DELAYED PHENOTYPIC EXPRESSION OF SPONTANEOUS MUTATIONS IN ESCHERICHIA COLI*

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INTRODUCTION

THE quantitative study of mutations occurring at low rates requires an organism that can be grown conveniently in large numbers, and mutations that can be readily detected. Furthermore, where information is desired concerning the effect of a gene change shortly after its occurrence, it is necessary to be able to examine the phenotype of the organism immediately following the change and at intervals thereafter.

Bacteria are superior to higher organisms for these purposes, since (1) large populations can be handled, (2) there are numerous mutants that can be readily detected and counted, and (3) individual organisms result from each cell division, enabling the phenotype to be determined at any time after a gene change. It was the purpose of this investigation to discover the rate of spontaneous mutation of the bacterium *Escherichia coli*, strain *B/r*, from sensitivity to resistance to the phage *T1*, and also, by indirect means, to determine the interval between time of occurrence of the mutation and phenotypic expression.

The reasons for investigating spontaneous mutation rate and time of phenotypic expression are as follows:

Two previously developed methods of estimating mutation rate in bacteria have yielded discrepant results (LURIA and DELBRÜCK 1943). This discrepancy has been ascribed to an error in the assumptions on which one of the methods is based; but, which assumption and which method is in error is not known.

One of the methods rests on the assumption that a gene mutation expresses itself immediately in the individual cell in which it occurs; and one of the possible interpretations of the discrepancy is that this is not the case, but that on the contrary one or more generations of growth are required before the mutation is expressed.

Delayed phenotypic expression, or "cytoplasmic lag" as it has been termed, has been observed in Paramecium (see SONNEBORN 1947); and if something of this nature occurs also in bacteria it is important from the standpoint of understanding gene action.

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Methods of Previous Workers

The early experiments of LURIA and DELBRÜCK (1943) referred to above will now be considered in detail.

To find out the number of phage-resistant mutants in a phage sensitive liquid culture, the whole culture if its population size is small, or a sample of it if it is large, is spread on agar together with the particular phage under consideration. On incubation, all bacteria that are sensitive to the phage are lysed, leaving only the resistant mutants. Each such mutant will eventually form a separate colony; and from the number of colonies the number of mutants in the liquid culture, or the sample, can be estimated.

By using this technique it is possible to determine the rate of mutation to phage resistance. As stated, two methods have been devised by LURIA and DELBRÜCK (1943). The experimental procedure in both of the methods is to grow a series of similar liquid cultures from small inocula, and to determine the numbers of resistant bacteria in each, as well as in the average population. The two estimates are derived from these primary data, the first using the proportion of cultures in which resistant mutants have appeared, and the second using the average number of resistant mutants per culture.

These methods are of course only strictly applicable where it is possible to eliminate the original type without affecting the mutant type, and where there are no selective differentials between mutant and original types.

In their experiments these test cultures were started with small inocula (50 to 500 bacteria from a growing culture of *E. coli*, strain *B*) and were grown to saturation either in broth or in synthetic medium. (The volume of the cultures was 10 cc in some experiments and 0.2 cc in others, the final numbers of bacteria being of the order of 3×10^{10} and 3×10^{8} respectively.) At the end of growth, samples of the cultures—or in some cases whole cultures—were tested to determine the numbers of bacteria resistant to the phage *T1*.

The cultures from which the inocula were taken contained between 1 and 1,000 resistant individuals per 10^8 total bacteria. Thus, the chance of introducing a resistant bacterium into the test cultures via the inoculum was small and in the rare event of one being introduced the fact would be indicated by an excessive proportion of resistant bacteria in the fully grown test culture. In practice, any resistant bacteria found at the time of testing—that is, after the cultures are fully grown—will therefore be the mutant offspring of one of the sensitive bacteria in the inoculum.

An estimate of mutation rate per bacterium per division cycle can be obtained if the numbers of mutations, and the average number of cell divisions, occurring in a series of cultures are known. The latter may be calculated from the final population in the series and the former from the proportion of cultures containing no mutants. The greatest accuracy is obtained when this proportion is neither too large nor too small, and the method cannot be used if every culture contains a mutant. Since the proportion of cultures having no mutants is a function of the number of cell devisions in a culture, it may be adjusted by altering the volume of medium, 0.2 cc being the amount used

in the experiments under consideration. To determine this proportion it is of course necessary to test whole cultures, as distinct from samples.

This method will be known as *method 1* throughout the present paper. It should be noted that it is based upon an estimate of the number of resistant clones developing in the series (this estimate being obtained from the proportion of cultures in which no resistant mutants have developed), and that it takes no account of the numbers of individuals in these clones at the end of the growth. For the purpose of this paper, the term "mutant clone" will refer to those individuals carrying genetic factors for phage resistance which have a common origin in a single mutation. Within a mutant clone individuals which are phenotypically resistant to phage will be collectively termed a "resistant clone." A culture may contain one or more mutant clones of varying age.

The second method of LURIA and DELBRÜCK uses the average number of resistant bacteria in a series of similar cultures and calculates mutation rate from this value, the average population, and the number of cultures. The number of mutants arising during the growth of a culture is of course, on the average, a function of the mutation rate. But there are very large variations in the number of mutants present in different cultures grown under identical conditions, these being due to chance variations in the time of occurrence of the mutations. Thus the occasional occurrence of a mutation early in the growth of a culture, at a time when the population is small, will result in a much higher than average number of mutants in that culture. Because of these statistical fluctuations, mutation rate cannot be calculated from the number of mutants in a single culture started from a small inoculum. It can, however, be estimated from the average number of mutants in a series of similar cultures; and the mathematical details of the method have been worked out by LURIA and DELBRÜCK (1943). This method will be known as *method 2* throughout the present paper. It differs from method 1, which utilizes the number of resistant clones occurring in liquid cultures, in that it takes into consideration the number of resistant individuals. Furthermore, in the event of any change in the mutation rate during growth, method 2 would give an average of the mutation rates obtaining in each of the generations during which mutations had occurred—equal weight being given to the early generations when the population and the number of mutations occurring were small, and to the later generations when both these values had increased. In contrast to this, in method 1 changes in the mutation rate during growth would give an estimate strongly biased in favor of the rate obtaining during the later period when the population and the absolute number of mutations occurring was large. Moreover, a delay in the phenotypic appearance of a mutation would reduce the rate as estimated by method 1, because recent mutations would not be detectable. The rate as estimated by method 2 would be affected less, since early mutations, which have a greater number of generations in which to become phenotypically resistant, are represented by larger numbers of descendants than are the later mutations. The possibility that mutation rate is

not constant throughout growth, and the possibility that phenotypic resistance does not appear for one or more generations after mutation to resistance has taken place, will now be considered.

A striking and unexpected finding of the LURIA and DELBRÜCK experiments was that the rates estimated by these two methods differed by a considerable factor, that from method 1 (utilizing the number of resistant clones developing in liquid cultures) being lower than that from method 2 (utilizing the average number of resistant individuals per culture). The averages of their estimates are $.32\times10^{-8}$ and 2.4×10^{-8} per bacterium per division cycle, respectively. This difference has since been confirmed by DEMEREC and FANO (1945), who have in addition shown that it is not peculiar to experiments using *T1* but is also true of rates of mutation to resistance to other phages (*T3*, *T4*, *T5*, *T6*, and *T7*) when estimated by methods 1 and 2.

A statistical bias in method 2 (which gives the high estimate) may contribute to the discrepancy, but the work of LURIA and DELBRÜCK suggests that its contribution is small. (For a discussion of this point the reader is referred to the original publication. Also, a method of estimating mutation rate, which avoids this source of error will be considered in a later section of the present paper.)

Since the two methods will give the same estimate if, and only if, (1) the rate of mutation is constant throughout growth and (2) the occurrence of a mutation gives rise to a phenotypic mutant without delay, it was concluded that one of these two conditions did not obtain. There was no critical evidence to indicate which one, however, since the discrepant estimates could be explained by assuming either a high mutation rate during the greater part of the growth period, dropping during the last few divisions, or a delay of one or more generations between mutation and phenotypic expression.

LURIA and DELBRÜCK did not favor the latter interpretation, since a fixed delay of one or more generations before the development of phenotypic resistance would mean that mutant clones would number two or more individuals at the time when phenotypic resistance appeared. It was therefore assumed that cultures with just one resistant individual would be rare if there were a delay; and these, instead of being rare, had been observed in considerable numbers (see LURIA 1946). It has been pointed out, on the other hand, that if some lines of descent within the clone were to develop resistance earlier than others, mutation plus delay could give rise to cultures having only one phenotypically resistant individual (SONNEBORN 1946). Thus the assumption of a delay is permissible provided it is also assumed that phenotypic expression is earlier in some lines of descent than in others within the same mutant clone.

Two alternative possibilities therefore exist: (1) that of a relatively high mutation rate during all but the last few generations, and (2) that of a variable delay in phenotypic expression. The possible significance of the second of these two alternatives should be considered. If there is a delay in phenotypic expression, then some cultures which showed no phenotypically resistant bacteria would contain mutants that could not be detected. Also, the end number of resistant bacteria in a culture would represent only part of the genetic mutants

present, some not having developed resistance by the time growth stopped. Thus the mutation rates calculated by methods 1 and 2 would both be underestimated, and the extent of the underestimate would depend upon the magnitude of the delay.

The information available so far sets no limit on the suspected delay or its variability within clones of mutants, and it is even possible that an extreme situation exists in which both are considerable. If this is true, mutation rate is greatly underestimated by the methods outlined.

It should be noted at this point that a delay similar to that suspected in the case of spontaneous mutations does in fact occur in irradiated material (DEMEREC 1946; and DEMEREC and LATARJET 1946). Although there is at present no certainty that spontaneous and induced mutations behave in precisely the same manner, it is of interest to consider the nature of the delay in the one case in which it has been established, that is, in induced mutations.

To determine the time of appearance of induced mutations, DEMEREC irradiated bacteria in liquid suspension, using ultraviolet radiation in some experiments and X-rays in others. These treated bacteria were spread on agar, incubated for varying periods of time to permit cell reproduction, sprayed with phage *T1*, and incubated again until colonies appeared. The spraying caused all susceptible bacteria to be infected and lysed; but where a mutation to resistance had occurred and had been expressed phenotypically, the resulting bacteria continued to grow after phaging, giving rise to one visible colony for each such mutation.

Irradiation caused an enormous increase in the number of resistant clones that appeared during growth, over the number that appeared during the same number of generations in untreated bacteria. The delay between irradiation and phenotypic change was such that less than one percent of the induced mutants appeared prior to the first division, 50 percent appeared after about five divisions, and some did not appear until after 11 or 12 divisions.

These observations give some support to the possibility that there is also a delay in the expression of spontaneous mutations.

The experiments described here were designed to distinguish between this and the alternative possibility of a change in the rate of mutation during growth. They show that there is in fact a delay, and that it is the cause of the discrepant estimates of rate obtained by using the LURIA and DEBRÜCK methods 1 and 2. Some indication of the extent and variability of the delay has been sought, and an attempt has been made to obtain a more accurate estimate of mutation rate.

MATERIALS

The bacterium *Escherichia coli* strain *B/r* was used in these investigations. This is a mutant derived from strain *B* (WITKIN 1946, 1947), and is more resistant than *B* to the action of radiations.

The mutations studied are those resulting in resistance to phage *T1*. There are at least two different categories of mutant: those resistant to *T1* but not to any other of the known phages, and those resistant to phages *T1* and

T5. These mutant categories are designated *B/r/1* and *B/r/1,5* respectively. Within each there occurs a number of morphologically distinguishable colony forms, and it is possible that these represent a number of mutations of dissimilar origin; but in this study no attempt has been made to distinguish between the various types of mutation that give rise to resistance to *T1*.

DISCREPANT ESTIMATES OF MUTATION RATE FROM NUMBERS OF RESISTANT CLONES (METHOD 1) AND OF RESISTANT INDIVIDUALS (METHOD 2)

In view of the possibility that *B/r* may differ from *B* in the rate with which it mutates, rates for *B/r* were determined by each of the LURIA and DELBRÜCK methods, using phage *T1*.

Eight separate experiments were carried out, and for each experiment 25 broth cultures of 0.2 cc were grown. Small inocula were used, and the cultures were incubated for 18 hours, by which time growth had stopped.

The inocula contained approximately ten bacteria per culture in four of the experiments, and approximately 10^4 in the other four. These numbers were small enough so that the chance carry-over of a mutant in the inoculum would be readily detected.

Method 1 was used to calculate mutation rate, a, from the proportion of cultures having no resistant bacteria, P_0, and the average number of bacteria at the end of growth, N, using the formula:

$$a = -(\ln 2)(\ln P_0)/N. \tag{1}$$

The above formula is derived from formulas (4) and (5) of LURIA and DELBRÜCK (1943), ln being the natural logarithm.

Method 2 was used to calculate mutation rate, a, from the average number of resistant bacteria per culture, r, the average number of bacteria at the end of growth, N, and the number of cultures, C, using the formula:

$$r = (aN/\ln 2) \ln (CaN/\ln 2). \tag{2}$$

This is derived from formula (8) of LURIA and DELBRÜCK.

The natural logarithm of 2 appears in these formulas because the mutation rate refers to the rate per bacterium per division cycle, as distinct from the rate per bacterial division. The significance of this distinction is best visualized by using a concrete example. If a population of 10^8 bacteria passes through one division cycle and one mutation takes place, the number of bacterial divisions is 10^8 and the mutation rate per bacterial division is 1×10^{-8}. The mean population throughout the cycle, however, is $10^8/\ln 2$, so that the rate per bacterium per division cycle is $\ln 2\times10^{-8}$, which is $.693\times10^{-8}$.

The first of these two methods of expressing mutation rate would be applicable if mutation took place only at the time of cell division, and affected just one of the offspring. The second would be applicable if mutability were continuous throughout the division cycle. In the absence of information on this point the choice is arbitrary, and since the latter method has been used by previous authors its use is continued in this paper to facilitate comparisons.

These considerations are of course based on the assumption that each bacterium divides, an assumption which will be discussed later in the paper.

Methods 1 and 2 have been used with strain *B/r* in order to determine (a) whether, as with strain *B*, estimates obtained by method 1 are lower than those obtained by method 2, and (b) whether the estimates from these two methods are the same for *B/r* as for *B*.

The data from these experiments and the estimated mutation rates are given in table 1. Those obtained using method 1 average $.40 \times 10^{-8}$ and, those using method 2 average 3.6×10^{-8}.

It will be seen from table 9—in which the results of previous workers, using strain *B*, have been quoted—that the discrepancy between the estimates of mutation rate given by the two methods as applied to *B/r* is similar to the discrepancy using strain *B*. It is also evident that the estimate of mutation rate for strains *B/r* and *B* are similar.

THE ELIMINATION OF A POSSIBLE UPWARD BIAS IN METHOD 2 BY THE USE OF LARGE INOCULA (METHOD 3)

The formula for calculating mutation rate from the average number of mutants per culture (method 2) disregards the early divisions, when the population is small and it is unlikely that a mutation will occur. The divisions which enter into the calculation are those occurring after an arbitrary time, this time being chosen so that on the average one mutation will occur prior to it in the whole series of cultures. LURIA and DELBRÜCK point out that the chance occurrence of this early mutation might account for part of the discrepancy between the estimates of rate obtained with the two methods. For a detailed discussion of this point the reader is referred to their paper.

It was therefore necessary to arrive at an estimate which, like that obtained by method 2, would utilize the number of resistant bacteria arising during growth in liquid culture, but which would not be biased by the chance occurrence of early mutations. This was done by growing the test cultures from inocula of sufficient size to ensure that an appreciable number of mutations would take place during the first division. Since much of the statistical fluctuation in end numbers of resistant bacteria is thus eliminated, mutation rates may be estimated from single cultures. (An experiment similar to this has been proposed by SHAPIRO 1946.)

The method can be used only if the proportion of resistant bacteria in the inoculum is small, since otherwise the relatively small increase due to mutation during growth could not be accurately determined. To serve as inocula, therefore, cultures containing very small proportions of resistant bacteria were chosen.

Five 50 cc and five 300 cc aerated cultures were grown from inocula of 2.6×10^9 and 2.1×10^8 bacteria, respectively. Synthetic medium, the M-9 of ANDERSON (1946), was used because the bacteria can be grown to a higher number per unit volume in it than in broth. In two separate sets of experiments growth resulted in increases in the numbers of individuals of approximately a hundredfold and three thousandfold, respectively.

TABLE 1

Estimates of mutation rate of B/r to resistance to phage T1, using the methods of LURIA *and* DELBRÜCK (*1943*) (*methods 1 and 2 in the present paper*), *calculating from the number of cultures with no resistant bacteria, and from the average number of resistant bacteria per culture, respectively, in series of similar cultures started from small inocula.*

EXPERIMENT	A	B	C	D	E	F	G	H
Inoculum (no. of bact.)	10	10	10	10	10^4	10^4	10^4	10^4
Number of cultures	25	25	25	25	25	25	25	25
Vol. of cultures, cc	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Culture Number	Number of Resistant Bacteria							
1	140	2	2	1	1	1	134	4
2	0	41	57	5	9	21	18	1
3	0	45	1	1	2	34	34	0
4	43	11	6	4	0	287	380	6
5	60	8	1	4	242	46	3	149
6	13	1	1	71	2	33	15	176
7	48	30	1	5	12	23	2	31
8	3	1	2	12	0	3	6	1
9	4	1	14	1	2	102	4	0
10	36	0	0	1	2	84	2	48
11	55	11	3	49	11	148	5	0
12	447	0	139	9	29	0	18	45
13	1	1	40	0	17	120	0	3
14	9	7	142	0	42	10	33	2
15	27	1	1	1	7	4	725	134
16	14	4	154	1	15	0	1	25
17	30	6	3	60	14	0	5	34
18	160	15	4	37	45	13	0	14
19	231	9	1	1	110	0	0	131
20	35	0	158	7	32	376	118	22
21	37	1	5	44	0	1	1	60
22	0	21	1	18	32	133	15	0
23	1	0	0	0	8	4	0	158
24	8	27	2	12	36	42	151	6
25	3	221	0	79	0	4	9	263
Method 1								
Cult. with no resist. b.	3	4	3	3	4	4	4	4
Bact. per cult., $\times 10^8$	3.1	4.6	2.5	2.8	4.2	3.7	3.2	3.8
Mutation rate, $\times 10^{-8}$	.42	.28	.59	.53	.31	.34	.40	.33
Method 2								
Av. resist. b. per cult.	56.1	18.5	29.5	16.9	26.8	59.5	68.6	52.6
Mutation rate, $\times 10^{-8}$	5.1	1.5	3.8	2.1	2.1	4.5	5.8	3.9

Assays were made of total bacteria and of numbers of resistant individuals in the inocula and in the fully grown test cultures. These four values are designated N_1, r_1, N_2 and r_2, respectively. For the sake of accuracy, five or ten independent assays were made in each case. The number of generations of growth, g, was determined for each of the cultures from the values N_1 and N_2.

Where the mutation rate per bacterium per division cycle is a, and the rate per bacterial division is a/(ln 2), the proportion of mutant bacteria in a culture will rise during growth by a fixed increment of a/2(ln2) per generation, provided the inoculum is of sufficient size so that there are no appreciable statistical fluctuations in the numbers of mutations occurring in the first division. Thus mutation rate can be obtained from the formula

$$a = 2(\ln 2)(r_2/N_2 - r_1/N_1)/g. \quad (3)$$

TABLE 2

Mutation rates of B/r to resistance to phage T1, calculated from the increase in the proportion of resistant bacteria in cultures grown from large inocula (method 3 of this paper). Short growth period.

TEST CULTURE	A	B	C	D	E	INOCULUM
Replicate assays	10	10	10	10	10	10
Inoculum, bact., $\times 10^8$	26	26	26	26	26	—
Vol. of culture, cc	50	50	50	50	50	—
Incr. in no. of bact.	114×	75×	112×	135×	46×	—
Resist. bact., $\times 10^{-8}$	19.6	20.8	24.4	20.9	15.4	7.0
Standard deviation	3.7	4.7	2.6	3.5	2.7	1.3
Generations growth	6.8	6.2	6.8	7.1	5.5	—
Incr. resist. b., $\times 10^{-8}$	12.6	13.8	17.4	13.9	8.5	—
Mutation rate, $\times 10^{-8}$	2.6	3.1	3.5	2.7	2.1	—

TABLE 3

Mutation rates of B/r to resistance to phage T1, calculated from the increase in the proportion of resistant bacteria in cultures grown from large inocula (method 3 of this paper). Long growth period.

TEST CULTURE	F	G	H	I	J	INOCULUM
Replicate assays	5	5	5	5	5	10
Inoculum, bact., $\times 10^8$	2.1	2.1	2.1	2.1	2.1	—
Vol. of culture, cc	300	300	300	300	300	—
Incr. in no. of bact.	3600×	2910×	2780×	2770×	3470×	—
Resist. bact. $\times 10^{-8}$	37.3	30.3	29 8	42.4	24.7	4.4
Standard deviation	6.9	3.9	3.5	6.2	5.6	1.1
Generations growth	11.8	11.5	11.5	11.5	11.8	—
Incr. resist. b., $\times 10^{-8}$	32.9	25.9	25.4	38.0	20.3	—
Mutation rate, $\times 10^{-8}$	3.9	3.1	3.0	4.6	2.4	—

The data from two sets of experiments (involving 6 generations and 11 generations of growth) are given in tables 2 and 3, together with the mutation rates obtained. These rates average 2.8×10^{-8} and 3.4×10^{-8}, respectively—values which are not appreciably different from each other, or from those obtained by method 2, using the average number of resistant bacteria per culture in cultures started with small inocula.

Since there is no question of a statistical bias in calculating mutation rate by method 3, the agreement may be interpreted as confirming the higher

value obtained by means of method 2. This is important, inasmuch as there has been no estimate of the magnitude of the bias in method 2, or of the extent to which the discrepancy between methods 1 and 2 is a product of it.

The above experiments demonstrate that this discrepancy is due to biological rather than to statistical causes.

There are two reasons for measuring mutation rate over periods of 6 and of 11 generations. First, had it been measured over the shorter period only, it would be possible for the results to be biased by a high mutation rate during the first generation or so. The obtaining of similar estimates of rate from both a short and a long period, however, eliminates this as an appreciable source of error.

Second, formula (3) assumes that the mutants multiply at the same rate as the parent strain. That this assumption is approximately true is indicated by certain experiments of of LURIA and DELBRÜCK and later of DEMEREC and FANO. Somewhat more critical evidence however is obtainable from experiments such as the above in which close agreement between estimates of mutation rate from short and from long periods of growth indicate that there is no appreciable bias arising from differential increase during logarithmic growth. A mathematical demonstration of this will be found in a paper by SHAPIRO (1946).

For present purposes it is sufficient to state the argument in general terms. From formula (3) it is evident that mutation acting alone causes the proportion of mutants r/N to rise arithmetically with each successive generation. If, however, the mutants were to increase more (or less) rapidly than the parent strain there would be superimposed upon this an exponential increase (or decrease) in r/N, and estimates of rate obtained using formula (3) would tend to rise (or fall) correspondingly with increasing periods of exponential growth. Thus it is clear that close agreement between estimates of mutation rate over periods of 6 and 11 generations constitutes evidence that the high estimates obtained using methods 2 and 3 are not the result of a differential favoring the mutant during periods of logarithmic growth.

It would be quite possible to supplement the above evidence on the relative rates of increase of mutant and non-mutant strains, by preparing mixed cultures and determining the change in proportion which takes place as the result of competitive growth, as in the above mentioned experiments of LURIA and DELBRÜCK, and DEMEREC and FANO. However the results of such experiments are not entirely critical in the case of the spontaneously occurring mutants, since the mutant strain must first be selected by growing in the presence of phage, and then must be freed from all phage particles by suspending a resistant colony in liquid, streaking the suspension on agar, and incubating until visible colonies are formed, the process being repeated a number of times. Such prolonged growth offers considerable opportunity for further mutation and selection, and it is impossible to be certain that the strain which is finally obtained will not have changed with regard to its ability to compete with the non-mutant.

This difficulty may be avoided, however, by the use of radiation induced

mutants of a similar kind, since these occur much more frequently, and cultures containing mutants and non-mutants in suitable proportions for competitive growth experiments can be obtained without the necessity for first isolating the mutant strain. Evidence has been obtained in this manner in connection with a separate study (NEWCOMBE and SCOTT) which will be published later. Six independent mutant clones were tested over prolonged periods of growth (18 to 20 generations) in competition with the corresponding non-mutant strain. In some cases the proportion of mutants was found to remain unchanged and in others to have declined slightly, the factors of change ranging from 1.20 ± 40 down to $0.16 \pm .05$. In no case did the proportion of mutants increase significantly during growth.

Thus, provided one assumes that the radiation induced mutants are identical to those occurring spontaneously, evidence from this source is in agreement with the above conclusion that the high estimates of mutation rate from methods 2 and 3 cannot be due to a differential favoring the mutants during the period of rapid growth.

These experiments do not eliminate, however, the possibility that during the approach to saturation, when the environment has altered considerably, there may be conditions favoring the mutant strain. An increase in the proportion of mutants during this period of approximately six times, would be sufficient to produce the observed high estimates of mutation rate arrived at from the numbers of individual resistant bacteria.

In order to test this possibility, an experiment was designed in which the conditions associated with the approach to saturation could act repeatedly on a bacterial population passing through approximately five to six generations, thus accentuating any selection.

Five replicate test cultures, each containing in the first instance one cc of broth, were incubated over a period of three days, during which time the amount of liquid medium was doubled at regular intervals and finally brought up to fifty cc. If the approach to saturation in the test cultures used in connection with methods 2 and 3 is accompanied by a six-fold increase in the proportion of mutants, one would expect from the above treatment an increase of much more than six times, in addition to any mutants which arose from spontaneous mutation. Also, estimates of mutation rate obtained from these cultures using method 3, should be very much greater than the corresponding estimates from normal test cultures.

Such estimates of mutation rate from the above experiment are presented in table 4. It will be seen that these are not appreciably increased by keeping the cultures under conditions approaching saturation throughout the whole of the growth period.

In evaluating these data it will be noted that all assays are of numbers of viable bacteria, and that if there is appreciable death due to the conditions of growth, the number of cell generations will be underestimated. This problem is considered in detail in a later section, where it will be shown that the effect of undetected cell death is to increase the estimate of mutation rate obtained, to a value above that of the true rate. Thus the experiment is weighted against

the argument, and the results can be considered as critical evidence that there are no appreciable differentials favoring the mutants during the latter part of the growth of a culture.

The conclusions from the experiments discussed above are applicable to differentials, both of division and of survival, and eliminate all possibility that the widely different estimates of mutation rate obtained using method 1

TABLE 4

Mutation rates of B/r to resistance to phage T1, calculated from the increase in the proportions of resistant bacteria in cultures grown from large inocula (method 3 of this paper). Entire growth under conditions approaching saturation.

TEST CULTURE	K	L	M	N	O	INOCULUM
Replicate assays	5	5	5	5	5	5
Inoculum, bact., $\times 10^8$	29.1	29.1	29.1	29.1	29.1	—
Initial vol. of cult., cc	1	1	1	1	1	—
Final vol. of cult., cc	50	50	50	50	50	—
Incr. in no. of bact.	33×	24×	48×	60×	46×	—
Resist. bact., $\times 10^{-8}$	15.9	15.5	17.2	18.7	21.6	5.0
Standard deviation	2.0	2.9	1.3	1.2	1.8	0.7
Generations growth	5.1	4.6	5.6	5.9	5.5	—
Incr. resist. b., $\times 10^{-8}$	10.9	10.5	12.2	13.7	16.6	—
Mutation rate, $\times 10^{-8}$	4.3	4.6	4.4	4.6	6.5	—

on the one hand, and methods 2 and 3 on the other, are due to such differentials.

Method 3 thus provides critical confirmation of the estimates obtained by method 2.

VARIATIONS IN MUTATION RATE WHICH WOULD BE REQUIRED TO EXPLAIN THE DISCREPANT ESTIMATES FROM METHODS 1 AND 2

It thus seems certain that the high estimates of mutation rate from the numbers of resistant individuals are not the product of an upward bias, and we may now turn to the possibility that the low estimates from the numbers of resistant clones are due to a downward bias. Two alternative possibilities have been suggested which would account for these lower values: (1) a change to a low rate of mutation during the later part of growth, and (2) a delay in the phenotypic expression of a mutation.

The problem of distinguishing between these will be simplified if we consider the first and determine the time at which the supposed transition would have to occur, and whether this time is related to the number of generations from resting stage or to the approach to saturation.

To determine whether the supposed transition would be a function of the number of generations from resting stage the data of table 1 may be used. Half the cultures were inoculated with 10 and half with 10,000 bacteria, and

these passed through 25 and 15 generations respectively (the relevant data are given in table 5). The mutation rates calculated by methods 1 and 2

TABLE 5

Generations between resting stage and the approximate time of the first mutation in series of liquid cultures started from widely different inocula showing that mutation rate is independent of this variable over the range of 6 to 18 generations. (Data from table 1.)

EXPERIMENT	A	B	C	D	E	F	G	H
Inoculum (no. of bact.)	10	10	10	10	10^4	10^4	10^4	10^4
End no. of bact. $\times 10^8$	3.1	4.6	2.4	2.8	4.2	3.7	3.2	3.8
Generations growth	24.9	25.5	24.6	24.7	15.4	15.2	15.0	15.8
Max. resist. b. in series	447	221	158	79	242	376	725	263
Gen. after first mutation	8.8	7.8	7.3	6.4	8.0	8.6	9.5	8.1
Gen. to first mutation	16.1	17.7	17.3	18.3	7.4	6.6	5.5	7.7
Mut. rate (method 1), $\times 10^{-8}$	.42	.28	.59	.53	.31	.34	.40	.33
Mut. rate (method 2), $\times 10^{-8}$	5.1	1.5	3.8	2.1	2.1	4.5	5.8	3.9

showed no effect due to the difference in number of generations from resting stage. Thus the time of the supposed transitions would have to be a function of the approach to saturation, and not of the number of generations from resting stage. This means that mutation rate would have to be of the order of 3×10^{-8} during the whole of the logarithmic growth phase, dropping to something like $.4\times10^{-8}$ during the last few divisions.

It will be shown later that there are apparent variations in mutation rate during early growth, but that they are associated with the first few divisions after resting stage and occur too early to have any bearing on the immediate problem.

It is thus evident that the possible effect of a low mutation rate during the later part of growth can be eliminated by confining one's tests to the period of rapid growth; and critical evidence for or against a possible delay can be obtained from estimates of mutation rate based on the number of resistant clones appearing during rapid growth.

EVIDENCE FOR A DELAY BETWEEN MUTATION AND PHENOTYPIC EXPRESSION (METHOD 4)

It now remains to determine the rate of appearance of resistant clones during rapid growth, when no approach to saturation is involved.

If the rate of appearance of resistant clones is high (3×10^{-8}), the discrepancy between methods 1 and 2 can be interpreted without assuming a delay between mutation and phenotypic expression. If, on the other hand, rate of appearance of resistant clones is low ($.4\times10^{-8}$), then from the previous evidence the number of individuals in an average resistant clone must be greater than expected on the basis of the number of generations passed through after

its first appearance. This would be interpreted as indicating that the mutant clone had its origin one or more divisions prior to its becoming phenotypically detectable.

One obvious alternative to this interpretation should be mentioned, namely that the excess numbers of mutants are due to more rapid division in these than in the parent strain. This is rendered unlikely however by the evidence of DEMEREC and FANO (1945) that these mutants do not divide more rapidly, and in addition more critical evidence against the possibility has been obtained from the experiments described under method 3.

The experimental procedure was essentially that used by DEMEREC (1946) in his work on mutation rates in *E. coli* following irradiation. Bacteria are grown on agar for varying periods of time, sprayed with phage, and incubated until colonies appear. Mutations occurring during growth, and gaining phenotypic expression, will give rise to resistant clones. Since individual bacteria cannot move about on the agar, the members of a clone are confined to a particular locality. These resistant clones survive the application of phage, and eventually form colonies of visible size. Thus each mutation which gives rise to phage resistance is in the end represented by one colony.

An estimate of mutation rate is obtained by dividing the number of resistant clones appearing in a given period by the number of bacterial divisions times $1/\ln 2$. Thus, if R_1 and R_2 are the numbers of resistant clones present at times 1 and 2, the number of resistant clones arising during the interval between times 1 and 2 is $R_2 - R_1$. Similarly, if N_1 and N_2 are the numbers of bacteria present at times 1 and 2 respectively, the increase during the interval will be $N_2 - N_1$. Since each division of a bacterium increases the total number by one, this value is equal to the number of bacterial divisions during the period. Mutation rate per bacterium per division cycle, a, will therefore be obtained from the formula:

$$a = (\ln 2)(R_2 - R_1)/(N_2 - N_1). \tag{4}$$

Since the values of R_1, R_2, N_1 and N_2 represent viable cells only, an assumption is involved, namely that all bacteria divide. It will be shown later that this assumption is approximately correct for the early stages of logarithmic growth, and it is assumed that no appreciable increase in the proportion of cells which fail to divide, takes place until the phase of declining growth rate is approached. In these experiments precautions were taken to ensure that growth is limited to the period of exponential increase.

It will also be shown that the effect of the presence of cells which do not divide further, will be to increase the estimated rate of mutation. The present experiments can therefore be considered critical if the estimates of mutation rate obtained using method 4 are found to be low relative to those obtained using methods 2 and 3.

Estimates of the values of R_1, R_2, N_1, and N_2 are obtained as follows. Four plates are inoculated with a suitable number of bacteria, two being incubated until time 1 and two until time 2. One plate from each incubation period is sprayed with phage and then incubated further. The numbers of colonies

developing on these plates represent the numbers of resistant clones present at the time of spraying, that is, R_1 and R_2, respectively. The remaining plate from each incubation period is washed with ten cc of normal saline and the numbers of bacteria present (N_1 and N_2, respectively) determined by colony counts.

Where time 1 is the time of plating the bacteria, and no divisions can have taken place, N_1 is determined in a more direct manner. Instead of plating and then washing off the bacteria plated, an equivalent quantity of the culture from which the inoculum was taken is diluted and colony counts made.

An estimate of mutation rate is thus obtained from four plates. In all experiments these four plates were replicated several times, and a corresponding number of independent estimates of rate obtained. These independent estimates have been averaged, and the standard deviations calculated.

In all experiments in which growth was determined by the use of duplicate plates, care was taken to ensure the same amount of growth on both plates. All plates were warmed in the incubator before plating the bacteria, and when removed for plating were kept warm on a thermostatically controlled warm table until returned to the incubator. All platings and removals from the incubator followed an accurately timed schedule. The temperature in the incubator was kept as uniform as possible by circulating the air rapidly with fans. At the end of incubation, growth was stopped abruptly by chilling plates in contact with the cold metal of a refrigerator freezing unit. This chilling did not affect the survival of the bacteria or the phenotypic expression of the mutants.

Additional precautions were required with respect to (1) the choice of cultures from which to inoculate the plates, (2) the number of bacteria plated, and (3) the amount of phage applied by spraying.

(1) When relatively large numbers (of the order of 10^8) of bacteria are plated, there will be a certain number of resistant cells in the inoculum. These resistant cells have occurred by mutation during the growth of the culture from which the inoculum is taken. Cultures vary widely in the number of mutants present at the end of growth, and in these experiments the number present in the inoculum (R_1) was determined by spraying with phage immediately after the bacteria had been plated. Where the number is excessive it is apt to obscure the increase in resistant clones resulting from growth, or to render the determination of the increase less accurate. For this reason, cultures having an excessive number of resistant cells were not used as inocula. The cultures that were used contained from 5 to 50 resistant bacteria per 10^8 sensitive.

There is no evidence that this selection biased the results, since mutation rates obtained using these inocula were the same regardless of the number of resistant bacteria present.

(2) The size of the inoculum was adjusted so that the end number of bacteria on the plate would be approximately 2×10^9. With end numbers of less than this the number of resistant colonies was reduced, and at the same time the accuracy of the method. With excessively large end numbers of bacteria there is a reduction in the apparent mutation rate. The precise interpretation

of the phenomenon is uncertain. In the absence of evidence to the contrary it has been assumed that mutations do occur at the normal rate, but that they fail to develop visible colonies owing to the presence of large numbers of sensitive bacteria that are not lysed by the phage—a situation which occurs if phage is applied after bacterial growth has passed the logarithmic phase.

Tests showed that this apparent reduction in mutation rate occurred only if the end number of bacteria exceeded 5×10^9.

(3) It is known that bacteria which are infected during rapid growth have a latent period of 13 minutes—at the end of which time they burst, liberating on the average 180 phage particles (DELBRÜCK and LURIA 1942). If some of the bacteria fail to be infected at the time of spraying, it is unlikely that they will escape infection once lysis of the others starts. Any uninfected individuals would, on the average, pass through somewhat less than one division during the 13-minute latent period. Thus, with a large proportion of the bacteria uninfected at the time of spraying, a somewhat less than twofold increase in population would be expected before all the bacteria became infected, and the apparent mutation rate from such an experiment would be increased proportionally. Where all but a small proportion of the bacteria are infected at the time of spraying, the apparent mutation rate would not be appreciably greater than in the case of 100 percent infection.

In the present experiments the number of phage particles applied was equal to the number of bacteria, or slightly in excess. Large excesses were not used since these involve long periods of spraying, with resultant wetting of the surface of the agar and a tendency for the bacteria to be moved about by the moisture. Larger numbers of phage particles have been used, however, by BEALE (1948), who concentrated the phage by centrifuging. The estimates of mutation rate that he obtained in this manner do not differ appreciably from those obtained in the present experiments, and it may be assumed that the quantities used in the latter were adequate.

By the method described in this section it was possible to determine the rate with which resistant clones appear during the period of rapid growth from the resting stage onward.

The results of four separate experiments, each with eight independent replicates, are given in table 6. In these experiments the bacteria were grown over a period of approximately eight generations; and the mutation rate obtained is that for the whole growth period.

It will be seen that the average of estimates of rate from all experiments is low ($.59 \times 10^{-8}$) and is in close agreement with the low estimates from method 1 (average $.40 \times 10^{-8}$). As pointed out earlier, this constitutes evidence that resistant clones arise from mutations occurring one or more generations prior to their first becoming detectable.

It should be mentioned at this point that subsequent experiments, described in the next section and summarized in figure 1, have enabled mutation rate to be calculated from a tenfold increase in bacterial titer onward. This eliminates the contribution of the first few divisions, during which an excessive number of

TABLE 6

Mutation rate of B/r to resistance to phage T1, estimated from the number of resistant clones appearing during bacterial multiplication on agar (method 4 of this paper).

EXPERIMENT	A	B	C	D
Inoculum (no. of bact.), $\times 10^8$	.115	.119	.106	.055
Resist. b. in inoc., $\times 10^{-8}$	30.2	30.2	10.0	38.0
Incr. in no. of bact.	264×	250×	345×	317×
Generations of growth	8.1	8.0	8.5	8.3
Replicates	Mutation Rate, $\times 10^{-8}$*			
1	.49	.54	.48	.53
2	.47	.61	.69	.38
3	.56	.70	.57	.49
4	.42	.49	.59	.71
5	.70	.54	.89	.50
6	.69	.46	.44	.52
7	.45	.51	.69	.63
8	.53	.66	1.04	.89
Av. mutation rate, $\times 10^{-8}$	.54	.57	.67	.58
Standard deviation	.098	.078	.205	.156
Mut. rate from 10× increase onward, $\times 10^{-8}$†	.45	.48	.60	.52

* Each replicate mutation rate is calculated from an independent single-plate estimate of each of the following four values: (1) number of bacteria in the inoculum, (2) end number of bacteria, (3) number of resistant bacteria in the inoculum, and (4) end number of resistant clones, using formula 4 of this paper.

† This mutation rate is calculated from a tenfold increase onward using the information in figure 1 and the method outlined in the section on method 4. It is a more accurate estimate of the rate of formation of resistant clones during logarithmic growth, since the bias from the high early rate of appearance of resistant clones is removed.

resistant clones appears, and reduces the average rate obtained from these experiments from $.59 \times 10^{-8}$ to $.51 \times 10^{-8}$.

RATE OF APPEARANCE OF RESISTANT MICROCOLONIES DURING THE EARLY DIVISIONS ON A SOLID MEDIUM

Where there is a delay between mutation and phenotypic expression such that the first phenotypically resistant individual appears n generations after the mutation has occurred, there will be 2^n bacteria in the mutant clone when it first becomes detectable. When a mutation occurs, a delay of n generations in the time of its appearance will correspond to a 2^n-fold increase in the population. The apparent number of bacterial divisions in which the mutation has occurred will then be 2^n times the true number, and when an estimate is made from the number of detectable mutant clones, the apparent rate will be reduced to $\frac{1}{2}^n$ of the true value.

From the interpretation here adopted, it follows that a sample from a culture will contain some mutant individuals which are not yet phenotypically resistant. When such a sample is spread on agar and allowed to grow (as in method 4) these hidden mutants develop phenotypic resistance during the first few divisions and thus give rise to resistant microcolonies. Since a mutant clone may contain a number of these hidden mutants, and since these are dispersed over the surface of the agar, a number of resistant microcolonies can

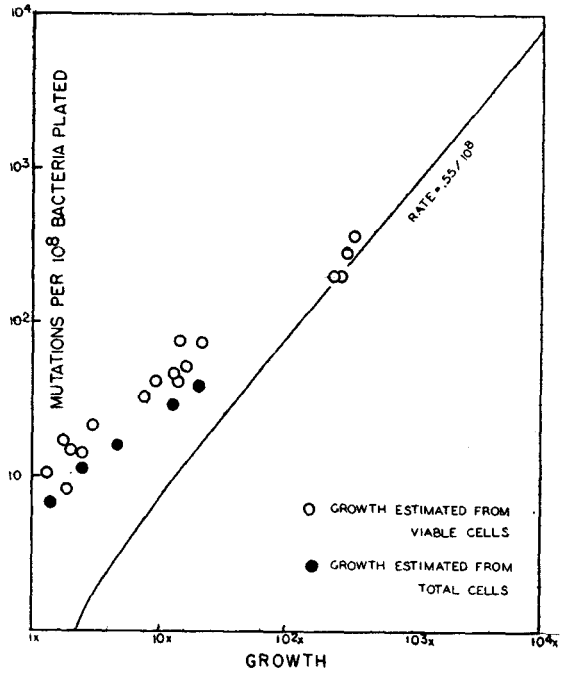

FIG. 1.—Numbers of mutations to phage (*T1*) resistance arising during bacterial multiplication, as estimated from the numbers of resistant microcolonies present after varying periods of growth.

result from a single mutation prior to plating. This is of course not true for mutations occurring after plating, because the products of these are confined to one locality.

Thus the rate of appearance of resistant microcolonies during the early divisions would be expected to be high, approaching the true mutation rate; and the rate of appearance during later divisions would be expected to be low, approaching 2^n times the true mutation rate.

Evidence has been obtained on this point, using method 4 and plotting the numbers of resistant clones arising during varying periods of incubation ($R_2 - R_1$, where time 1 is the time of plating) against growth in terms of factor increase in the number of bacteria (N_2/N_1). The absolute increase in resistant clones with growth depends, of course, upon the original number of bacteria plated, and where data are obtained from a number of separate experiments they are comparable only if expressed in terms of the number of individuals in

the inoculum. It is therefore convenient, when plotting a curve using data from a number of experiments, to express the increase in terms of resistant clones per 10^8 bacteria in the inoculum. This has been done in figure 1, where $(R_2-R_1)/N_1\times10^8$ is plotted against growth (N_2/N_1), time 1 being the time of plating. Each point shown in figure 1 was obtained by averaging at least eight independent estimates of increase in number of resistant clones and of growth.

Two experimental procedures for estimating growth were used in this case. One was that mentioned earlier, in which bacteria are washed from the plate and the number determined by dilution and assay. The other was by direct count of the numbers of bacteria in the developing microcolonies.

For the latter purpose small numbers of bacteria were plated on agar, and the plates were incubated at the same time and for the same period as those that were to be sprayed with phage. These growth assay plates were then chilled to stop division and examined under a high-power dry objective. The numbers of bacteria in 50 microcolonies were counted and averaged. The method gave accurate results up to an increase in number of bacteria of approximately 64 times.

In figure 1, points obtained by calculating growth by means of assay of bacteria washed from duplicate plates are shown as hollow dots. Those for which growth was calculated from the average number of bacteria per microcolony are shown as solid dots.

Something similar to the expected high early rate of appearance of resistant clones is observed in these results. In figure 1 the numbers of resistant clones appearing during growth are plotted on a logarithmic grid against growth expressed in terms of factor increase in numbers of bacteria. For comparison, a curve is drawn showing the expected numbers—assuming a rate of $.55\times10^{-8}$ and immediate phenotypic expression. The experimental curve does show a high early rate, declining as growth proceeds. This is qualitatively what would be expected where there is a delay, though the rate is actually higher than expected.

The increase in resistant clones when material is sprayed with phage after a twofold increase in population is approximately 12 per 10^8 bacteria plated, which represents an apparent mutation rate of 8.3×10^{-8}; whereas the rate expected during the first division is the same as that obtained from liquid cultures, that is, something between 2.8 and 3.4×10^{-8}.

Furthermore, by extrapolating the curve backward it would appear that about seven per 10^8 bacteria become resistant during the lag phase before any division has taken place.

This point has been studied by incubating bacteria with a known lag phase of 70 minutes for 30, 60, and 90 minutes on agar and then spraying with phage. The results are given in table 7, and show an appreciable increase in resistant colonies from plates sprayed just before the onset of the first division and during the very early part of the division.

The points just considered are not directly related to the main issue. The important contribution of these experiments is to show that the rate of appearance of resistant clones declines during the first few divisions on agar.

A METHOD OF REDUCING THE DOWNWARD BIAS IN METHOD 2 DUE TO THE DELAY IN PHENOTYPIC EXPRESSION (METHOD 5)

As mentioned in the introduction, a delay between mutation and phenotypic expression must be variable within a mutant clone, expression occurring in some lines of descent earlier than in others. This leaves open the questions of the extent of the delay, the nature of the variation, and the rate of gene muta-

TABLE 7

Increase in number of bacteria resistant to phage T1 during early growth, using strain B/r grown on agar, and spraying with phage during the lag phase and early part of the first division.

EXPERIMENT	A	B	C
Replicate plates	10	10	10
Bact. in inoculum, $\times 10^8$	11.0	11.0	11.0
Resist. bact. in inoc., per 10^8	10.1	10.1	10.1
Incubation, minutes	30	60	90
Incr. in no. of bact.	1.0×	1.0×	1.3×
New resist. bact., per 10^8	0.1	5.8	10.7
Standard deviation	1.89	1.87	2.05

tion, the last being to a greater or smaller degree underestimated by the methods dealt with so far. Method 5 is designed to obtain a less biased estimate of mutation rate, and, from this, some idea of the extent of the delay.

With a variable delay it would be expected that mutant clones arising from mutations early in the growth of a culture would contain a higher proportion of phenotypically resistant cells than would younger mutant clones, and that a higher and less biased estimate of mutation rate would be obtained if it could be calculated from these older clones alone.

To do this the following method has been devised. In a series of similar test cultures a few of the cultures contain many times the average number of resistant cells, because of the chance occurrence in these cultures of an early mutation. In such cases the precise numbers of resistant bacteria from the earliest mutation and from subsequent mutations cannot be determined directly. The probable number from later mutations, however, is approximately equal to the mean number in the whole series. This is, incidentally, a very slight overestimate, and a more precise approach will be considered later.

Using this method, the probable number of resistant bacteria descended from the earliest mutation in a series of cultures is obtained by subtracting the mean number of resistant bacteria per culture, r, from the highest number occurring in any one of the cultures, h. At the probable time of occurrence of this mutation the population in the culture would have been $N/(h-r)$, where N is the end population; and the population in the whole series of cultures would have been $CN/(h-r)$, where C is the number of cultures in the series. Thus the first mutation in the series occurred when the population was $CN/(h-r)$, and since the inocula were small the number of bacterial divisions

giving rise to this population would also be $CN/(h-r)$. The mutation rate, a, when one mutation occurs in this number of bacterial divisions is of course given by the formula:

$$a = (\ln 2)(h-r)/CN. \quad (5)$$

Mutation rate is very slightly underestimated by this formula, since r is an overcorrection for the probable number of resistant cells from mutations subsequent to the first. A more precise correction would be obtained by averaging the number of resistant cells per culture, exclusive of those from the first mutation in the series. This complication has not been introduced, however, since the gain in accuracy would not be appreciable.

The rates calculated in this manner may be considered as approaching the true rate if most of the members of these older clones have become phenotypically resistant. If only a small proportion have become resistant, this rate is still an underestimate, although it would be closer to the true rate of gene mutation than the estimates using method 2. This method will be known as method 5.

The rates obtained in this manner, using the data from table 1, are presented in table 8, together with the values required for the calculations. Data of

TABLE 8

Mutation rate of B/r to resistance to phage T1, calculated from the maximum number of resistant bacteria in any one culture of a series, using method 5 of this paper, and the data in table 1.

EXPERIMENT	A	B	C	D	E	F	G	H
Number of cultures	25	25	25	25	25	25	25	25
Bact. per cult., $\times 10^8$	3.1	4.6	2.5	2.8	4.2	3.7	3.2	3.8
Av. resist. b. per cult.	56.1	18.5	29.5	16.9	26.8	59.5	68.6	52.6
Max. no. resist. bact.	447	221	158	79	242	287	725	263
Mutation rate, $\times 10^{-8}$	3.6	1.3	1.3	.83	1.5	2.3	5.7	1.5

LURIA and DELBRÜCK, and DEMEREC and FANO, obtained using phage *T1*, have been treated similarly, and a detailed comparison is made in table 9 of rates from methods 1, 2, and 5, using information from all sources. For the sake of convenience, the rates obtained by methods 2 and 5 have been expressed in the last two columns of the table in terms of the smaller value from method 1, as ratios—rate (2)/rate (1), and rate (5)/rate (1).

Taking into consideration all available information on mutation to resistance to phage *T1*, rates from methods 2 and 5 differ only slightly and are between four and nine times the rates from method 1.

The application of method 5 has also been extended to the data obtained by DEMEREC and FANO using phages other than *T1;* and averages of mutation rates from methods 1, 2, and 5, and of the ratios rate (2)/rate (1), and rate (5)/rate (1), have been worked out for all available data on strains *B* and *B/r* and phages *T1*, *T3*, *T4*, *T5*, *T6*, and *T7*. They are given in table 9.

The purpose of these calculations was to determine whether mutation rate when estimated from the older mutant clones only is higher than when estimated from the average numbers of resistant bacteria per culture. If the delay is sufficiently variable and of sufficient magnitude, it would be expected that

TABLE 9

Mutation rates of strains B and B/r to resistance to phage T1, from series of similar liquid cultures started with small inocula, using methods 1, 2, and 5 and calculating from data of LURIA *and* DELBRÜCK *(1943, table 3, experiment 23),* DEMEREC *and* FANO *(1945, table 4), and the present paper (tables 1 and 8). All the experiments which are suitable for the application of the three methods have been used.*

EXPER.	STRAIN	CULTURES	RATE (1) $\times 10^{-8}$	RATE (2) $\times 10^{-8}$	RATE (5) $\times 10^{-8}$	RATIO (2)/(1)	RATIO (5)/(1)
LURIA and DELBRÜCK							
A	*B*	87	.32	2.4	1.5	7.5	4.4
DEMEREC and FANO							
A	*B*	53	.63	1.4	1.8	2.2	2.9
B		53	.33	2.3	6.1	7.0	18.5
C		17	1.15	3.9	9.2	3.4	7.9
D		25	.71	3.1	4.1	4.4	5.8
E		19	.66	3.2	3.0	4.8	4.5
F		38	1.26	6.1	7.8	4.7	6.2
G		45	.24	0.8	1.3	3.3	5.4
H		44	.63	3.2	6.0	5.7	9.6
I		45	.31	2.0	2.9	6.4	9.4
J		45	.92	1.3	1.3	1.4	1.4
		Average	.68	2.7	4.4	4.3	7.2
Present investigation							
A	*B/r*	25	.42	5.1	3.6	12.2	8.6
B		25	.28	1.5	1.3	5.4	4.4
C		25	.59	3.8	1.3	6.6	2.1
D		25	.53	2.1	8.3	4.0	1.6
E		25	.31	2.1	1.5	6.8	4.7
F		25	.34	4.5	2.3	13.2	6.7
G		25	.40	5.8	5.7	14.5	13.8
H		25	.33	3.9	1.5	11.8	4.6
		Average	.40	3.6	2.3	9.3	5.8

in these older clones a higher proportion of the mutants would have become phenotypically resistant.

Not all experiments show a difference between estimates of rate from the average resistant bacteria (method 2) and estimates from the highest number of resistant bacteria, of which the majority are presumably descended from an early mutation (method 5). Examining data from all sources, the estimates from method 5 and from method 2 do not differ significantly.

With the exception of the mutations to *B/3, 4* and *B/3, 4, 7*, estimates from method 5 are of the order of six to seven times those from method 1, and the lower ratios in the case of these exceptions are in all probability due to the high proportion of slow-growing *B/3, 4*, and *B/3, 4, 7* mutants—DEMEREC and FANO having shown in their experiments on competitive growth that these mutants grow much more slowly than the parent strain *B*.

A crude average of the ratio rate (5)/rate (1) from mutations other than those to *B/3, 4*, and *B/3, 4, 7* shows that estimates obtained by method 5

TABLE 10

Mean mutation rates of strains B and B/r to phage resistance, estimated by methods 1, 2, and 5; data of LURIA *and* DELBRÜCK, DEMEREC *and* FANO, *and the present investigation (table 9), all from series of similar liquid cultures started with small inocula.*

PHAGE	STRAIN	RESISTANT BACTERIA	NO. OF EXPER. AVERAGED	AVER. RATE (1) $\times 10^{-8}$	AVER. RATE (2) $\times 10^{-8}$	AVER. RATE (5) $\times 10^{-8}$	AVER. RATIO (2)/(1)	AVER. RATIO (5)/(1)	SOURCE
T1	*B/r*	*B/r/1,5; B/r/1*	8	.40	3.6	2.2	9.3	5.8	table 9
T1	*B*	*B/1,5; B/1*	1	.32	2.4	1.5	7.5	4.4	L. & D.
T1	*B*	*B/1,5; B/1*	10	.68	2.7	4.4	4.3	7.1	D. & F.
T5	*B*	*B/1,5*	5*	.38	1.5	2.6	4.3	7.5	D. & F.
T6	*B*	*B/6*	3	5.0	30.8	42.0	5.3	7.1	D. & F.
T3	*B*	*B/3,4,7; B/3,4*	7	5.1	10.0	12.5	2.3	2.9	D. & F.
T4	*B*	*B/3,4,7; B/3,4*	3	5.5	12.9	34.8	2.7	5.8	D. & F.
T7	*B*	*B/3,4,7*	3	4.4	12.2	9.6	2.8	2.4	D. & F.

* Note: One experiment containing an exceptional culture with 20,000 resistant bacteria has been omitted from these averages.

are approximately 6.4 times those obtained by method 1. The significance of this ratio in relation to the extent and variability of the delay between mutation and phenotypic expression will be considered in the discussion.

THE INFLUENCE OF DIVISION MORTALITY UPON ESTIMATES OF MUTATION RATE

All the methods of estimating mutation rate are subject to a small error because some of the bacteria resulting from a division fail to divide again (WILSON 1922). The number of mutations occurring during a division is thus underestimated, and the number of divisions, where calculated from numbers of viable bacteria, is also underestimated.

It might be assumed that the apparent variations in the mutation rate during the early part of the growth cycle are due to corresponding changes in the mortality associated with division. That this is not the case will be demonstrated; and corrections to be applied to mutation rates which have been based upon the assumption of unity survival will be considered.

Where growth has been estimated from the numbers of *viable* bacteria, a correction may be obtained in the following manner. Let S be the chance of

survival of one of the bacteria resulting from a division. In a population of N viable bacteria, one division will raise the number from N to 2NS viable individuals, and, where A is the true rate of mutation, will result in the production of ANS/ln 2 viable mutant individuals. If the increase in population is determined from assays of viable bacteria, the apparent mutation rate, a, during this division will be (ln 2) (ANS)/(ln 2) (2NS−N), which reduces to AS/(2S−1). The true mutation rate may thus be obtained from the apparent rate by the formula:

$$A = a(2S - 1)/S. \tag{6}$$

The relationship between the true mutation rate and an apparent rate, a_1, obtained by utilizing the increase in *total* population as distinct from viable population, may be derived in a similar manner and is expressed by the formula:

$$A = a_1/S. \tag{7}$$

Where estimates of mutation rate have been made in both ways, that is, using both viable population increase and total population increase, the value of S may be determined from the apparent mutation rates a and a_1. The formula for this as obtained from formulas (6) and (7) by simultaneous solution and is:

$$S = (a + a_1)/2a. \tag{8}$$

The true mutation rate also may be obtained from the values a and a_1, using equation (8) and substituting for S. This yields the formula:

$$A = 2a \cdot a_1(a + a_1). \tag{9}$$

These formulae may be applied to the data from figure 1 in order to determine whether the high apparent mutation rate during the first few divisions could be due to a low survival. It will be noted that points on figure 1 obtained by estimating growth from viable numbers of bacteria do not differ greatly from those obtained by estimating growth from total bacteria. This suggests that mortality due to division is not excessive, and we may calculate the chance of survival of the individual products of one division, by using formula (8). Thus, over the period from a twofold to a tenfold increase in population, the rates estimated from the two curves would be approximately 2.6×10^{-8} and 1.4×10^{-8}, respectively. From these two values the calculated survival is 0.75. Therefore the high rate of appearance of resistant clones during the early divisions on a solid medium discussed earlier cannot be due to an excessive division mortality.

It should also be noted that the estimate of rate which would have been obtained over this period of growth by either method, had survival been unity, is given by formula (9) and is 1.9×10^{-8}. This latter figure, however, has little bearing on the present discussion, as it is in any case intermediate between the estimates of rate obtained from numbers of resistant clones and from numbers of resistant individuals.

It is realized that this early low division mortality may not persist throughout the whole of the growth cycle but may rise as the population in a culture approaches the saturation density. In order to minimize the possibility of this effect, the end population of cultures grown on agar (method 4) was not allowed to reach that period at which growth rate begins to decline (approximately 5×10^9 bacteria on a 100 millimeter plate of broth agar). If, despite these precautions, there was an increase in division mortality, then as pointed out earlier, the estimates of mutation rate obtained would be somewhat greater than the true rate. It will be noted that this would tend to weight the data obtained by method 4 against the interpretation arrived at. We may therefore conclude that errors from this source do not affect the main conclusions.

DISCUSSION

From the experiments described, it is apparent that those methods which take into account only the number of resistant clones developing in a given period, and not the number of resistant individuals in these clones, give a low estimate of the mutation rate (see table 11 for summary).

Method 1 falls in this category, since it is based upon the proportion of cultures in which no phenotypic mutants have appeared—this being a function of the total number of phenotypically resistant clones in the series of cultures—and is independent of the size of the clone.

Method 4 also falls in this category, since the members of a mutant clone are confined to one spot on the agar and after one member has become resistant others may change in the same manner without being detected. Method 4, which uses the number of resistant clones appearing during rapid growth, provides a necessary check on method 1, which uses the number of resistant clones developing during growth to saturation, and which would be strongly biased if resistant clones appeared at a different rate during the few divisions prior to the cessation of growth. The estimates of rate obtained by these two methods are, within the limits of experimental error, similar, and in the present experiments—using resistance of strain *B/r* to the phage *T1*—average approximately 0.5×10^{-8}.

Methods that are based on the increase in numbers of resistant individuals developing during growth, however, as distinct from the numbers of resistant clones, produce much higher estimates of mutation rate. The following fall in this category: method 2, which uses the average number of resistant bacteria in a series of similar cultures started with small inocula; method 3, which uses the number of resistant bacteria in a single culture started with a large inoculum; and method 5, which uses the highest number of resistant bacteria in any one culture of a series.

Method 3, which is based on a relatively direct calculation, provides a necessary check on method 2, which is subject to a statistical bias. Similarly, method 5, which is based on the early mutations, provides a check on method 2, which would be biased if the delay in developing phenotypic resistance were excessive in an appreciable proportion of the lines of descent within a mutant clone. Method 3 is also of use in this connection in the one experiment in which

TABLE 11

Averages of mutation rates for strains B and B/r to resistance to phage T1 obtained by methods 1 to 5. Summary of calculations using data, or mutation rates already calculated, from the present investigation, LURIA *and* DELBRÜCK *(1943),* DEMEREC *and* FANO *(1945), and* BEALE *(1948).*

METHOD	FORMULA	MUTATION RATE, $\times 10^{-8}$ *Present*	L. & D.	D. & F.	BEALE
Estimates based on numbers of resistant clones					
(1) Series of liquid cult., no resist. b.	$a=-(\ln 2)(\ln P_0)/N$	0.40	0.32	0.68	—
(4) Solid medium, resist. clones	$a=(\ln 2)(R_2-R_1)/(N_2-N_1)$	0.51	—	—	0.49*
Estimates based on numbers of resistant individuals					
(2) Series of liquid cult., av. res. b.	$r=(aN/\ln 2)\ln(CaN/\ln 2)$	3.6	2.4	2.7	—
(3) Liquid cult. from large inocula	$a=(\ln 2)(r_2/N_2-r_1/N_1)/g$	3.1	—	—	—
(5) Series of liquid cult., max. res. b.	$a=(\ln 2)(h-r)/CN$	2.2	1.5	4.4	—
Early growth					
(4a) Solid med., res. clones, first div.	$a=(\ln 2)(R_2-R_1)/(N_2-N_1)$	8.3	—	—	—

Symbols:

a, mutation rate per bacterium per division cycle.

r, average number of resistant bacteria per culture.

h, highest number of resistant bacteria in any one culture of a series.

N, average number of bacteria per culture; N_1 and N_2, average number at times 1 and 2 respectively.

C, number of cultures.

R, average number of resistant clones per plate; R_1 and R_2, average numbers at times 1 and 2 respectively.

g, number of cell generations.

P_0, proportion of test cultures in which there are no resistant mutants.

* Note: the value $.7\times10^{-8}$ bacterial divisions obtained by BEALE has been converted to the rate per bacterium per division cycle by multiplying by ln 2 (=.693).

the growth period is long, and in which the majority of the resistant individuals are from mutations occurring many generations previous to the cessation of growth.

The estimates of rate obtained by these methods are all considerably greater than those which took into consideration only the numbers of resistant clones and not the numbers of resistant individuals, and differ only slightly among themselves. In the present experiments they average approximately 3×10^{-8}.

It is thus evident that the numbers of resistant individuals in a mutant clone must be greater than would be expected on the basis of the time of phenotypic appearance of that clone. One simple interpretation of this—namely,

that the growth rate is higher in the mutant clones than in the parent strain—is ruled out by the results obtained using method 3. The only plausible interpretation, therefore, seems to be that a mutation to resistance is not phenotypically expressed until after a number of divisions have taken place.

Two possibilities exist with regard to such a delay: (1) that after a fixed period of one or more generations all of the offspring of the original mutant become phenotypically resistant at the same time, and (2) that the delay is variable within the mutant clone, some lines of descent becoming resistant earlier than others. As pointed out by LURIA (1946), the presence of cultures having only one resistant bacterium eliminates the first of these. Thus the delay must be variable within the mutant clones, and a single individual may develop phenotypic resistance in the first instance, being followed later by other members of the clone.

As pointed out, the presence of phenotypically susceptible individuals in the younger mutant clones, and the occurrence of changes in these to phenotypic resistance, results in a high apparent mutation rate when the members of the clone are dispersed and the changes are thus rendered detectable in each individual, and in a low apparent mutation rate when the members of a clone are grouped together and only the first change to phenotypic resistance is detectable. In the early growth on agar a situation exists in which the members of mutant clones in the inoculum are dispersed over the plate but the products of subsequent divisions of individual bacteria are grouped together. In such circumstances the apparent mutation rate would be expected to be high during the first division, as in liquid cultures, and to decline during subsequent generations to the apparent rate obtained when only the numbers of resistant clones are considered. This, in fact, has been observed and lends support to the conclusions reached.

In this connection, however, it should be noted that the rate of appearance of resistant bacteria during the first division on agar following a resting stage is higher than expected, by a factor of approximately three or four. This does not affect the main line of reasoning, although it is of interest in itself and appears to be well substantiated by the data.

Attempts to interpret this as due to the bacteria's failing to adsorb phage during the early stages of growth after a resting stage have not been successful, since one would have to suppose complete failure of adsorption over a period of at least two generations.

Two possibilities remain: (1) that of a genuine high rate of mutation during this division, together with physiological conditions favoring immediate phenotypic expression, since otherwise the products of these mutations would not be detectable; and (2) that of physiological conditions favoring phenotypic expression of mutants which had not hitherto become resistant. So far, no methods have been devised to distinguish between these two possibilities.

Thus the rate of appearance of resistant clones during early growth on agar is in agreement with the concept of a delayed phenotypic expression, although an additional phenomenon appears to be involved.

The size of a mutant clone at the time of appearance of the first resistant in-

dividual may be obtained by comparing the estimates of mutation rate from method 1, utilizing the number of resistant clones developing in liquid cultures, with those from method 5, utilizing the number of resistant individuals in old mutant clones. Table 9 may be used for this purpose, and the ratio rate (5)/ rate (1) considered. If one omits the data on mutations to *B/3, 4* and *B/3, 4, 7*, because of the bias resulting from the known low growth rate of these mutants (DEMEREC and FANO 1945, table 7), the ratios in the cases of the remaining mutations appear to be similar. A crude average of these is 6.4. This indicates that there are approximately six or seven individuals in a mutant clone at the time of appearance of the first resistant individual; that is, that the delay between mutation and phenotypic expression in at least one member of the resulting clone is of the order of 2.7 generations.

The extent of the delay in individual lines of descent within a mutant clone other than the first one to become resistant, cannot be determined directly. However, the absence of any striking difference between the estimates of mutation rate obtained by methods 2 and 5 suggests that the delay is not excessive; and the similar values, 2.8×10^{-8} and 3.4×10^{-8}, obtained by method 3 with growth periods of six and eleven generations respectively, indicate that no very large proportion of a mutant clone changes to phenotypic resistance after six generations from the time of the mutation.

The possibility that the delay in expression of irradiation-induced changes is of the same origin as that observed in spontaneous mutations should be considered at this point, although a positive answer cannot be given, since strictly comparable studies of the induced changes have not yet been carried out, and, in particular, nothing is known of the numbers of resistant individuals developing in the late-appearing resistant clones resulting from irradiation.

If irradiation results in the immediate induction of gene changes similar to those occurring spontaneously, and the delay is one of phenotypic expression similar to that in untreated material, then the numbers of resistant individuals in the late-appearing resistant clones should rise rapidly after the clones first become detectable, because of susceptible members becoming phenotypically resistant, and should eventually approach the numbers in the resistant clones that appeared soon after treatment. Variation in the time of occurrence of phenotypic expression in different lines of the same mutant clone would also be expected in irradiated material.

Until information of this nature is obtained it is not possible to say with certainty that the observed delays in appearance of induced and spontaneous mutations are of the same origin. In the absence of more detailed information, one can only speculate on the basis of the variation between clones. Since in untreated material there is no striking difference between the apparent mutation rates as determined over periods of six and eleven generations (method 3), it would seem that six generations is adequate both for phenotypic expression in most of the mutants within a clone and also for expression in at least one individual in most mutant clones. In irradiated material an appreciable proportion of the resistant clones do not appear until after the sixth generation;

and it is therefore tentatively suggested that the two delays are not of the same origin.

It should also be noted that a delay in phenotypic expression has been observed in Paramecium following hybridization (KIMBALL 1937; SONNEBORN 1943; SONNEBORN and LYNCH 1934) and has been termed "cytoplasmic lag." This phenomenon is at least superficially similar to that described in the present work, in that "phenotypic expression . . . commonly appears first at slightly different times in different lines of descent within the clone"(SONNEBORN 1947). The extent of the delay appears to vary in Paramecium with the nature of the change, and in two instances of alteration in type there is no detectable delay. (These are the alterations from mating type I to mating type II in variety 1, and from mating type V to mating type VI in variety 3. For a discussion of this, see SONNEBORN 1947.) It will therefore be of interest to determine whether a similar diversity in the delay exists in bacteria, since the apparent differences in the mutations so far studied, all of which mutations are to resistance to one or another of the phages, are relatively small and can be explained as due to known differences in the growth rates of the mutants. Until more information of this nature is available it is not profitable to speculate further about whether "cytoplasmic lag" in protozoa and "delayed phenotypic expression" in bacteria have a common basis.

CONCLUSIONS

Evidence of a delay in the phenotypic expression of spontaneous bacterial mutations to phage resistance, using *E. coli*, strain *B/r*, has been obtained. This evidence comes from a comparison of the rate of appearance of phenotypically resistant clones during bacterial multiplication with the rate of gene mutation, the latter being estimated from the numbers of resistant individuals arising during similar periods of growth.

The rate of appearance of resistant clones per bacterium per division cycle is approximately 0.5×10^{-8}, whereas the numbers of resistant individuals correspond to a much higher rate of mutation, a rate of approximately 3×10^{-8}.

Since it is known that the mutants do not divide more rapidly than the parent strain, the excess number of individuals composing a mutant clone indicates that the clone was formed sometime prior to its becoming phenotypically detectable. Thus there appears to be a delay between mutation and phenotypic expression.

The possibilities of alternative interpretations of the data of previous workers on the basis of a statistical bias in one of the methods, and of a change in the mutation rate during the later part of growth, have been eliminated by using methods which are not susceptible to these sources of error.

A delay in phenotypic expression would be expected to result in a high rate of appearance of resistant clones during the first few divisions on agar, declining to the value 0.5×10^{-8} with further growth. This has been observed, although an additional phenomenon appears to be present which results in the appearance of a higher-than-expected number of resistant clones during the

first division. This phenomenon, although of interest in itself, does not affect the main conclusions.

It is estimated that phenotypic expression occurs in at least one member of a mutant clone approximately two or three generations after the mutation, and that in many cases it affects at first only one individual, others of the mutants becoming resistant during subsequent divisions.

Expression does not appear to be delayed in any appreciable proportion of a clone beyond approximately six generations after the mutation.

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