SPONTANEOUS MUTATION IN NON-DIVIDING BACTERIA

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IT has usually been supposed that spontaneous mutations in bacteria take place only among dividing organisms (LURIA and DELBRÜCK 1943). Although NOVICK and SZILARD (1949) showed that in the chemostat the rate of mutation per bacterium per hour could be independent of the rate of growth, the mutations were studied only while the bacteria were dividing. As a consequence of these and other facts it has been proposed that mutations result from rare errors in gene duplication (cf. NEWCOMBE 1953).

When more sensitive methods, to be described in this paper, are brought to bear on the problem, it appears that mutations occurring at a rate of ca. 10^{-9} per bacterium per hour can be detected during the stationary phase. Since this stationary phase does not seem to be dynamic and since the mutations seem to involve the initiation of genotypic changes, it is concluded that mutation can take place in non-dividing bacteria.

MATERIALS AND METHODS

The mutation studied in these experiments was that from a histidineless (h^-) to a non-histidine requiring (h^+) condition in *Escherichia coli* (strain 15). The basic techniques are either described or referred to by RYAN and WAINWRIGHT (1954). Modifications in these procedures will be referred to in relevant places in the text.

RESULTS

The fundamental finding in this investigation was the continued appearance of h^+ mutants in h^- cultures that had ceased multiplying. It has long been appreciated that after a culture of auxotrophs has been plated in agar devoid of the required growth factor, new mutations to prototrophy can take place if the plate allows enough residual growth (RYAN and SCHNEIDER 1949). In this event the final number of prototrophs is not directly proportional to the size of the population introduced into the plate. When the agar and medium do not contain traces of the required growth-factor and appreciable residual growth does not occur, for several days the number of prototrophic colonies is approximately proportional to the total number of bacteria put in the plate (table 1). But with time a few new prototrophic colonies appear which tend to disturb this proportionality by being somewhat too frequent in the lower concentrations. Nonetheless, the number of new prototrophic colonies is usually greater the higher the concentration of parental bacteria.

Under the conditions of the experiments reported in table 1, washed h^- bacteria undergo a negligible residual growth. For example, in six experiments where ca. 5×10^4 washed h^- bacteria were plated on agar medium devoid of histidine (H^-), washed off at different times and replated (RYAN, FRIED and SCHWARTZ 1954), they

TABLE 1

The number of h^+ *mutants found at different times in agar devoid of histidine and inoculated with a series of dilutions of* h^- *cultures*

Dilution	Number of h^+ mutant colonies									
	Experiment (50 hours)					Experiment (180 hours)				
	A	B	C	D	E	A	B	C	D	E
10^0	286	172	134	190	56	288	174	148	194	77
10^{-1}	32	24	18	17	6	34	28	21	21	12
10^{-2}	3	1	1	5	1	3	2	3	9	3
10^{-3}	0	0	1	0	0	1	0	1	1	4
10^{-4}	0	0	1	0	0	2	1	3	0	1
10^{-5}	0	0	0	0	0	0	1	0	0	0
10^{-6}	0	0	0	0	0	0	0	0	0	0

ceased to increase in number after about 24 hours when they had barely doubled (to ca. 1×10^5). This was true whether washed or unwashed agar was employed, although only in recent years has Difco-bacto agar been so clean as to give this result. Furthermore, washed h^- bacteria suspended in liquid H^- medium show no increase in numbers whatsoever, indicating that the very slight residual growth on agar, to a level of ca. 10^5 cells per plate, is not due to the slow synthesis of histidine by h^- bacteria but rather to a very slight contamination of the agar, even when washed (ca. 10^{-1} μg histidine per gram of dry agar). Since the rate of mutation during growth from h^- to h^+ is ca. 3×10^{-8} per bacterium per generation (RYAN and WAINWRIGHT 1954), only one colony per 500 plates could be expected to arise from this cause. Thus, the very slight growth on H^- agar is insufficient to account for the new h^+ colonies found.

Nonetheless, new h^+ colonies continue to appear in appreciable numbers with time on H^- plates as is shown in figure 1. This rise in number cannot be due to contamination during storage, since the colonies are found deep within the agar. It is possible, especially with plates containing large numbers of colonies, that some counted for the first time on the third day were present on the second, but not observed. However, the likelihood of this error can be judged insignificant in view of the fact that h^- colonies found on H^+ agar are usually counted in exactly the same numbers on the second and succeeding days. Furthermore, the new h^+ colonies when first observed are usually smaller than those which were counted the day before.

Before accepting the mutational origin of the new h^+ colonies, not due to residual growth on h^- bacteria on the plate, it is necessary to entertain another possibility. Perhaps the mutations occurred during the growth of the h^- inocula in histidine but, when the h^+ mutants giving rise to the late-appearing colonies were placed on H^- agar devoid of histidine, they grew more slowly than other homogeneous h^+ bacteria and hence formed colonies later (WRIGHT 1953a and b). In this event the new h^+ colonies should exhibit the same sort of clonal variation as do the early-arising h^+ colonies, since in both cases mutations occurring early in the growth of the in-

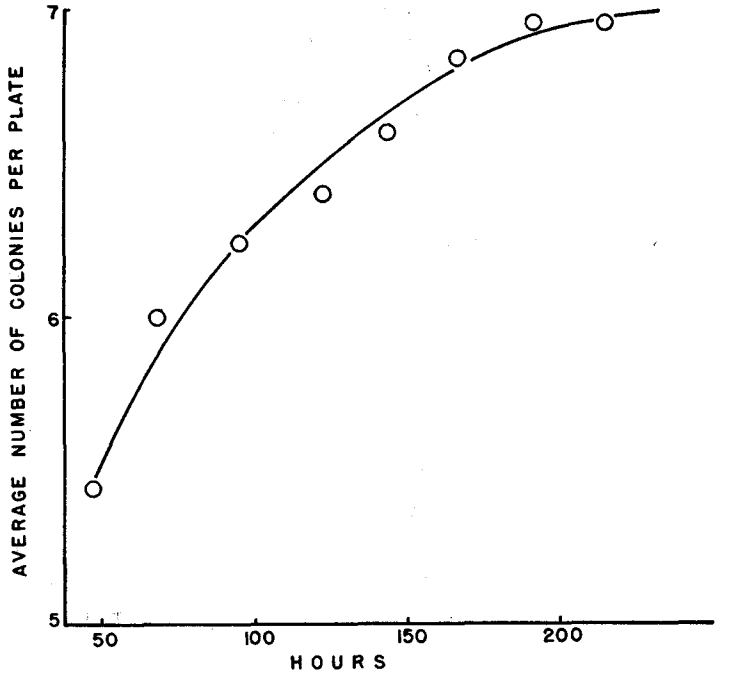

FIGURE 1.—The rise in numbers of h^+ colonies on 25 H^- plates with time. Each plate was inoculated with the whole contents of separate 2 ml cultures containing an average of ca. 3.2×10^7 h^- bacteria. The rate of mutation from h^- to h^+ during growth, calculated from the fraction of plates without colonies at 47 hours, was 3.9×10^{-8} per bacterium per generation.

oculum in histidine would give rise to more mutants than would later-occurring mutations. Table 2 shows that this is not the case. The variances for early-arising h^+ colonies are from 10 to 100 times the mean number of colonies per culture and there are insignificant probabilities that the different cultures were homogeneous with regard to the h^+ mutants they contained. This finding is consistent with the hypothesis that the mutations occurred during the growth of the inoculum in histidine. The late-arising colonies, on the other hand, showed variances approximately equal to the mean numbers and, in most cases, there was a high probability that they formed a homogeneous population. This is inconsistent with the hypothesis that the late-arising h^+ colonies came from h^+ mutants that arose during inoculum growth but grew slowly on the H^- plates.

In experiment 3 of table 2 each of the replicate cultures contained a mixture of h^- lac^- (lactose non-fermenting) and h^- lac^+ bacteria, and the h^+ colonies arising early or late were tested for their *lac* character. The distribution of early-arising lac^+ and lac^- colonies among the different cultures did not fit (χ^2 test; $P = <0.01$) that expected on the hypothesis that each had a chance of being lac^- or lac^+ equal to the overall proportion of lac^- and lac^+ colonies found. Rather the h^+ mutants that gave rise to the lac^- and lac^+ colonies appear to have arisen independently at different times during growth and to have multiplied into larger or smaller clones

TABLE 2

Variance analysis of the numbers of early- and late-arising h^+ *colonies on* H^- *plates inoculated with the entire contents of replicate* h^- *cultures*

	Experiment							
	1	2	3	4	5	6	7	8
Number of cultures	10	25	27	26	50	22	41	45
Bacteria per culture ($\times 10^7$)	3.2	2.2	3.4	3.6	3.8	3.2	38	24
Early-arising h^+ colonies								
Time in hours	55	49	52	51	55	47	48	53
Average number of h^+ colonies	3.3	6.3	28	1.2	17	5.4	52	66
Variance	31.2	642	2218	11.4	844	92.3	5009	701
P of homogeneity (χ^2 test)	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Late-arising h^+ colonies								
Time in hours*	142	192	218	191	170	214	192	101
Average number of h^+ colonies	0.8	0.5	1.3	0.7	1.2	1.5	3.3	5.5
Variance	0.9	0.7	2.1	0.8	1.4	1.4	8.1	9.9
P of homogeneity (χ^2 test)	0.22	0.14	0.02	0.68	0.37	0.46	<0.01	0.04

* Minus time at which early-arising colonies were enumerated.

accordingly. The late-arising colonies, on the other hand, did not show a clonal nature and their distribution fit (χ^2 test; $P = 0.29$) the hypothesis that they had a chance of being *lac*$^-$ or *lac*$^+$ according to the overall proportion found. Therefore, the late-arising h^+ colonies show no evidence of a clonal origin during growth. It can be concluded that the clones of h^+ mutants had their origin among the non-dividing h^- bacteria on the H^- plate.

Clones of h^+ mutants also arise among h^- bacteria kept in the stationary phase in liquid. This was discovered in the course of using, for the determination of mutation rates, liquid cultures inoculated with ca. 50 h^- bacteria whose growth was limited by the exhaustion of histidine. If one or more mutations to h^+ occurred during the limited growth of the h^- bacteria, the h^+ mutants, unrestricted by the exhaustion of histidine, overgrew the culture and caused visible turbidity. From the proportion of cultures that did not show turbidity the average number of *mutations* can be calculated by the zero term of the Poisson formulation. It was observed that many cultures showed adaptive overgrowth at about 50 hours but that thereafter, at a low rate, new adaptive overgrowths occurred in other cultures for as long as 2 weeks. In order to determine how much time was required for the adaptive overgrowth of an h^+ mutant once it had appeared, single h^+ mutants which were also *lac*$^-$ were introduced into the unadapted h^- *lac*$^+$ cultures from a Poisson experiment. These mutants overgrew in about 27 hours. The time required for the h^- inoculum to grow to the level limited by the exhaustion of histidine (ca. 2.0×10^7 h^- bacteria per culture containing 0.25 μg L-histidine monohydrochloride) was between 20 and 24 hours. Since most of the cultures had no mutations, most of the

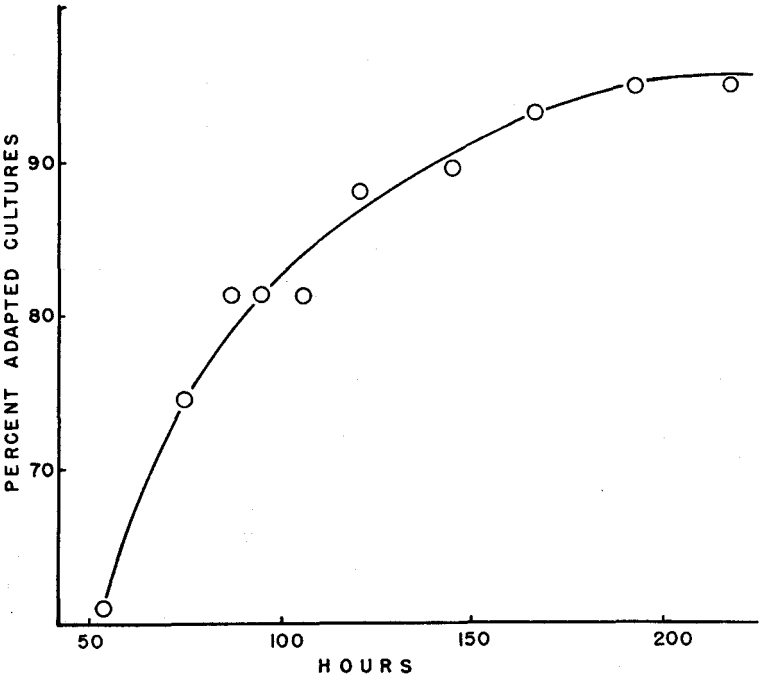

FIGURE 2.—The continued appearance of h^+ adaptations among h^- cultures in a histidine-limited stationary phase. The data are from 59 two ml cultures with 0.36 μg histidine which supported the growth of 3.2×10^7 h^- bacteria. The rate of mutation from h^- to h^+ during growth, calculated from the fraction of non-adapted cultures at 54 hours, was 2.0×10^{-8} per bacterium per generation.

mutants came from mutations which took place in the last generation. Therefore, the time at which an h^+ mutant arising during growth should be seen overgrowing the culture is about 50 hours.

But what about those mutants that overgrow some cultures after that time? Figure 2 shows the pattern of appearance of cultures with such mutants. The total number of h^- bacteria in these histidine-limited cultures remained absolutely constant when determined with a Petroff-Hauser counting chamber. For example, in 2 experiments, where the average total number of bacteria was 3.5×10^7 per tube after 1 day, that number was retained throughout the next 10 days at which time the average total number was 3.3×10^7. Hence, residual growth cannot have yielded the h^+ mutants that overgrew. Furthermore, the late adaptations involve growth of the h^+ mutants, from levels as low as 50 h^+ per culture, at a rate of ca. 56 minutes per generation. Since this is the maximal rate of growth of h^+ bacteria, the late adaptations cannot be said to be due to slow-growers. Like the late-arising h^+ colonies on H^- plates, the late adaptations appear to involve clones of h^+ mutants that arose among h^- bacteria in the stationary phase of the culture.

During the course of these long-term experiments attempts were made to retard evaporation; plates were stored at 37°C in a humid incubator; 2 ml liquid cultures were kept at 37°C in small tubes in a moist chamber. Nonetheless, some evaporation did occur. It did not reduce the volume of 2 ml cultures more than 10 percent

until after about 150 hours; later on it became more serious and calculations were corrected when it was observed to occur.

The rate of increase of the number of new h^+ mutant clones (m) due to mutation from h^- in the stationary phase can be written as:

$$\frac{dm}{dt} = \mu N \tag{1}$$

where μ is the chance of mutation per h^- bacterium per hour and N is the number of viable h^- bacteria present at time t which is measured in hours.

The rate of change of N because of mutation to h^+ alone can be expressed as:

$$\frac{dN}{dt} = -\mu N \tag{2}$$

and the rate of change of N because of death of the h^- bacteria alone (fig. 3) can be expressed as:

$$\frac{dN}{dt} = -kN \tag{3}$$

where k is the chance of death per hour. Thus the rate of change of N for these two reasons is:

$$\frac{dN}{dt} = -(\mu + k)N \tag{4}$$

The integral of equation 4 is:

$$N = N_o e^{-(\mu+k)t} \tag{5}$$

However, $\mu + k$ can be taken from the line in figure 3 as 1.8×10^{-2} and μ is less than 10^{-8}; therefore, $\mu + k$ is approximately equal to k and equation 5 can be approximated by:

$$N = N_o e^{-kt} \tag{6}$$

Equation 6 can be substituted into equation 1 to give

$$\frac{dm}{dt} = \mu N_o e^{-kt} \tag{7}$$

whose integral is:

$$m = N_o \frac{\mu}{k}(1 - e^{-kt}) \tag{8}$$

which transpsosed, give:

$$\mu = \frac{km}{N_o}(1 - e^{-kt}) \tag{9}$$

With equation 9 the rate of origin of new h^+ clones (mutation to h^+) can be calculated throughout the stationary phase.

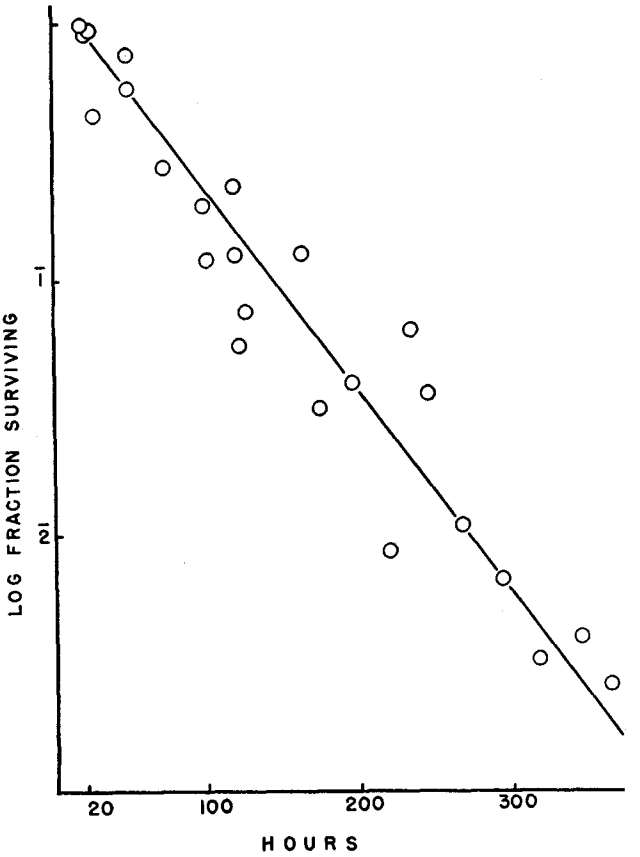

FIGURE 3.—The rate of death of h^- bacteria kept at 37°C in the stationary phase by the exhaustion of histidine.

The value N_o was calculated as follows. At about 50 hours an observation of the early-arising adaptations was made. At intervals thereafter the number of new adaptations was observed. Since 27 hours had to elapse between the origin of an h^+ mutant clone and its identification as an adaptive overgrowth, the adaptations observed during the interval had their mutational origin in the period between 27 hours before the beginning of the interval and 27 hours before its end. N_o was taken from figure 3 as the number of bacteria surviving at the time the interval began, minus 27 hours.

Table 3 shows, as an example, mutation rates calculated from the data in figure 2. It will be observed that the rate of appearance of new h^+ clones remains relatively constant throughout the stationary phase. This constancy was observed in eight other experiments, some of which involved stationary phases more than 300 hours long. The average rate of mutation in all these experiments was 1.2×10^{-9} per h^- bacterium per hour.

If it is assumed that death of h^- bacteria on H^- agar occurs at the same rate as in liquid, equation 9 can be used to calculate the rate of appearance of h^+ colonies

TABLE 3

Mutation rates from h^- *to* h^+ *in populations of* h^- *bacteria in a histidine-limited stationary phase in liquid medium*

Interval in hours	Average number of new mutations (m) from fraction of cultures without mutants	Chance of mutation per bacterium per hour (μ)
54–75	0.43	0.9×10^{-9}
75–87	0.31	1.6×10^{-9}
87–95	0	0
95–106	0	0
106–121	0.46	3.2×10^{-9}
121–145	0.15	0.9×10^{-9}
145–167	0.40	4.2×10^{-9}
167–193	0.30	3.9×10^{-9}
193–217	0	0
		—
		average = 1.6×10^{-9}
54–193	2.05	1.5×10^{-9}

TABLE 4

Mutation rates from h^- *to* h^+ *in populations of non-dividing* h^- *bacteria on* H^- *plates*

Interval in hours	Average number of new h^+ colonies per plate (m)	Chance of mutation per bacterium per hour (μ)
47–68	0.56	1.6×10^{-9}
68–95	0.24	0.8×10^{-9}
95–122	0.16	0.9×10^{-9}
122–143	0.20	2.2×10^{-9}
143–166	0.24	3.6×10^{-9}
166–191	0.12	2.5×10^{-9}
191–215	0	0
		—
		average = 1.7×10^{-9}
47–191	1.52	1.3×10^{-9}

among the h^- bacteria surviving on the plate. The early-arising colonies were counted after 2 days and new colonies were counted at intervals thereafter. N_o was taken from figure 3 as the number of bacteria surviving at the time at which the interval began, minus 4 hours. This was done because the colonies observed had an origin about 24 hours beforehand while death of the h^- parents on the plates began immediately and not after 20 hours of growth as was the case in liquid. Table 4 shows the mutation rates calculated from the data in figure 1. The relative constancy of the mutation rates observed is consistent with the assumption that h^- bacteria were dying on the plates. This constancy was found in six other experiments and the average mutation rate was 0.91×10^{-9} per h^- bacterium per hour.

This rate is indistinguishable from that found in liquid cultures and implies that the new h^+ colonies on H^- agar and the new adaptations in histidine-limited liquid cultures of h^- bacteria have the same origin.

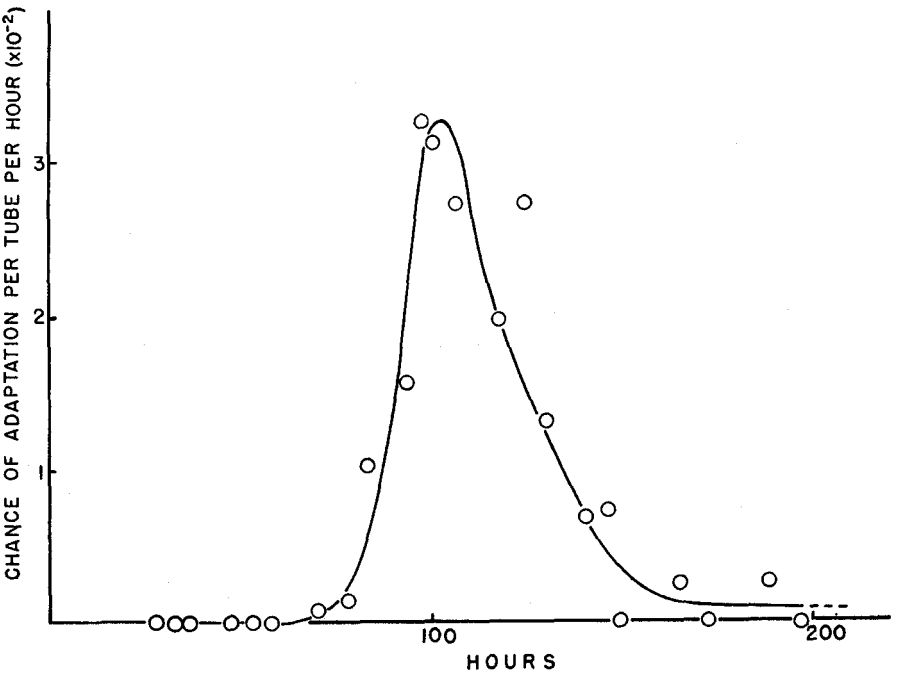

FIGURE 4.—The fraction of unadapted cultures adapting per hour as a function of time. The data are from an experiment in which 92 tubes containing 10 ml of H^- medium each received 0.1 ml of a suspension of h^- bacteria which had been subjected to a dose of ultraviolet light giving a survival of 1.1×10^{-5}. The frequency of induced h^+ mutants was 2.3×10^{-4} per survivor; the frequency of spontaneous h^+ mutants is ca. 2×10^{-7}.

It is now necessary to consider the possibility that the late-appearing clones of mutants are due to mutations which arose during the growth of the inoculum but which, even in the presence of histidine, ceased growing as soon as they arose but did not die. They would, consequently, not show a clonal distribution and would not be observed on H^- agar or in H^- medium until the onset of their growth which may not take place until well into the stationary phase of their h^- parents. This hypothesis in its simplest form would predict that there would be some average time at which the delay in the onset of division of the mutant would end. Thus the pattern of appearance of new mutant clones would be a distribution about some median. On the contrary, new mutant clones appear at a constant rate.

There is one instance known in which new mutants have a unique delay in the onset of division. This is the case of h^+ mutants induced by high doses of ultraviolet light (RYAN 1954). The pattern of termination of this delay was experimentally determined by tubing aliquots of a washed suspension of heavily irradiated h^- bacteria in H^- medium. If the dilution was so adjusted that each tube received on the average about one h^+ mutant, then the times at which they were observed to overgrow the cultures would very nearly reflect the times at which individual mutants begin to grow. Figure 4 shows how these times are distributed about a median. In this and similar experiments the h^- bacteria inoculated underwent some growth

from ca. 6×10^3 to ca. 5×10^5 per culture, presumably on histidine released from the dead cells present. Among the tubes still unadapted at the time this growth was observed to have occurred, one spontaneous mutation to h^+ could have occurred at the rate normal for growing h^- bacteria; an insignificant correction was accordingly made.

Most of the adaptations due to induced mutants occurred between 4 and 6 days; thereafter adaptations continued at the relatively low and constant rate of stationary phase mutation. At 6 days 26 percent of the tubes were unadapted, indicating that an average of 1.35 induced mutants had been added per tube. This is exactly the number of mutants calculated to have been added from a determination of the h^+ present in the irradiated h^- suspension by plating on H^- agar. In two other experiments, where the induced mutants added were also recovered as adaptations, a distribution of times of onset of growth around a median was found as in figure 4. The median time was shorter when survival was greater and the frequency of induced mutants lower; but the chance that an induced mutant would begin to grow was not constant with time. It can be concluded, therefore, that it is unlikely that spontaneous h^+ mutants, if they do have a long delay in the onset of growth, will have a constant chance to begin to divide with time. Hence this hypothesis for the appearance of new mutant clones in the stationary phase may be rejected.

DISCUSSION

There remain two hypotheses alternative to that of mutation in non-dividing bacteria which might still explain the data. The first is that genotypic changes occur during the growth of the inoculum in histidine but that the phenotypic change which allows the mutant to be recognized as h^+ occurs without division at some time during the stationary phase of the h^- parents (WRIGHT 1953b and c). WITKIN (DEMEREC *et al.* 1949) has interpreted the increased frequency of phage resistant mutants of *E. coli* during the "stationary" phase in broth when sodium nucleate is present as due to the acceleration of phenotypic expression by this compound. But, first, evidence has not been presented that this compound did not stimulate bacterial division and, second, the hypotheses that the nucleate hastened the delayed onset of division of mutants or raised the rate of mutation in the "stationary" phase were not excluded. Further, if nucleate simply accelerated phenotypic expression it would not be expected to produce zero-point mutants, a result reported to have occurred. It cannot be considered, then, that release from a phenotypic delay has been shown to occur in the stationary phase. With regard to the experiments reported in the present paper, this hypothesis, like that of the delayed onset of growth of the mutant, predicts a distribution of late-appearing mutants around a mean. This does not occur. Neither is there a clonal distribution of late-arising mutants which would ensue if the late-appearing mutants resulted from the delayed phenotypic expression of mutations occurring during growth of the inoculum in histidine. Therefore, phenotypic lag can be rejected as an explanation of the results.

The second hypothesis contends that bacteria in a histidine limited stationary phase are in a dynamic state and subject to turnover. Some cells may lyse and

provide histidine with which new cells are formed. In the course of this replacement by growth, mutations may occur at the rate normal for growing bacteria. Yet the total number of bacteria would remain the same, the growth being cryptic. It is possible to calculate what this rate of turnover must be if the mutations observed were actually occurring during growth at a rate of ca. 3×10^{-8} per bacterium per generation. Since there were on the average 2×10^{7} bacteria present per culture and mutations were occurring at a rate of ca. 1×10^{-9} per hour per bacterium, there were 2×10^{-2} mutations per hour per culture. When mutations occur at a rate of 3×10^{-8} per bacterium per generation, there are, on the average, 2×10^{7} bacteria per mutation. When the number of bacteria per mutation is multiplied by the number of mutations per hour, it is found that 4×10^{5} bacteria must divide per hour. This, divided by the number of bacteria per culture gives the turnover rate as 2×10^{-2}. Therefore, about one cell in fifty would have to lyse and be replaced each hour in order to account for the mutation rate observed in terms of mutation at the normal spontaneous rate during cryptic growth. This hypothesis demands not only an extraordinarily rapid spontaneous lysis for which there is no evidence, but also a remarkable efficiency of utilization of histidine released by lysis. Nonetheless, because it is amenable to experimental analysis it cannot be summarily rejected; a final decision on this point must wait until the completion of experiments now in progress.

In the meanwhile, the fact that mutations can arise in populations of bacteria whose numbers are not increasing must be accepted. In the present case the stationary phase was *bona fide* inasmuch as the total number of bacteria remained constant; thus, it is unlike the cases discussed by LWOFF and AUDUREAU (1941), ENGLESBERG and STAINER (1949) and RYAN (1952), where cryptic changes in population size were occurring. It may be similar to the case reported by KAPLAN (1947), where new mutants accumulated at a constant rate in colonies of *Serratia marcescens* in the stationary phase. Analogous cases appear to exist with organisms other than bacteria. For example, spontaneous mutations occur in spores of Streptomyces where the nuclei are not dividing (WAINWRIGHT unpublished), in the stored seeds and pollen of plants (STUBBE 1935, 1936; BLAKESLEE 1954) and in the stored sperm of Drosophila (TIMOFEEF-RESOVSKY 1937; MULLER 1946).

If these stationary phase mutations are actually occurring in the absence of a cryptic division, they occur as a constant function of physical time. This is the same relation as that found by NOVICK and SZILARD (1949) for mutations to phage resistance among bacteria dividing at different rates, although it is not yet certain how general this relation is (WITKIN 1953; DE ROTHSCHILD 1954). The magnitude of the mutation rate could be some function of the rate and nature of bacterial metabolism which is certainly lower and different during the stationary phase.

The reason mutation in the stationary phase of bacteria has not been more frequently observed is probably its low rate of occurrence. The simple observation of the total number of mutants before and after a short period in the stationary phase is not a sensitive enough method (RYAN and WAINWRIGHT 1954); there are too many mutants present after growth and too few added by stationary phase mutation to make the detection of the latter easy. Nor do stationary phase mutations

occur rapidly enough to increase the numbers of mutants in stock cultures significantly.

It is appropriate to point out here what constitutes a mutation like that from h^- to h^+ in asexually reproducing *E. coli*. What is observed is an hereditary change in cell type; from this is inferred a mutation in a genetic determinant. This is formally the same as any designation of genes as mutable units in sexually reproducing forms, such as strain K12 of *E. coli* or higher organisms. There are numerous and obvious resemblances between mutable genetic units in all organisms; the problem of the degree of similarity between mutable units and other gene concepts such as recombination units is one that cannot as yet be analyzed in the asexual h^- strain. As a consequence it is not known whether the mutable h^- genetic units are chromosomal or otherwise. It does appear, however, that they reside in the deoxyribonucleic acid "nuclear" bodies (RYAN, FRIED and SCHWARTZ 1954).

As a consequence, it is possible to imagine that an increase occurs in the genetic deoxyribonucleic acid in stationary phase cells that are not undergoing division. Thus, these stationary phase mutations could arise as errors in duplication. It is not, however, necessary to assume this, inasmuch as mutations are known to be induced by naturally occurring metabolites (cf. NOVICK and SZILARD 1952 and WRIGHT 1953). Although certain stages in the division cycle may be more sensitive, even non-dividing cells can respond to chemical mutagens. Natural radiation does not seem sufficient to account for rates of mutation as fast as those occurring among bacteria (cf. MULLER 1930).

SUMMARY

New clones of mutant h^+ bacteria appear during the stationary phase of parental h^- bacteria in both liquid and agar media. During this time there is no increase in the number of parents. The new h^+ clones are not the result of the slow growth of, or the development of phenotypes by, mutants which arose during growth. Nor can the new h^+ clones result from the delayed onset of growth of mutants arising during previous growth. The origin of the new h^+ clones occurs at a rate of ca. 1×10^{-9} per bacterium per hour which may be constant for many days. This rate is one fortieth of that which is observed during growth (ca. 4×10^{-8} per bacterium per hour).

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