although not all females will do so. Eggs were collected daily, observed carefully for 4 days, then discarded. Fertilized eggs normally hatch, under laboratory conditions, 24 to 48 hours after deposition.

During these experiments 1001 females laid 618 egg rafts containing 85,851 eggs. Three first-stage larvae hatched. The three hatched larvae were in three different rafts; thus, in each case only one of approximately 140 eggs hatched. It cannot be argued, therefore, that a male had somehow escaped detection, for if fertilization had in fact occurred, all or nearly all of the eggs in the raft should have been fertile.

All three larvae were weak. One survived the first molt but died soon thereafter; the other two died a few hours after hatching.

Species crosses in *Culex* (*4*) usually produce some eggs which are fertile but which fail to hatch. Such eggs, if sufficiently developed, can be identified under the dissecting microscope. A sampling of 150 egg rafts (containing an average of 125 eggs per raft) from the parthenogenesis experiments failed to reveal any such fertile but unhatched eggs.

Parthenogenetic development, therefore, apparently does occur in *Culex fatigans,* but the frequency of such occurrences is low (*5*).

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16 October 1958

Selection of Auxotrophic Bacterial Mutants by Tritium-Labeled Thymidine

Abstract. A method is proposed for the efficient isolation of auxotrophic mutants of cells of diverse origin by the use of radioactive materials. An example is described in which mutants of *Escherichia coli* B were selected by growing irradiated cells in the presence of tritium-labeled thymidine.

Bacterial mutants with specific nutritional requirements have been used extensively in biochemical and genetic studies. These auxotrophic mutants are ordinarily selected with efficiency by incubation of a mixed culture in minimal medium with penicillin. The growing wild-type are killed, and the dormant mutant cells are spared (*1*).

Many cell species which can be grown in culture are not sensitive to penicillin. No convenient method exists, with few exceptions, for selecting auxotrophic mutants of these cell species (*2*). It seemed possible that an alternative approach might be provided by radioactive materials, such as tritium-labeled thymidine (thymidine-H^3) of high specific activity. Growing cells of both plants and animals incorporate thymidine into deoxyribonucleic acid (DNA) (*3*), and localization of thymidine in the nucleus has been demonstrated by autoradiographs of thymidine-H^3 (*4*). The addition of thymidine-H^3 to HeLa cells growing in tissue culture has recently been shown to decrease viability by as much as 50 percent (*5*).

This report (*6*) describes an initial test of the proposed method in a system which has been studied extensively. First a reconstruction experiment was done with wild-type *Escherichia coli* B and a histidine auxotroph. These were grown separately on *enriched* medium [medium A (*7*) supplemented with 0.2 percent Difco yeast extract and 0.2 percent Sheffield tryptic casein hydrolyzate]. After washing, a suspension of each was placed in 1 ml of *minimal* medium A to which 0.1 ml of thymidine-H^3 was added (*8*). After dilution, the activity of tritium was 40 μc/ml, and the concentration of carrier thymidine about 4 μg/ml (*9*). The cells were incubated at 35°C for 3.5 hours, by which time the count of viable wild-type cells per milliliter had increased from 10^6 to 10^7. Half of each sample was removed, and the remainder was reincubated. After an additional 2.5 hours, wild-type had increased to $5 \cdot 10^7$. The number of histidine auxotrophs was not significantly changed by incubation. A control without thymidine-H^3 was included. After incubation, all samples were stored at 5°C.

Table 1. Decrease in viability after growth in the presence of tritium-labeled thymidine.

Tube No.	Thymidine-H^3 (μc/ml)	Duration of incubation (min)		Viable bacteria after cold storage (No./ml)		
		Before addn. of thymidine-H^3	After addn. of thymidine-H^3	0 days	7 days	13 days*
1	45	0	130	$6.6 \cdot 10^7$	2,600	190
2	45	45	85	$7.5 \cdot 10^7$	1,800	340
3	45	90	40	$6.0 \cdot 10^7$	3,000	210
4	15	0	130	$16.0 \cdot 10^7$	14,000	5200
5	15	45	85	$9.6 \cdot 10^7$	6,600	2100
6	15	90	40	$9.0 \cdot 10^7$	17,000	2400
7	0	130		$11.0 \cdot 10^7$	$3.0 \cdot 10^6$	
8	0	130		$15.0 \cdot 10^7$	$1.4 \cdot 10^6$	

* In all samples at 13 days, the fraction of mutant colonies exceeded 50 percent.

Viability was determined at intervals by plating appropriate dilutions on enriched agar plates. In the absence of thymidine-H^3, the viability of both the wild-type and the mutant fell to approximately 10 percent during 6 days of cold storage. After incubation with thymidine-H^3 the mutant showed a similar decrease, but the count of wild-type fell much more sharply.

The fraction of mutants surviving, divided by the fraction of wild-type surviving, can be expressed as an enrichment ratio. Without thymidine-H^3 this ratio was approximately 1. For the tubes incubated with thymidine-H^3 for 3.5 hours, the enrichment ratio at the end of 6 days of storage was about 4000. In the samples incubated with thymidine-H^3 for 6 hours, the ratio was only 70.

In a mutant isolation experiment, wild-type *E. coli* B were treated with ultraviolet radiation to approximately 1 percent survival. One-half of one milliliter was added to 5 ml of enriched medium and incubated for 24 hours. The cells were washed, diluted by 10^3, and 2.5 ml of the suspension placed in each of eight tubes. The amount of thymidine-H^3 and the time of addition were systematically varied, as is shown in Table 1. During the incubation period of 130 minutes, two to three divisions occurred. The tubes were then stored at 5°.

In this experiment, survival of bacteria in the tubes lacking thymidine-H^3 was 1 to 3 percent after 7 days at 5°C. In the presence of thymidine-H^3, survival was about 1/100 to 1/1000 as great. Each tube was carefully kept cold when samples were removed. After 7 days a search by the replica plating technique (*10*) showed that mutants were present at a frequency of 5 to 10 percent. At 13 days, the yield of mutants was above 50 percent, and in a few cases as high as 90 percent. Although killing of bacteria was greater with 45 μc (tubes 1 to 3) than with 15 μc (tubes 4 to 6), the fractions

of mutant colonies in these two groups were similar. No mutants were recovered from colonies not exposed to thymidine-H^3 (tubes 7 and 8). The number of mutants isolated can be accounted for by supposing that thymidine-H^3 acts as a selective lethal agent for cells that have grown in its presence.

On theoretical grounds, it does not seem likely that much of the selective killing of wild-type could be caused by radiation from thymidine-H^3 in the surrounding solution. The emitted beta particle has an average energy of 5.7 kev and a range in water of about 1 μ. In these experiments the ratio of fluid volume to bacterial volume was about 10^4, and almost all the radiation originating in the surrounding fluid would fail to reach the bacterial cells. This argument is supported by the results of the reconstruction experiment, in which the presence of thymidine-H^3 did not accelerate killing of the histidine auxotroph.

In addition, aliquots from tubes 1 to 8 of the mutant isolation experiment were diluted sixfold after the period of incubation and stored at 5°C. Almost as many mutants were isolated from these samples as from the undiluted tubes. This suggests that thymidine-H^3 is incorporated into the cells in a form unable to diffuse out during cold storage.

In a third experiment, the bacterial suspension was diluted considerably, so that the count at the end of incubation was only 10^6 per milliliter. Killing was rapid, presumably because the amount of thymidine-H^3 per cell was high, and after 5 days of cold storage the yield of colonies which were mutants ranged from 10 to 50 percent.

Among the auxotrophic mutants isolated by this procedure the following requirements have been identified: arginine, cystine, glycine, histidine, isoleucine, leucine, methionine, proline, serine, threonine, tryptophane, tyrosine, valine, isoleucine plus valine, and multiple aromatic compounds. These experiments show that thymidine-H^3 is an efficient selective agent for the isolation of auxotrophic bacterial mutants. The method may be applicable to other species of cells.

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6. This investigation began as a result of a series of provocative lectures on bacterial physiology given by Dr. B. D. Davis at Woods Hole. I am grateful to him for offering the facilities of his laboratory, instruction in bacteriological methods, and a number of discussions throughout the course of this work. This work was supported by U.S.P.H.S. grant H-1498C, U.S.P.H.S. senior research fellowship SF-83, the Muscular Dystrophy Associations of America, Inc., and the Lalor Foundation.
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15 October 1958

Mutagenic Effect of Azaserine in Relation to Azaserine Resistance in Escherichia coli

Abstract. For demonstration of the mutagenic effect of azaserine (mutation from streptomycin dependence to nondependence), higher concentrations of this antibiotic are required with azaserine-resistant *Escherichia coli* than with the sensitive, parental strain. At barely toxic concentrations of azaserine, however, the mutagenic response of the resistant strain is many times higher than that of the sensitive strain.

The suitability of azaserine as a chemical mutagen in elucidating the mutagenic process in *E. coli* has been evaluated and described in an earlier publication (*1*). Like most other mutagens, azaserine induces mutations with increasing frequency at concentration levels that are increasingly bactericidal. Nevertheless, it is one of the few known chemical mutagens which is appreciably mutagenic at levels relatively nonbactericidal. This poses the important question as to whether the process of azaserine-induced mutagenesis is related to the bactericidal property or whether the two phenomena are independent of each other. With this question in mind, a comparative study of the mutagenic response in mutants of a streptomycin-dependent strain of *E. coli*, showing varying degrees of azaserine resistance, was initiated (*2*).

From the parent Sd-4 strain (A) (*3*), mutants (B, C) resistant to varying levels of azaserine (*4*) were derived by methods based on the gradient plate principle (*5*). These were maintained on nutrient agar containing 100 μg of streptomycin per milliliter and concentrations of azaserine increasing from 0 to 1000 μg/ml. Washed cells from agar slants were used as inocula for the experiments. Details of the methods were the same as those described in an earlier paper (*1*). The effect of exposure to varying concentrations of azaserine for a period of 2 hours at 32°C on the survival of azaserine-sensitive (A) and azaserine-resistant (B, C) strains and on the mutation rate to streptomycin independence was studied.

The results, selected as representative of several independent experiments, are presented in Fig. 1. They suggest that azaserine is able to induce mutations in azaserine-resistant strains of *E. coli* only at concentration levels much above that required for mutagenesis in the parent, sensitive strain. It is apparent that, in strains resistant to azaserine, an increase in the mutation rate comparable to that produced in the more sensitive parental culture is obtained only at higher concentrations of the mutagen. Comparable results were obtained with several additional, independently isolated azaserine-resistant mutants. A closer analysis of the data, however, reveals that the resistant strains treated at barely bactericidal concentrations of azaserine show a higher mutagenic response than the sensitive parent under comparable conditions.

The data presented suggest that although the mutagenic and bactericidal effects of azaserine are correlated, the quantitative responses are not identical. If the bactericidal effects of azaserine on sensitive and resistant cells are regarded

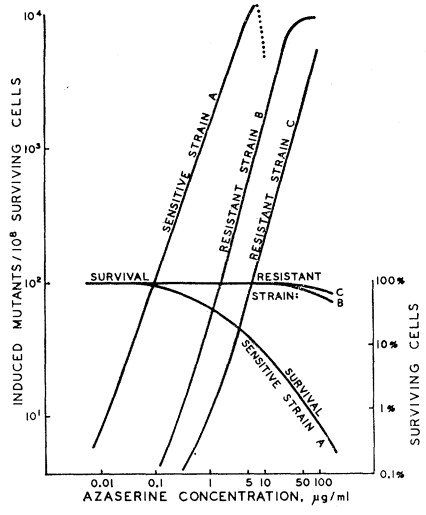

Fig. 1. Effect of azaserine concentration on the number of induced mutants (mutation from streptomycin dependence to independence) and on the percentage of survivors, assessed with azaserine-sensitive strain Sd-4 of *E. coli* and two azaserine-resistant mutants derived from that strain. Two hours' exposure in distilled water at 32°C.