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Inactivation of Bacteriophage T4 by Ethyl Methanesulfonate: Influence of Host and Viral Genotypes

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Inactivation of bacteriophage T4 by ethyl methanesulfonate (EMS) is a complex process which depends critically upon the conditions of treatment and upon both the viral and the host genotypes. EMS-inactivated particles are capable of multiplicity and cross-reactivation, indicating the need for caution in using EMS in certain types of mutation studies. The pyrimidine dimer excision systems of the phage and the host do not affect the EMS sensitivity of T4, but the T4x^+y^+ system does. Mutational defects in the deoxyribonucleic acid (DNA) ligase and the DNA polymerase systems both of the virus and of its host also affect viral EMS sensitivity.

Monofunctional alkylating agents such as ethyl methanesulfonate (EMS) introduce many more chemical lesions than lethal hits into the deoxyribonucleic acid (DNA) of cells and viruses (12, 34). Most of the chemical lesions are innocuous, either because they fail to interfere with DNA replication (34) or because they are repaired (8, 9, 16, 39-41). Cellular repair systems appear sometimes (but not always) capable of acting upon both alkylation and ultraviolet (UV) lesions (8, 9, 40).

EMS inactivation of a T-even bacteriophage was first studied by Loveless (29, 30). Unlike many viruses, bacteriophage T4 often fails to benefit from cellular repair systems, perhaps because its DNA contains glucosylated 5-hydroxymethylcytosine instead of cytosine. The *Escherichia coli uvr* and *rec* systems, for instance, are intimately involved in the control of cellular UV sensitivity but do not affect the UV sensitivity of T4, although the *phr* and *polA* (37) systems certainly do. In partial compensation, T4 elaborates certain UV repair systems of its own such as *v*, *x*, and *y* (10, 11, 22).

We examined the ability of a number of mutations residing either in the viral or in the host genome to affect the EMS sensitivity of bacteriophage T4. We concluded that the control of EMS sensitivity in T4 involves components from both genomes. Some overlap was observed between alkylation and UV sensitivity systems.

MATERIALS AND METHODS

Strains of bacteriophage T4 are listed in Table 1. Various *E. coli* strains were employed. The wild-type strains B and BB were obtained from S. Benzer (7) and were the standard host cells. B/r, obtained from R. Uretz, is resistant to ionizing and UV irradiation and to bifunctional alkylating agents (30). Bs-1, Bs-3, Bs-8, Bs-11, and Bs-12, obtained from R. Setlow, are UV-sensitive mutants (20, 24), Bs-1 being *fil$^-$ exr$^-$ hcr$^-$* (24, 33). KB, obtained from S. Benzer, is a K-12 wild-type lambda lysogen and was used as a differential host for T4*rII*$^+$. P3478, obtained from J. Cairns, lacks DNA polymerase I and is sensitive to UV and to methyl methanesulfonate (MMS; references 13, 21); W3110 is its *thy*$^-$ parent. AB3027, obtained from P. Howard-Flanders, is a K-12 multiauxotroph sensitive to MMS. A number of K-12 *rec* strains, obtained from J. Weil, carried *recA13*, *recB21*, *recC22*, or various combinations thereof (42): JC2926 = *recA*, JC5412 = *recB*, JC5489 = *recC*, JC5495 = *recA recB*, JC5544 = *recA recC*, JC5519 = *recB recC*, and JC5547 = *recA recB recC*. Ligase mutants (19), obtained from M. Gellert, were the parental K-12 strain, N953; N1323, ligase-overproducing *lop8*; and N1325, ligase-defective *lop8 lig2*.

Media, plating procedures, and methods for growing stocks have been described previously (1, 2, 27). Plating was occasionally performed with 0.3% Oxoid Ionagar No. 2 in the top agar, which greatly expands plaque sizes.

EMS was obtained from Eastman Organic Chemicals. Solutions were prepared immediately before use and were not stored. Certain batches of EMS have been reported to contain toxic impurities which vaporize at lower temperatures than does EMS (28). We collected two fractions at a pressure of 15 to 20 mm of Hg, about 40% at 150 to 155 C, and about 40% at 170 to 175 C; the tared residue was discarded. Neither fraction differed from the starting material in specific lethality.

Unless otherwise indicated, T4 stocks were treated at 43 C with 0.4 M EMS in 0.2 M phosphate buffer

TABLE 1. *T4 strains*

Strain	Relevant characteristics[a]	Source
B	Wild type (1, 14); parent of *rII* and *o* mutants	S. Benzer
D	Wild type (1, 14); parent of *ts* and UV[b]-sensitive mutants	S. E. Luria and W. Harm
v	UV-sensitive; unable to excise pyrimidine dimers (17, 22, 44)	W. Harm
x	UV-sensitive; weakly *rec*⁻ (22, 23)	W. Harm
y	UV-sensitive; epistatic to *x* (10, 11)	N. Symonds
Gene 30 *ts*	Temperature-sensitive DNA ligase (15)	R. Edgar
Gene 43 *ts*	Temperature-sensitive DNA polymerase (2)	R. Edgar
o	Resistant to osmotic shock	C. Lundeen and J. W. Drake
r1231	Deletion of *rII* region (6) and beyond	S. Benzer
rUV, *rSM*	Point *rII* mutants	J. W. Drake

[a] Numbers in parentheses refer to references.
[b] Ultraviolet.

(*p*H 7.0). (When prepared at room temperature, a 4% EMS solution is 0.4 M.) The reaction was stopped by diluting 20-fold or more into cold broth containing 0.16 M $Na_2S_2O_3$; carry-over of the thiosulfate into the assay system did not affect plating efficiencies. The samples were then plated immediately or were stoppered and incubated further for post-treatment incubation. In 37 control experiments, the average survival of many different T4 stocks in 0.2 M buffer alone was 0.96 after 100 min at 43 C.

EMS is quite unstable, hydrolyzing to ethanol plus methylsulfonic acid. [A mixture (0.4 M) of ethanol plus sodium methylsulfonate, however, was neither inactivating nor mutagenic to T4 over 100 min at 43 C at *p*H 7.] The half-life of EMS in water at 37 C is reported as 10.4 hr (36), and we have observed a half-life in water at 43 C of about 6 hr and in 0.2 M phosphate buffer (*p*H 7) of about 4 hr. About 25% of the EMS was hydrolyzed in a typical 100-min treatment, and at the same time the *p*H dropped from 7.0 to about 6. When T4 was treated for 80 min at 43 C with 0.4 M EMS, survivals were about sixfold lower in 0.4 M than in 0.2 M phosphate buffer, and the average *p*H was 6.43 and 6.25, respectively; presumably alkylation proceeds slightly more rapidly at the higher *p*H values. A buffer effect of this type may partly explain why Krieg (25) observed somewhat greater EMS killing than we did; additionally, however, he did not employ sufficient thiosulfate to neutralize unreacted EMS.

Treatment in broth, or in 0.1 M phosphate buffer, typically resulted in a final *p*H of approximately 3. Survivals are then extremely low, in part because of acid inactivation (as determined by reconstruction experiments) and presumably in part because of the very rapid depurination of ethylated purines (26, 36). The low *p*H probably affected the experiments of Ronen (35), who used 0.04 M phosphate buffer with 4% EMS and observed very large inactivation rates. His main conclusion, that secondary reactions are not important components in immediate inactivation, is therefore not established by his data. To clear up this point, we repeated his experiments with 0.2 M buffer

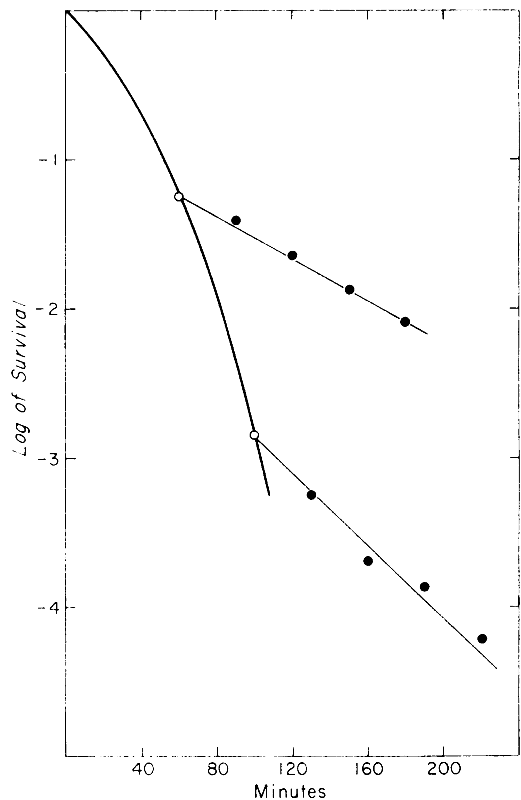

FIG. 1. *Immediate versus delayed inactivation of T4D. The sample was treated with 0.4 M EMS at 43 C (○), and post-treatment incubation (●) was also conducted at 43 C.*

(Fig. 1). When post-treatment inactivation was approximated by a single-hit curve and the instantaneous rate of inactivation at 60 and 100 min of EMS treatment was estimated from the tangent to the immediate

inactivation curve, post-treatment inactivation was observed to occur at a substantial rate compared to the rate of immediate inactivation: about 23% at 60 min and about 27% at 100 min. It is therefore clear that secondary events contribute a substantial minority to the lethal events detected upon immediate plating. A similar conclusion has also been reached by Loveless with bacteriophage T2 (see p. 109 and 135 of reference 30).

RESULTS

Paragenetic parameters. Some lethal agents, particularly UV irradiation (31), reduce burst sizes, extend latent periods among the virus particles which survive treatment, or both. (These effects, however, are usually nonheritable.) As a result, plaque sizes become highly variable. When T4 was treated with EMS to a survival of about 0.006, the majority of the survivors exhibited an extended latent period but a normal burst size (Fig. 2). The survivors of EMS treatment sometimes showed reduced average plaque sizes, particularly at the higher doses.

UV-irradiated T4 particles are capable of strong multiplicity reactivation (32). EMS-inactivated T4 particles, on the other hand, are capable of definite but not very strong multiplicity reactivation (Table 2); T4 inactivated to a similar extent by UV irradiation would have produced factors 15- to 75-fold larger. [A previous investigation (35) did not discern multiplicity reactivation among T4 particles inactivated by EMS, presumably because of extensive low *p*H inactivation; see above.] Marker rescue from inactivated genomes was also readily observed (Fig. 3). These results indicated that care must be taken in mutation experiments when large numbers of particles are plated on selective indicators.

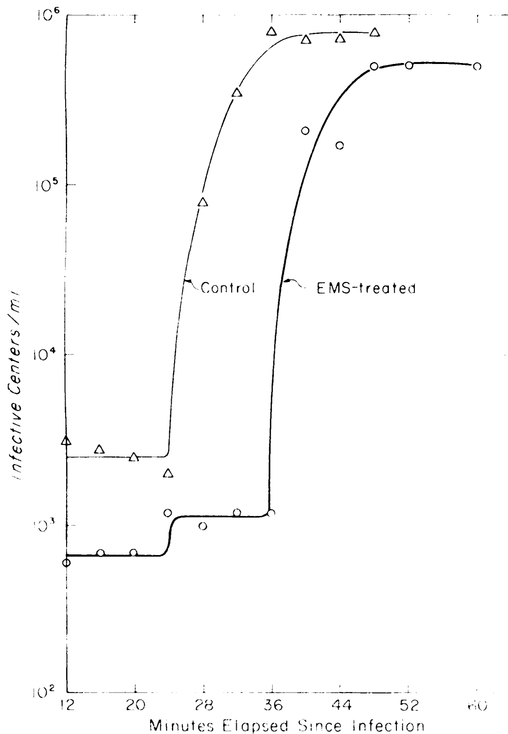

FIG. 2. *One-step growth curves of normal and EMS-treated T4B. T4B was treated with 0.4 M EMS for 75 min at 43 C (surviving fraction 0.0064). Virus was adsorbed to B cells in L broth at a multiplicity of 0.22 per cell, and the complexes were incubated at 37 C.*

Since we wished to examine the effects on EMS lesions of repair systems known to operate upon UV lesions, we also tested the combined effects of EMS and UV on T4. Their lethal effects were simply additive, no synergistic effects being observed regardless of which agent was applied first.

Influence of viral genotype. We examined various strains of T4 carrying mutations which might affect their sensitivities to the lethal effects of EMS. These strains included mutants exhibiting increased sensitivity to UV irradiation (*v*, *x*, *y*, and combinations thereof); mutants defective in DNA synthesis (*ts* mutations in DNA polymerase gene 43 and in DNA ligase gene 30); two mutants not obviously involved in DNA repair or synthesis (osmotic shock resistance, *o*; insusceptibility to lysis inhibition, *rII*); and the two standard wild-type strains, T4B and T4D. The effects of the *ts* mutations were tested by comparing the survival of EMS-treated particles when plated at a "permissive" temperature (32 C) and at "semi-permissive" temperatures (35 to 43 C); at the higher temperatures the efficiency of plating re-

TABLE 2. *Multiplicity reactivation*[a]

Expt	Surviving fraction	MOI	Specific infectivity
1	0.0014	0.0057	1.0
		0.057	1.2
		0.57	2.2
2	0.00053	0.073	1.0
		0.73	2.5
		7.3	8.3
		14.6	11.0

[a] T4B (experiment 1) or T4D (experiment 2) was treated for 100 min at 43 C with 0.4 M EMS and, after the thiosulfate step, was adsorbed to BB cells at the indicated multiplicity of infection (MOI) and plated before lysis. The titers were normalized in each experiment to the sample with the lowest MOI to obtain the specific infectivity.

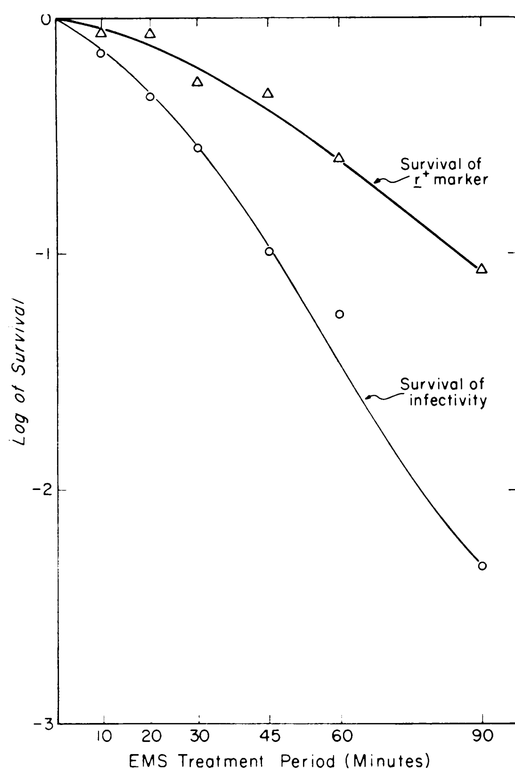

FIG. 3. *Cross-reactivation of T4B. Wild-type particles were treated with 0.4 M EMS at 43 C and adsorbed to B cells at a multiplicity of 0.4, together with an average of six particles of untreated r1272. The complexes were plated on KB cells before lysis.*

mains high (≥ 0.75), but the plaques become very small.

The following genotypes produced identical survival curves in 0.4 M EMS at 43 C over 100 min: T4B and T4D, with or without tryptophan (which is an adsorption cofactor for T4B) at 50 μg/ml; *v*, which is defective in the initial step in the excision of UV-induced pyrimidine dimers (17, 44); *r1272* and many other *rII* mutants; and *o*. An independently arising *o* mutant was earlier reported to respond similarly (25).

T4*x* exhibited increased sensitivity to EMS (Fig. 4). Indistinguishable curves were obtained with T4D*x*, T4B*x* (backcrossed eight times), T4*y*, T4*vx*, and T4*xy*. The *x* and *y* mutations are also nearly completely epistatic with respect to UV inactivation (10, 11).

Mutational lesions in the genes for DNA polymerase were reported to increase UV sensitivity both in T4 and in its host (3, 21). The reported effects of T4 DNA polymerase mutations upon UV sensitivity, however, resulted from plaque size artifacts: the "missing" plaques corresponding to the very slightly increased UV sensitivity reappeared when plaque sizes were expanded (38; I. Albrecht, C. Schnitzlein, and J. W. Drake, *unpublished data*). DNA polymerase defects also increased MMS sensitivity in *E. coli* (13) but again not in T4 (4). We tested the effects of plating EMS-treated T4 mutants carrying *ts* mutations in gene 43, using both permissive and semipermissive temperatures (Fig. 5 and Table 3). Mutant *tsA58* maps at the beginning of the gene, *tsA69* maps about a third of the way into the gene, and *tsL74*, *tsL91*, and *tsL106* map at a common site at the end of the gene (2). The first two mutants exhibited a slightly increased EMS sensitivity at 39 C, and the last three mutants were distinctly more sensitive even at 32 C. Expanding plaque sizes very substantially by plating with soft agar containing 0.3% Ionagar No. 2 failed to restore the missing plaques. Among 14 spontaneous ts^+ revertants of *tsL91*, nine displayed the wild-type sensitivity to EMS, whereas five were at least as sensitive as *tsL91* itself. All of the revertants displayed the wild-type sensitivity to UV irradiation. The revertants *rUV13-tsL91R5*, *rUV58-tsL91R9*, *rUV58-tsL91R10*, and *rUV304-tsL91R14* were backcrossed against their *rII* component, and the progeny were examined for *ts* segregants. In the first three instances, reversion clearly occurred by an event located within 0.07 map unit (roughly seven base pairs) of *tsL91* itself. The fourth revertant contained a suppressor, which was either ts^+ or lethal, located 0.5 map

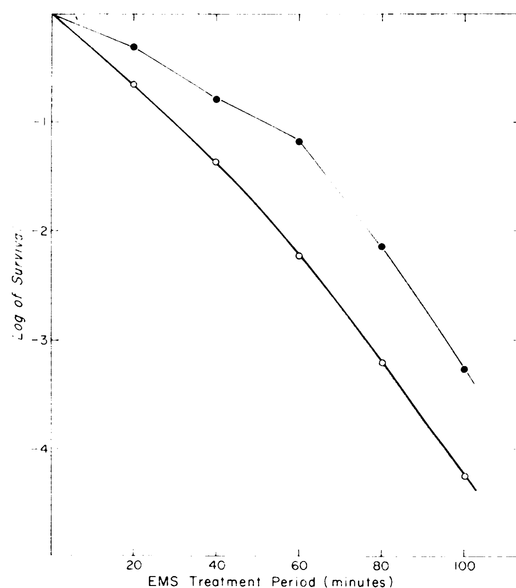

FIG. 4. *Survival of T4D (●) and T4Bx (○) treated with 0.4 M EMS at 43 C.*

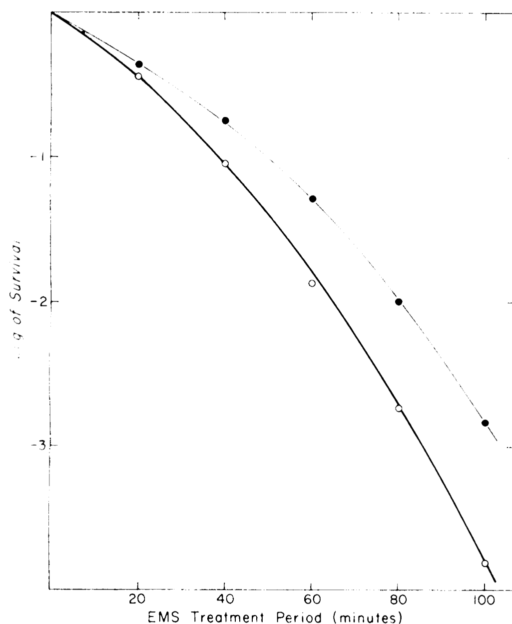

FIG. 5. *Survival of T4D (●) and tsL91 (○) treated with 0.4 M EMS at 43 C and plated on BB cells at 32 C.*

unit (roughly 50 base pairs) from *tsL91* and therefore probably within gene 43. We also performed complementation tests with *tsL91* and *tsA69* at 43 C, which confirmed the inclusion of the *tsL91* cluster within gene 43. Our results therefore implicated the T4 DNA polymerase in the control of T4 EMS sensitivity. The observation that a few of the (presumably intracistronic) ts^+ revertants retained their increased EMS sensitivities shows that EMS sensitivity does not depend upon temperature sensitivity itself but rather upon some other alteration in enzyme function.

Mutational lesions in the gene for DNA ligase were reported to increase UV sensitivity both in T4 (3) and in its host (18) and to increase MMS sensitivity in T4 (4). Under standard plating conditions, we sometimes observed markedly increased EMS sensitivity in gene 30 *ts* mutants of T4 plated at semipermissive temperatures, but when plating was performed with 0.3% Ionagar, most of the "missing" plaques reappeared. We still, however, observed slightly increased sensitivities to EMS in two *ts* mutants of gene 30 (Table 4), and the increased sensitivities were augmented by plating on host cells defective in host ligase, as detailed below. (In the experiments reported in Table 4, qualitatively similar results were obtained at 60 and 100 min of treatment. The differential between the wild type and the *ts* mutants was more often of the magnitude shown in the bottom of the table than in the top.) Several ts^+rII^+ revertants of *tsA80* and *tsB20* were also tested for EMS sensitivity. Some, such as those described in Table 4, had regained most or all of the wild-type resistance, whereas others were just as sensitive as their *ts* parents.

A search was conducted for new T4 mutants with altered EMS sensitivities. Mutants with decreased sensitivities were sought by multiple cycles of direct selection. Mutants with increased sensitivity were sought by mutagenizing T4 with both 5-bromodeoxyuridine and 2-aminopurine (final *r* frequency, 0.7%), picking and purifying 960 random isolates, and testing each for EMS sensitivity in a semiquantitative screening test. No nontrivial mutants were recovered. (Several mutants with altered sensitivities were isolated, but in each case the character was phenotypically unstable and probably represented a mutation in a somatic viral protein.)

Influence of host genotype. EMS-treated T4 particles were plated on various cell strains defective in repair or DNA metabolism. Untreated particles plated normally on all of the strains employed, the K-12 strains producing the usual efficiency of plating of about 0.8 compared to B strains. Survival curves of T4D were identical on all of the strains employed which were wild type for repair systems.

TABLE 3. *Relative survivals of T4 gene 43 mutants treated with ethylmethanesulfonate (EMS)*[a]

Genotype	Relative survival
T4D	1.00
tsA58	1.14
tsA58 (39 C)	0.79
tsA69	0.47
tsA69 (39 C)	0.15
tsL74	0.06
tsL91	0.08
tsL106	0.10
tsL91R1	0.97
tsL91R2	1.03
T4B	0.60
rUV58	0.73
rUV58-tsL91	0.01
rUV58-tsL91R9	0.02
rUV58-tsL91R10	0.72
rUV48-tsL91R8	0.02
rSM94-tsL91R3	0.75
rUV13-tsL91R5	0.004
rUV304-tsL91R14	0.60

[a] Stocks were treated for 100 min at 43 C with 0.4 M EMS; survival of T4D was 0.9 $\times 10^{-3}$ to 1.5 $\times$ 10^{-3} in various experiments. Plating was performed at 32 C, except where indicated.

TABLE 4. *Effects of viral and host DNA ligase defects*[a]

T4 strain	EMS	Host cell	Surviving fraction
T4D[b]	−	any	1.00
	+	*wt*	0.00064
		lop	0.0015
		lig	0.00035
tsA80	−	*wt* or *lop*	1.00
		lig	0.88
	+	*wt*	0.00052
		lop	0.00090
		lig	0.000091
tsB20[b]	−	*wt* or *lop*	1.00
		lig	0.83
	+	*wt*	0.00050
		lop	0.0015
		lig	0.000043
T4D[c]	−		1.00
	+		0.0015
tsA80[c]	−		1.00
	+		0.00040
tsA80R1[c]	−		1.00
	+		0.0016
tsA80R3[c]	−		1.00
	+		0.0017
tsB20[c]	−		1.00
	+		0.00038
tsB20R1[c]	−		1.00
	+		0.0013
tsB20R3[c]	−		1.00
	+		0.00088

[a] Stocks were treated at 43 C with 0.4 M ethylmethanesulfonate (EMS) for 100 min.

[b] T4D and *tsA80* were plated at 37 C, *tsB20* was plated at 39 C; *wt* (wild type) = N953, *lop* (ligase overproducer) = N1323, and *lig* (ligase defective) = N1325.

[c] Host cell was BB and the plating temperatures were 37 C for *tsA80* and 39 C for all others. In all cases, the *ts* mutants were plated with 0.3% Ionagar.

A variety of B and K-12 strains, exhibiting altered UV sensitivities, decreased abilities to generate recombinants, or both, plated EMS-treated T4D normally. The B strains included the radiation- and alkylation-resistant strain B/r and the UV-sensitive strains Bs-1, Bs-3, Bs-8, Bs-11, and Bs-12; most of these strains have not been extensively characterized genetically, but Bs-1 contains both *hcr* and *exr* defects (33), whereas Bs-8 and Bs-12 contain *hcr* and Bs-11 contains *exr* defects (20). The K strains included all of those listed previously carrying lesions in *recA*, *recB*, or *recC* and all possible combinations thereof. UV-irradiated T4 also plates normally on all of these B and K strains (C. Schnitzlein, *personal communication*).

The K strains P3478 and AB3027 exhibit increased sensitivity to MMS (13; P. Howard-Flanders, *personal communication*). In our studies, both strains also exhibited increased sensitivity to EMS. Both of these strains plated EMS-treated T4 with reduced efficiencies (Fig. 6). The survival of EMS-treated T4 during post-treatment incubation, however, was normal on these two strains; the plating differential observed upon immediate plating was simply maintained thereafter.

P3478 lacks host DNA polymerase I. Since polymerase defects in both the phage and the host increase the EMS sensitivity of T4, we tested whether the two defects exhibited synergism. None was detected: plating *tsL91* (treated for 100 min with 0.4 M EMS, plated at 32 C) on P3478 cells produced the survival expected from the independent effects of plating T4D on P3478 cells (compared to BB cells) and plating *tsL91* (compared to T4D) on BB cells.

The effects of alterations in the host DNA ligase were also examined (Table 4). Untreated

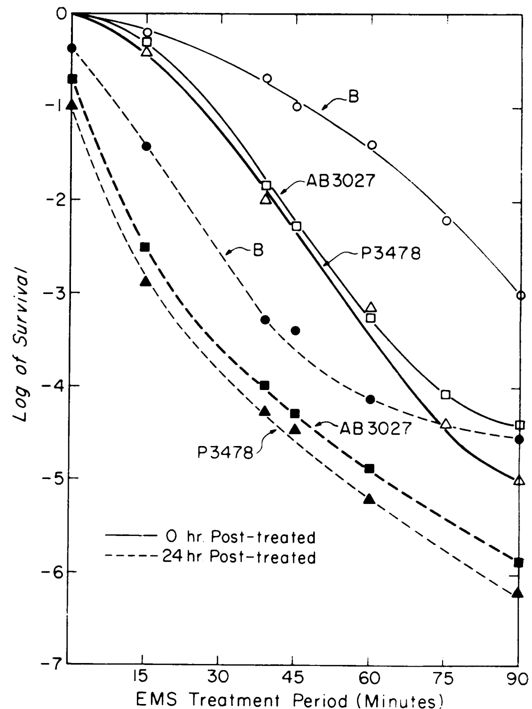

FIG. 6. *Survival of T4B treated with 0.4 M EMS at 43 C on various cells upon immediate plating (solid lines) and after 24 hr of post-treatment incubation at 37 C (broken lines).*

T4 plated equally well on all of the host cells tested over 32 to 39 C. The gene 30 *ts* mutants also plated equally well, except for a small reduction in plating efficiency at the semipermissive temperature on the ligase-defective host. The survival of EMS-treated T4 was slightly increased by plating on the ligase-overproducing host and was slightly decreased by plating on the ligase-defective host. The survival of the EMS-treated gene 30 *ts* mutants was also slightly increased by plating on the ligase-overproducing host but was markedly reduced by plating on the ligase-defective host. It appears that either the viral or the host ligase alone is nearly sufficient for optimal EMS resistance and that both must be made defective to observe a marked effect on the survival of EMS-treated T4. (It should be noted, however, that the rate constant differences which are implied by the data of Table 4 are probably rather small. In the absence of an adequate kinetic description of EMS inactivation, it is unfortunately impossible to calculate rate constants unequivocally.)

DISCUSSION

The immediate inactivation of T4 by EMS is in part composed of the types of damages which also occur when treated particles are further incubated in the absence of EMS, in mild disagreement with the conclusions of Ronen (35). The multiplicity and cross-reactivation which we observed indicate that much of the damage resides in the DNA, in agreement with earlier chemical studies (12, 26). The occurrence of these reactivation processes also indicates that mutational studies must be carefully controlled when the reversion is studied by differential plating.

The lesions which accumulate during post-treatment inactivation are clearly different from those induced immediately, as shown, for instance, by their differing susceptibilities to genetic control and, presumably, multiplicity reactivation (35). These lesions are usually attributed to depurination, strand breakage, or both (5, 26, 30), but the number of depurinations per lethal hit was rather large, and the other types of chemical events certainly cannot be excluded.

Among the several T4 mutants exhibiting increased UV sensitivity, both *x* and *y* (but not *v*) also exhibited increased EMS sensitivity. The v^+ function determines an endonuclease which appears to be specific for pyrimidine dimers (17, 44). Little has been reported concerning the properties of *y*, except that it is largely epistatic to *x* (10, 11), as we also observed. The *x* mutation, in addition to increasing UV sensitivity, also produces a threefold decreased rate of recombination (23), a sixfold increased MMS sensitivity (4), and perhaps also a twofold increased sensitivity to photodynamic inactivation (18). Certain of these properties make *x* similar to the bacterial *rec* mutants (43), although it would be misleading to push the analogy very far.

T4 DNA polymerase is clearly involved in the repair of EMS damages, although its possible involvement in the repair of UV damages is yet to be established unequivocally. This result supports the speculation that the T4 DNA polymerase is both a replicase and a repair enzyme (2). The host DNA polymerase, however, is also involved in the repair of EMS lesions in the T4 chromosome. Joint defects in both polymerases appear to have independent effects, suggesting that the two enzymes work independently and not as sequential elements.

T4 DNA ligase is clearly (but not markedly) involved in the repair of EMS damages, as well as in the repair of UV damages (3). The host DNA ligase is also involved in the repair of EMS lesions in the T4 chromosome, as well as in the repair of UV lesions in the host chromosome (19).

The failure to detect additional EMS-sensitive T4 mutants, despite the fact that the screening program appeared sufficient to recover more than two mutants from every EMS sensitivity cistron of average mutability, implies the absence from the genome of cistrons like v^+ which encode a highly specialized function which is dispensable for normal growth. The mutated function in *E. coli* AB3027 which affects the survival of EMS-treated T4 remains undescribed but appears to be different from *polA*; perhaps it involves the alkylation- and depurination-specific endonuclease II (16; S. M. Hadi and D. A. Goldthwait, Fed. Proc. **30**:1156, 1971).

ACKNOWLEDGMENTS

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