. This gradient would decrease going from left to right on the genetic map since the early genes (on left) are injected first. We observed such a gradient (Fig. 2 and 3).

Pao and Speyer (13) interpreted their data for T7 phage treated with X-rays as showing that the DNA of this phage is injected in a unique fashion. Our results with monofunctional alkylating agents support this hypothesis of unidirectional injection. The recent report by Saigo (15) that the part of T7 DNA corresponding to the left end of the genetic map is ejected first from the phage tail when the phage is disrupted in formamide is in full agreement with this hypothesis.

Because of the gradient in recombination frequencies, we can reject the all-or-none model to explain the defect in DNA injection due to alkylation. The magnitude of this defect must thus be greater than that which can be calculated from the all-or-none hypothesis. The present data do not, however, allow estimation of the fraction of immediate inactivation due to the injection block since the decrease in recom-

bination frequency depends not only on the injection defect but also on the effect of alkylation on recombination itself.

The results obtained with T7 *am*208 (mutation in gene 4) are surprising because, for certain markers, recombination is stimulated by alkylation. At similar survival values (around 10%), the cross alkylated *am*37 × *am*208 gives 23% of normal recombination, whereas the cross alkylated *am*208 × *am*37 yields 155% normal recombination (Tables 2 and 3). Pao and Speyer (13) did not report such a stimulation after X-irradiation of this same mutant, *am*208. Gene 4 is implicated in DNA replication and recombination (10), but the mechanism of the apparent stimulation of recombination by alkylation is presently unknown.

ACKNOWLEDGMENTS

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