DELAY IN THE APPEARANCE OF CAFFEINE-INDUCED T5 RESISTANCE IN *ESCHERICHIA COLI*¹

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IT is well known that the application of a mutagen usually does not produce the mutant phenotype immediately. A delay may be inevitable, for the expression of a genic change should require metabolic changes in the cell which cannot occur instantaneously, and which in turn may await other processes such as nuclear segregation. Indeed, if a variant phenotype were to be shown to follow treatment immediately, a more probable interpretation according to the present-day viewpoint would be that the variant arose through a cytoplasmic change.

Most studies of delayed expression have been made with bacteria, mainly with *Escherichia coli*. Some of the factors which must be considered for their possible contribution to delay have been listed by WITKIN (1956): the delay could occur in (a) *gene mutation* itself, (b) *nuclear, or genic, segregation*, (c) *phenotypic expression* due to prerequisite metabolic activity, (d) *irregularity of the growth pattern* when the mutagen interferes with the division rate of cells in the culture, and (e) *irregularity in the onset of division* of the mutant cells. All of these have been invoked as probable sources of delay in one previous study or another.

Induced delays range from about one to three divisions for streptomycin resistance (NEWCOMB 1953) to some 12 divisions for the appearance of the mutant phenotype of phage resistance (DEMEREK and LATARJET 1946; WITKIN 1953) and for an auxotrophic back mutation (Demerec and Cahn 1953). Furthermore, widely different delays have been observed in the expression of a given phenotype depending upon variation in culture conditions (NOVICK 1956; LABRUM 1953) and upon dose of mutagen (RYAN 1954). NOVICK observed a delay with the chemostat in the appearance of T5 resistance which depended upon the growth rate of the culture. LABRUM found that full expression of T5 resistance had a delay varying from about three generations in a chemostat to about 12 generations upon nutrient agar plates. RYAN inferred from studies of back mutation to histidine independence that the factor of phenotypic expression in the delay was less than one generation when induced by small exposures to ultraviolet light, but that large exposures could cause delays greater than 30 generations. He suggested that the long delays were caused by the delayed onset in division due to injury. Furthermore, NEWCOMB (1953) suggested that the relatively long delays observed in the expression of phage resistance were due to a selection artifact in the plate culture method.

In view of the unusually long delays obtained for phage resistance with the

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plate culture method, as well as variations occurring in this and other systems with change in culture conditions and mutagen, it might be supposed that there can be no significant measure of delay. Nevertheless, it is possible to assume that different experimental methods should reveal the same factors contributing to the delay despite different growth conditions, and, indeed, under similar growth conditions and at low doses of mutagen these factors should contribute to the same extent.

Conceptually, it is convenient to group the factors contributing to the delayed appearance of the mutant phenotype in the following manner. After the mutagen is applied to a cell, the *initial* component of the delay is that time required to alter the state of the genetic apparatus so that the mutation of the gene occurs, or will occur without further intervention by the mutagen. Once in this state, *primary* genetic and physiological factors can add their contributions to the delay. If large doses of the mutagen are used, it is reasonable to expect a change in the delay period due to concomitant physiological effects of the mutagen upon the cells. This change, which to our knowledge has only been observed as an extension of the delay (RYAN), may arise because of direct physiological injury to the cell by the mutagen, as well as through secondary physiological effects generated by cell products and cell debris not present at low doses of the mutagen. At vanishingly small doses of mutagen the primary factors are expected to give a constant contribution to the delay regardless of the mutagen used.

With this viewpoint, although delay may have a complex basis in any particular kind of experiment, measurement of the primary component would be expected to give the same result under similar growth conditions. If the time taken to establish the mutated gene following the addition of mutagen in low dose is variable but relatively short compared to the total delay, then the delays measured by different kinds of experiments should give similar values. Thus induced delays would be expected to give values similar to those for delays in which the genetic change is brought in by transduction or recombination.

In order to obtain the primary component of the delay it is probably necessary to observe additional experimental restrictions. Transferring cells to an alternate growth medium may give a different delay, especially if the average cell size changes. We find, for example, that the average cell volume is increased about fivefold when *E. coli*, B or B/1,t is transferred from the steady growth state in a lactate medium to the steady growth state in nutrient broth. For the same reason it is advisable to maintain a culture within a constant growth phase during a given experiment.

The use of the chemostat, or another method of continuous culture, has several distinct advantages over standard plate culture methods for the measurement of delay periods. Bacteria are in a steady growth phase under constant environmental conditions, except for a small perturbation upon addition of the mutagenic agent. Samples of the culture withdrawn from the chemostat have a constant concentration, so that in studies of phage resistance it is easy to maintain a fairly uniform multiplicity of phage to bacteria. Again, the bacteria are dispersed in

liquid medium which prevents shielding of a part of the population from the phage, as occurs when phage is sprayed on culture plates containing micro-colonies. Thus the time of adsorption of the phage is more precisely determined in liquid media. Finally, in the subsequent growth of the phage-bacterial samples upon plates, only a small fraction of sensitive bacteria can survive infection, and these only for a short period of time. Since this fraction is constant, it does not affect the measurement of mutation rates or delay.

We report here measurements with the modified chemostat (KUBITSCHER 1954) of the delay in the appearance of resistance to the bacteriophage T5 by *E. coli*, strain B/1,t. We find a mean delay of nearly 3.4 generations, which agrees well with the measurement of HAYES (1957), who observed that the full expression of resistance in recombinants required some four generations of growth of the recombinant genotype. In addition, we find the delay appears independent of the growth rate of the culture, the concentration of the mutagen, caffeine, and is the same at 26°C and 37°C. In these experiments we used tryptophan to limit the growth rate of the cultures. We are indebted to Dr. A. NOVICK for the parent culture.

During the course of the experiments described in this paper we found two forms of the phenotype B/1,t/5 (resistant to the bacteriophages T1 and T5 and requiring tryptophan). The first of these, designated strain E (early), has a growth rate on nutrient agar which appears independent of the presence of T5 up to phage concentrations as great as 10^{11} ml⁻¹. The other, strain L (late), has a growth rate greatly reduced upon nutrient agar in the presence of large concentrations of T5. E strain bacteria grow to countable colonies within 16 hours at 37°C in the presence of 10^9 T5 particles ml⁻¹, but the L strain colonies are smaller and cannot be counted reproducibly at this time. We customarily counted L strain colonies during the second day of incubation since the count remained stationary from that time on. In the absence of T5 the growth rates of the two strains appear equal. These characteristics of the two strains are reproduced in their progeny. The L variant had been found previously by A. NOVICK (personal communication). In addition, he found that the effect of T5 upon the delay in the appearance of L strain colonies essentially could be nullified by the addition of iron to the nutrient agar.

We observe both strains to accumulate linearly in the chemostat before as well as after the addition of caffeine as a mutagen. Thus, in the chemostat both strains have growth rates equivalent to that of the parent culture. However, strain E predominates until the addition of caffeine to the growth tube, after which the L strain accumulates more rapidly. We have measured the mean delay in the appearance of resistance to the bacteriophage T5 for both of these strains.

Theory

In steady state operation, mutants accumulate linearly in the growth tube of a chemostat when their growth rate is equivalent to that of the parent strain (NOVICK and SZILARD 1950) provided the mutant population is but a small frac-

tion of the culture. The application of a mutagen to the culture increases the rate of appearance of mutants after a time lag (NOVICK and SZILARD 1952). Examples of this delay period are shown in Figures 5 and 6 where the initial linear portion of each curve is extended to intersect the asymptote to the final portion.

We shall now show that this intersection occurs at that time following application of the mutagen which is equivalent to the mean delay period required for the appearance of the mutant phenotype. Let $p(t)$ represent the distribution function giving the probability for the appearance of the mutant phenotype at a time t following the mutational stimulus. The mutational stimulus is considered to be that action of the mutagen upon the cell which initiates the chain of events leading to the mutant genotype. For the moment we neglect the spontaneous background mutation rate. We assume that in the presence of the mutagen, which is added to the culture at time zero, the mutational stimulus has a small probability μ of occurring in any cell in unit time. Then the differential number of mutants induced between times ϕ and $\phi + d\phi$ for which the mutant phenotype appears between times θ and $\theta + d\theta$ is given by

$$d^2m = \mu N p(\theta - \phi) d\phi d\theta, \quad (1)$$

provided $m \ll N$, where N is the bacterial concentration, and m is the concentration of mutant bacteria. However, the test for the presence of these mutants will usually occur at some later time t , when the concentration of mutants in the population will then be

$$m = \mu N \int_0^t d\phi \int_0^t p(\theta - \phi) d\theta \quad (2)$$

It is convenient to make the transformations $a \equiv t - \phi$ and $\beta \equiv \theta - \phi$. Then we can rewrite Equation 2 as

$$m = \mu N \int_0^t da \int_0^a p(\beta) d\beta; \quad (3)$$

and integration by parts gives

$$m = \mu N t \int_0^t p(\beta) d\beta - \mu N \int_0^t \beta p(\beta) d\beta. \quad (4)$$

For times greater than that required for the expression of, say, 95 percent of the mutants, for which it may be assumed that the probability distribution function $p(t)$ either vanishes or decreases more rapidly than t^{-2} , the number of mutants approaches the asymptotic value

$$m_a = \mu N t \int_0^\infty p(\beta) d\beta - \mu N \int_0^\infty \beta p(\beta) d\beta. \quad (5)$$

The intersection of this asymptote with the abscissa, $m_a = 0$, gives

$$t_a = \frac{\int_0^\infty \beta p(\beta) d\beta}{\int_0^\infty p(\beta) d\beta}. \quad (6)$$

But formally, Equation 6 is also the definition of the mean delay in the appearance of the mutant phenotype.

When the spontaneous mutation rate, μ_0 , is considered, then the number of mutants before the application of the mutagen is

$$m = m_0 + \mu_0 N t, \quad (7)$$

where m_0 is a constant. The asymptote to the curve after the mutagen has been added is

$$m_a = m_0 + \mu_0 N t + \mu N t \int_0^\infty p(\beta) d\beta - \mu N \int_0^\infty \beta p(\beta) d\beta \quad (8)$$

Again, these intersect at the mean delay time.

MATERIALS AND METHODS

All measurements were made with cultures of strain B/1,t in the chemostat growing in Friedlein medium with tryptophan added in growth-rate-limiting amount (DL-tryptophan 100 or 1000 γ /l). In any given experiment, growth rate, temperature, and medium (except for the addition of caffeine) were not disturbed, although these and other parameters were varied between experiments. Caffeine was added both to the culture vessel and nutrient solution, usually at 450 mg/l, after the steady growth state had been reached.

Unless otherwise indicated, the parent population and the T5-resistant fraction were determined by standard methods described by ADAMS (1950). In testing for resistant bacteria the phage-bacterial mixtures were maintained for five minutes at 45°C before plating in an attempt to obtain phage adsorption upon all sensitive cells before pouring and solidification of the agar. There was no detectible decrease in the viability of parental or T5-resistant cultures in nutrient agar in the sol state at 45°C within the first ten minutes. In these mixtures phage concentrations were in the range 4×10^9 to 10^{10} ml⁻¹.

The phage-bacterial mixtures were often plated both upon Difco nutrient agar supplemented with 3 gm/l yeast extract and upon this same medium additionally supplemented with FeCl₃·6 H₂O at 100 mg/l. The agar layer technique with an additional sterile layer poured over the culture layer was used in determining the number of cells, primarily to increase the distinguishability between E and L strain colonies. E strain counts upon nutrient agar were made the day after plating. These plates were recounted the following day to determine total resistant cells (E + L strains). The addition of iron to the nutrient agar gave almost the same counts (E + L) on both days.

In order to compute the mean delay, the data from a given experiment must be fitted with two straight lines (as shown, for example, in Figure 5). The initial linear response and the asymptote to the final portion of the curve were fitted to the data by least mean squares. A mutation rate of 1.42×10^{-8} hr⁻¹ bact⁻¹ was assumed for the initial linear response. This value, with a standard error of 0.045 mutants hr⁻¹ bact⁻¹, represents the average of six measurements of the spontaneous mutation rate. The slope of the asymptote was not assumed.

Following the introduction of the mutagen, a major difficulty in fitting the data is that of determining that initial point of the curve which may be considered to be indistinguishable from the asymptote to the tail of the curve. This difficulty

arises from the errors involved in determining bacterial concentrations by plating, and is in turn the major source of some of the larger errors estimated in Figure 1. In further discussion, it will be referred to as the "initial point difficulty".

RESULTS AND DISCUSSION

Figure 1 indicates the mean delay $\bar{D}$ observed for the E strain with chemostats operated under standard conditions: 37°C, caffeine added to a concentration of 450 mg/l, and the parent culture at 2 to 3×10^8 ml⁻¹. The variations shown are graphical estimates of the error of the individual delays determined in the following manner. For each experiment of the kind shown in Figure 5, the initial linear portion of the curve is replaced by a new line of reduced slope and reduced mean. The slope is reduced by the slope standard error, 0.045×10^{-8} mutants hr⁻¹ bact⁻¹. The mean is reduced by the standard error of the mean calculated from the data.

FIGURE 1.—The mean delays for the E strain observed under standard conditions.

Next the asymptote is replaced by a new line with reduced slope and reduced mean, but in this case the slope standard error is computed from the data. The intercept of these new lines gives a time shorter than the observed delay by approximately its standard error. In the absence of initial point difficulty preliminary calculations indicate that this estimate of the standard error is valid within 10 percent for our experiments. The standard error was also estimated in the same fashion by increasing the mean and the slope of both lines.

The data of Figure 1 are consistent with a constant delay, in terms of the number of generations (elapsed time/generation time) required for the expression of resistance to T5, for a generation time τ greater than three hours. One generation time is the time required to wash out a culture volume equivalent to that of the growth tube (NOVICK and SZILARD 1950); it is also the time required for the number of fissions which have occurred within the growth tube to be equivalent to the number of bacteria. Since a weighted average would contain the initial point difficulty, and thereby weight some data unduly, we present the unweighted average for the mean delay period for these data, $\bar{D} = 3.64 \pm 0.15$ generations for $\tau > 3$ hours.

An apparent increase in the delay was observed at generation times less than three hours. This increased delay is associated with the formation of filamentous bacteria at these more rapid growth rates. The filaments are readily demonstrated by microscopic observation, as well as by an apparent drop in plate count, but not in turbidity. Figure 2 indicates the plate count as a function of generation time in the presence of 450 mg of caffeine per liter. The formation of filaments also occurs at rapid growth rates in the absence of caffeine, but only rarely at generation times greater than $2\frac{1}{2}$ hours.

Our interpretation of the increased delay is that T5 resistance is recessive to sensitivity, that segregation of T5 resistance is not possible in a nondividing filament, and that the development of the new phenotype must await the formation of more or less normal (homokaryotic?) cells by the reduction of the filament to cells of customary size. The hypothesis that T5 resistance is recessive is consistent with the observation that T1 resistance is recessive (LEDERBERG 1949). If, instead, T5 resistance were dominant, the culture fraction of mutant bacteria would appear to be unduly great in the presence of filaments and the mean delay period would appear shortened at rapid growth rates.

Since the formation of filaments increases abruptly at generation times less than three hours, we consider only those data for generation times greater than three hours.

It was realized that T5 concentrations which are too low should give too small a value for the mean delay: some of the cells with latent (cryptic) resistance will not be exposed to T5 before resistance is expressed, and the measured level of T5-resistant cells will be falsely high. To test that our data were not subject to this error three different concentrations of phage were applied to successive samples from a single chemostat containing caffeine. The results, shown in Figure 3, indicate that the phage concentration of 4×10^9 ml⁻¹ was as effective as the higher

FIGURE 2.—The decrease in bacterial concentration, as determined by plate count, at rapid growth rates in the chemostat with a caffeine concentration of 450 mg/l.

A: limited with about 500 γ of L-tryptophan per liter.

Multiply ordinate by 10^8 .

B: limited with about 50 γ L-tryptophan per liter.

Multiply ordinate by 10^7 .

concentration of $1.5 \times 10^{10} \text{ ml}^{-1}$; however, the ten-fold dilution to $4 \times 10^8 \text{ ml}^{-1}$ gave a delay period which was obviously shorter. The corresponding multiplicities of infection were estimated to be about 5, 30, and 0.5 phage adsorbed per bacterium, respectively. The two-day plate counts, which are not shown, gave parallel results. These experiments indicated that the phage concentration customarily used during adsorption, $4 \times 10^9 \text{ ml}^{-1}$, was sufficiently high so that, within the accuracy of measurement, all cells with latent resistance were infected before division could ensue.

When the mean delay was measured by including the L strain colonies in the plate count it again appeared constant for $\tau > 3$ hours and not significantly different from the previous value for $\bar{D}$. This is indicated in Table 1 where the mean delay periods for the E strain and the E + L strains may be compared. A further comparison was made with delays for the L strain alone, the points being determined by difference between the one- and two-day plate counts on nutrient agar; again the delay agrees with that of the E strain. The E and L strains are similar also in that both appear highly resistant to T5: we were not able to demonstrate adsorption of T5 onto heat-killed cells of either strain in nutrient broth over a

period of four hours. However, the inhibition of the development of L strain colonies by T5 indicates an interaction, the nature of which has not been ascertained.

Measurement of the two strains together to determine the delay provides an advantage over a similar measurement for E or L strain alone. This advantage is not so much the increased number of resistant cells available, since these often must be diluted to avoid overcrowding of colonies upon plates, but results primarily from the increased angle at which the asymptote rises, which reduces the dispersion Δ in a measurement of the delay. When Δ is very small compared to

FIGURE 3.—The effect of several concentrations of T5. Data taken under standard conditions; $B/1,t$ at 2.0×10^8 ml $^{-1}$, generation time of 7.5 hrs. The concentrations during adsorption were the following: ●, 4.3×10^8 phage ml $^{-1}$; ○, 4.3×10^9 phage ml $^{-1}$; for each, $0.6 - 2.9 \times 10^7$ bact ml $^{-1}$; □, 1.5×10^{10} phage ml $^{-1}$ and $0.3 - 1.0 \times 10^8$ bact ml $^{-1}$. Caffeine added at time zero. A, from plate counts upon nutrient agar; B, from plate counts upon nutrient agar supplemented with ferric chloride.

TABLE 1
Measurements of mean delay periods

Experimental parameters varied	Range of generation times, hr.	Number of measurements	Mean delay, generations	Standard error, generations
All measurements under all conditions	3.2-16.6	70	3.42	.10
Measurements under standard conditions* only				
E strain on NA†	3.4-13.2	30	3.64	.15
E + L strain on NA	4.0-10.3	6	3.5	.2
E + L strain on Fe-NA‡				
Initial count	5.6-10.3	3	3.5	.0
Final count	5.6-10.3	3	3.0	.8
Standard conditions except for bacterial titer:				
1-2 × 10 ⁷ ml ⁻¹	3.5, 11.3	8	3.1	.3
Measurements at 26° C. (titers: 2.0 × 10 ⁸ ml ⁻¹ , 1.8 × 10 ⁷ ml ⁻¹)	7.7-16.6	8	3.6	.3
All measurements of				
E strain	3.2-16.6	41	3.53	.14
E + L strain	3.5-16.6	29	3.26	.13
L strain	3.5-16.6	17	3.45	.18

* Standard conditions: caffeine at 450 mg/l, 37° C., and B/1, t concentration 2-3 × 10⁸ ml⁻¹.

† Nutrient agar.

‡ Nutrient agar supplemented with ferric chloride.

the time over which the final asymptote is measured, then it can be shown that

$$\frac{\Delta}{D} \sim \frac{1}{\sin A}$$

where A is the angle of intersection between the initial linear portion of the curve and the asymptote, and D is the mean delay for that measurement. At 37° C the caffeine-induced rate to T5 resistance for both strains is greater than twice that for the E strain alone. However, consideration of the final entries in Table 1 shows that the standard error was reduced very little. A greater reduction may have been prevented by the initial point difficulty, which contributes error to D asymmetrically.

The delay also is interpreted to be constant, in terms of generations of growth of the culture, for the following ranges of parameters: caffeine concentration, 75 to 450 mg/l (Figure 4); temperature, 37° C and 26° C (Table 1); bacterial concentration, 10⁷ to 3 × 10⁸ ml⁻¹ (Table 1); and culture volume, 22 to 250 ml. Table 1 displays the mean delays and their standard errors under the assumption that each determination has the same validity. The constancy of the mean delay period at the two temperatures, 26° C and 37° C, agrees with the constancy, but not the extent of delay obtained for T1 resistance by WIRKIN (1953). Measure-

FIGURE 4.—The mean delay as a function of caffeine concentration at 37° C.

The generation times at which the delays were measured are indicated with the number of measurements at each time shown in parentheses when more than one measurement was made. Both E and E + L strain measurements were made. The solid horizontal line indicates the average 3.46 generations, of all measurements on the graph and the dotted lines the extent of the standard error, 0.10 generations.

ments with the chemostat have the advantage that growth rate and temperature can be uncoupled.

Figures 5 and 6 compare typical experiments at low bacterial concentration for 37° C and 26° C respectively, and also indicate the effect of iron upon the plate counts. At both temperatures the colony count increase occurring between the initial count (one day) and the final count (two days) is smaller upon medium supplemented with ferric chloride, because of the appearance of the L strain colonies in the initial count on iron-supplemented nutrient agar. It also may be noted that the spontaneous mutation rate is reduced from 1.4×10^{-8} bact⁻¹ hr⁻¹ at 37° C to about 0.5×10^{-8} bact⁻¹ hr⁻¹ at 26° C, while the relative reduction in the rate of mutation to the E strain in the presence of caffeine is even more marked, about 80 percent.

Figures 5 and 6 also show the advantage of reducing the bacterial density in the chemostat to about 10^7 ml⁻¹. Normally, with cultures of about 2×10^8 bact ml⁻¹, variants of greater growth rate appear within some 5 to 8 days, with the consequent displacement of the more slowly growing mutants from the chemostat (NOVICK and SZILARD 1950). This event terminates the experiment. But with reduced density, the parental strain B/1,t rarely is displaced within eight days by the more rapidly growing strain B/1,t/f, and displacement may be delayed for several weeks. Operationally, low bacterial concentrations permit a more precise

determination of mutation rates and mean delay periods with a single chemostat than would be possible with more dense cultures.

Finally, the data for all determinations averaged over unit intervals of generation time give the results shown in Figure 7. For generation times in excess of three hours the average of all mean delays (the E and E + L strain measurements), 3.42 ± 0.10 generations, can be seen to agree with the data, reinforcing the previous interpretation of a constant mean delay period. This result is at variance with Novick's study (1956), which gave delays of about two generations or less at comparable growth rates. (However, his measurements also included the use of antimutagens.) A shorter delay could be interpreted most simply as

FIGURE 5.—The T5-resistant population in a chemostat with a bacterial concentration of 1.5×10^7 ml $^{-1}$ at 37° C.

Generation time, 11.3 hr; caffeine, 450 mg/l. The two lower curves show the plate count increase between the initial and final counts on nutrient agar, the two upper curves for iron-supplemented agar. The lowest curve displays the appearance of the E strain only.

FIGURE 6.—The T5-resistant population in a chemostat with a bacterial concentration of 1.8×10^7 ml $^{-1}$ at 26° C.

Generation time, 16.6 hrs; caffeine, 450 mg/l. The two lower curves show the plate count increase between the initial and final counts on nutrient agar, the two upper curves for iron-supplemented nutrient agar. The lowest curve displays the appearance of the E strain only.

FIGURE 7.—The independence of the mean delay period with respect to generation time.

The data for all determinations have been averaged over unit intervals of generation time. The vertical bars indicate the extent of the standard error both above and below the average mean delay for the interval.

arising from the incomplete exposure of cells with latent resistance to bacteriophage. An alternative possibility is that the studies involve different strains of bacteria, but such an interpretation is not strongly suggested, since in both studies the spontaneous and caffeine-induced mutation rates are very similar.

The mean delay period of 3.42 generations is about half that usually observed for the mean delay to the phage resistant phenotype by the plate culture method. Thus these experiments also demonstrate that an artifact has been removed by using cultures in suspension at the time of application of the phage. This artifact was not present, of course, in the work of LABRUM (1953), who observed that the delay in T5 resistance induced with manganous chloride in a chemostat was not constant, but varied as the generation time was changed. As NOVICK (1955) has already pointed out, LABRUM's work is open to the difficulty that the bacteria were emerging from a long lag period, during which the mutagen had been applied, and the question of the irregularity in the onset of division now becomes predominant. However, our mean delay period can be seen to be in fair agreement with the delay measured for the expression of T1 resistance introduced by recombination (HAYES 1957), where full expression of resistance is delayed until the fourth recombinant generation. A more precise comparison of the two methods awaits the determination of the initial component of the induced delay and the number of divisions required after formation of the zygote to establish the initiation of the first division of the recombinant cells.

At this point it is tempting and customary, to speculate upon the immediate mechanisms controlling the delay period. Such speculation here would be premature, since our measurements of the delay have not established the delay in divisions of the newly-forming mutant, but have only measured the delay in terms of the average number of divisions undergone by the parent culture. Although the growth rate of the established T5-resistant population is equal to that of the parent culture, it cannot be expected *a priori*, that the growth rate of the fraction with latent resistance is also the same. At present the constancy of the delay, in terms of generations of growth, is as little understood as the constancy, in terms of chronological time, of the mutation rate in the same bacterium (NOVICK and SZILARD 1950).

However, the interpretation of a delay constant under a variety of conditions requires additional explanation beyond the allocation of time spent by the cell in, for example, nuclear segregation. In our estimation this constancy may imply that tryptophan limits the rate of transformation of the mutant to the same extent that it limits the growth rate of the cell. That is, just as tryptophan is required for bacterial growth ending in division, so is there a requirement for tryptophan by the newly formed mutant in order that the new phenotype may be expressed. Furthermore, this constancy of the mean delay period also implies that despite varying culture conditions, there is little or no change in the number of generations that are required for the cell to pass through nuclear segregation, or through any other state preliminary to the establishment of the mutant phenotype, when tryptophan limits the growth of the culture.

SUMMARY

When caffeine is added to the medium of a continuous culture (chemostat) of *Escherichia coli*, strain B/1,t, the mean delay period for the appearance of bacteria resistant to the phage T5 is 3.42 ± 0.10 generation times of the parent culture. No difference in delay is observed for several caffeine concentrations, bacterial concentrations, growth rates at two temperatures, and for the appearance of two mutant strains of T5-resistant bacteria, designated E and L, and differing in their growth rates in the presence of large concentrations of T5. These results indicate that the use of the chemostat has eliminated a selection artifact commonly observed when phage is sprayed upon cultures of agar.

In addition, for the case (these experiments) where the mutant culture has the same growth rate as the parent culture, it is shown mathematically that the mean delay period is measured by the time given by the intersection of two straight lines fitted to the data.

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