ETHYL METHANESULFONATE-INDUCED REVERSION OF BACTERIOPHAGE T4*r*II MUTANTS

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ETHYL methanesulfonate (EMS[2]) is an alkylating agent that can react with extracellular virus particles to produce many mutations with rather little killing (LOVELESS 1958). The induced mutations may be delayed by several generations (GREEN and KRIEG 1961). The mutagen reacts with three of the four bases naturally occurring in DNA (REINER and ZAMENHOF 1957; BROOKES and LAWLEY 1960, 1961a, 1962; PAL 1962), yet there is reason to suspect that it might be quite specific as to the type of base pair changes it usually induces. The purpose of this investigation was to compare the frequencies of EMS-induced mutations at various sites within the *r*II region of bacteriophage T4, and to test the notion that predominantly one type of base pair substitution might be induced.

Four years ago in this laboratory, during a search for strongly EMS-revertible point mutants. D. M. GREEN found that some AP mutants (mutants which had been produced from standard r^+ phage by AP) were quite EMS-revertible, but that none of the 18 EMS-produced mutants he examined were induced by EMS to revert to a comparable extent. This prompted the working hypothesis that one of the base pair transitions—either GC to AT or AT to GC—was much more strongly inducible by EMS than was the other transition. It was decided to test this possible specificity by checking a group of EMS mutants for EMS-induced reversion by a more sensitive test than had been previously employed, and to test similarly additional base analog and proflavin mutants.

While this work was in progress, the results of another investigation with similar intent were reported (BAUTZ and FREESE 1960; FREESE 1961). The results differed in several respects for mutants that were included in both investigations; most striking was that the other group of workers found much less induced reversion. Although the other workers used EES, the chemistry of the two ethylating agents would be expected to be very similar. It seemed that the difference must have arisen from the different techniques employed. This possibility led to an expansion of our work, and the chief reason for the conflicting results—which will be discussed in context in this paper—has now been clarified. We shall

[1] Operated by Union Carbide Corporation for the U. S. Atomic Energy Commission.

[2] The abbreviations used are: G, guanine; C, cytosine or 5-hydroxymethylcytosine; A, adenine; T, thymine; EMS, ethyl methanesulfonate; EES, ethyl ethanesulfonate; AP, 2-aminopurine; BU, 5-bromouracil or 5-bromodeoxyuridine; P, proflavine; and DNA, deoxyribonucleic acid.

conclude that EMS induces the GC to AT transition at a much higher rate than the AT to GC transition. As previously concluded, transversions—the replacement of a purine-pyrimidine pair by a pyrimidine-purine pair—may also be induced to some extent by EMS (BAUTZ and FREESE 1960; FREESE 1961), but we do not consider the existing evidence to be compelling. We shall describe several hypothetical molecular mechanisms of EMS-induced mutation, including one that was not considered by the other group and that would generate only GC to AT transitions by pairing errors in replicating DNA. BAUTZ and FREESE (1960) concluded that any base could be incorporated opposite a "gap" in the DNA template produced by the hydrolytic removal of 7-ethylguanine from DNA prior to replication. We suggest that, if such gaps are not lethal, they might lead to base pair deletions.

MATERIALS AND METHODS

General: Bacteriophage T4B and derivative *r*II mutants were obtained from SEYMOUR BENZER, ERNST FREESE, and JOHN DRAKE. An *r*II mutant is defined as one that has the distinctive *r*-type plaque morphology on plates with strain B bacteria and that does not normally form plaques when plated with bacteria lysogenic for phage lambda. All such mutants are closely linked and occur in two adjacent cistrons. *Escherichia coli* strain Bb, originally isolated from Berkeley B by SYDNEY BRENNER and obtained from BENZER, was used to prepare phage stocks and as the host for nonselective growth. Strain B was used for nonselective platings, and two lambda-lysogenic strains, both previously designated K and now redesignated KB (K-12 from BENZER) and KT [112–12 (λ *h*) No. 3, from the California Institute of Technology collection] were used for selective platings (BENZER and CHAMPE 1961). Growing bacteria were centrifuged and resuspended in cold broth for use as plating bacteria. Host bacteria were grown to about 5×10^7 cells/ml, and cyanide was added before centrifuging.

H-broth (8 g Nutrient Broth, 5 g NaCl, 1 g dextrose, 5 g Peptone/liter H_2O) was used for growth of plating and host bacteria. The medium used for production of progeny phage in experiments was identical to H-broth except that NaCl was omitted, and indole was usually added at 0.1 g/liter. M9 buffer contained, per liter of distilled H_2O: 5.8 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl, and 0.01 g gelatin. The modified M9 medium used for growth of phage stocks was made by adding, to M9 buffer, separately autoclaved solutions to give 4 g/l dextrose, 1 mM $MgSO_4$, 2 μM $FeCl_3$, 25 mg/l L-tryptophan, and either 100 mg/l histidine or 2.5 g/l vitamin-free casamino acids. (It was found that phage were inactivated in the presence of dextrose unless either histidine or casamino acids were added (KLIGLER and OLENICK 1943); this inactivation is also prevented by materials present in lysates). Plating media were those described by HERSHEY and ROTMAN (1949), except that peptone was used instead of tryptone.

Procedure for standard reversion experiments: A phage lysate was diluted in M9 buffer to 1×10^{10}/ml, and an equal volume of 1 M phosphate buffer at pH 7 was added. Phage were exposed at 45°C by adding this buffered preparation to EMS in the ratio 1 ml to 0.025 ml. Exposures were terminated by chilling and

dilution into four volumes of cold M9 buffer containing $Na_2S_2O_3$ at a final concentration of 0.04 M. For controls, the procedure was similar except for addition of EMS, and dilution was made with 19 volumes to give the same titer of viable phage. The dilutions were kept cold until used, then incubated ten to 30 minutes at 37°C to allow residual EMS to be eliminated by the thiosulfate. An equal volume of resuspended host bacteria was added, and the mixture was gently aerated ten minutes for adsorption. The ratio of viable phage to bacteria was about 0.6, and 0.001 M NaCN was present to synchronize infection. Adsorption of EMS-exposed phage is impaired, but about 85 percent of the viable phage are adsorbed under these conditions. Growth bottles were made by adding 0.5 ml aliquots of the adsorption mixtures to 100 ml of salt-free broth. After two hours incubation at 37°C the growth bottles were shaken with chloroform and kept cold. Assays were made with B bacteria for total progeny phage and with KB or KT bacteria for revertant phage. Any plaques found on KB or KT are attributed to revertants, without necessarily implying that they represent true back mutants. Platings of standard r^+ phage were made routinely to determine the efficiency of plating for the bacterial strains. Usually, two growth bottles were made from each adsorption mixture, and the results were averaged. In the earliest experiments a single growth bottle was made, and at 30°C with a slightly longer incubation. The mean burst size was usually more than 100. In a few experiments a much lower progeny titer was found in one or more growth bottles, and the mutants were checked again; since similar revertant frequencies were found, both experiments are reported.

EXPERIMENTAL

Relation between duration of exposure, extent of killing and induced mutation

Figure 1 shows the fraction of phage found to be viable at various times after EMS was added to a phage suspension. The rate of phage killing increases uniformly throughout the entire period studied. Since a constant slope is not achieved, this curve is not of a "multiple hit" or "multiple target" type. The increasing rate of killing is apparently not due simply to a progressive diffusion of the poison into the phage; when EMS was added to a cold phage suspension and the mixture was kept in the refrigerator for one or 24 hours, during which killing was 12 percent or less, a survival curve of the same general shape was generated once the reaction mixture was brought to 45°C. Moreover, T4o5 stocks, having a mutant gene for osmotic shock resistance and thus a higher permeability to ions, give a similar survival curve. Additional experiments (not shown here) established that the progressively steepening survival curve is not due to the gradual production of a toxic substance in the reaction mixture. When additional phage were added to the reaction mixture 80 minutes after EMS was put into solution, the newly added phage were killed no faster than was initially observed. A survival curve of the same general shape was generated, but with a somewhat lower initial rate of killing, a result suggesting that the effective concentration of the poison was decreasing rather than increasing.

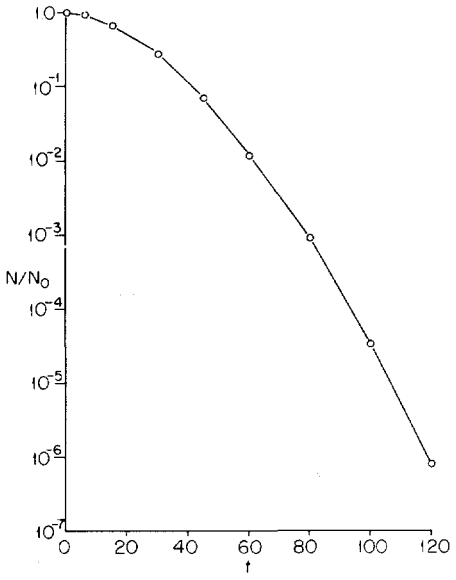

FIGURE 1.—Semilogarithmic plot of the fraction of phage surviving an exposure to EMS for t minutes. N = viable phage titer at time t, N_0 = original phage titer.

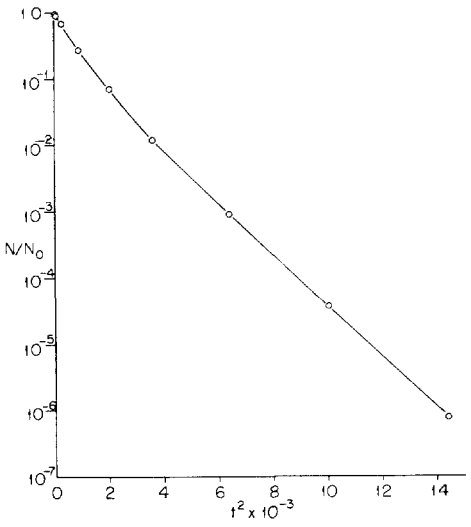

FIGURE 2.—Semilogarithmic plot of the fraction of phage surviving versus the square of the duration of exposure to EMS.

Interpretation of survival curve: The survival curve can be interpreted according to the following hypothesis. We assume that each phage has a large number, S, of sites of potential lethality that are ethylated with pseudo-first order kinetics at a rate a, proportional to the EMS concentration. We further assume that an ethylated site is not in itself lethal, but that it can be converted to a lethal damage by a first order reaction that has a rate constant b. For a duration of exposure, t, that is small relative to $1/a$ and $1/b$, the survival curve predicted by this model is approximately given by $\log (N/N_o) = -(abS/2)\ t^2$, where N_o is the initial titer and N the titer of viable phage at time t.

To test the applicability of this equation, the data presented in Figure 1 are replotted in Figure 2, with t^2 as the abscissa. As predicted, an approximately straight line is found. The gradually decreasing slope may reflect a gradual reduction in the effective concentration of EMS. The compound is unstable in aqueous solution, and Figure 2 suggests a half-life of several hours for EMS. This postulated two-step mechanism of killing accounts for the instability of ethylated phage after an exposure to EMS, which was reported previously (LOVELESS 1959; BAUTZ and FREESE 1960; STRAUSS 1961).

Kinetics of induced mutation: The relation between induced mutation and duration of EMS exposure was determined for reversions of the *r*II mutant AP72 and is shown in Figure 3. It can be seen that although killing is approximately proportional to t^2, the frequency of induced mutation is approximately proportional to t and, therefore, is not proportional to the number of phage-lethal damages.

This suggests that mutations are produced by ethylated bases contained within the phage at the time of infection and that killing is due to a secondary event that also occurs during the exposure interval, such as a breakdown or hydrolysis of ethylated molecules.

It should be noted that the frequency of induced reversions was reported to be proportional to the frequency of lethal damages for two other mutants, AP156 and EES66 (BAUTZ and FREESE 1960; FREESE 1961). It is not known whether the different dose dependence reported is due to the different mutants or to the different techniques employed.

Comparative survey of EMS-induced revertibility of AP, BU, EMS and P mutants

An exposure of 30 minutes was chosen as the standard condition for a comparative survey of mutants. This exposure allowed convenient production of progeny populations with a titer of about 10^8/ml, and since it induced revertants at a frequency of about 10^{-4} for AP72, it offered a highly sensitive test of low-level revertibility.

The mutants initially chosen for analysis included a set of nine produced by AP and seven produced by 5-bromouracil (indicated here by the prefix BU, instead of their former prefix, N). These were selected to permit comparison with the studies by FREESE and coworkers of their induced revertibility by various agents, including base analogs (FREESE 1959), nitrous acid (FREESE and FREESE 1961), and by DRAKE (personal communication) for UV-induced reversion. A set of 11 EMS-produced mutants (indicated by the prefix EM) and ten proflavin-produced mutants (indicated by the prefix P) were obtained from BENZER, as a random sample of those he mapped in the B cistron (BENZER 1961). Mutants added for various reasons after the survey had been begun are: AP50, BU90, EM126, EES66, and *r*207.

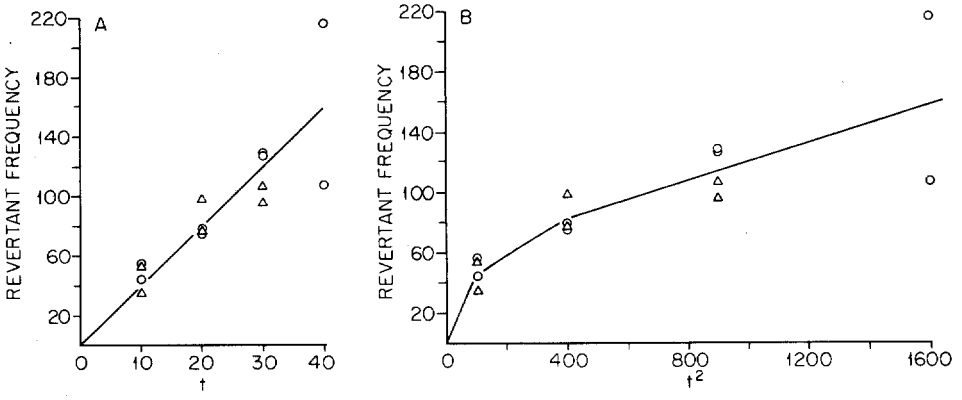

FIGURE 3.—Frequency of induced AP72 revertants per 10^6 progeny phage as a function of duration of exposure to EMS, *t*. Triangles are for exposure of *r*AP72 and circles are for exposure of *r*AP72*o*5, which carries a gene for osmotic shock resistance. A separate point is indicated for the duplicate growth bottles made for each exposure.

Table 1 presents the results of all standard reversion experiments performed. Induced revertant frequency refers to the difference between the results for the treated and the control populations.

Plaque morphologies of revertants: The plaques produced by revertants on the plates with a selective strain of bacteria were generally indistinguishable from those produced by the standard r^+ phage under those plating conditions. Generally, a small fraction of the plaques had an aberrant plaque morphology, but this could reasonably be expected from separate plaque morphology mutations incidentally produced in the same line of descent as the *r*II reversion, so no notation is made in the tables if the frequency of aberrant plaque morphologies amounted to less than ten percent of the revertants. Some mutants, however, regularly produced revertants that had distinctively smaller plaques. In these

TABLE 1

EMS-induced reversion

Mutant	Site*	Frequency of revertants per 10^6 progeny phage†	
		Control	Induced
AP114	DAP56	2.8 s, 0.9 t	290. s, 30. t
AP72	607	1.7	143
		1.2	129
		1.4	153
		1.1	183
		0.4	144
AP12	F102	0.9 s, 18. t	81. s, 37. t
		0.8 s, 24. t	111. s, 32. t
AP41	AP41	2.6 s, 44. t	77. s, 154. t
AP275	N38?	1.4	85.
		1.0	50.
		1.4	55.4
		0.4	71.
		1.5	49.
AP50	AP50	0.74 s, 16. t	37. s, 42. t
AP61	AP61	0.2	0.6
AP156	EM9	0.02	0.4
		0.02	0.4
		0.03	0.36
AP70	?	0.00	0.5
		0.00	0.15
AP83	EM9	0.03	0.2
BU90	N90	0.8 s, 0.4 t	23.2 s, 5.6 t
BU24	N24	0.7	1.3
BU7	N7	0.01	0.6
BU29	N29	0.2	0.6
		0.15	0.00
BU19	N19	0.07	0.18
BU12	N12	0.04	0.15
BU21	N21	0.00 s, 0.06 t	0.05 s, 0.09 t
BU101	NT71	0.005	0.02

TABLE 1—Continued

Mutant	Site*	Frequency of revertants per 10^6 progeny phage†	
		Control	Induced
EM126	607	0.35	147.
EM34	EM34	0.4 s, 15. t	22. s, 36. t
EM43	N24	0.3	2.3
		0.2	0.3
EM11	BC35	0.17 s, 0.10 t	1.1 s, 2.7 t
EM3	EM3	3.3	0.9
EM9	EM9	0.02	0.5
EM30	117	0.0	0.3
		0.5	0.5
EM41	163	0.04	0.02
		0.12	0.55
EM7	EM7	0.03	0.16
EM26	EM26	0.96	0.11
		0.03	0.15
EM87	EM87	0.01	0.00
EM15	N7	0.28	—0.05
P85	326?	1.7 s, 27. t	60.8 s, 76. t
P42	P42	12. s, 1. t	—1. s, 35. t
		10.6 s, 1.6 t	0.3 s, 24. t
P28	NT23	0.3	0.7
P67	P67	0.35	0.60
P83	P83	0.07	0.10
P48	P48	0.05	0.1
P79	P79	0.06	0.08
P87	P87	0.07	0.07
P13	1074	0.27 s, 0.1 t	0.04 s, 1.0 t
P31	EM113	0.11	—0.02
*r*207	?	85.4	32.4
		85.6	31.3
EES66	?	8.4	4.2
		9.1	3.7

* As designated by BENZER (1961).
† s = standard sized plaques on selective platings; t = tiny plaques on selective platings.

cases, the tabulated results distinguish between apparently standard size plaques (designated *s*) and the "tiny" plaques (designated *t*). It must be emphasized that this criterion refers to the appearance of the plaques on the strain initially used for the detection of revertants. In certain cases, including all those in which EMS-induced revertants had a frequency of more than one per 10^6 progeny, five or more revertant plaques were picked and their contents plated with the bacterial strains B, KB, and KT. Some revertants were indistinguishable from standard r^+ phage with respect to plaque morphology and efficiency of plating under all conditions that were tried. Some revertants that produced full-size plaques with one selective strain produced tiny plaques or had a reduced efficiency of plating with the other strain, and sometimes this detection depended on the

physiological state of the plating bacteria. Some revertants were indistinguishable from standard r^+ phage on both selective strains but were found to give distinctively different plaque morphologies with strain B. For instance, the BU90 revertants designated *s* were found to give nearly *r*-like plaques with strain B. Most *s*-type revertants from AP50 resemble standard r^+, as do a fraction of those from AP41 and AP114; but none resembling standard r^+ were observed from AP12 or EM34.

Back-crosses of apparent wild-type revertants: AP72, AP275, and AP156 regularly gave EMS-induced revertants which were indistinguishable from standard r^+ phage. Stocks were made of one such revertant from each, and these were back-crossed to standard-type phage, with UV irradiation to enhance recombination. If the revertants were caused by suppressor mutations, the original *r*-type phage would be segregated out, and perhaps the isolated suppressor might also be recognized as an *r* plaque-former (E. B. FREESE 1962; CRICK, BARNETT, BRENNER and WATTS-TOBIN 1961). The frequency of *r* plaque-formers among the progeny was not greater than 0.1 percent in any of these backcrosses, but as a further test of closely linked suppressor mutations, all *r* plaques found among 3500 to 8000 progeny were picked and spot-tested (BENZER 1957) with the original *r* mutant. In each case there was a positive indication of recombination showing that it was a different mutant. Hence, the EMS-induced revertants from AP72, AP275, and AP156 are either true back mutants or due to very closely linked suppressors. In contrast to these results, when a back-cross was made between standard phage and an *s*-type revertant from BU90, *r* plaques were found at a frequency of 0.1 percent and four out of the six *r* plaques found had phage that were indistinguishable from BU90 by the criteria of spontaneous revertibility and standard crosses to BU90. The presumed suppressor was not recovered, but it is not known whether that is because insufficient *r* plaques were tested or because the isolated suppressor fails to make a plaque type distinguishable from standard phage and the *s* revertant. (The backcross progeny in this case were preabsorbed on B bacteria and plated on a mixture of B and KB bacteria, on which the *r*-like plaques of the *s* revertant could be distinguished by their clear centers from the more turbid plaques of *r*II mutants.)

Contrast of induced revertant frequencies with previously published data: When the results shown in Table 1 for EMS-induced reversion of base-analog-produced mutants are compared with the results reported for EES-induced reversion of the same mutants (BAUTZ and FREESE 1960; FREESE 1961), two features are especially striking. First, the mutants that we find most strongly revertible were also the most revertible in their data, but the absolute values are much higher in our study. For instance, the induced revertant frequencies we find for AP72 and AP275 are 149 and 62 per 10^6, while they report only 1.0 and 1.5 per 10^6, respectively. Second, mutants they found to give induced revertants at a lower frequency are also revertible in our studies, but the absolute values are not enhanced to the same extent. For instance, AP156 and AP70 give 0.4 and 0.3 per 10^6 in our study, and 0.08 and 0.065 per 10^6 in their study.

The implications of these findings for inferring base-pair changes induced by

ethylating agents will be discussed later. The results prompted the next group of experiments to determine the reasons for the differences observed.

A comparison of EMS-induced revertant frequencies obtained from direct selection of exposed phage and selection of progeny

It seemed likely that the direct plating of exposed phage with selective bacteria might give only a partial recovery of the induced revertants, and that this could account for the lower values observed in the experiments of FREESE and coworkers. We tested this by directly plating a portion of phage exposed under our standard conditions and subjecting another portion of the same phage preparation to our standard cycle of reproduction in a nonselective host bacterial strain before selective platings for revertants. Table 2 presents the results of such experiments. It also includes the results of a reconstruction experiment performed with a mixture of AP156 and standard r^+ phage. The mixture was subjected to

TABLE 2

Recovery of induced revertants from direct selective platings of exposed phage compared to platings of progeny phage

	Frequency of revertants per 10^6				
	Direct platings		Progeny platings*		Induced revertants (direct/progeny)
Mutant	Control	Induced	Control	Induced	
AP72	0.8	4.6	1.1	183	0.03
	0.4	2.4	0.4	143	0.02
EM126	0.1	7.2	0.4	147	0.05
AP275	1.9	10.2	0.4	71	0.14
	2.7	9.2	1.5	49	0.19
*r*207	39	—11	85.4	32.4	...
	53	—14	85.6	31.3	...
EES66	3.9	0.8	8.4	4.2	0.2
	4.8	—1.4	9.1	3.7	...
AP156	0.005	0.67	0.03	0.36	1.9
Mixture of AP156 and r^+	3.69	0.60	4.60	0.57	...
BU29	0.04	0.00	0.15	0.00	...
AP114	3.9 s	40. s	2.8 s	290. s	0.14 s
	1.6 t	9. t	0.9 t	30. t	0.30 t
AP12	0.25 s	3.1 s	0.8 s	111. s	0.03 s
	2.6 t	7.9 t	24. t	32. t	0.25 t
AP41	0.3 s	9.3 s	2.6 s	77. s	0.12 s
	15. t	11. t	44. t	154. t	0.07 t
P85	0.5 s	5.2 s	1.7 s	60.8 s	0.09 s
	15.2 t	24.0 t	26.8 t	75.7 t	0.32 t
AP50	0.3 s	30. s	0.7 s	37. s	0.81 s
	9.5 t	6. t	16. t	42. t	0.14 t
EM34	0.36 s	0.3 s	0.4 s	22. s	0.01 s
	3.7 t	1.3 t	15. t	36. t	0.04 t

* These data are also included in Table 1.

standard EMS exposure and control procedures, and dilutions for assays, etc., were identical with those used for AP156 alone in a parallel experiment. This experiment was designed to evaluate the reliability of detection of r^+ phage among a large exces of *r*II phage, since it seemed possible that some plaques of spurious origin might appear on the selective platings of exposed phage. For instance, if enough plating bacteria were infected by more than one phage, plaques might arise from killed r^+ phage by multiplicity reactivation or cross reactivation. The data show that this is negligible under these conditions and the increased frequency of r^+ plaques among exposed phage can be attributed to induced revertants.

The data of Table 2 indicate there is indeed a markedly reduced recovery of potential induced revertants in direct platings of exposed phage on selective bacteria. The ratio of the revertant frequency detected in this way to that observed among progeny differs from mutant to mutant. (This variability does not simply come from daily variations in plating conditions, since it was found for different mutants checked at the same time.) While our most revertible mutants show a ratio as low as a few percent of the potential induced revertants, AP156 regularly gives about the same frequency by the two techniques; indeed, it appears that a slightly higher frequency may be found on direct platings. These results will be discussed later in terms of mechanisms of induced reversion, but it may be noted here that the apparent relative revertibility of different mutants from our direct platings agrees approximately with the results of direct platings of EES-exposed phage (BAUTZ and FREESE 1960; FREESE 1961). The fact that our data still give generally larger rates may arise from other differences of procedure that affect the various mutants to a similar extent; for instance, we did not use the 24 hour incubation at 37°C which the other group used after EES exposure.

Some technical comments seem to be appropriate. There was a wide range of plaque sizes on selective platings of exposed phage, grading from full size plaques like those typically made by standard r^+ phage down to very small plaques. This was true even for AP275, AP72, AP156 and the reconstruction experiment involving AP156, although progeny populations from the same exposed phage preparations gave typically *s*-type plaques and no "tiny" revertants. The reduced plaque sizes can probably be partly attributed to nonheritable damage from the EMS, for instance, reduced burst size or extended latent period. Delayed revertants might also be expected to give a smaller plaque, since only a fraction of the phage issuing from the first infection—in some cases, a single phage particle—would be revertant and would thus get a relatively "late start" at making a plaque. Counting all the revertant plaques becomes a greater problem for a mutant like AP12, which gives two types of revertants. Generally, the *s* and *t* types are fairly distinct in selective platings in progeny. However, the variable reduction in plaque size in direct platings of exposed phage results in a blurring of the distinction: some standard revertants probably make plaques as small as a typical tiny revertant, and plaques from the tiny revertants grade down to a size so small as to be overlooked. Poor reproducibility among experi-

ments for the same mutant suggests that differences in plating conditions from day to day may also be more of a problem in platings of such mutants.

DISCUSSION

Possible molecular mechanisms of EMS-induced mutations

Alkylating agents like EMS react with guanine, adenine, cytosine, and 5-hydroxymethylcytosine when the free bases or nucleotides are exposed in solution (BROOKES and LAWLEY 1960, 1961a, 1962; PAL 1962). The reaction of this agent with DNA produces 7-ethylguanine (BROOKES and LAWLEY 1961b), and 3-ethyladenine and other products may occur to a lesser extent. We shall consider two distinct mutagenic mechanisms that may be postulated for 7-ethylguanine, and one mechanism for 3-ethyladenine.

1. *GC to AT transitions mediated by 7-ethylguanine pairing errors:* Whereas guanosine has a pK of 9.2 for the dissociation of the hydrogen atom at the N_1 position, the corresponding pK for a 7, 9-substituted guanine is shifted closer to 7 (LAWLEY and BROOKES 1961; PFLEIDERER 1961). Thus, ionization of the N_1 may occur while the DNA strand is serving as a template for DNA synthesis, which would permit pairing of 7-ethylguanine with thymine (Figure 4A) as well as with cytosine. This would generate a GC to AT transition. If a template molecule of 7-ethylguanine was not ionized at the moment of DNA replication it would pair like guanine with cytosine and hence make a normal, nonmutated copy. The possibility of making a later copy error would be retained by the DNA

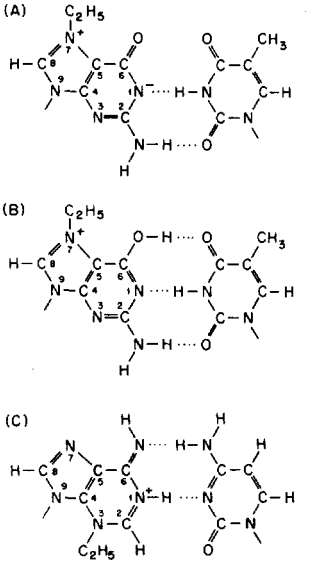

FIGURE 4.—Pairings postulated to occur during replication of DNA containing ethylated purines. Hydrogen bonds are shown by dotted lines. A: 7-ethylguanine and thymine. (The negative charge may be located on C_6O as well as on N_1.) B: Enol tautomer of 7-ethylguanine and thymine. C: 3-ethyladenine and cytosine.

strand containing the analog, regardless of the number of normal copies made first. The probability of a pairing error would be a function of the pH at the time and place of replication and of the effective pK of the analog present in a DNA strand: if the pH were one unit lower than the effective pK, the chance of a pairing error might be about 0.1; if the pH were two units lower, about 0.01. However, we do not know the actual pK of 7-ethylguanine in DNA, nor the effective pH during replication. The effective pK for 7-ethylguanine could well be several units higher in an ordered DNA molecule than for the nucleotide, by analogy with observations on guanine and thymine (CAVALIERI and STONE 1955). Thus, the absolute probability of the pairing error may be quite small. Nevertheless, the effective pK of 7-ethylguanine should still be lower than that of guanine in DNA. Thus, the probability of a pairing error with thymine would be larger for the guanine analog than for guanine.

Another mechanism by which 7-ethylguanine in DNA might make pairing errors during replication can be mentioned as a variant of this hypothesis. At pH values below the pK for ionization, this analog and also guanine may possibly exist to some extent in the enol rather than the keto tautomeric form. If so, the enol tautomer would be expected to pair with thymine by three hydrogen bonds (Figure 4B). If the frequency of the presumably rare enol tautomer were higher in DNA for 7-ethylguanine than for guanine, it would permit another mechanism for pairing errors which would be nearly indistinguishable in its effects from the mechanism outlined above.

2. *Replication errors from gaps produced by 7-ethylguanine hydrolysis:* Alkylation of the N_7 of deoxyriboguanylic acid produces a quaternary ammonium, and the positive charge may be shared by the N_9 atom. This weakens the glycoside linkage, which may lead to hydrolysis of the 7-ethylguanine from the DNA prior to replication (LAWLEY 1957; BAUTZ and FREESE 1960). The biological effect on DNA synthesis of a "gap" from a missing base is not known and it may be lethal. However, if replication can proceed past this point it may result in a base pair deletion or, as has been previously suggested, in a base pair substitution at the site formerly occupied by the GC pair (BAUTZ and FREESE 1960). This hypothesis is supported by the observed mutagenicity of low pH on phage (FREESE 1961). The chance of a 7-ethylguanine producing a replication error by this mechanism will be the product of the incidence of hydrolysis at that site and the probability of the replication error occurring at such a gap. If any of the four types of precursor molecules may be incorporated randomly opposite a gap, there may be a comparable chance of obtaining a normal copy, a GC to AT transition, or a GC to CG or GC to TA transversion. However, when the strands of DNA separate and serve as a template for replication, the forces normally tending to "stack" adjacent bases may draw together the two previously separated bases neighboring the gap produced by 7-ethylguanine hydrolysis. Replication then might result in the production of a single base pair deletion, equivalent to one of the types of "reading frame shift" mutations postulated for acridine mutagenesis (CRICK *et al.* 1961).

3. *AT to GC transitions mediated by 3-ethyladenine:* 3-Methyladenosine has

been shown to have an imino group in place of the amino group of adenine, and to be positively charged at pH 8 and below (PAL and HORTON 1962). The presumed structure indicates that during DNA replication, pairing would be possible between 3-ethyladenine and cytosine by two hydrogen bonds (Figure 4C), and that pairing with thymine would not occur. This would imply that the pairing error from 3-ethyladenine would have a probability near one.

Some implications of different copy error probabilities

The probability of copy error (c) characteristic of a given mutational mechanism will determine whether the frequency of induced mutations is as high as or lower than the fraction of the bases that have been altered at a given site in the DNA during exposure to the ethylating agent, whether the induced mutations are immediate or delayed, and how the frequency M_p of mutants among progeny derived from exposed phage is related to the fraction M_s of survivors that give an induced mutation. We shall illustrate these relations by considering several cases and then consider what this suggests as to the relative importance of the three hypothetical mechanisms.

If c is equal to one, the induced mutation occurs at the first duplication. If half the clone produced by a phage is descendant from the strand of the DNA duplex that contains the altered base, and this subclone is entirely mutant, then $M_p = 0.5\ M_s$. This is the case for most nitrous acid-induced mutations (TESSMAN 1959). As will be discussed in connection with AP156, we infer that this is the case for AT to GC transitions mediated by 3-ethyladenine.

If c is smaller than one, and a normal nonmutated copy can be made, then normal duplications may precede the occurrence of a copy error at the site of an altered base. A population of phage containing altered bases producing mutations by such a mechanism would generate mutant subclones of various fractional sizes distributed as $\frac{1}{2}^n$ of the total clones with n reflecting the duplication at which a particular copy error took place. We refer to this as a pattern of delayed mutation. If c is very small, the altered bases could generate an equal incidence of half-, quarter-, eighth-mutant clones, etc. (GREEN and KRIEG 1961). This distribution implies that half of the total mutants in the progeny population would be contained in half-mutant clones produced in the first duplication and that the mean fractional mutant clone size would be 0.14 (GREEN and KRIEG 1961). Hence with such a pattern of delayed mutations, $M_p = 0.14\ M_s$ and the ratio of the frequency of mutant progeny to the fraction of bases ethylated would be equal to c, even smaller than 0.14.

EMS-induced mutants, produced and detected by methods similar to those employed in this study, occur as delayed mutations (GREEN and KRIEG 1961). Thus we infer that the mechanism accounting for all or most EMS-induced mutations is characterized by a low probability of copy error, and we should regard as most important a mechanism that might have this characteristic. We have indicated that this could be true for (1), based on the ionization of 7-ethylguanine, and that it is probably not true for (3), based on 3-ethyladenine. The

implications of (2) are not so clearly predictable, but if gaps were produced by hydrolysis prior to or early in infection it seems unlikely they would produce the observed pattern of delayed mutations. The possibility also exists that a pattern of delayed mutations would be generated if hydrolysis yielded mutants only if it occurred after DNA synthesis had began.

An explanation for the low recovery of EMS-induced revertants in direct selection

The foregoing discussion leads us to an hypothesis to account for the various ratios found between the frequency of revertants observed in direct platings of exposed phage with selective bacteria and the frequency of revertant progeny (Table 2).

When an *r*II phage infects a bacterium of a selective strain, the chance of any phage being produced is normally quite small; this value is called the transmission coefficient and is about 0.0004 for AP72 in KB. For a revertant frequency of 140 per 10^6 progeny, as is found for our standard exposure of AP72, we infer that the frequency of reversions per survivor is about 1000 per 10^6 ($M_s = M_p/0.14$). The frequency of reversions per survivor observed on direct plating with KB bacteria is about 5 per 10^6, so the efficiency of their recovery is about 0.005, considerably larger than the normal transmission coefficient. The corresponding efficiency of recovery for AP275 revertants is about 0.02. These calculations suggest that the production of revertant phage by an infected KB bacterium requires not only the production of a revertant gene but also one or more revertant gene products, and that a revertant gene product is produced in only a small fraction of the bacteria infected by a phage with an ethylated AP72 or AP275 site. If the production of a revertant gene product involves the production of an r^+ messenger RNA molecule by an induced pairing error on the DNA template of the infecting phage, the probability may be directly related to c, the probability per DNA duplication of a pairing error at an ethylated mutant site. (Uracil, like thymine, could pair with 7-ethylguanine.) As we have pointed out, the occurrence of delayed mutations implies that c is much lower than one, and this could be expected for 7-ethylguanine. Hence, a low recovery of induced revertants on direct selection may be a characteristic feature of EMS-induced GC to AT transitions.

If the probability of a copy error from 3-ethyladenine is nearly one per duplication, both during RNA and DNA synthesis, then there may be a characteristically high recovery of EMS-induced AT to GC transition revertants on direct selection. This may account for the high ratio observed for AP156.

Revertants that are not true back mutants

As mentioned with the results, many of the revertants can be distinguished from standard r^+ phage and thus are not true back mutants. We have attempted to record all revertant plaques observed, distinguishing revertant types where possible. We are using reversion analysis primarily in the attempt to characterize representative individual EMS-induced mutational changes, and excluding rever-

sions that are not back mutations could bias the survey. For most purposes it may not be important whether the reversion reverses the original base pair change, is another type of change at the same site, or occurs at another site.

Although the frequencies have not usually been tabulated, partial revertants ("tiny" or "false" revertants) have also been observed in other *r*II reversion studies (FREESE 1959; FREESE 1962; FREESE, BAUTZ and FREESE 1961; FREESE and FREESE 1961; CHAMPE and BENZER 1962). In some cases they have been shown to be due to suppressor mutations within the *r*II region (R. P. FEYNMAN, personal communcation; CRICK *et al.* 1961). A detailed account of suppressor mutations has also been reported for another phage locus (JINKS 1961).

YANOFSKY, HELSINKI, and MALING (1961) found proteins from revertant bacteria that differed from normal tryptophan synthetase by one or two amino acid substitutions but still had partial or even full enzymatic activity. The interpretation was that the original mutant had one base pair substitution and that the partial reverants had either a different base pair substitution at the same site or at another site in the same or a different amino acid coding unit. This interpretation may be applicable to our results, but an alternative is mentioned below with our discussion of proflavin mutants.

The types of base pair changes involved in induced reversion of different r*II mutants*

GC to AT transitions: The results presented in Table 1 show that base analog-produced mutants are generally revertible by EMS, and that they fall into two fairly distinct groups: those with revertant frequencies close to those of AP72 and AP275 (about 60 to 150 revertants per 10^6 progeny), and those giving about one per 10^6 or less. There are several lines of evidence that indicate that the high EMS-induced revertant frequencies may occur at a GC site: 1. EMS exposure of DNA produces more 7-ethylguanine than other ethylated bases. 2. EMS-induced AP72 reversions, and most EMS-induced *r* mutations under our conditions, are delayed mutations. This would be expected from 7-ethylguanine but not from 3-ethyladenine. 3. These high revertant frequencies are associated with a low efficiency of recovery on direct selection, as expected from copy errors at the site of ethylated guanines but not from ethylated adenines. 4. These highly EMS-revertible mutants are also highly revertible by hydroxylamine, which reacts with cytosine and 5-hydroxylmethylcytosine more rapidly than with thymine (FREESE, BAUTZ and FREESE 1961; CHAMPE and BENZER 1962).

These lines of evidence would be applicable whether or not the induced revertants were true back mutations at the site originally mutated. The revertants from AP72 and AP275 appear to be true back-mutants, however, and since base analog-induced mutants are thought to be produced by transitions, these highly inducible reversions may be presumed to be GC to AT transitions. The possibility that other types of base pair changes may also be induced at GC sites will be discussed shortly, and we will simply conclude here that EMS-induced GC to AT transitions occur at a high frequency.

AT to GC transitions: Some members of the less revertible class of base analog-produced mutants, like AP156, have a spontaneous reversion frequency so low that it is clear that EMS induces a significant increase in revertants. We tentatively infer that EMS induces AT to GC transitions, at frequencies about two orders of magnitude lower than the highly inducible GC to AT transitions.

The conclusion that both types of transitions are induced by EMS, at widely different rates, is supported by our results for EMS-induced reversion of EMS-produced mutants. As predicted, most EMS-produced mutants are induced to revert at rates of one per 10^6 or less. Occasional mutants would be expected to revert at a much higher rate, if they had been originally produced by an AT to GC transition. BENZER found an EMS-produced mutant (EM126) that mapped at the site of AP72. When we tested this mutant, it was found to give the same high EMS-induced reversion frequency.

Additional evidence that AP156 reverts by mutation of an AT site has been presented by SETLOW (1962). This mutant, and AP61, can be induced to revert by ultraviolet irradiation of infected bacteria, with an action spectrum for induced reversion resembling the absorption spectrum for thymine. Mutants apparently identical to AP156 are regularly produced by AP and by EMS (BENZER 1961), and our data include several at this site (EM9 and AP83) that have the same revertibility. Since the analysis of the revertants indicates that they may be true back-mutants, we infer that these reversions involve transitions.

There are several reasons, therefore, for concluding that EMS-induced AT to GC transitions occur, but at a low frequency.

Proflavin mutants: There is evidence that acridines such as proflavin act as mutagens by adding or deleting single base pairs, and that addition mutants are subject to reversion by closely linked suppressors arising by base pair deletions, and conversely for deletion mutants (BRENNER *et al.* 1961; CRICK *et al.* 1961).

Most proflavin-produced mutants we tested, including P13 which CRICK *et al.* used as the starting point for their analysis (and redesignated FCO), were not particularly subject to EMS-induced reversion. However, P42 and P85 were quite EMS-revertible. It may be that these two mutants are caused by base pair additions and that EMS can induce base pair deletions by the mechanism we have previously outlined.

We have recently found, however, that most of the mutants giving EMS-induced partial revertants (including P85, AP12, AP41, AP50, AP114, BU90 and EM34) are also induced to revert both by 5-bromouracil and 5-aminoacridine (unpublished results). ORGEL and BRENNER (1961) also found a spontaneous mutant that was induced to give apparently standard type revertants by 5-bromouracil and tiny revertants by proflavin. They report that 5-aminoacridine and proflavin have generally the same mutational characteristics. These results are not easily compatible with previous interpretations of mutagen specificity and the action of suppressor mutations. Perhaps there are partly functional genes that differ from the standard base sequence by one base pair substitution and by one base pair addition or deletion.

Transversions: FREESE (1961) concluded that transversions can be induced by

ethylating agents and by low pH. The accuracy of this conclusion can be important in distinguishing between the several mechanisms that have been described for the role of 7-ethylguanine in inducing mutations. We shall summarize here the evidence that was offered for this conclusion, together with reasons why we do not find the evidence to be compelling.

1. A small fraction of EES-produced mutants (about 20 percent) and of mutants produced by a heat and acid treatment (about 10 percent) were not inducible to revert by base analogs. This was taken to imply that they could not be transition mutants. This implies the assumption that all transitions should be base analog inducible. However, CHAMPE and BENZER (1962) designate about 30 percent of the BU- and AP-produced mutants that they tested as giving weak, false or no base analog-induced back mutation. This indicates that not all transitions are base analog-inducible and/or that not all base analog-induced mutants are transitions.

2. FREESE (1961) selected five EES-produced and 15 spontaneous mutants that were not base analog revertible and found that about half were induced to revert both by EES and by a pH 4 treatment. Two levels of induced reversion frequencies were observed: about 50 per 10^6 and about four per 10^6 (corrected to treatments that gave 37 percent survival). However, the spontaneous revertant frequency of each of these mutants is correspondingly high. We checked two of these mutants, *r*207 and EES66, by our standard progeny procedure and found that EMS also induced about a 50 percent increase over the spontaneous levels. The increases may be significant, but the analysis and interpretation are difficult. FREESE (1961) also reported that no nitrous acid-induced revertants were detectable for these mutants. However, direct plating of nitrous acid-treated phage is not very efficient in detecting induced revertants of presumed transition mutants (FREESE and FREESE 1961) and low induced rates could have been masked by the high levels of spontaneous revertants.

3. Four of the mutants in the above mentioned group mapped at sites where other distinguishable mutants were also known, and since in two cases one of the other mutants was identified by reversion data as the member of the set that reverted by an AT to GC transition, it was inferred that the EES-revertible mutant could not also be a transition (FREESE 1961). However, the conclusion that two mutants occur at the same site is drawn from their inability to give wild type recombinants, and the high levels of spontaneous revertants characteristic of the mutants in question could mask low recombination frequencies so the site identities of these mutants is only tenative. TESSMAN (1962) reports finding recombination frequencies for some other mutant pairs to be much lower than the level of sensitivity in these cases.

Additional evidence is needed to evaluate the question of EMS-induced transversions. FREESE and coworkers had concluded that ethylation-induced mutations result from the hydrolysis of 7-ethylguanine, the second molecular mechanism we discussed previously. On this basis, transversions might be expected to be at least as frequent as transitions. The first molecular mechanism we discussed would generate only GC to AT transitions, however. The occurrence of a small

fraction of EMS mutants that are highly EMS-revertible might be adequately explained by 3-ethyladenine-induced *r* mutations that revert by GC to AT transitions. More direct tests are needed to evaluate the alternative molecular mechanisms hypothesized for 7-ethylguanine-induced mutation.

SUMMARY

Killing and induced reversion were investigated for bacteriophage T4*r*II mutants exposed to ethyl methanesulfonate (EMS).

1. The survival curve is approximately a parabola. That is, the frequency of lethal damages is approximately proportional to the square of the duration of exposure of phage to EMS. This could result from a two-step (not a two-hit) mechanism of killing. The frequency of induced revertants from a highly revertible mutant was found to be proportional to the first power of the duration of exposure. It is concluded that ethylated molecules are produced within the phage by a first-order reaction, and that induced mutations result from their presence within the phage DNA. The second step in the mechanism of killing could be the hydrolysis or breakdown of ethylated phage components prior to infection.

2. Many base analog-produced mutants, and some EMS-produced mutants, are induced to revert at high frequencies by EMS. It is concluded that these represent base pair substitutions at a GC site, probably GC to AT transitions.

3. Most base analog and EMS mutants that are not induced to revert at high frequencies by EMS are induced to revert but at much lower frequencies. These are believed to represent AT to GC transitions.

4. Induced revertants from the highly revertible mutants are recovered with low efficiency if the exposed phage are subjected to a selective assay procedure directly, without prior opportunity for nonselective growth. This phenomenon is related to delayed mutation and a low frequency of induced "mispairings" of ethylated guanine with thymine or uracil.

5. The possibility is discussed that EMS may also induce transversions of GC to CG or of GC to TA. The evidence is considered inconclusive.

6. Several proflavin-produced mutants were found to be reverted at fairly high frequencies by EMS. It is possible that these revertants are caused by deletions of single GC pairs.

7. Some of the induced revertants appear to be true back mutants, while others are definitely distinguishable from standard r^+ phage. This further confirms the evidence that a variety of base sequences may specify a functional gene product. Various intracistron suppressor mutation mechanisms are known that may account for these observations.

8. Three possible mechanisms of EMS-induced mutation are described: pairing errors of 7-ethylguanine with thymine, pairing of 3-ethyladenine with cytosine, and replication errors at the site of gaps produced by the hydrolysis of 7-ethyl-guanine.

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