PENICILLIN-SCREENED AUXOTROPHIC MUTATIONS IN SALMONELLA TYPHIMURIUM AND THEIR RELATION TO X-RAY DOSAGE[1]

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The demonstration by Davis (1948) and by Lederberg and Zinder independently that penicillin can be used as a screen to eliminate unchanged or "prototrophic" organisms from a radiated suspension of *Escherichia coli* while leaving the mutated or "auxotrophic" bacteria, which have specific growth requirements, has been a great stimulus to investigations in bacterial genetics. The technique has been described in detail by Davis (1949), and it is apparently applicable to many different species of bacteria since nearly all show susceptibility to inhibition by penicillin, if it is sufficiently concentrated. Variation in the penicillin susceptibility of the individual mutant strains is not involved, but only their ability to divide on a particular medium. If they divide, the concentrated penicillin will inhibit and eventually kill most of them. If they do not divide, they remain dormant and will grow when transferred to penicillin-free medium containing the particular nutrilite required.

We have applied the method to several different prototrophic strains of *Salmonella* and have isolated a large number of different auxotrophic strains that have lost the ability to synthesize one or more of the amino acids, nucleic acid fractions, or growth factors, which the parent strains can synthesize for themselves. Most of these mutant strains are constant, or revert to the prototrophic parental type with a low frequency, whereas a few revert rapidly. All those that revert can be recovered by rescreening the cultures through penicillin, and they can ordinarily be maintained on complete media in which no selection in favor of the reverted strain takes place. These studies are making available a whole new series of assay organisms besides giving information on mutation rate in bacteria, on the mode of action and interaction of genes in the bacterial cell, and especially on the chemical processes involved in the synthesis of amino acids, purines, and pyrimidines. There are apparent interrelations also with such general problems as the mode of action of certain antibiotics, virulence, and antigenic specificity.

The report of Roepke, Libby, and Small (1944) first established that mutations to specific food requirements in *E. coli* could be induced by radiation, and their observations were confirmed and extended by Tatum (1946), who showed that not only X-rays but ultraviolet light and mutagenic chemicals like the

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nitrogen mustards produced them. The inducing agents are the same as those causing gene mutations in the chromosomes of fungi, higher plants, and animals, so that the presumption is strong that bacterial genes are being acted on. The most interesting additional and independent evidence of gene action in bacteria is that of Tatum and Lederberg (1947), extended further by Lederberg (1947*a*), apparently indicating (for the K12 strain of *E. coli*) fusion of cells, gene transfer, and recombination, in a way that is most easily accounted for by linear arrangement of the genes in some sort of a chromosome.

We believe that the pathogenic *Enterobacteriacea* offer more favorable material for continued study of bacterial genetics than does *E. coli*. This is particularly true of *Salmonella*, in which the extensive knowledge of antigenic differences shown by the White-Kauffmann schema, as extended particularly by Edwards and Bruner (1942), presents a unique and readily testable tree-like interrelationship. Such a set of related strains or species, together with extensive serological studies such as those of Wheeler, Stuart, *et al.* (1943) showing interrelationship throughout the whole family of the *Enterobacteriacea*, should receive independent support from investigations of induced mutations derived from single strains of *Salmonella*. It should be possible to secure evidence whether serological types are gene-controlled or whether some other (possibly cytoplasmic) cellular elements determine them. There is the added important advantage that serological tests make possible frequent checks of the purity of the strains being investigated. A survey of existing strains of *Salmonella* for biochemical mutations has already been reported by Lederberg (1947*b*). Finally the genetic bases of virulence in the pathogenic organisms may be further studied along lines already laid down by the important basic work of Zelle (1942) and of Gowen (1945).

Our own studies are being made on *S. typhimurium*. Already a number of our students and assistants have contributed to the work. We wish especially to acknowledge the help of the following, all of whom have taken part in the laborious work of screening and testing the mutations: Myron Saltz, Martin L. Vogel, Tilford Miller, Dorothy L. Farley, and A. Marie McCarthy.

METHODS

Stocks of *S. typhimurium* have been received from several different sources, and they have been serologically typed by the Connecticut State Laboratory through the kindness of Dr. E. K. Borman. Only two have been used for the tests reported in this paper: no. 519, isolated from an enteric infection and kindly furnished by Dr. E. Seligmann of the New York Salmonella Center at the Beth Israel Hospital, and no. 533, received from Dr. J. W. Gowen of the Department of Genetics, Iowa State College. The latter is the strain used by Gowen (1945) in his studies of mutations in virulence and was derived from a single-cell isolation made by Zelle. Both are smooth strains giving the typical biochemical reactions and having a high virulence for mice. Strain no. 519 produces a relatively small amount of gas, whereas 533 is a very active gas former. Another strain, no. 511, on which some preliminary work was done, was originally isolated in a U. S. Army Laboratory in Manila, P. I., as typical. This has turned out to be *S. newport*, and it has been used in the preliminary tests only.

Both ultraviolet radiation and X-radiation have been used to induce mutations, but the discovery by Kelner (1949) that visible light corrects the effects of ultraviolet renders the dosages of ultraviolet unreliable, and so discussion of the ultraviolet effects will be deferred. X-radiation was done at the Marine Biological Laboratory at Woods Hole, and bacterial suspensions were subjected to an intensity of 2,860 roentgens per minute for from 4 to 20 minutes. This is the same apparatus utilized by Burkholder and Giles (1947) for inducing mutations in spores of *Bacillus subtilis*, but they used up to ten times higher dosage.

Stock strains are subjected to frequent dilution plating and to single-colony isolation, and this was always done before the radiation. The growth of a 24-hour agar slant is suspended in 5 ml sterile saline and brought up to a total volume of 22 ml, and then 5-ml samples of this suspension containing approximately 10^9 bacteria per ml are transferred to several sterile 50-mm petri plates, giving fluid samples about 5 mm in thickness. One of these plates is placed under the X-ray target, with the cover removed during the actual radiation period. We have found this method more reliable than the use of quartz flasks. Contaminations are infrequent and can be easily checked.

After radiation, the suspensions and the nonradiated controls are either diluted for immediate growth tests or subjected to screening with penicillin. In all our earlier tests the first method was followed, namely, dilution and plating in minimal medium, then, after incubation, layering with complete medium, and isolation for tests of small colonies that appear after the supplement is added (Plough, 1950). More recently we have found the Davis-Lederberg penicillin screening technique far more productive of mutations, and we are now utilizing that exclusively. First, 1-ml amounts of the radiated suspension are transferred to tubes containing 4 ml of complete nutrient broth and incubated for 24 hours. Then the tubes are centrifuged and the growing organisms resuspended in 3 ml of saline and diluted 1:100; then 0.1 ml of this dilution is transferred to synthetic minimal medium containing 100 units per ml of penicillin G and reincubated for 24 hours. The culture is plated on enriched nutrient agar by means of the layering technique to give up to 500 colonies per plate (cf. Davis, 1949). One hundred colonies are then picked at random and each transferred with the same inoculating wire to two broth tubes, one containing minimal and one complete medium (figure 1). If growth occurs in the second but not in the first, the strain isolated is an auxotrophic mutant.

Our method of making the determination of the specific nutrilite required has proved so rapid and accurate that it is described here. It is the reverse of the usual Beijerinck technique used by Pontecorvo (1949) and others. Paper disks such as those sometimes used for antibiotic assay are cut out of filter paper with a punch, and 50 or more are placed in each of a number of small, covered stender dishes and autoclaved. Five ml of distilled water are inoculated with the test organism by transfer with a straight wire from a 24-hour broth culture. After thorough mixing, 1 ml of this suspension is placed in one of the dishes containing the paper disks. Agar plates are previously prepared containing minimal medium plus 10 μg per ml of each of 20 amino acids (L-form, or 20 μg DL-form), appropriate dilutions of 10 vitamins, or a mixture of purines and pyrimidines. Tests

are made by removing with flamed forceps one paper disk from each of the bacterial suspensions and placing it in a similar position on each of the test agar plates. The plates are incubated and read at 24 hours and again at 48 hours. Failure of growth around any particular disk on any medium shows that the specific nutrilite required is absent. If the required substance is contained in any one of the three combinations on the test plates (amino acids, vitamins, or nucleic acids), growth will appear as a halo about the disk carrying the particular

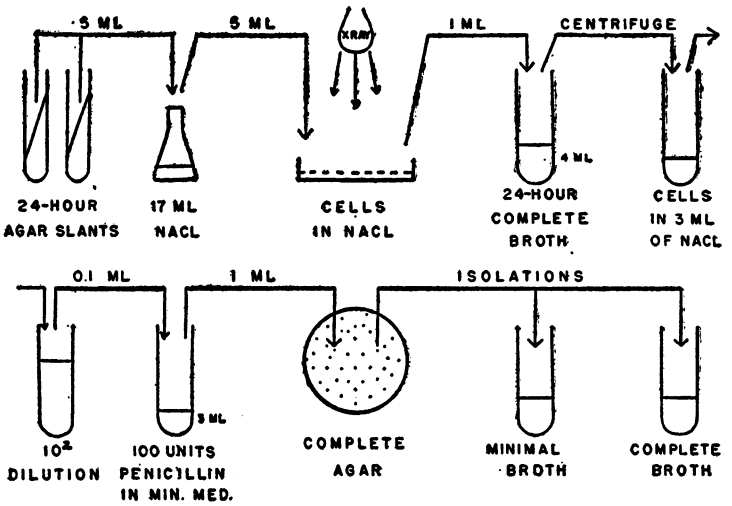

Figure 1. Isolation of auxotrophic mutants by penicillin screening.

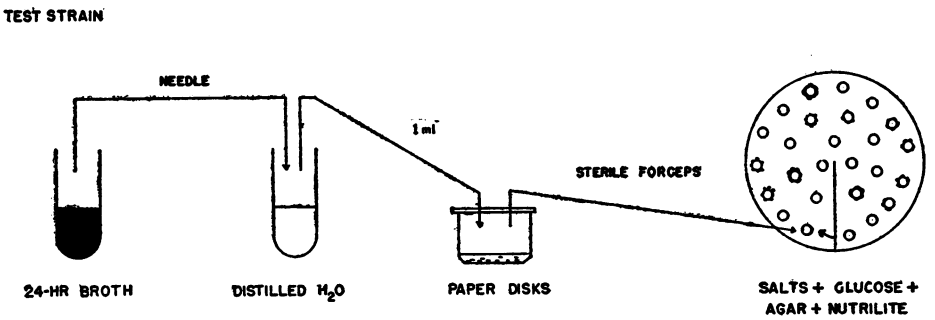

Figure 2. Paper-disk method for testing biochemical requirements.

organism (figure 2). If, for instance, any particular disk shows growth on the amino acid mixture and no growth on vitamins and nucleic acid fractions, we know that it requires some one of the amino acids for growth. The specific amino acid is then determined by making a similar set of plates containing each one of the amino acids separately. The method is rapid since up to 50 disks can be placed on the surface of one plate. If growth occurs on none of the test plates, we know that the organism has a multiple requirement and must be tested on combinations of the nutrilites or by omitting one after another from a complete mixture until the required combination is found.

The minimal medium used throughout this study is that of MacLeod (1940), which is a synthetic solution containing sugar as energy and carbon source and no organic source of nitrogen: NaCl 5.00 g, $(NH_4)_2SO_4$ 4.72 g, KH_2PO_4 2.72 g, glucose 2.0 g, plus 1 ml of solution containing 1 g each of $FeCl_2$, $MgCl_2$, and $CaCl_2$ in 600 ml H_2O. Glass-distilled H_2O is added to make 1,000 ml, and the pH is adjusted to 7.00 with NaOH. Most newly isolated strains of *S. typhimurium* will give suboptimal growth on this medium, and the addition of organic nitrogen in the form of asparagine, as is done in the *E. coli* medium or by Braun (1948) in his synthetic medium for diagnosis of *Salmonella*, only adds a complicating factor in this work.

Only washed vitamin-free agar is used, and tests show it will not support growth without added nutrilites. We have found Difco Noble agar entirely satisfactory. The complete medium was either an infusion agar made from lean beef or the Difco brain heart infusion with yeast extract added. Amino acids and vitamins added to the minimal medium were tested products supplied by various manufacturers.

TABLE 1

	NO. 511 (ULTRAVIOLET)	NO. 519 (X-RADIATION)	TOTALS
Control colonies isolated for test.....	452	227	679
Deficient mutants.....	0	0	0
Radiated colonies isolated for test.....	972	488	1,460
Deficient mutants.....	1	1	2

MUTATION FREQUENCY IN DIRECT ISOLATIONS

In order to indicate the approximate frequency of occurrence of biochemical mutations that have lost the ability to synthesize particular nutrilites, we shall summarize our earlier work in which we used the method of pouring dilution plates in minimal agar medium, layering with complete medium, and isolating slow-growing colonies. It is clear now that this method is too time-consuming and laborious to be feasible if large numbers of auxotrophic mutations are desired, even though it reveals many different kinds of less extreme variants (cf. Plough, 1950). For these tests we used two stocks: no. 519, and no. 511, the latter of which has now been identified as *S. newport*. Twenty-four-hour agar slants were suspended in 10 ml of sterile water diluted 1:100 and divided into two samples, one reserved as a control and the other used for radiation. Portions to receive ultraviolet radiation were pipetted into sterile quartz flasks of 35-ml capacity and rotated under a 15-watt General Electric germicidal lamp for a period of 1 to 2 minutes. It was calculated that the energy received was 300 to 600 ergs per mm^2. X-rayed suspensions were exposed to a total of 2,700 r units at the Marine Biological Laboratory at Woods Hole. It has been shown since that both these radiation dosages are too low to have any considerable mutagenic effect. The ultraviolet dosage killed only about 40 per cent of the organisms, and the X-radiation killed about 40 to 50 per cent. The results of these preliminary isolations are shown in table 1.

As table 1 shows, only two mutant strains having specific biochemical deficiencies were discovered in this series, although only small slow-growing colonies were selected for test—about 10 per cent of the total number. The first was 511-10-1, which is a cysteine-requiring mutant already briefly described (Plough and Grimm, 1949). Besides requiring cysteine—or S in reduced form—it is more sensitive to penicillin than the parent strain. The other, 519B-21-26, appears to have a complex requirement, which has not yet been analyzed. About 15 other strains isolated showed chemical deficiencies of some degree, but the majority of these reverted to normal after one or two transplants.

The table allows a rough estimate of the frequency of occurrence of auxotrophic mutations. Since about 10 per cent of the colonies were picked for tests as slow-growing colonies, we may multiply the total number of tests by 10 for a figure of the total number examined. Thus there are 0 mutants from close to 700 control colonies examined, and there are but 2 from about 15,000 in the radiated series (lumping together ultraviolet and X-ray treated). As the data in the next section indicate, we cannot expect that either radiation dosage used here would increase mutation frequency more than 5 to 10 times. If we assume the latter figure, and it is probably too high, we may place the frequency of spontaneous mutations of this kind among unradiated or control organisms at something like 2 in 150,000 or 1 in 75,000 dividing cells. This is a figure of the same order as that estimated by Lincoln and Gowen (1942) for spontaneous mutations in *Phytomonas stewartii* (46 per million). It will serve as a rough base for comparison with the results reported in the next section.

MUTATION FREQUENCY AFTER PENICILLIN SCREENING

In contrast with the extremely small number of mutants secured by the method of isolating colonies showing delayed growth on supplemented medium, we find that the use of the Davis-Lederberg method of screening with penicillin isolated numerous auxotrophic mutations, and the numbers show a definite relation to the X-ray dosage. A preliminary series of plate counts was made to establish the percentage of organisms surviving the different dosages used, and the results are shown in columns 1 and 2 of table 2. The lowest dosage used, 11,400 r units, was about four times that used in the preliminary tests summarized in the previous section.

After the penicillin screening and the preliminary and final tests, the mutant cultures were transferred to agar stabs, and all were retested after 4 weeks. A negligible number had reverted to the original wild type, and most of these have since been recovered by rescreening. The final summaries are shown in table 2, columns 3 and 4, where it will be seen that out of a total of 459 colonies tested, 234 proved to be auxotrophic mutants of some sort. In addition, a comparison of the relative numbers of the percentages of the mutations isolated following the successive dosages (table 2, column 5) shows a clear relation between mutation frequency and the X-ray dosage (table 2, columns 1 and 5, and figure 3). Although Davis (1949) states (p. 7), "The method as developed so far does not appear to yield quantitative survival of mutants, and hence cannot be used for

TABLE 2

S. typhimurium mutations that require specific nutrilites after X-radiation and penicillin screening

1 X-RAY DOSAGE AND TIME	2 % BACTERIA SURVIVING	3 TOTAL NO. TESTS	4 TOTAL NO. MUTANTS	5 % MUTANTS	6 NO. DIFFERENT MUTANTS	7 % DIFFERENT MUTANTS
Strain 533						
I Controls	100	135	7	5.1	3	2.2
II 11,400 r, 4 min	40	62	16	25.9	4	6.4
III 17,100 r, 6 min	25	86	18	20.9	4	4.7
IV 22,800 r, 8 min	14	41	11	26.8	3	7.3
V 28,500 r, 10 min	6	25	19	76.0	6	24.0
VI 34,300 r, 12 min	1.5	94	64	68.1	8	8.4
VII 45,600 r, 16 min	0.9	99	72	72.6	17	17.2
VIII 57,000 r, 20 min	1	50	34	68.0	12	24.0
Totals		459	234			
IX II + III + IV		189	45	23.8	11	5.8
X V + VI + VII + VIII		268	189	70.5	43	16.0
Strain 519						
XI 45,600 r, 16 min	2.5	100	22	22.0	9	9.0

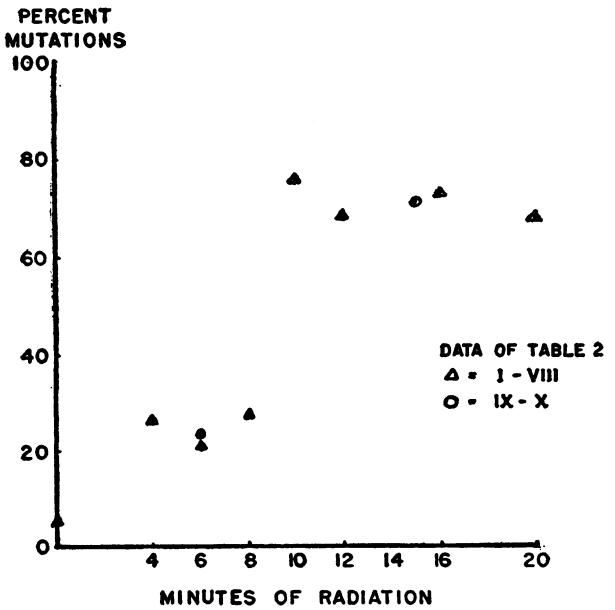

Figure 3. Relation between percentage of mutations and X-ray dosage in strain 533.

accurate determination of mutation frequencies," the results here shown suggest that this can be done with a fair degree of accuracy. We can properly com-

bine the results for 4, 6, and 8 minutes of radiation, which give an average of 23.8 per cent mutants (table 2, line IX). Similarly combining the results from 10, 12, 16, and 20 minutes of exposure (table 2, line X) the average is 70.5 per cent. A chi square test done on these numbers indicates a P, or probability, value of less than 1 in 10,000 that two such samples could be drawn from the same population. Even though the mutant cells have divided many times during the initial 24-hour period in the complete medium, so that duplications must have arisen from the same initial mutant, nevertheless each of the radiated samples received the same treatment except for the increased dosage. The clear difference between the number of mutations isolated from the summed lower dosages compared with those from the higher dosages can be explained as due to the correlation between dosage and mutation frequency, as has been proved many times for other organisms.

This relation is further and perhaps more convincingly substantiated if comparisons are made between the numbers of different mutations at successive X-ray dosages. In table 2, column 6 lists the numbers and column 7 the percentages of different mutations at each successive interval of radiation. Again combining results from the first three against the last four dosages, we find that, although in the controls 2.2 per cent of the mutants were different, an average from 4 through 8 minutes of radiation shows 6.1 per cent were different, whereas the average of the 10 through 20 minutes of dosages gives 18.4 per cent different. Thus, even if every repetition of the same mutant in the observations totaled for columns 4 and 5 should be due to multiplication of the same initial X-ray-induced mutation, the relation between X-ray dosage and mutation frequency would still hold. It is more reasonable to conclude that both the numbers of individual mutants and the variety are increased in a direct relation to X-ray dosage. Inspection of the spread of the points showing the percentage of the tests that were mutations at the successive dosages of radiation suggests a sigmoid curve, although data for other organisms usually give a straight-line relation.

All of these comparisons summarized in table 2, lines I to X, concern the results found using strain 533. In line XI are given the data for one test of strain 519 radiated at the same time for an interval of 16 minutes. The results, columns 5 and 7, show that this strain is considerably more resistant to radiation than is 533; whereas at 16 minutes of radiation 533 gave 72.6 per cent mutations of which 17.2 per cent were different, 519 gave only 22 per cent mutations, of which 9 per cent were different. It is clear that for comparable data showing the effects of radiation dosage in producing mutations the same strain must always be used (cf. Witkin, 1947).

THE SPECIFIC MUTATIONS ISOLATED

A summary of the actual auxotrophic mutations isolated in the different series of tests with the frequencies of each is given in table 3. It will be seen that over 30 different mutations have been found, which fall into at least four different classes. The largest class includes those that require one specific nutrilite, either an amino acid or a purine (adenine). Of these by far the most frequent is a re-

quirement for cysteine. Studies of a series of these cysteine-requiring strains has already shown that they include at least two different mutants and perhaps more. The requirements for certain ones can be satisfied by inorganic sulfides, though they cannot reduce sulfates; hence they are not amino acid requirers but need rather an SH radical only (cf. Lampen, Roepke, and Jones, 1947). Others need cysteine or cystine as such. The next most frequent mutants require histidine,

TABLE 3

Frequency of biochemical mutations in S. typhimurium

NO.	STRAIN 533 SINGLE AMINO ACIDS	NO.	STRAIN 533 ALTERNATIVE AMINO ACIDS	NO.	STRAIN 519 SINGLE AMINO ACIDS
103	Cysteine	20	Cysteine or methionine	8	Histidine
52	Histidine	2	Cysteine or proline	3	Cysteine
13	Leucine	1	Cysteine or leucine	3	Methionine
11	Proline	1	Cysteine or serine	3	Proline
5	Tyrosine	1	Cysteine or isoleucine	1	Leucine
2	Threonine			1	Tryptophan
2	Methionine	2	Methionine or glutamic		
1	Valine				
		1	Proline or threonine		
		1	Proline or arginine		
		1	Proline or hydroxyproline		Alternative amino acids
	Nucleic acid fraction	1	Histidine or leucine	1	Cysteine or methionine
		1	Histidine or threonine		
5	Adenine	2	Cysteine or methionine or phenylalanine	1	Phenylalanine or hydroxyproline
		1	Cysteine or methionine or glutamic		
		1	Histidine or threonine or isoleucine		
		1	Histidine or arginine or hydroxyproline		
	Multiple amino acids	1	Valine or arginine or lysine or aspartic		
1	Valine and isoleucine				
4	Unanalyzed				

cysteine or methionine, leucine, proline, tyrosine, threonine, methionine, valine, and tryptophan in that order.

Next there is a series of mutant strains whose requirements are less precise in that they will grow if any one of two or three alternative amino acids are present. The alternations of certain of these, as cysteine or methionine, are easily understandable in the light of the chemical structures involved, but others, as cysteine or proline, are harder to follow and will require considerable biochemical study. Finally several strains having multiple requirements have appeared. One of the clearest of these will grow only if both valine and isoleucine are available. It has not as yet been established whether there is any sparing action of the one on the other as in the similar *Neurospora* mutant (Bonner, 1946).

When the mutations are classified according to the various X-ray dosages certain added relations appear (table 4). Among those isolated from nonradiated control cultures, which probably represent accumulated mutations for a number of cell generations, the proline requirement is the most frequent. In all radiated samples through 12 minutes, mutations to a cysteine requirement are by far the most numerous. However, at 16 and 20 minutes of radiation, mutations to a histidine requirement become the most frequent. These differences are no less significant statistically than are those between the total numbers of mutations at the lower and upper levels of X-ray dosage. The chi square values calculated for the numbers of cysteine- and histidine-requiring mutants at 12 and 16 minutes of radiation indicate a probability of less than one in a thousand that two such

TABLE 4

Biochemical requirements in order of frequency

X-RADIATION DOSAGE	TOTAL NO. TESTS	1			2			3		
		NO.		%	NO.		%	NO.		%
Strain 533										
Untreated	135	4	Proline	2.9	1	Cysteine	0.7	1	Histidine	0.7
4 min	62	7	Cysteine	11.2	3	Cysteine or methionine	4.6	2	Histidine	3.2
6 min	86	9	Cysteine	10.4	4	Cysteine or methionine	4.6	4	Histidine	4.6
8 min	41	9	Cysteine	20.5	1	Histidine	2.4	1	Threonine	2.4
10 min	25	9	Cysteine	35.0	6	Histidine	24.0	1	Cysteine or methionine	4.0
12 min	94	45	Cysteine	47.0	10	Cysteine or methionine	10.6	1	Histidine	1.0
16 min	99	25	Histidine	26.0	13	Cysteine	13.8	10	Leucine	10.0
20 min	50	12	Histidine	24.0	8	Cysteine	16.0	3	Leucine	6.0
Strain 519										
16 min	100	8	Histidine	8.0	3	Cysteine	3.0			
					3	Methionine	3.0			
					3	Proline	3.0			

samples could be taken from the same population. Even the frequencies of the mutations other than cysteine and histidine show significant differences in these two series. Since all other conditions except the radiation dosage were constant, it appears that mutations to a cysteine requirement are more likely at lower dosages of radiation, whereas above 40,000 r others, especially histidine-requiring mutants, are more common.

Such a result is difficult to interpret in terms of differences in frequencies of mutation of different genes and could perhaps come about as a differential effect of wave length. It may be significant that in parallel experiments with ultraviolet a histidine-requiring mutant is by far the most frequent.

EFFICACY OF PENICILLIN SCREENING AND MUTATION FREQUENCY

As already noted the data given in table 2 and shown graphically in figure 3 indicate increased mutation frequency in the radiated lines as compared with

the controls and a larger total number of mutations at the higher X-ray dosages than at the lower. Comparing the percentage of mutants in the controls with those in the two combined groups of radiated lines (table 3, column 5, lines I, IX, and X), it appears that the control value has been multiplied close to 5 times at the lower intervals and 14 times in the combined total for the upper. The order is similar if we compare the percentages of the tests that gave different mutants (column 7), but the differences are somewhat less marked, namely, the lower dosage levels combined are about 3 times and the upper level about 7 times the control number. These figures do not admit of any more exact determination of mutation frequency. If our rough estimate given in an earlier section of 1 mutation in 75,000 tests in unradiated controls is used, this would become about 1 in 15,000 for the lower X-ray dosage and about 1 in 5,000 for the higher dosage level.

The form of the curve shown in figure 3 resembles a sigmoid curve rather than a straight line such as has usually been found for the relation between mutation

TABLE 5

Effect of growth in complete medium followed by penicillin screening on artificial mixtures of control and a cysteine mutant

MIXTURE	1 PROPORTIONS MIXED		2 PERCENTAGES BEFORE SCREENING		3 PERCENTAGES AFTER SCREENING	
	533	533-169	533	533-169	533	533-169
A	90	10	70	30	8	92
B	50	50	33	67	2	98
C	10	90	5	95	2	98

frequency and X-ray dosage. It appears to have been influenced by two factors in addition to the radiation dosage: first, differential growth of mutant lines during the 24-hour period on the complete medium; and, second, the differential elimination of the original prototrophic bacteria by the penicillin in the minimal medium. In an effort to test the effects of these two factors the following simple tests were set up.

Two approximately similar suspensions were made up as for X-radiation (figure 1), one of unradiated prototrophic 533 and one of a cysteine-requiring mutant, strain 533-169. They were of approximately the same optical density as measured in the "lumetron" turbidometer. These were mixed in the three proportions indicated in column 1, table 5, and then run through the penicillin screening process as already described. Plates were made and 100 colonies tested and identified as prototroph or mutant after 24 hours in complete medium (column 2) and again after 24 hours more in minimal medium containing 100 units of penicillin (column 3). The results are shown in table 5.

The counts shown in table 5 indicate that both factors mentioned above do in fact operate, at least with the mutant line tested. Under the conditions noted the cysteine-requiring mutant tends to outgrow the parent line in complete medium, though not very rapidly. In minimal medium with penicillin added the differential killing of the parent line is very rapid. It will be noted, however,

that more prototrophs survive in the mixture that started with the largest number. To that extent then the preliminary conclusions about mutation frequency appear justified, for the initial number of mutant bacteria after irradiation was much smaller than it was in any of the tests given.

STEPS IN SYNTHESIS REVEALED BY THE AUXOTROPHIC MUTANTS

Obviously each of the different classes of mutations summarized in table 3 requires separate biochemical study. As visualized by Beadle (1949) and his associates in the *Neurospora* studies, each of the auxotrophic mutants is most easily interpreted as having lost one enzyme or enzyme system that produces a substance essential in the process of synthesis. The chain of reactions may be broken at any one of a series of steps. If the break occurs late in a synthetic chain, the final product only may be required by the organism. If earlier, either the final product or one of the precursors may satisfy the requirement for growth.

Lampen, Roepke, and Jones (1947) have already shown that breaks at an early stage in sulfur utilization show themselves as cysteine or methionine requirements in *E. coli*. Preliminary tests indicate that certain of our *Salmonella* mutants showing alternative requirements for cysteine or methionine are in this same category, although they grow more slowly on methionine. The methionine-requiring strains appear to have a block much later in the synthetic chain of reactions. The many cysteine-requiring strains are at present more difficult to fit into a scheme that postulates the synthesis of methionine from cysteine. One such strain 533-169 used in table 5 will not utilize methionine, and it will give optimal growth when supplied with sulfides, but not sulfates, showing that the break in the chemical chain is concerned with the inorganic reduction. These and other tests are going on and will be reported separately.

Finally we have made preliminary tests for possible sexual fusion and genetic recombination between strains of *S. typhimurium* similar to those which have given positive results in the K12 strain of *E. coli* as reported by Lederberg. Although more than 20 such recombination tests have been made so far, with both single and multiple strains, the results are so far entirely negative. If such recombinations occur at all in *Salmonella*, they must be extremely rare.

SUMMARY

Suspensions of *S. typhimurium* have been exposed to X-radiation up to 57,000 roentgen units, then screened with penicillin on minimal medium containing essential salts and glucose according to the technique of Davis and Lederberg. Of the 457 strains isolated and tested, 234 proved to be auxotrophic mutant strains having a constant requirement for one or more food substances that the parent strain can synthesize for itself. The frequencies of these mutations are directly related to the X-ray dosage, suggesting that they are gene mutations similar to those found in higher organisms.

Most of the mutant strains required one of the amino acids or one of the purines, but alternative and multiple requirements were found. The data indicate that at radiation dosages up to 35,000 r units a cysteine-requiring mutant is the most numerous, whereas above that level the mutant most frequently isolated requires histidine.

A test of the efficacy of the penicillin screening of mixtures of wild type and X-ray-induced mutations is given.

The value of auxotrophic mutations for studies of the steps in bacterial synthetic processes is suggested.

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