FORWARD AND REVERSE MUTATION IN A HISTIDINE-REQUIRING STRAIN OF ESCHERICHIA COLI [1]

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THERE is an increasing volume of evidence that bacteria resemble higher organisms in their growth and reproduction (LURIA 1946; LEDERBERG 1948, 1949; BOIVIN 1950; GOTTSCHEWSKI 1950). Their small size and rapid multiplication make bacteria ideal material for investigations of spontaneous and induced mutations, and of the relationship between the gene and its intracellular and extracellular environment. In the present study, a comparison is made in *Escherichia coli* between the rate of mutation of a form having no special growth factor requirements to a form requiring histidine for growth, and the rate of reverse mutation resulting in the restoration of the prototrophic condition. These mutation rates have been studied during the normal growth of the culture (spontaneous mutation) and after treatment with ultraviolet radiation. The experiments were also designed to detect related phenomena such as phenotypic lag and nuclear segregation. Although this study was limited to a single character, in the absence of recombination in this strain (LEDERBERG 1947) it is not possible to determine the exact genetic nature of the mutations involved.

Because of the technical difficulties, there have been few comparisons of forward and reverse mutation rates. GOWEN (1941) found that under the influence of X-rays, the ordinary tobacco mosaic virus apparently mutated to aucuba with a higher frequency than aucuba reverted to wild-type. In Serratia, BUNTING (1946) determined a rate of 10^{-4} per bacterium per generation for spontaneous mutation from dark red to bright pink, and a spontaneous reversion rate of 2×10^{-3} per bacterium per generation. Comparisons of X-ray-induced forward and reverse mutation rates have been made for individual genes in Drosophila (PATTERSON and MULLER 1930; TIMOFEEFF-RESSOVSKY 1934) but no consistent trend for mutation to occur in either direction has been established.

A quantitative study of mutations induced in bacteria by different conditions has been made by NEWCOMBE (unpublished). Comparing the effects of spontaneous, ultraviolet- and X-ray-induced mutation, NEWCOMBE found that the ratio of mutants resistant to phage T1 to streptomycin-resistant mutants was 1:1500 for spontaneous mutation, 1:10 and 1:40 for mutation after ultraviolet and X-ray treatment. In studies of streptomycin resistance and

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dependence DEMEREC (1950) found that strains of *E. coli* with high and low rates of spontaneous mutation retained their relative mutability when treated with ultraviolet radiation. Additional evidence is required to determine whether the spontaneous mutability of a character is an indication of its ability to be mutated by ultraviolet radiation or other agents.

In their studies of phenotypic or "phenomic" lag, DEMEREC and LATARJET (1946), NEWCOMBE (1948) and NEWCOMBE and SCOTT (1949) have shown that in strain B/r the majority of both spontaneous and induced mutations to T1 resistance are not expressed until two or three additional cell divisions have taken place. After ultraviolet treatment there are apparently some "zero-point" resistant mutations which do not require cell division for expression, but these form only a small fraction of the total number of mutants. No phenotypic lag has been detected for the spontaneous mutation of streptomycin resistance in the same *E. coli* strain (NEWCOMBE and HAWIRKO 1949) while several hours' growth was necessary for the expression of all X-ray and ultraviolet-induced mutations (NEWCOMBE, unpublished). DAVIS (1950) has reported that cell division is required for the expression of several types of ultraviolet-induced auxotrophic and prototrophic mutants.

MATERIALS AND METHODS

The strains used in the following experiments were derived from a histidine-requiring (h^-) mutant (No. 148-334) of strain 15 secured after X-irradiation by DRS. J. O. LAMPEN and R. R. ROEPKE of the AMERICAN CYANAMIDE COMPANY. RYAN (1948) has shown that h^- mutates spontaneously to histidine-independence (h^+) and application of the variance analysis (LURIA and DELBRÜCK 1943) to data obtained in connection with the present investigation indicates that the mutation of h^+ to h^- also occurs spontaneously. In one of the serially transferred cultures of h^- maintained for many months by RYAN and SCHNEIDER, a type of h^- distinguishable from the parent type by its phage sensitivity pattern was found. From this h^-, h^+ strain "15-2" was obtained by spontaneous mutation; the h^- strain "A-11" was derived from a spontaneous h^- mutant selected from 15-2 by the penicillin technique. Strain 15-2 was used for all the studies of mutation from h^+ to h^- and A-11 in all the reversion experiments. Both strains have a division time of about 45 minutes in aerated synthetic medium at 37°C. Two h^- strains unable to ferment lactose (Lac^-) were also used: Lac^-A, recovered from 148-334 by DR. J. LEDERBERG after ultraviolet treatment, and Lac^-II, isolated from A-11 after ultraviolet treatment. Stocks were maintained at about 5°C on slants of 1 percent nutrient broth plus 2 percent agar and transferred about every four months.

For all the experiments to be described, bacteria were grown in a "minimal" medium composed of a mixture of reagent-grade salts described by GRAY and TATUM (1944). Trace elements and asparagine were omitted. Cultures grown in this medium with the addition of 0.05 percent glucose are limited in size by the carbon source rather than by pH and rapidly resume

growth when transferred to fresh medium. In experiments where a dense suspension of bacteria in the logarithmic phase of growth was desired, the phosphate concentration was increased four times, and the glucose concentration ten times. The plating medium contained Gray and Tatum's salts with the addition of 0.5 percent glucose and 1.5 percent washed agar (RYAN and SCHNEIDER 1949). 25γ per cc of histidine monohydrochloride · H_2O was added to the medium in which h^- cells were grown, unless otherwise noted. Cultures were washed in 12-cc centrifuge tubes by resuspending three times with sterile 0.9 percent saline. Sterile distilled water was used for all dilutions. Liquid cultures for inocula were grown in tubes containing 10 cc of medium which were aerated by continuous rolling at 37°C. Individual cultures for experiments on spontaneous mutation rates were inoculated with about 50 newly grown cells. Large cultures for irradiation experiments were aerated at 37°C on a Warburg shaker. For reproducibility, cultures in the logarithmic phase of growth were used in all the experiments to be described.

CALCULATION OF MUTATION RATE

Forward and reverse mutation rates were calculated by three different methods. The derivations of the equations and methods for the calculation of the variance of each method are contained in the papers of LURIA and DELBRÜCK (1943), NEWCOMBE (1948) and LEA and COULSON (1949). All the methods involve the determination of the number of mutants in each of several cultures of similar size. By Method I, the average number of *mutations,* m, per culture is calculated from P_0, the proportion of cultures with no mutants:

$$P_0 = e^{-m} \qquad \text{I}$$

By Method II, m is obtained from r, the average number of mutant bacteria per culture and C, the number of similar cultures using the estimate

$$r = m \cdot \ln(Cm) \qquad \text{II}$$

to plot a family of curves relating r to m for different values of C. The average number of mutations may be estimated with greater statistical efficiency from r_0, the number of mutants in the median culture of the series using the equation

$$\frac{r_0}{m} - \ln m = 1.24 \qquad \text{III}$$

To obtain the mutation rate, the number of mutations per culture is divided by n/ln 2, n being the total number of cells per culture (NEWCOMBE 1948). Because of the high variance expected when m is estimated by Method II, conclusions as to mutation rates have been based on Methods I and III. However, Methods II and III give very similar mutation rates when applied to the date below or to the phage-resistance data published by NEWCOMBE (1948, table 1). These experiments were apparently not extensive enough to include cultures containing large clusters of mutants arising from exceptionally early mutations; such clusters lead to the calculation of excessively high mutation rates by Method II.

There are assumptions inherent in all three methods, and in each series of experiments controls were set up to test them. It is necessary to verify the expectation that mutations occur at random with a fixed probability. Since a very large series of similar cultures were not run, it is not practicable to compare the observed frequency of cultures with 1, 2, etc., mutants with the expected frequency. If, however, the mutation rate as calculated by Method I is constant regardless of culture size, one can assume that the first mutation in each culture, although not necessarily all subsequent mutations, occurs with a fixed probability. Methods II and III will give erroneous mutation rates unless new mutants divide at a rate equal to the division rate of the parental cells. If g equals the number of generations of growth after the appearance of the first mutation, the number of mutants at any time should

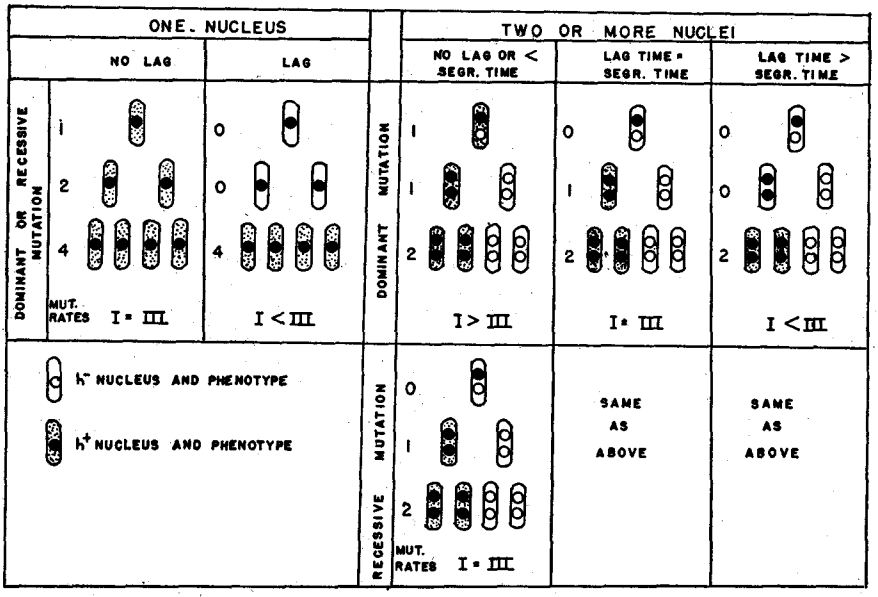

FIGURE 1.—Mode of increase of phenotypically mutant cells in mutant clones.

be $(g+1)2^g$ while the number of parental cells has increased by the factor 2^g. The effect of phenotypic lag on the mode of increase in detectable mutants within a single clone is illustrated in figure 1. If the bacteria are multinucleate, the calculated mutation rates will usually be lower than the true figure. Delay in phenotypic expression in either mono- or multi-nucleate cells will be reflected in differences between mutation rates calculated by Method I and rates obtained by Methods II and III and the duration of the period of phenotypic lag can be estimated from the extent of the discrepancy.

SPONTANEOUS REVERSION OF h^- TO h^+

Small cultures are required for the determination of mutation rates by Method I since some of the cultures must contain no mutants. Therefore, h^- cells were inoculated into small tubes containing medium in which the

amount of histidine limited the final size of the population to the desired level. The h^+ mutants which arose in some of the cultures continued to multiply, eventually producing turbidity in the culture tubes. Although one h^+ cell will multiply to give visible turbidity in 24 hours or less under the experimental conditions (RYAN and SCHNEIDER, personal communication), the number of turbid tubes continues to increase over a period of several days without an increase in the number of viable parental cells. Only the cultures becoming turbid within 24 hours after the end of h^- growth were counted. Table 1 lists experiments performed by the tube turbidity method, and includes data obtained by RYAN and SCHNEIDER (in press). The mutation rates as determined by Method I on cultures varying in size from 1.0×10^6 to 9.5×10^7 do not appear to be correlated with culture size ($t = .816$, D.F. = 11, $p = .4$). Thus, population size does not affect the probability of appearance of the first mutant in each culture.

TABLE 1

Estimation of mutation rate from h^- to h^+ using Method I.

	N Number of cultures	n h^- cells per culture $\times 10^7$	P_0	a Mutation rate $\times 10^{-8}$	Standard deviation* $\times 10^{-8}$
1	55	.10	.962	1.29	1.40
2	32	.17	.906	4.14	2.29
3	69	.22	.855	4.99	1.59
4	198	.41	.828	3.18	.59
5	69	.56	.793	2.86	.77
6	14	1.24	.571	3.12	1.28
7	60	1.30	.583	2.87	.58
8	57	2.40	.579	1.57	.33
9	26	3.04	.269	2.98	1.06
10	59	3.20	.390	2.03	.35
11	59	6.30	.068	2.94	.53
12	48	6.32	.208	1.72	.30
13	64	9.48	.016	3.01	.73
			Weighted average	2.86×10^{-8}	
			Standard deviation	1.77×10^{-8}	

* Standard deviation after LEA and COULSON (1949). These estimates of S.D. do not take into account the variance due to diluting and plating errors and are thus lower than the S.D. of the weighted average.

For the determination of the number of h^+ mutants in larger populations, cultures were grown in medium containing an excess of histidine. When the population had reached the desired size, the cells were washed and the entire cultures plated on minimal agar. Table 2 lists mutation rates obtained by Methods II and III. There is apparently a correlation, in this case, between increase in culture size and decrease in mutation rate ($p = .03$). The average mutation rate is not significantly lower than that obtained by Method I, but it varies with the size of the culture being tested.

A selection pressure against h^+ would progressively lower the ratio of h^+ to h^- from that expected, and Method III would then give decreasing muta-

TABLE 2

Estimation of mutation rate from h^- to h^+ using Methods II and III.

	N	n	r	r_0	Mutation rate		Standard deviation*
	Number of cultures	h^- cells per culture $\times 10^8$			Method II $\times 10^{-8}$	Method III $\times 10^{-8}$	III $\times 10^{-8}$
1	12	.37	1.6	1	1.7	1.68	.81
2	24	.63	6.9	5	2.2	2.63	.85
3	12	.67	3.8	3	1.3	1.75	.46
4	7	.68	15.3	9	4.1	3.60	1.37
5	12	1.26	9.8	9	1.6	1.88	.56
6	9	1.49	15.1	9	1.8	1.64	.56
7	11	1.63	21.6	12	2.2	1.86	.53
8	6	2.28	14.6	12	1.4	1.41	.52
9	5	2.39	11.8	11	1.1	1.16	.61
10	7	11.79	99.0	95	1.2	1.25	.35
11	3	12.39	69.0	73	.9	1.01	.39
12	9	12.71	109.4	110	1.1	1.32	.30
				Weighted average		1.88×10^{-8}	
				Standard deviation		$.72 \times 10^{-8}$	

* Standard deviation after LEA and COULSON (1949).

tion rates with increasing culture size. To test the possibility of selection for or against h^+ cells in h^- cultures, one or more h^+ cells were introduced into some cultures at the time of inoculation. Ten different h^+ strains derived from spontaneously arising mutants and grown under the same conditions as were the h^- inocula were used in the various tests. The numbers of h^- and h^+ cells were determined after 10–15 generations and a calculation was made of the percent recovered of the number of h^+ expected if h^- and h^+ grow at the same rates. As shown by table 3, there is, on the average, no selection for or against h^+ cells.

The negative correlation between culture size and mutation rate could be

TABLE 3

Growth of h^+ in the presence of h^-.

	Total number of cultures	Cultures with h^+ added	t_1 Cells per culture		t_2 Cells per culture		Percent recovery of h^+
			h^- $\times 10^4$	h^+ $\times 10^0$	h^- $\times 10^8$	h^+ $\times 10^4$	
1	12	4	.22	.41	.68	1.37	110
2	12	6	.58	.69	2.29	3.38	124
3	24	12	.63	.69	.09	.08	81
4	12	3	1.77	.29	1.49	.31	126
5	11	10	2.27	2.40	1.25	1.74	132
6	23	18	5.10	1.57	2.39	1.02	138
7	12	9	6.54	1.39	12.39	2.39	91
8	11	10	12.00	2.40	4.26	.78	92
9	12	5	16.96	.54	11.79	.31	82
10	12	3	26.40	.29	12.71	.08	56
						Average	103

attributed to segregation if one assumes that h^+ is a dominant mutation occurring in one nucleus of a multinucleate cell. As in the case of selection against the mutant, the mutant frequency would not increase with culture size in the manner expected. Giemsa staining of h^+ and h^- cells in the logarithmic phase of growth reveals two to four chromatinic bodies per cell; ROBINOW (1945) and others have suggested that these bodies are analogous to cell nuclei, and thus the segregation hypothesis appears to have some morphological basis. Although h^+ is assumed to be dominant, the new mutant might require one or more cell divisions for the conversion of the h^- phenotype to h^+. As NEWCOMBE and HAWIRKO (1949) have pointed out, and as shown in figure 1, when phenotypic lag and segregation require the same period of time, no difference is expected in mutation rates calculated by Methods I and II. The data are in accord with the assumption that segregation extends over a period of about one generation. They would be equally consistent, however, with the hypothesis that segregation and the development of the h^+ phenotype occur simultaneously for a period of one or more generations but that segregation continues for at least one additional generation.

SPONTANEOUS MUTATION FROM h^+ TO h^-

The original penicillin selection method of DAVIS (1948) and LEDERBERG and ZINDER (1948) has been modified to increase the recovery of h^- mutants and to obtain an estimate of the percent of total mutants that are recovered. The optimal recovery of h^- consistent with the killing of the great majority of h^+ cells occurs with a penicillin concentration of 100 to 150 units per cc and an exposure of 8 to 10 hours at 37°C. No substance enhancing the selective killing action of penicillin under these experimental conditions has yet been found. The optimal concentration of h^+ cells is 5×10^6 per cc; when higher concentrations are used, the recovery of h^- is significantly decreased. For the most rapid killing, cultures to be tested should be in the logarithmic phase of growth. The penicillin method was made quantitative by adding a known number of h^-Lac^- cells to some of the cultures in each series and determining the percent recovery of the Lac^- cells after penicillin treatment. Assuming that a similar proportion of marked h^- cells and spontaneous h^- mutants is lost, it is possible to calculate the original number of mutants in each culture.

For example, 3 cc cultures of h^+Lac^+ cells were grown to the desired population size in medium containing histidine, washed, and resuspended in minimal medium. After assay of the h^+, penicillin was added to each culture to give a final concentration of 140 units per cc. At the same time, a certain number of h^-Lac^- cells were added to some or all of the cultures. After 10 hours at 37°C, the cultures were plated directly in minimal agar, each culture being divided between two plates. The plates were layered with minimal medium according to the technique of LEDERBERG and TATUM (1946). After 36 hours, the visible colonies, derived from h^+ cells not killed by penicillin,

TABLE 4

Comparison of recovery of h^- Lac^- and h^- Lac^+.

	h^+ per cc $\times 10^6$	No. of cultures	% recovery of h^-		S.E. of diff.	t	P
			Lac^+	Lac^-			
1	.2	12	29	32	15.3	.20	>.5
2	.6	10	42	49	6.5	1.08	.3
3	3.0	4	61	54	31.0	.22	>.5
4	3.6	12	26	50	6.9	3.48	.01

were marked, and a layer of minimal agar plus histidine was added. The additional colonies, presumably h^-, that appeared were streaked to Endo agar to determine the number of h^-Lac^- cells recovered. To avoid testing large numbers of h^-, the number of h^-Lac^- added per culture was approximately equal to the number of newly arisen h^- expected. The Lac^+ colonies were streaked from Endo to minimal agar to make certain that they were h^-; the percent that proved to be h^- varied from 50 to 100 percent, recovery being most efficient when the h^+ had been reduced to 50 or less per culture. The determined number of new h^- per culture was corrected by dividing by the percent survival of the h^-Lac^-.

To test the assumption that h^-Lac^- and h^-Lac^+ cells are killed at the same rate under similar conditions, experiments were set up in which equal numbers of (a) a mixture of spontaneously arising h^-Lac^+ or (b) h^-Lac^- cells were added to aliquots of minimal medium containing washed h^+ cells. The numbers of h^+ and both types of h^- cells were so adjusted that the contribution to the h^-Lac^+ class due to spontaneous mutation was negligible. The data from several experiments, summarized in table 4, show that in only one case was there a significant difference (at the 5 percent level) between the recovery of h^-Lac^- and h^-Lac^+ cells.

A number of h^- cells, derived from spontaneously arising mutants, were added to some of the h^+ cultures at the time of inoculation to determine whether there is selection against h^- mutants in predominantly h^+ cultures.

TABLE 5

Growth of h^- in the presence of h^+.

	N No. of cultures	Cultures with h^- added	% recov. from penicil.	t_1 Cells per culture		t_2 Cells per culture		% recov. h^-
				h^+ $\times 10^3$	h^- $\times 10^{-1}$	h^+ $\times 10^6$	h^- $\times 10^2$	
1	34	5	61	2.24	1.59	.36	.05	20
2	34	9	75	3.77	3.08	.74	.34	56
3	17	2	18	4.52	1.26	2.92	.75	107
4	17	1	18	4.52	.61	9.03	.85	70
5	17	8	39	6.20	6.37	5.94	5.78	95
							Average	70

The h^- recovery, calculated as in the experiments on h^+ recovery, averaged 70 percent of that expected (table 5), indicating that selection would slightly lower the mutation rate as estimated by Methods II and III.

Table 6 lists the data obtained in experiments in which the penicillin method was used to estimate the frequency of h^- cells in h^+ cultures. The

TABLE 6

Estimation of mutation rate from h^+ to h^- using Methods II and III.

	Percent recovery of h^-	N No. of cultures	n h^+ per culture $\times 10^6$	r	r_0	Mutation rate Method II $\times 10^{-6}$	Mutation rate Method III $\times 10^{-6}$	Standard deviation III $\times 10^{-6}$
1	80	28	.36	1.6		1.27		
2	67	27	.38	1.4		1.09		
3	31	18	.62	4.5		1.55		
4	83	25	.74	2.3	1.2	.78	.90	.30
5	34	29	1.04	3.4		.73		
6	37	15	1.46	14.2	5.4	1.61	1.18	.34
7	38	11	1.85	23.7	7.9	2.16	1.23	.38
8	18	14	2.92	21.1	11.1	1.13	.98	.25
9	64	8	3.26	48.4	32.8	2.22	1.95	.54
10	42	9	5.94	41.9	23.8	1.07	.84	.23
11	39	11	10.98	133.3	120.5	1.54	1.63	.32
				Weighted average			1.17×10^{-6}	
				Standard deviation			$.36 \times 10^{-6}$	

experimentally observed P_0 cannot be used to determine the mutation rate by Method I because the "0" class includes some cultures which contained one or more h^- cells prior to treatment with penicillin. The probability that a culture containing r mutants before treatment will have no mutants after treatment is a function of r and S, the fraction of h^- surviving penicillin. Thus, for each culture

$$P_{0,c} = (1 - S)^r \qquad \text{IV}$$

This value, $P_{0,c}$, for each individual culture could then be applied as a correction factor if the frequency, P_r, of cultures having r mutants were known. Thus, the P_0 expected after penicillin treatment of a group of cultures is given by

$$P_0 = \sum_{r=0}^{r=n} (1 - S)^r (P_r)$$

LEA and COULSON (1949) have calculated P_r for various values of r from m, the average number of mutations per culture. An estimate of m can be obtained by Method II or, preferably, by Method III.

The values for the expected and observed P_0 agree closely, as shown in table 7. If an appreciable amount of phenotypic lag were present, the ob-

served P_0 would be higher than the expected P_0 which is based on the average or median number of mutants per culture. A statistical test has shown no correlation between culture size and the mutation rate obtained by Method III, indicating that mutant frequency increases with culture size in the manner expected in the absence of phenotypic lag. Adhering to the assumptions put forth to account for the discrepancies in mutation rates from h^- to h^+, it is necessary to postulate that if h^+ bacteria are multinucleate, a mutation to h^- cannot be detected in a cell simultaneously containing one or more h^+ nuclei. The absence of any detectable lag thus indicates that when h^- has segregated from h^+, the h^- phenotype appears rapidly.

ULTRAVIOLET-INDUCED MUTATION OF h^- TO h^+

Cultures to be irradiated were grown to 10^8–10^9 cells per cc and then washed twice with saline at room temperature over a period of an hour or more to stop bacterial multiplication. Ten-cc aliquots of the saline suspension were pipetted into Petri dishes which were rotated on a small turntable during irradiation. The ultraviolet source was a G.E. " Sterilamp " about 80 percent of whose ultraviolet radiation consists of the 2537 Å wave length. Non-reactivating yellow light from G.E. " Bugaway " bulbs was used throughout all experiments with irradiated cells. Since the bacterial concentration was not kept constant from experiment to experiment, dosage was measured in percent survival. After irradiation, the bacteria were transferred to centrifuge tubes and resuspended in sterile saline. A part of each treated culture was plated on minimal agar to determine the number of h^+ mutants present at zero time, and another part was diluted with medium containing histidine and allowed to divide one or more times. After growth, each culture was washed and assayed for h^- and h^+.

The technique of plating in medium with limited histidine enrichment in order to reveal induced mutants (DAVIS 1950) was unsuccessful; fewer h^+ colonies appeared on plates containing irradiated h^- cells than on control plates containing equal numbers of viable h^-. When h^+ cells were added to irradiated cultures before plating, there was 100 percent recovery of the

TABLE 7

Comparison of expected and observed P_0.

	Percent recovery of h^-	n h^+ per culture $\times 10^6$	m Mutations per cult.		P_0	
			From r	From r_0	Exp.	Obs.
1	80	.36	.66	....	.520	.500
2	67	.38	.60	....	.551	.704
3	31	.62	1.40	....	.490	.556
4	83	.74	.84	.96	.417	.440
5	34	1.04	1.10	....	.550	.553
6	37	1.46	3.40	2.50	.246	.200
7	38	1.85	5.80	3.25	.157	.273
8	18	2.92	4.80	4.15	.212	.285

added cells, showing that h^+ cells are not prevented from forming colonies. A possible reason for the failure of the limited enrichment method is the removal of the histidine present by the large numbers of "killed" irradiated cells which continue to metabolize but do not divide or give rise to viable h^+ cells. Although more tedious, the technique of growing irradiated cultures in liquid medium with optimal histidine also allows the cells to be tested at any interval after the initiation of growth.

Repeated tests have shown that h^- and h^+ cells are equally sensitive to irradiation when grown and irradiated under identical conditions. In order to determine whether there was selection for either type when h^- and h^+ were grown after irradiation, aliquots of a mixed culture were irradiated for various lengths of time. The irradiated cultures were grown in histidine-supplemented medium for 18 generations and then assayed for h^- and h^+. When approximately equal numbers of h^- and h^+ are irradiated there is no evidence of selection for either type. To test for selection under conditions more closely resembling those in irradiated h^- cultures, a small number of h^+Lac^- cells were added to irradiated cultures which were then grown for several generations. In six experiments in which h^+Lac^- cells were mixed with irradiated h^- in ratios of about 1 to 10^6 and grown for 15 generations, there was 89 percent recovery of the marked cells. Expected recovery was determined from cultures containing h^+Lac^- and unirradiated h^- cells. Selection is apparently not great enough to alter the major conclusions concerning induced reverse mutations.

When cultures are carefully washed before irradiation to halt cell division, "zero-point" h^+ mutations are not detected (fig. 2). Exceptions were found in the two earliest experiments of the series, but it has been impossible to repeat these observations in many trials. In all other experiments, there was a downward trend in h^+ frequency with increasing ultraviolet dose. Mutant frequency after growth (end-point mutation) was determined after one division and after five or more divisions of the irradiated cultures. To indicate the amount of variation in mutant frequency in different cultures before irradiation, the total h^+ frequency is plotted and thus includes both spontaneous and induced mutants. Apparently most induced mutants are expressed after one division but two or more divisions may be necessary for the expression of all h^+ mutants. In evaluating the data to determine the approximate duration of the lag in mutant expression, it is not necessary to consider the effects of irradiation-induced delay in division which is present in strain B/r (DEMEREC and LATARJET 1946; NEWCOMBE and SCOTT 1949). In both h^- and h^+ cultures, the surviving cells exhibit no greater delay than do unirradiated cells. This conclusion is based on determinations of cell increase in liquid cultures and on the surface of agar plates.

Because of the scatter between data from different experiments, it is difficult to determine whether the h^+ frequency increases linearly with dose. The log percent survival is inversely proportional to ultraviolet dose except for doses allowing over 50 percent survival. If mutant increase were to be plotted

against dose, the curve would show a slight flattening near the origin. Doses allowing less than 0.5 percent survival of h^- do not further increase the h^+ frequency, and the limited number of data for higher doses suggests that there may be a decrease in mutant frequency as has been observed for phage resistant mutants (DEMEREC and LATARJET 1946). NEWCOMBE and WHITEHEAD (1951) have found in *E. coli* that the frequency of color response mutants on mannitol-tetrazolium agar remains constant at doses over 500 ergs per sq. mm. A decline in mutant frequency with high doses of ultraviolet radiation occurs for the production of morphological and auxotrophic mutants in molds (HOLLAENDER and EMMONS 1941, 1946) and in Drosophila

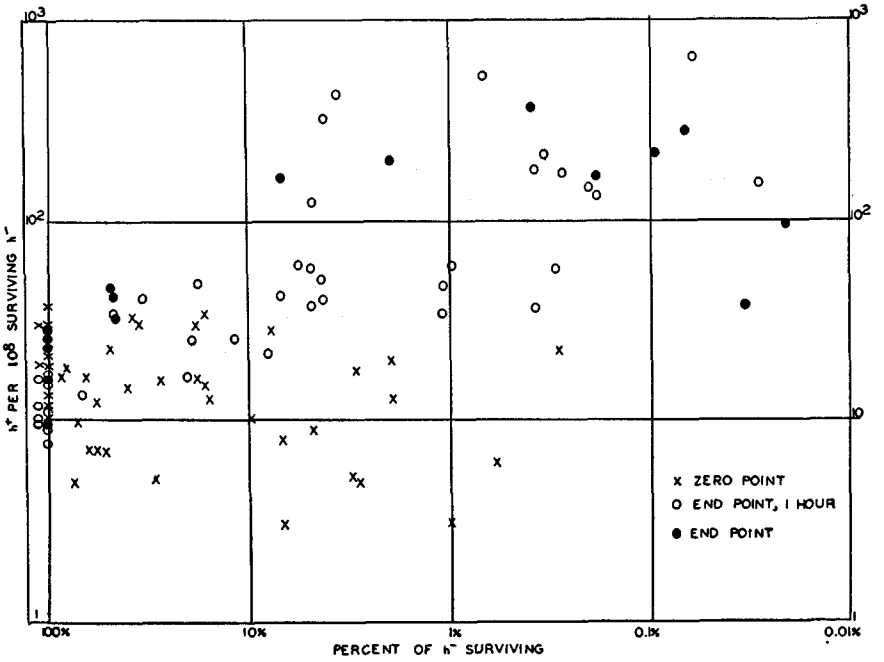

FIGURE 2.—Increase in the frequency of h^+ after ultraviolet irradiation.

(ALTENBURG, ALTENBURG, MEYER and MULLER 1950). This change in the relation of lethality to mutagenicity at high doses appears to be characteristic of ultraviolet treatment.

POST-IRRADIATION DEATH

In the course of the irradiation experiments, it was found that when irradiated non-growing cultures are plated immediately, the percent survival is many times greater than that observed if the cultures are assayed after a short period (30–50 minutes) of growth in liquid medium. The bacteria surviving this post-irradiation death divide at a normal rate unless the culture consists of h^- cells in a medium devoid of histidine, in which case the viable

count becomes stable. A careful study of the data suggests that the ultraviolet survivors immediately after irradiation consist of two types: "liquid resistants," which will divide at a normal rate in liquid medium, and "liquid sensitives," which die when placed in liquid medium but survive when plated. The ratio of percent survival immediately after ultraviolet to survival after growth in liquid is not constant, but increases with increasing dose. These observations are introduced here because post-irradiation death occurs between the measurement of "zero-point" and end-point mutants. Although it has been shown that h^- and h^+ cells do not differ in sensitivity to ultraviolet treatments that include post-irradiation growth in liquid, the possibility that there is a relationship between post-irradiation death and the production of mutations has not been ruled out.

ULTRAVIOLET-INDUCED MUTATION FROM h^+ TO h^-

The study of ultraviolet-induced mutation to h^- is limited to a low range of doses because the penicillin-selection method cannot be used effectively when the concentration of living plus ultraviolet-killed cells exceeds 5×10^6 per cc. The h^+ cells were washed and irradiated as were the h^- cells in the reversion experiments. Part of each irradiated suspension was grown in minimal medium containing histidine, part was tested immediately. After resuspension of the irradiated cells in minimal medium, penicillin and h^-Lac^- cells were added and the number of new h^- mutants determined as previously described in the section on spontaneous mutation. In addition to penicillin-killing, post-irradiation death, which affects both h^- and h^+ cells, occurs when bacteria are placed in liquid medium and penicillin after irradiation. Thus, it is impossible to determine the number of radiation survivors in these cultures either by assaying before the addition of penicillin or by assaying after the completion of post-irradiation death. A good approximation may be obtained by placing part of the irradiated suspension in liquid medium and assaying after one hour or by using a curve, obtained by pooling the data from several experiments, relating survival immediately after ultraviolet treatment to survival after growth in liquid medium. Penicillin has no effect on the post-irradiation death of h^- cells, and thus it may be expected that the death of h^+ is similarly unaffected.

Figure 3 shows that ultraviolet irradiation increases the frequency of mutation from h^+ to h^-. There is no detectable increase in mutant frequency when irradiated h^+ cultures are grown for one or more generations, so all mutations appear to be "zero-point" mutations. When evaluating these results, it should be borne in mind that penicillin does not kill all growing cells instantly. After one hour in penicillin, 90 percent of h^+ cells are incapable of dividing to form colonies even though they are freed of the drug. However, it is theoretically possible that an h^+ cell which has mutated to h^- might become phenotypically h^- during the early minutes of penicillin action, and later recover from the effects of penicillin. There is also the possibility that h^+ cells which have been washed thoroughly are essentially h^- in phenotype,

and remain so unless the h^+ gene(s) resumes its functions when the cells are replaced in culture medium. The term " zero-point " may be misleading and is, in any case, inaccurate, since a measurable length of time must elapse between mutagenic treatment and selection of the mutants.

The increase in h^- frequency relative to dose is very similar to the end-point h^+ increase in irradiated h^- cultures. Again, there appears to be a plateau or peak above which the mutant frequency does not rise although the

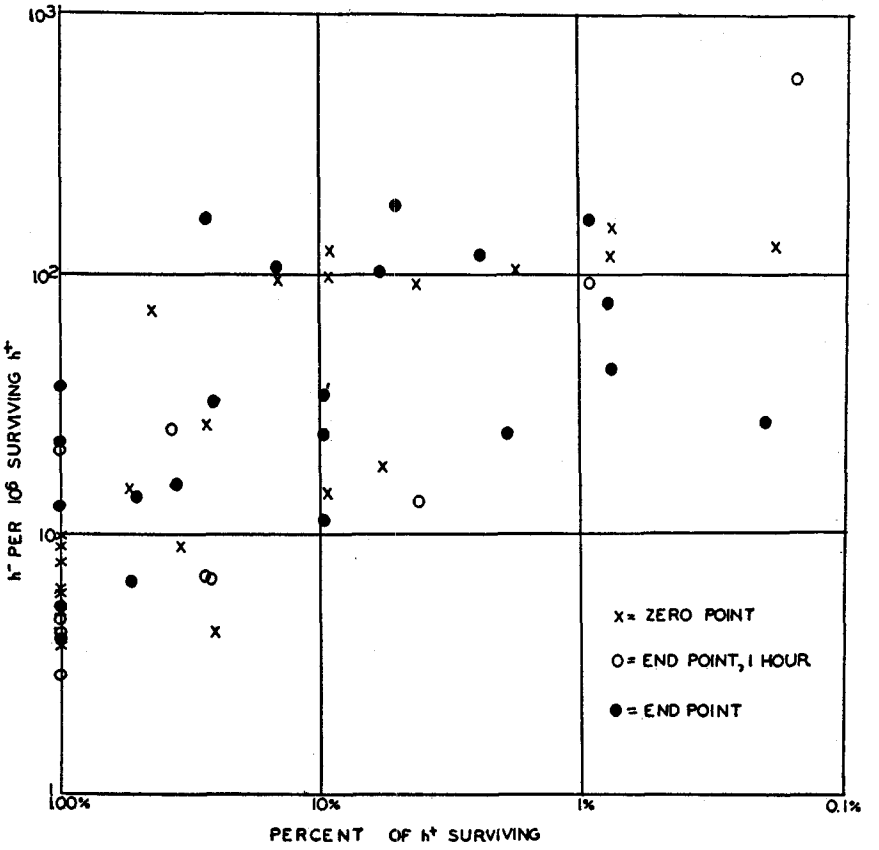

FIGURE 3.—Increase in the frequency of h^- after ultraviolet irradiation.

dose is increased. A considerable amount of variability is observed in the mutant frequencies at comparable doses; this was also observed by DEMEREC and LATARJET (1946).

COMPARISON OF INDEPENDENTLY ISOLATED MUTANTS

The growth rates of 22 spontaneous and induced h^+ mutants, each independently isolated, were compared turbidimetrically. In minimal medium, the growth curves of all strains appeared identical and were indistinguishable from the growth curve of the parent h^+ strain. The tests in which h^+ were

grown with h^- cells (table 3) provide further evidence of the similarity of the h^+ mutants. Nineteen h^+ strains were tested for the frequency of h^- mutants, which varied from 3.3 per 10^6 to 7.5 per 10^5 h^+. The similarity of mutant frequencies strongly suggests that the rate of mutation from h^+ to h^- is almost identical in all the tested strains. DAVIS (1950) found growth curves differing from wild-type for the majority of back mutants from several different auxotrophs. Many of these prototrophs were "partial back mutants," requiring a growth factor for optimal growth. If h^- strain A-11 gave rise to such mutants, these slow-growing prototrophs should have been detected, for the h^+ mutants to be tested were often isolated from minimal agar several days after plating. The similarity of the h^+ strains suggests that h^- strain A-11 reverts to h^+ by a single mutation.

The growth rates of 28 h^- strains, both spontaneous and induced, were studied in medium containing histidine. Although the curves varied somewhat, the final level of growth was the same for all mutants. A slight difference in growth rates among various strains is reflected in the results of selection tests (table 5). The frequency of h^+ mutants varied from 0 per 10^9 to 1 per 10^5, indicating that the rates of mutation to h^+ and/or selection of h^+ mutants differ significantly in the various h^- strains. At least three different h^- types appear to be present on the basis of reversion frequency; apparently strain A-11 carries only one of several possible histidine-requiring mutations, which may represent allelic mutations or mutations of different genes.

DISCUSSION

A comparison of spontaneous mutation rates with ultraviolet-induced mutation frequencies indicates that the h^+ and h^- characters retain their relative mutability when subjected to ultraviolet radiation. However, while the rate of spontaneous mutation to phage T1 resistance in strain B/r is similar to that of h^- and h^+ in strain 15, the frequency of phage-resistant mutants was increased to a far greater extent by ultraviolet treatment. One cannot be certain whether this inconsistency is a reflection of a difference in the particular genetic system involved or of differences in the entire genetic constitutions of the two strains. The majority of the data now available suggest that, in *E. coli,* the relative spontaneous mutability of a character is an indication of its relative mutability when treated with ultraviolet radiation.

The relation between dose and the production of h^- and h^+ mutations differs considerably from that found for T1 resistance (DEMEREC and LATARJET 1946). If the h^+ data are corrected for mutations present before irradiation, the curve of mutant increase shows a steady rise to a maximum, in contrast to the sharp initial rise in the end-point mutant curve for T1 resistance. This initial rise has not been observed in any other studies of induced mutation and may be unique for phage resistant mutants or associated with the conditions of the experiment (LEDERBERG 1949).

The assumption has been made that the bacteria contained in this study

contained about two nuclei, and that one h^+ nucleus is dominant to one or more h^- nuclei. This conclusion is based primarily on the discrepancies in mutation rate calculations for the spontaneous mutation of h^- to h^+. If h^+ is dominant, segregation cannot be detected in a study of spontaneous mutation from h^+ to h^-, but should be observed as a delay in the appearance of induced h^- mutants. Some of the possible causes for the failure to detect any lag have been discussed above, and one should also consider the possibility that ultraviolet radiation produces a considerable amount of nuclear killing, so that many of the cells surviving radiation may be effectively mononucleate. Since the duration of the expected segregation lag is only about one division, the failure to detect it with present methods cannot be considered conclusive evidence of its absence.

Phenotypic lag, unless obscured by nuclear segregation, is apparently not present in the spontaneous mutation of h^- to h^+ or h^+ to h^-. However, a "priming" (DAVIS 1950) period of about one division is required for the expression of induced h^+ mutations. Since h^+ is apparently dominant, lag cannot be attributed to the necessity for nuclear segregation. The lag in h^+ expression is not one of time alone, since induced h^+ mutations never appear if the cells are plated in minimal medium without previous growth in histidine. This suggests that for h^+ expression, there must be the production of a new material, involving cellular growth, rather than the decay of some product(s) of the h^- character. In the case of spontaneous mutation, phenotypic lag may be of shorter duration, or may be obscured by segregation.

SUMMARY

1. A method has been developed for the quantitative recovery of growth-factor-requiring mutants from their parental cultures.

2. The rate of spontaneous mutation from h^+ to h^- in the prototrophic strain studied is 1.2×10^{-6} per bacterium per division cycle, and the reversion rate of a derived h^- strain is 2.9×10^{-8} per bacterium per division cycle.

3. Equivalent doses of ultraviolet radiation increased both mutation rates by approximately the same factor. The mutant frequency increased with dose, reaching a maximum of about $100 \times$ the original frequency at 0.5 to 1.0 percent survival.

4. No phenotypic lag was detected for the expression of spontaneous h^- and h^+ mutations. After irradiation, h^+ mutations were not expressed unless the culture was placed for about one hour in a culture medium containing histidine, but h^- mutations did not require this treatment for expression.

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