

## GENETIC EFFECTS OF LSD-25 ON *E. COLI*\*

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(Received May 4th, 1970)

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### SUMMARY

A technique originally described for yeasts in which equilibria established between wild and mutant cells in culture can be used to estimate mutation rate, was tested on wild-type *E. coli* cells which were treated with varying concentrations of lysergic acid diethylamide. Significant numbers of auxotrophic mutations were produced. The dose-response was found to be linear: the slope of the regression of mutation rate on dose indicates that for each 0.1 mg increment of LSD added to the culture medium, the mutation rate increases by about 0.0001% over the spontaneous level. If a threshold for dose exists, it most likely lies at a concentration below 0.1 mg LSD per ml medium. LSD treatment was also seen to increase generation time although the pattern to this relationship is not clear at this time.

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### INTRODUCTION

Although many studies have been made of the effect of D-lysergic acid diethylamide (LSD-25) upon chromosomes, full details of its mutagenic action have yet to be developed. For example, from karyotype analyses of humans who have ingested LSD, some investigators have concluded that it is not mutagenic<sup>7,13</sup> though a considerably larger number have reported a high incidence of chromosome aberrations in human subjects<sup>1,5,9</sup> while COHEN *et al.*<sup>2</sup> demonstrated such aberrations in human chromosome cultures as well. Because of its long-standing utility in the detection of induced mutations, *Drosophila* was also tested. GRACE *et al.*<sup>4</sup> found that LSD did not induce germinal mutations when injected directly into *D. melanogaster* males in concentrations as great as 500  $\mu\text{g}/\text{ml}$  (in a volume equivalent to about 1 l of this concentration in a human subject), although VANN<sup>14</sup> found it can produce both X-linked and autosomal recessive lethal mutations with concentrations of at least 2000  $\mu\text{g}/\text{ml}$  when introduced either by injection or the less effective mode of ingestion.

LSD is clearly mutagenic in *Drosophila* and, most likely, in man. Unfortunately, from the data published to date it is not possible to assess its general mutagenic characteristics. For example, the effects of this chemical upon mutation rate have

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\* Research supported in part by a grant from the National Institute of Mental Health.

Abbreviations: MA, minimal agar; MB, minimal broth; TSA, trypticase soy agar.

not been quantified in any organism. Nor has it been determined if these effects are strictly dose-dependent or exhibit a threshold (see MARKOWITZ *et al.*<sup>8</sup> for a discussion of the latter questions). Similarly, while WAGNER<sup>15</sup> showed that LSD intercalates with DNA *in vitro*, nothing is known of its *in vivo* mode of action, *e.g.*, if it is dependent on or exclusive of cell division.

To examine these questions, *Escherichia coli* was selected as the experimental organism and mutations from proto- to auxotrophy as the system for study. An organism such as this can provide the large samples necessary for a sensitive test of effect upon mutation rate as well as an ideal system for determining (empirically, at least) the mode of action of this chemical in the following way: If the rate of mutation from wild to mutant cell type stays constant at different growth rates of the wild-type cells, the mutants must arise as a result of cell division. However, if the rate of mutation is inversely proportional to growth rate of the wild-type cells, the mutants most probably arise exclusive of cell division<sup>11</sup>.

#### MATERIALS AND METHODS AND RESULTS

A strain of *E. coli* (ATCC # 14948) well adapted to growth on minimal broth (type M-9 described in *Carnegie Inst. Wash. Publ.*, 1955, No. 607) which produced a maximum density of  $2.3 \cdot 10^9$  cells per ml, was used.

*Estimate of mutation rate to auxotrophy.* The procedure was based on that developed by OGUR *et al.*<sup>12</sup> who showed that when growth rates of wild and mutant yeast cells are unequal and the rate of back mutation is zero, a population equilibrium can be established in which the frequency of mutant cells is numerically equal to the rate of origin by mutation. (See NORTHRUP AND KUNITZ<sup>10</sup> for mathematical proof.) By growing wild-type cells on a minimal medium, the conditions necessary for the establishment of such an equilibrium have been fulfilled.

*A. Spontaneous mutation rate.* In order to obtain a reasonably accurate measure of the spontaneous rate of mutation to auxotrophy and to test the applicability of OGUR's procedure to *E. coli*, several 1-1 replicate cultures of wild-type cells were begun in aerated MB with an initial inoculum density of  $10^2$  cells per ml. Exponential growth began after a short lag phase. Periodically two samples were withdrawn from one of the replicate cultures. One sample (usually 1 ml in volume) was plated out on TSA providing an estimate of total number of viable cells at that time. The second sample (of volume varying from as much as 300 ml early in incubation when total cell counts were low, to 10 ml when cell densities were at least  $10^6$ /ml) was treated as follows. The sample was diluted to three times its original volume in MB containing 3000 units of penicillin-G/ml and incubated with aeration for 3 h during which time the vast majority of prototrophs were killed. After washing in NaCl to remove the penicillin, the cells were resuspended in 10 ml of NaCl and plated onto TSA as 100 0.1-ml aliquots. This means that the reconstituted volume could range from 1/30th to 1.00 portion of the original sample volume. After clone formation on this medium replicate plates were made on MA using the LEDERBERG<sup>6</sup> technique. The numbers of clones forming on TSA but not on the MA replicates provide an estimate of the frequency of mutant cells among all viable cells of that generation

(see Table I). Due most likely to the relatively small numbers of cells used to begin each of the replicate cultures and the relatively low frequency of mutants in the early hours of culture, early counts were quite variable and not considered reliable estimates of mutant frequency. However, these estimates increased with time and became far less variable until an equilibrium between mutant and wild-type cells became apparent by the 6th h (about generation 11) of culture. Exponential growth

TABLE I

THE RELATIVE FREQUENCIES OF WILD-TYPE AND SPONTANEOUSLY OCCURRING AUXOTROPHIC MUTANT CELLS GROWN IN MB

<i>Time after inoculation (h)</i>	<i>Total viable cells</i>	<i>Mutant cells</i>	<i>Ratio mutants:total</i>
0.5	$1.3 \cdot 10^2$	0.0	—
1.0	2.5	0.0	—
2.5	6.9	0.0	—
3.0	$1.2 \cdot 10^3$	$2.3 \cdot 10^{-3}$	$1.9 \cdot 10^{-6}$
4.0	3.1	6.2	2.0
5.0	9.7	$2.9 \cdot 10^{-2}$	3.0
6.5	$4.9 \cdot 10^4$	$1.8 \cdot 10^{-1}$	3.6
8.0	$2.1 \cdot 10^5$	7.1	3.4
9.0	6.0	$2.2 \cdot 10^0$	3.6
10.0	$1.9 \cdot 10^6$	6.8	3.6
12.0	$1.2 \cdot 10^7$	$4.4 \cdot 10^1$	3.7
13.5	6.0	$2.2 \cdot 10^2$	3.6
14.0	$1.1 \cdot 10^8$	3.9	3.6
15.0	3.1	$1.1 \cdot 10^3$	3.5
16.0	8.0	2.9	3.6

was maintained by transferring  $10^4$  cells to fresh medium after 8 h. The equilibrium frequency of mutant cells was found to be  $3.6 \cdot 10^{-6}$  which was taken to equal mutation rate per cell per generation. One can calculate the time required to reach equilibrium frequency from the formula

$$t_{0.50} = \frac{\ln 2}{A - B}$$

where  $A$  and  $B$  are the growth rates of wild and mutant cells, respectively, and provided the mutation rate to the mutant form is  $\ll 1$  (ref. 10). In this system one could expect to approach to about 1% of equilibrium after 2.75 h of exponential growth but total cell counts and, hence, the proportion of mutants at this time was so low as to be undetectable in this system.

*B. Mutation rate at various concentrations of LSD.* The procedure used was like that described just previously with a few exceptions. For each concentration of LSD used, two replicate inocula of  $10^2$  cells per ml were begun in MB. One was a control while the other contained the quantity of the chemical to be tested.

The test at a concentration of 0.1 mg LSD per ml medium can serve as the illustrative case. The experimental culture was limited to an initial volume of 20 ml due to the short supply of the chemical. Periodically two samples of generally dissimilar volumes were withdrawn; the smaller of them (1 ml) was used to estimate total number viable cells, the other (from 10 ml after 6 h of incubation to 1 ml

TABLE II

THE RELATIVE FREQUENCIES OF WILD-TYPE AND AUXOTROPHIC MUTANT CELLS GROWN IN MB CONTAINING 0.1 mg LSD/ml

<i>Time after inoculation (h)</i>	<i>Total viable cells</i>	<i>Mutant cells</i>	<i>Ratio mutants:total</i>
1.0	$1.9 \cdot 10^2$	0.0	—
2.0	4.0	0.0	—
4.0	$3.0 \cdot 10^3$	0.0	—
5.0	9.1	0.0	—
6.0	$2.6 \cdot 10^4$	0.0	—
8.0	$1.9 \cdot 10^5$	$8.6 \cdot 10^{-1}$	$4.5 \cdot 10^{-6}$
10.5	$3.0 \cdot 10^6$	$12.9 \cdot 10^0$	4.3
12.0	$1.3 \cdot 10^7$	$5.5 \cdot 10^1$	4.2
14.0	$1.0 \cdot 10^8$	$4.1 \cdot 10^2$	4.2
16.0	8.1	$3.5 \cdot 10^3$	4.3

after 12 h) to estimate frequencies of auxotrophic mutants. The results of this test are presented in Table II.

The other concentrations of LSD were tested in analogous fashion, the results of which are summarized in Table III. It can here be seen that all doses of LSD (except the smallest, 0.1 mg/ml) produce mutation rates that are significantly higher than the spontaneous level  $3.6 \cdot 10^{-6}$ .

Additionally, the response to dose is linear (see Fig. 1a) with the slope of the regression line equal to  $1.05 \cdot 10^{-6}$ ; that is, mutation rate/cell/generation is increased by this amount with each 0.1 mg increment of LSD.

*Chemical concentration and growth rate.* The effect on growth rate is not well defined. Table III shows generation time to increase with dose of LSD. In Fig. 1b the dotted line shows generation time in the experimental cultures as a function of chemical concentration and connects the observed points while the solid line is the one of best fit. Clearly the differences between experimental and control generation times are real and this chemical does in some manner inhibit cell division (in at least some of the cells of a culture).

*Mutation rate vs. generation time.* Two cultures of cells were begun in MB at 30° instead of the optimal 37°. Each was begun with  $10^2$  cells per ml but to the experimental culture was added 0.3 mg LSD per ml medium. Total viable cell counts were made from both cultures at varying intervals; however, an estimate of mutation rate was also made from the experimental culture. The estimated generation time in the control culture was 67 min and 70 in the experimental. Though generation time in the latter culture was about 50% longer than the comparable series run at 37° (*i.e.*, 70 vs. 44 min), the frequency of mutant cells in the 30° culture reached an equilibrium of about  $6.4 \cdot 10^{-6}$  (very close to that of the earlier series run at the higher temperature  $6.5 \cdot 10^{-6}$ ).

TABLE III

<i>Dose LSD (mg/ml)</i>	<i>Ratio mutants:total</i>	<i>Increase over spontaneous frequency (%)</i>	<i>Significance</i>	<i>Generation time (min)</i>
0.1	$4.3 \cdot 10^{-6}$	19	n.s. <sup>a</sup>	40
0.3	6.5	80	*	44
0.6	9.7	270	*	45
1.2	$1.7 \cdot 10^{-5}$	530	*	49

<sup>a</sup> n.s., not significant.

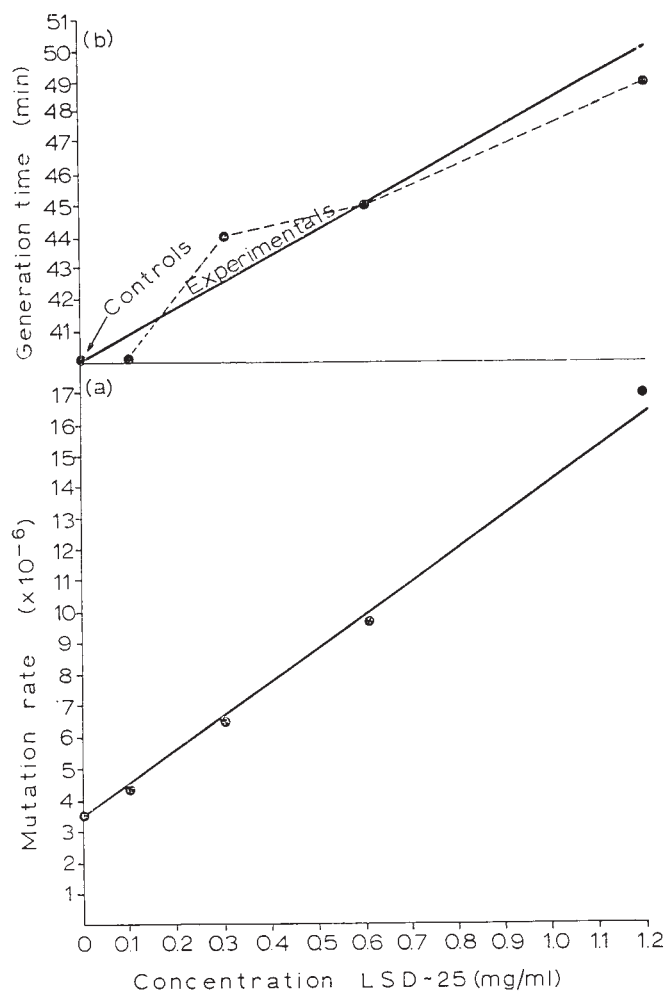


Fig. 1a. Mutation rate in experimental cultures regressed on dose of LSD administered.

Fig. 1b. Generation time in experimental cultures as a function of dose of LSD administered. The dotted line connects the observed points while the solid line is the one of best fit.

*Mutation rate in the absence of cell division.* A culture containing 0.6 mg LSD per ml medium was begun at the optimal temperature. After 6 h of incubation, estimates of total number viable and mutant cells were made. Then one-half of this culture was removed and the cells were washed and resuspended in the identical volume of a medium which was similar to the MB with LSD described earlier, except that it did not contain sugar. After 4 h of incubation in this medium estimates of total viable and mutant cells were again made. After 6 h in MB: wild  $1.3 \cdot 10^4$ , mutant  $1.3 \cdot 10^{-1}$ . Then 4 h in MB with sugar: wild  $6.5 \cdot 10^5$ , mutant  $5.5 \cdot 10^0$ . Or 4 h in MB less sugar: wild  $1.4 \cdot 10^4$ , mutant  $1.0 \cdot 10^{-1}$ .

It appears that no new mutant cells arose in the sugarless medium; that is, at a time in which growth of prototrophic cells was arrested. Consequently, from this and the previous section it appears most plausible that the origin of new mutants is directly dependent upon cell division.

#### DISCUSSION

The system for determining mutation rate by means of an equilibrium between mutant and wild-type cells in a selective medium appears to work for populations

of *E. coli*. The questions originally posed were three in number: is LSD mutagenic in *E. coli*; if such is the case what is the nature of the dose-response; and, are the mutational events induced in this system, related to cell division? It is unquestionable that this chemical is mutagenic in *E. coli*; the data shown in Fig. 1a in which frequencies of mutant cells are seen to increase with dose, confirm this.

The nature of the dose-response appears to be linear. When mutation rate is regressed on dose, the resulting line indicates that for each 0.1 mg increment of this chemical per ml medium, mutation rate increases by about 0.0001% over the spontaneous level. While the lowest dose of LSD tested provides a mutation rate that was seen not to differ significantly from the spontaneous, the opposite is true for the higher doses. However, one can note in Fig. 1a that the mutation rate at the lowest concentration lies very nearly on the regression line which suggests that even at the lowest dose we are likely measuring a real effect. Consequently, if a threshold is present, it likely lies at a concentration below 0.1 mg LSD per ml medium.

It is most probable that these mutation rates have not been measured without error, although they are likely reasonably conservative since one prime source of error, that due to phenomic lag, would cause these mutation rates to be underestimates of the true values; newly-mutated cells would be lost due to killing in the penicillin-MB medium. Another potential source of error is that attributable to differential killing of prototrophs by the chemical. It has been controlled in this experiment since auxotrophic cell frequency was measured relative to total *surviving* cells in each culture.

As was seen in the cultures in which (a) generation time increased with decreasing temperature and, (b) cell division was inhibited by lack of a metabolite, the rate of induction of mutations by LSD is a function of rate of cell division. Within the range of doses used (about 1 order of magnitude\*) the relationship between this variable and mutation rate is linear and possesses a rather low threshold, if any. In this sense, the chemical acts like ionizing radiation in a framework of "single hit" events. This observation has been noted previously in *E. coli*<sup>3</sup>. It can be justified in the present experiment if one assumes that (a) any *E. coli* prototroph has a given probability of mutating to auxotrophy at some concentration of LSD, (b) from each typically uninucleate cell which divides, only two cells form where there was one and, (c) mutants appear at the time of cell division. It follows then that each mutant cell will have arisen from the pair produced by only one cell division and, since a cell can become mutant only once and cannot again divide in the selective medium, the sort of dose-response observed is precisely what might be expected.

Assumption (a) above suggests a rather curious fact. All cells treated experimentally remained in the same LSD-containing culture medium for an 8-h period during which time no additional LSD was introduced. Since estimates of mutation rates were made at relatively frequent intervals during incubation, it does not seem possible that the dose-response of these cells changed drastically during this period.

Consequently, whatever the mode of action of this chemical, it does not appear to be rapidly depleted from the culture medium.

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\* Unfortunately, due to the relative scarcity and the time required to obtain supplies of this chemical, we were unable to test larger doses before the termination of the experiment.

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