Back Mutation of Leucine-Requiring Auxotrophs of *Salmonella typhimurium* Induced by Diethylsulphate

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SUMMARY

Ten leucine-requiring mutants of *Salmonella typhimurium* were induced to revert with the alkylating agent, diethylsulphate (DES). The pattern of reversion was studied by following the appearance of leucine-independent colonies on minimal plates, and of turbid (revertant) tubes among tubes containing 10^5 – 10^6 treated bacteria in minimal medium. Five mutants gave high (0.6 – 5×10^{-5}) frequencies of reversion. The other five gave lower (5×10^{-8} – 1.3×10^{-6}) frequencies immediately after plating on minimal agar or dilution in minimal medium, but reversion continued to take place until a high frequency was attained. This delayed mutation was found to be independent of cell metabolism. The possibility is discussed that the different patterns of reversion reflect alkylation of different base pairs. It is suggested that mutants having guanine:cytosine (G:C) base pairs at the mutant site revert mainly by pairing errors made by alkylated guanine. Mutants with adenine:thymine (A:T) sites are assumed to revert through the production of apurinic gaps, formed by the hydrolysis of ethyladenine.

INTRODUCTION

It has been proposed that ethylating agents exert their mutagenic effects mainly through the production of 7-ethylguanine (EG) in the DNA molecule (Bautz & Freese, 1960). The other major product of alkylation, 3-ethyladenine (EA), is assumed to have a lesser role in ethylation-induced mutagenesis (Brooks & Lawley, 1962; Pal, 1962; Krieg, 1963*a, b*). Ethylation-induced mutation of phage exhibits a pattern of delayed mutation, in which mutant subclones appear to be formed after normal non-mutant gene copies have been made (Green & Krieg, 1961). Two interpretations of this phenomenon have been advanced, based on different base-pairing properties of ionized or tautomerized EG, or based on hydrolysis of EG (Green & Krieg, 1961; Freese, 1963; Krieg, 1963*a*). Experiments to test the latter interpretation did not support it and favoured the pairing-error interpretation of the delayed mutant subclones (Löbbecke & Krieg, cited in Krieg, 1963*b*).

In experiments with bacteria it is common practice to count revertant colonies at an early time (36–48 hr), to avoid counting slow-growing colonies which may be due to suppressor mutations (Margolin & Mukai, 1961). Yet it is common experience to find a continuous appearance of new colonies on the selective plates after plating bacteria treated with alkylating agents.

Two years ago an experiment was designed by Professor F. J. Ryan and the present author to study diethylsulphate (DES)-induced recombination in *Salmonella*

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typhimurium. The results were indecisive because of a continuous reversion of some of the markers, although the treated bacteria were kept in buffer. This finding could not be accounted for by selection. Somewhat similar results were found by Strauss & Okubo (1960), who found increased reversion from auxotrophy to prototrophy upon incubation of DES-treated *Escherichia coli* at an elevated temperature before selective plating was done. In the present paper, a study of the pattern of mutant appearance after DES treatment is described. It shows that late appearance of revertant colonies after DES treatment is indicative of delayed mutation rather than slowly growing suppressor mutations, and is not due to a general 'physiological damage' of alkylation. It further shows that the different mutations can be grouped in two classes—delaying and non-delaying ones—which can be correlated with the pattern of mutation found after treatment with some base analogues.

METHODS

Organisms used. Leucine-requiring mutants of *Salmonella typhimurium* strain LT2 were used (we are indebted to Dr P. Margolin for these mutants) and are among 42 studied by Margolin & Mukai (1961) which were shown to be revertible by both diethylsulphate and 2-amino-purine (AP). They fell into two groups with respect to AP-induced reversion: those which showed a higher frequency of induced mutation when several cell divisions occurred after treatment (division-dependent, DD), and those whose frequency of induced reversion was not dependent on further cell division (division-independent, DI).

Media. Liquid minimal medium (MM) used in reversion experiments contained (g./l.): 10.5, K_2HPO_4 ; 4.5, KH_2PO_4 ; 1.0, $(NH_4)_2SO_4$; 0.98, Na-citrate; 8.0, NaCl; 2.0, glucose; 1 ml. M-MgSO₄; water to 1 l. Minimal agar (MA) contained, in addition, 20 g. agar/l. The same minimal agar, supplemented with 25 µg. leucine/ml., was used for viable counts. Tryptone broth contained (g./l.): 10, Bacto tryptone; 9, NaCl; 2, glucose. The buffer used for treatment as well as post-treatment incubation contained (g./l.): 5.8, Na_2HPO_4 ; 3, KH_2PO_4 ; 1, NH_4Cl ; 8, NaCl; and 1 ml. M-MgSO₄; water to 1 l.

Treatment. Overnight cultures grown in 100 ml. tryptone broth were washed twice and resuspended in 10 ml. buffer. After further incubation for about 3 hr at 37°, diethylsulphate was added to 5 ml. of suspension. The treatment mixture was shaken at 37° on a shaker. Treatment was stopped by diluting 0.05 ml. of the mixture into 5 ml. buffer supplemented with $Na_2S_2O_3$ to a final concentration of 0.04 M. Whenever large numbers of treated bacteria were needed, 1.0 ml. of the treated suspension was diluted in 100 ml. of buffer supplemented with $Na_2S_2O_3$. After incubation at 37° for 1 hr, the bacteria were collected by centrifugation and resuspended in 5 ml. buffer containing 0.04 M- $Na_2S_2O_3$.

Plating experiments. Plates of minimal agar (MA) seeded with 10^6 – 10^9 bacteria were incubated at 37°, and revertant colonies marked and counted. In each experiment, a 36 hr colony from a control MA plate (seeded with untreated bacteria) was chosen as a 'reference colony' (and the plate kept in a refrigerator). Revertant colonies were counted only after attaining the size of that colony.

Tube experiments. For tube experiments, treated bacteria were further diluted in chilled liquid minimal medium (MM) and distributed in small tubes. The tubes were incubated at 37° without aeration and examined routinely for the appearance

of turbidity caused by overgrowth of revertants. Whenever it was necessary to follow the optical density in those tubes, 4 ml. samples were distributed; otherwise, each tube contained 2 ml. A Bausch and Lomb 'Spectronic 20' spectrophotometer was used for readings of optical density at 540 m μ .

RESULTS

Alkylation of mutant leu-524

The effects of diethylsulphate (DES) treatment on viability and mutation in the *Salmonella typhimurium* mutant, *leu-524*, are shown in Fig. 1. Samples (5 ml.) of buffer suspension of bacteria were treated with different DES concentrations for 20 min., or with 0.04 ml. (final concentration of 0.06 M) of DES for various lengths of time. When the whole range of diethylsulphate concentrations used was drawn so as to match the whole range of treatment time, the two curves closely resembled one another within the ranges of times and doses shown; they deviated, however, with more severe treatments. This was because of rapid decomposition of DES in aqueous solution and because, with higher concentrations of DES, the oily DES drops in the treatment suspension become very large and remain attached to the bottom of the tube. A 20 min. treatment with 0.04 ml. DES/5 ml. bacterial suspension was arbitrarily chosen as standard treatment in further experiments.

The reversion data shown in Fig. 1 were obtained by treating 5 ml. bacterial suspension with different concentrations of DES for 10 and 20 min. Colonies growing on minimal medium agar (MA) were counted after 5 days. After that time, there was little change in colony count from day to day. One can, however, count the colonies on the same reversion plates every day, and plot the additive numbers against time. When this was done with mutant *leu-524*, the results shown in Fig. 2 were obtained. Spontaneous revertants form colonies which reach the size of the reference colony on MA after 30–40 hr, and there is little or no increase in their numbers after 60 hr. DES-induced revertant colonies lag behind the spontaneous colonies, and their number continues to increase considerably for another 96 hr. *A priori*, this increase in colony count could be accounted for by some physiological variability, slow-growing suppressor mutants, or delay in back mutation of *leu-524* itself. On the other hand, the possibility exists that the DES curves in Fig. 2 finally levelled off only because of environmental changes that took place on the plates while they were being incubated (such as bacteria becoming devoid of nutrients as the plates became too dry).

To test these possibilities, the following experiment was made. Treated bacteria of strain *leu-524* were diluted in MM to approximately 10^5 bacteria/ml.; 2 ml. samples were distributed in small tubes and allowed to stand at 37° without aeration. Control bacteria were diluted in MM to about 10^7 bacteria/ml. (very slight turbidity). The results are shown in Fig. 3. Most of the control tubes remained clear for about 3 weeks. However, some tubes became turbid because of the overgrowth of *leu*⁺ revertants. Pre-existing mutants required, under those conditions, about 48 hr for full overgrowth. After that time, no more mutants were found. (The rate of spontaneous reversion in *leu-524* is too low to become manifest at these conditions.) In the treated series, the number of turbid tubes continued to increase, although at a decreasing rate, throughout the experiment.

One can estimate, for any given time, the mean number (m) of mutant bacteria per tube (Ryan, 1955). After a time sufficient for full overgrowth (as made manifest by increase of turbidity), m represents the average number of pre-existing mutants originally distributed in the tubes. Thereafter, m also includes new mutants which

Fig. 1

Fig. 1. Viability and mutation frequency after various treatments with diethylsulphate. ● = Viability after treatment with 0.04 ml. diethylsulphate/5 ml. bacterial suspension for various lengths of time. ○ = Viability after treatment with various concentrations of diethylsulphate for 20 min. ▼ = The frequency of revertants among surviving bacteria after 20 min. treatment with various concentrations of diethylsulphate. ▽ = The frequency of revertants among surviving bacteria after 10 min. treatment with various concentrations of diethylsulphate.

Fig. 2

Fig. 2. The number of revertant colonies growing on minimal medium agar, as recorded at different times after plating. A culture of c. 10^{10} *leu-524* bacteria/ml. was treated with diethylsulphate and plated at final dilution of 10^{-2} , 10^{-3} and 10^{-4} . Control bacteria were plated undiluted.

originated after the bacteria were distributed in the tubes. m is estimated by determining the fraction of clear tubes among all the tubes (P_0), and applying it to the zero term of the Poisson distribution:

$$P_0 = e^{-m}. \quad (1)$$

In Fig. 3, typical m curves for control and DES-treated *leu-524* bacteria are shown. In the control m increased from 30 to 48 hr, after which there was no further increase. In the treated series m increased continuously for more than 400 hr.

'Suppressor' mutations

Inasmuch as some suppressor mutations can be distinguished from 'true' reversion by the slow growth rate of suppressor mutants, mutant *leu-524* gives, upon alkylation, mainly true revertants; only few slow-growing tubes were detected by routine measurements of optical density. Nor were many found in reconstruction

experiments. In such experiments, single bacteria were isolated from tubes which had become turbid at different times in the main experiment. This was done by diluting them (so as to give, on the average, less than one cell per tube) into tubes

Fig. 3. (a) The average number of mutations (m) in control (closed circles) and diethylsulphate-treated (open circles) leu-524 bacteria. The total numbers of tubes were 285 and 492, respectively. (b) Survival of control (closed circles) and diethylsulphate-treated (open circles) in the above experiment.

Fig. 4. The average number of mutations (m) in control (bottom curves) 524, +---+; 48 and 122, ●—●; and diethylsulphate-treated (upper curves) bacteria of mutants 48, x—x—x; 122, ●—●; 524, ○—○; 524 slow, ▼—▼. 524 slow was the slow growing tubes from leu 524.

containing 2 ml. liquid minimal medium with or without a 'background' population of about 5×10^5 mutant (untreated) bacteria/ml. The time for overgrowth in each tube was measured. The same observation, namely, that 'early' and 'late' mutants grew at the same rate, was confirmed in later experiments by the use of the spectrophotometer: with few exceptions (considered as slow growers), it took 6–10 hr from the time tubes showed any measurable turbidity until they became fully turbid. However, by using the spectrophotometer it was possible to secure a sufficient number of slow-growing revertants. After 120 hr from the time of dilution, the *m* curve for slow growers followed closely the *m* curve obtained with the normal growers (Fig. 4).

Table 1. *Delayed mutation in various leucine mutants of Salmonella typhimurium*

Mutant	Frequency of spontaneous mutation		Frequency of diethylsulphate-induced mutation		Division-dependence*	Delayed mutation
	At 48 hr	After 7 days	At 48 hr	After 7 days		
<i>leu-10</i>	1.7×10^{-9}	2.3×10^{-9}	8.8×10^{-8}	$> 10^{-4}$	+	+
<i>leu-25</i>	3.0×10^{-9}	5.0×10^{-9}	5.0×10^{-8}	2.8×10^{-6}	+	+
<i>leu-34</i>	4.6×10^{-9}	6.3×10^{-9}	1.4×10^{-7}	8.9×10^{-5}	+	+
<i>leu-44</i>	1.2×10^{-9}	2.3×10^{-9}	2.0×10^{-5}	5.1×10^{-5}	—	—
<i>leu-48</i>	1.0×10^{-9}	2.0×10^{-9}	2.8×10^{-5}	3.9×10^{-5}	—	—
<i>leu-122</i>	4.2×10^{-9}	7.8×10^{-9}	5.4×10^{-6}	5.9×10^{-6}	—	—
<i>leu-127</i>	1.3×10^{-9}	4.5×10^{-9}	3.8×10^{-5}	4.0×10^{-5}	—	—
<i>leu-130</i>	4.3×10^{-9}	8.3×10^{-9}	2.4×10^{-5}	2.5×10^{-5}	—	—
<i>leu-140</i>	1.7×10^{-8}	3.9×10^{-8}	6.5×10^{-7}	1.3×10^{-5}	+	+
<i>leu-524</i>	6.4×10^{-10}	9.5×10^{-10}	1.3×10^{-6}	2.4×10^{-5}	+	+

* As observed by Margolin & Mukai (1961): + = division-dependent; — = division-independent.

Experiments with other leu mutants

Having thus established that delayed mutation in mutant *leu-524* was not an artifact caused by slow growth, it became desirable to see whether other *leucine* mutants would show the same phenomenon after treatment with diethylsulphate (DES), or whether the delayed mutation was peculiar to mutant *leu-524*. Nine other DES-revertible *leu* mutants were selected for this purpose, on the basis of their patterns of reversion with 2-aminopurine (Margolin & Mukai, 1961) (Table 1, column 6). Buffer suspensions of these mutants were treated with diethylsulphate as described for mutant *leu-524*, and the numbers of revertant colonies were recorded repeatedly.

The results obtained with all 10 mutants are summarized in Table 1 and in Fig. 5. It can be clearly seen that all mutants did not show the delay effect. Mutants 44, 48, 122, 127 and 130 (namely, all division-independent mutants), after the appearance of the first burst of revertant colonies, show no further increase in the number of revertant colonies. On the other hand, all five division-dependent mutants did show such an increase for more than a week.

Delayed mutation on minimal medium agar and in buffer

A question arises about the relation between diethylsulphate-induced mutation and division-dependence after treatment with 2-aminopurine. To separate the time effect from the cell-replication effect, treated bacteria were kept in buffer before

plating was done on minimal medium agar. The results are shown in Fig. 6. *Leu-524* bacteria showed about a 40-fold increase in the number of revertant colonies over the first burst, when plated immediately after treatment. After incubation in buffer for 72 hr there was less than a 1.5-fold increase in the number of revertant colonies (Fig. 6a). This was even more pronounced in another experiment (Fig. 6b) where treated bacteria were kept in buffer for 140 hr before a tube test was made.

Fig. 5

Fig. 6

Fig. 5. Frequency of revertant colonies of the various mutants on minimal medium agar, as recorded at different times after plating. The frequencies of induced reversion of all 10 mutants are shown (10 upper curves). To make the figure more legible, only some of the control curves are shown. The others fall within the same limits and show the same pattern. The figures for *leu-524* are magnified ten times.

Fig. 6. (a) Reversion frequency of *leu-524* on minimal medium agar, as recorded at different times after plating. 0 = Cells plated immediately after treatment. 72 = Cells plated after 72 hr of post-treatment incubation in buffer. (b) The average number of mutants (m) among diethylsulphate-treated *leu-524* bacteria in a tube experiment. ●—● = 0 cells distributed immediately; ○—○ = 140 cells distributed after 140 hr of post-treatment incubation in buffer.

In contrast with the gradual increase in the m curve obtained with bacteria which were distributed immediately after treatment, the rise in m values for the bacteria which had been kept in buffer was rather abrupt, with no further increase. In both cases, however, the actual frequency of revertants was the same whether or not the bacteria had been aged in buffer before selection.

DISCUSSION

Margolin & Mukai (1961) studied 2-aminopurine-induced reversion of some 42 leucine-requiring mutants of *Salmonella typhimurium*, including 9 of the mutants used in the present work. They were able to demonstrate a distinction between continuous (division-dependent) and a single burst (division-independent) mutation. Both theoretical considerations (Freese, 1959) and the patterns of mutation after 2-aminopurine and 5-bromouracil found in *Escherichia coli* (Strelzoff, 1962) strongly suggest that the division-dependent mutants, 10, 25, 34, 140 and 524 have adenine-thymine base pairs at their mutant sites, while mutants 44, 48, 122, 127 and 130 have guanine-cytosine sites.

The present work shows that mutants of the two groups are diethylsulphate revertible and have different mutation patterns after diethylsulphate treatment. In spite of some degree of intra-group variability, mutants that are division-dependent after 2-aminopurine treatment show delayed mutation after alkylation, while division-independent mutants do not. However, from the experiments with incubation in buffer it follows that this delay is dependent only upon time, and not on replication.

At present, little is known about mechanisms that can account for the different patterns of alkylation-induced mutation (see review by Krieg, 1963*b*). Alkylation of native DNA was found to give mainly 7-alkylguanine and 3-alkyladenine (Lawley & Brooks, 1963).

Krieg (1963*a*) found that *r*II mutants of bacteriophage T4 that were highly revertible by ethyl methanesulphonate gave only a small fraction of their induced revertants when plated directly on selective host bacteria. The high frequency of induced revertants was found only when the treated phages were first allowed to reproduce in a non-selective host. (Neither procedure involved post-treatment storage of phage to promote depurination.) He attributed this phenomenon, and the delayed mutations occurring during replication (Green & Krieg, 1961), to pairing errors of 7-ethylguanine. Other T4*r*II mutants, assumed to have adenine-thymine pairs at their mutant sites, gave much lower or no ethyl methanesulphonate-induced reversion. Schwartz (1963), treating *lac*⁻ mutants of *Escherichia coli* with ethyl methanesulphonate, failed to induce the reversion of ethyl methanesulphonate-induced mutants at a high frequency. He attributed it to the fact that ethyl methanesulphonate induces mainly guanine-cytosine to adenine-thymine transitions. However, in the present study diethylsulphate was capable of reverting both guanine-cytosine and adenine-thymine mutant sites. Since diethylsulphate acts as a monofunctional ethylating agent like ethyl methanesulphonate, their mutagenicity should be expected to be similar.

Time-dependent delayed mutation in alkylation-induced bacteria is not necessarily identical with replication-dependent delay in phages. It is not clear, *a priori*, which of the alkylated bases should be implicated in interpreting the pattern of delayed mutation reported here. Nearly equal amounts of 7-ethylguanine and 3-ethyladenine are released initially during post-treatment storage (Lawley & Brookes, 1963). The apurinic gaps formed in this manner may account for the delayed mutations, by any of three mechanisms: (1) by taking in a 'wrong' base prior to replication (Ryan, Nakada & Schneider, 1961); (2) by the incorporation of the

latter opposite the gap, during replication; (3) by being capable of coding for the 'right' messenger RNA, thus initiating growth and replication. Such processes are quite speculative, but the idea of an apurinic gap leading to mutation is supported by the mutagenic effects of treatment at low pH values on phage (Freese, 1959), and of heat to bacteria (Greer & Zamenhof, 1962).

It is also possible that the depurination-dependent process may be masked at guanine-cytosine sites, due to the 'erroneous' pairing of 7-ethylguanine with thymine (or uracil, in RNA synthesis) (Brookes & Lawley, 1962). These hypothetical mechanisms are summarized in Fig. 7. Such a mechanism would be facilitated by

Fig. 7. Possible mechanisms of alkylation-induced reversion. Mutant 7-ethylguanine:cytosine (EG:C) sites are assumed to revert (immediately) through pairing errors during residual replication on minimal medium agar or in liquid minimal medium. 8-ethyladenine:thymine (EA:T) sites are assumed to revert through (delayed) depurination followed by insertion of guanine (G) in the apurinic gap, or by the polymerization of cytosine (C) opposite the gap. Of the other theoretical possibilities, delayed mutation through the production of apurinic gaps in EG:C sites is assumed to be masked by the process indicated here, while the ability of EA to pair with cytosine (Krieg, 1963*a*) has not been proven. O = Apurinic gap. Base pairs encircled by a broken line indicate a possible partial (phenotypic) reversion. Base pairs encircled by a continuous line indicate complete reversion.

the technically uncontrolled residual replication of the bacteria upon resuspension in minimal medium. Whatever the mechanism of delayed mutation, further complications may be expected to stem from indirect effects, such as the possible influence of neighbouring bases in the DNA molecule, on base substitution. It is not unlikely that similar mechanisms underlie spontaneous mutation in the stationary phase (Ryan, 1959) and of diethylsulphate-induced mutation. More direct evidence and better understanding of the role of base turnover in stationary-phase mutation (Ryan *et al.* 1961) seem necessary for the interpretation of alkylation-induced mutagenesis. Experiments bearing on the uptake by bacteria of bases alkylated *in vitro*, and the pattern of consequent mutation, are underway now.

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