LATENT MUTANTS IN CHEMOSTATS[1]

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THE correlation of spontaneous mutation with active growth in cell culture has led in the past to a common tacit assumption that division is required for mutation, an assumption exposed by the expression of mutation rates as some function of division. A ready explanation was provided by the error (copy-error) hypothesis which views mutation as an error in gene duplication, and which predicts therefore, that mutation rate is proportional to the rate of cell division. However, in measurements of the spontaneous mutation rates of bacterial cultures in chemostats in which growth was limited with tryptophan, NOVICK and SZILARD (1950) found that spontaneous mutation to resistance to bacteriophage T5 appeared independent of growth rate. Attempts were made to reconcile this result with the error hypothesis by assuming compensatory processes (NOVICK 1955), but the absence of experimental confirmation of the latter has caused their finding to be regarded as paradoxical, rather than fundamental, in most reviews on mutation.

On the other hand, when glucose is used to limit growth rate the accumulation of caffeine-induced mutants is proportional to growth rate (KUBITSCHEK 1960), seemingly providing *prima facie* evidence for the error hypothesis. Nevertheless, the error hypothesis is shown in this report to fail to account satisfactorily for newly-forming mutants when glucose limits growth. Furthermore, the constancy of mutation rate when tryptophan limits growth is not an isolated case peculiar to spontaneous mutation but occurs also for caffeine-induced mutations.

An examination of the nature of the difference between results from tryptophan-limited and glucose-limited cultures has been made possible by the development of a method of delayed selection that permits, for the first time, measurement of concentrations of cells which are latent mutants for T5 resistance in the chemostat in the absence of accumulated mutations arising spontaneously after plating (plate mutants). Since plate mutant concentrations are insignificant, this method allows simultaneous measurements of concentration of expressed and of latent mutants (those cells in which the mutational process has been initiated, but which have not yet expressed the mutant phenotype), making possible a study of the kinetics of mutation. Previously, with the time-course measurement of the appearance of mutant colonies introduced by DEMEREC (1946) it had been necessary to assume latent mutant concentrations as a difference between the maximum yield and those mutations already expressed. Correspondingly, there has been no clear determination of latent mutant concentrations in later modifica-

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tions, including those treatments with chloramphenicol or other agents which interfere with protein synthesis (WITKIN 1956, 1958; DOUDNEY and HAAS 1958). A technique of delayed selection similar to ours was developed for the detection of marker replication in transformation experiments (EPHRUSSI-TAYLOR 1959). Our method leads to the operational definition of a latent mutant as a cell with wild phenotype and unspecified genotype, in which, as a result of exposure to mutagen, the mutation can be displayed later, either in this cell or in its progeny.

THEORY

Although latent mutants may be genetic mutants as well as cells in which genetic material may not yet have been affected, we can distinguish at present only two steps in the mutational process: cells of wild genotype n must become latent mutants l before phenotypic mutants m can arise. If these steps are reversible, $n \rightleftarrows l \rightleftarrows m$. The corresponding concentrations, denoted by N, L, and M, respectively, will depend upon the presence or absence of back reactions (including reversion), selection, and segregation. These factors when present individually give kinetics of accumulation of mutants which are qualitatively different, as described below. The cases we consider most appropriate to our findings are presented in detail. These cases do not include the possibility that the induction of latent mutants might be modified by premutational treatments (premutational damage) since experiments of this kind were not performed. Premutational damage might exist, but it is not revealed in our experiments at different growth rates in which there have been no significant differences in yields of latent mutants.

Case I: No reversion, selection, or segregation of mutational potential or mutant character: Addition of a mutagen will induce the mutational process in parent cells n in the chemostat with a probability μ per cell per hour (averaged over a cell cycle), depending upon the intensity or concentration of the mutagen. In the absence of cell death, the rate of induction of latent mutants is

$$-dN/dt = \mu N = \mu (N_o - L - M),$$

where N_o is the total concentration of cells in the chemostat. In our experiments $L, M \ll N_o$, so to good approximation

$$-dN/dt = \mu N_o.$$

The concentration of latent mutants changes at the rate

$$dL/dt = \mu N_o - dM/dt. \tag{1}$$

At first the latent mutant concentration rises at the rate of induction μN_o, since $dM/dt = 0$, due to the delay in expression of the mutant phenotype. During the steady state which follows, dL/dt vanishes, since production of new latent mutants is balanced by phenotypic expression of others from the pool of latent mutants which has accumulated. From this time on, according to equation 1, mutations are expressed at the rate of induction, μN_o.

Equation 1 also shows that the sum of the rates of accumulation of expressed and latent mutants, $M + L$, has the same value, μN_o, at *all* times after adding mutagen. Thus M and the sum $M + L$ both accumulate linearly, $M + L$ immediately upon addition of the mutagen, but M only after a delay equivalent to the mean delay period (KUBITSCHEK and BENDIGKEIT 1958).

Case II: Death of some phenotypic mutants or reversion to latent mutants or wild-type cells; no segregation or selection: Here the rate of death or reversion is proportional to the concentration M of cells with the mutant phenotype, becoming appreciable only after some time has elapsed. Thus mutants at first increase relatively rapidly, later more moderately. Previous observations by NOVICK and SZILARD (1950, 1951) demonstrated a linear increase in spontaneous and chemically-induced mutants resistant to bacteriophage T5. Thus, death or reversion of these phenotypic mutants appears negligible.

Case III: Loss of mutational potential, reversion to wild type, or death of some latent mutants; no segregation or selection: Let $f(\lambda)$ represent the fractional rate of loss of latent mutants at the growth rate λ. Then

$$dL/dt = \mu N_o - Lf(\lambda) - dM/dt. \tag{2}$$

As before, dM/dt remains zero for a time after the addition of mutagen. Initially, L is also negligible, so latent mutants again accumulate at the rate of induction μN_o, an approximation which is good for $L \ll \mu N_o/f(\lambda)$. The concentration of latent mutants again rises to a maximum value, L_∞, during the steady state which follows. Then, according to equation 2, the rate of expression of mutants is

$$dM/dt = \mu N_o - L_\infty f(\lambda). \tag{3}$$

Since L_∞ and $f(\lambda)$ are constant at a given growth rate, equation 3 shows that M is again asymptotic to a straight line (which, as in Case I, intersects the time axis at the mean delay period), but the rate of accumulation is less than that in Case I by an amount proportional to the loss of latent mutants at the growth rate λ.

Here, also, the sum $M + L$ no longer accumulates linearly after adding mutagen, as it did in Case I, but increases first at the rate of induction of latent mutants, μN_o, then more and more slowly until it becomes parallel to the rate of expression given in equation 3.

Case IV: Growth of latent mutant cells at a slower rate than nonmutants or expressed mutants; no segregation, reversion, or death: M and $M + L$ are curves of the same shape as in the previous case due to washout of some latent mutants from the chemostat. However, the mean delay period does not have a constant value unless latent mutant growth rates are always proportional to the growth rate of the parent culture.

Case V: Segregation of the mutational potential or mutant character; no selection or reversion: Segregation again gives rise to a constant latent mutant pool, but changes in growth rate would not be expected to affect the pattern of segregation. Thus, neither mutation rate nor the number of generations of delay in expression of the mutant phenotype would be expected to change.

MATERIALS AND METHODS

Chemostats were inoculated with *Escherichia coli* B or B/l,*try*$^-$. All chemostats contained the following concentrations of salts in gm/l: NH_4Cl, 1; Na_2HPO_4, 3.5; KH_2PO_4, 1.5; $MgSO_4$, 0.1. When the growth rate of strain B/l,*try*$^-$ was limited with tryptophan, additional concentrations were 0.5 mg/l L-tryptophan and 10.6 gm/l lactic acid neutralized with 3.5 gm/l NaOH (tryptophan-supplemented Friedlein medium). When growth rate was limited with a carbon source, usually 100 mg/l glucose or 150 mg/l lactic acid, L-tryptophan concentration was increased to 1 mg/l.

For most experiments, plates contained nutrient agar (Difco-Bacto) supplemented with iron (190 mg/l monosodium ferric ethylene diaminotetraacetate, a gift of the Geigy Chemical Corporation, Ardsley, N. Y.), which permits rapid development of visible colonies from all T5-resistant cells in the presence of the phage. For some experiments cells were spread upon an iron-supplemented salts-lactate-glucose agar: Friedlein medium plus 2 gm/l glucose and 1.5 gm/l agar (Difco-Noble).

Viable cell concentrations N were determined from chemostat samples diluted to give about 100 cells per plate. Plates were incubated at 37°C. At least three plates were used for each determination of total concentration and of mutant concentration.

Concentrations M of phenotypically resistant cells were measured by exposing chemostat samples to T5. Aliquots containing about 2×10^7 bacteria were spread upon plates with sterile glass rods and sprayed with about 5×10^9 T5 particles within ten minutes.

Concentrations $M + L$ of all cells capable of giving rise to resistant colonies upon plates (i.e., phenotypic plus latent mutants) were determined by spreading cells upon plates as above, and allowing growth to microcolonies during an incubation period at 37°C before spraying with T5. Upon nutrient agar the incubation period was three hours, upon salts-lactate-glucose agar four hours. The justification for the incubation procedure is given in the following section.

RESULTS

Delayed selection for latent mutants: When chemostat samples were plated and allowed to incubate before spraying with T5 (delayed selection), the number of T5-resistant colonies arising was observed to depend upon the period of incubation. A characteristic response was observed in tests of samples taken from more than a dozen different chemostats. The data in Figure 1 illustrate the general features of this response, which is independent of cell strain, medium, or mutagen. Three segments of the curve are discernible: mutant concentrations appear constant at first, then rise to a plateau (which seems to be atypically long in Figure 1), and finally increase sharply. This response can be understood as the accumulation of mutants from three different sources.

(1) The initial constancy is due to mutations *(m)* that were already expressed in the chemostat. When a culture is exposed to mutagen, the magnitude of this

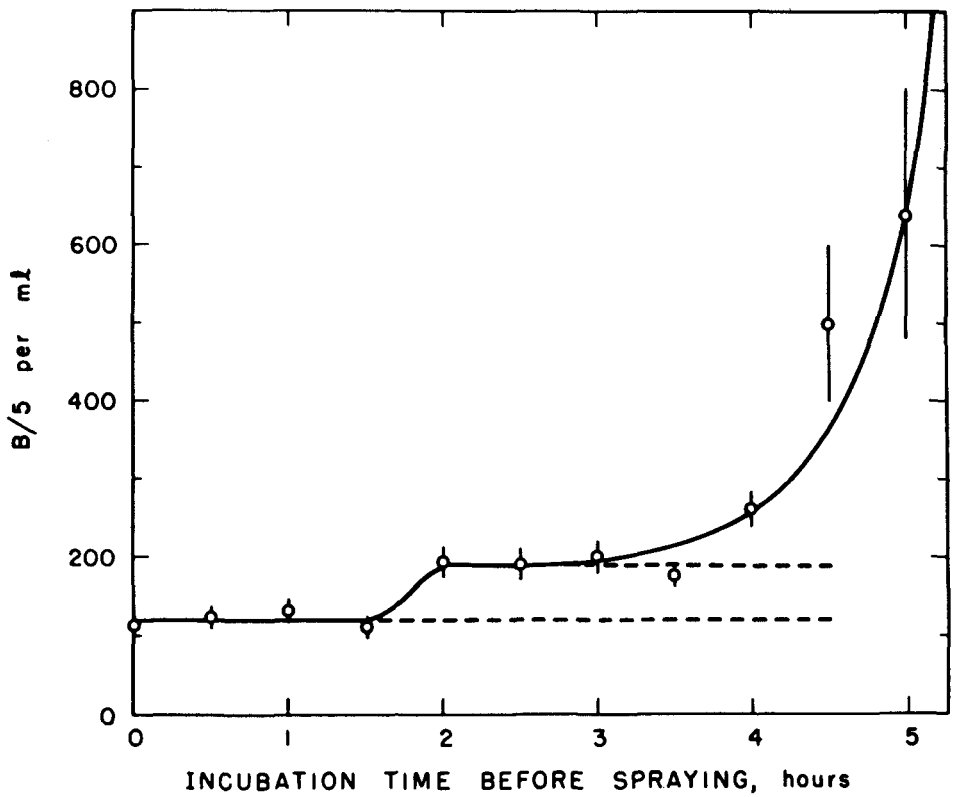

FIGURE 1.—Accumulation of mutants in a sample taken from a chemostat. *E. coli*, strain B, from a glucose-limited chemostat at 37°C; spread upon iron-supplemented nutrient agar plates and preincubated for times shown before spraying with T5. The bars above and below the points are calculated values of the standard errors from replicate plate counts or the Poisson standard deviation for the total number of colony counts, the larger of these two values being chosen.

initial level increases steadily, as expected for the accumulation of phenotypic mutants.

(2) The increase to the plateau is the expression of mutation after plating in the progeny of cells that were latent mutants *(l)* in the chemostat. This increase should rise to a constant value in a chemostat culture exposed to mutagen since latent mutants accumulate to a constant concentration, L_∞, during the steady state. The data of Figures 3, 6, and 7 (determined by spraying after a standardized incubation period of three hours) are in agreement with these predictions. The latent mutant concentration, L, is given by the difference between the corresponding upper and lower points in each figure.

(3) The final steep rise can be accounted for by mutations occurring spontaneously upon plates (plate mutants). During the early period of incubation plate mutants occur infrequently because of the relatively small number of cells upon the plates, and those that do occur probably fail to be expressed until after the lag phase. Later, there is an exponential increase in the number of cells in the

developing microcolonies. Since the probability of occurrence of a mutation in a colony is proportional to the number of cells it contains, the total number of mutant colonies increases exponentially. For *E. coli* B and B/l,*try*$^-$ the time required to double the number of cells on nutrient agar is about 20 minutes at 37°C. This value has been used to fit the final rise in Figure 1. The increase in the number of plate mutants at five hours of incubation might be no longer exponential because of a reduced division rate due to the overcrowding of cells upon the plates, but this would not affect the determination of latent mutant concentrations.

Confidence in this method for the detection of latent mutants is increased by the finding that essentially equivalent results were obtained when cells were plated upon salts-lactate-glucose agar. The characteristic response was observed, although both the latent mutant plateau and the final rise were longer due to the slower growth rate of cells upon this medium. Latent mutant concentrations were indistinguishable from those determined upon nutrient agar within a standard error of about 15 percent, since these concentrations were determined by difference between two kinds of plate counts.

The plateau for latent mutants from a chemostat containing caffeine is rather short when the cells are spread upon nutrient agar. Delayed selection experiments like that for Figure 1 were performed in order to determine the time at which all latent mutants became expressed. The data from several tryptophan-limited chemostat cultures of strain B/l,*try*$^-$ containing 0.45 mg/l caffeine are plotted in Figure 2 in terms of the fraction of latent mutants that had been expressed upon nutrient agar plates. According to this figure all latent mutants appear to be expressed within three hours. The rapid accumulations of plate mutants thereafter accounts for the variability observed in the number of latent mutants after four hours. Three hours was chosen as the optimum time of incubation before spraying. In most experiments a deviation of ten minutes would displace the value for latent mutant concentration no more than five percent, less than the usual errors involved in plating and counting.

Accumulation of caffeine-induced mutants in tryptophan-limited cultures: Figure 3 displays mutant accumulations in a tryptophan-limited culture to which caffeine was added at time zero and is representative of the results of three other experiments. The solid circles indicate mutant concentration M and are fitted with two straight-line segments intersecting at 3.4 generations, the mean delay period for phenotypic expression of T5-resistance (KUBITSCHEK and BENDIGKEIT 1958). The open circles indicate values obtained by the inclusion of latent mutant concentrations L, due to incubation of cells for three hours at 37°C before spraying with T5. The points for this curve are also fitted with two straight-line segments representing the steady state accumulations of mutants $M + L_\infty$, as discussed earlier, and are therefore drawn parallel to their corresponding members in the first curve. The steady accumulation of mutants in experiments of this kind agrees only with Case I, derived for the simple two-step reaction in which selection, segregation, and reversion are absent. In further support of Case I is the constancy

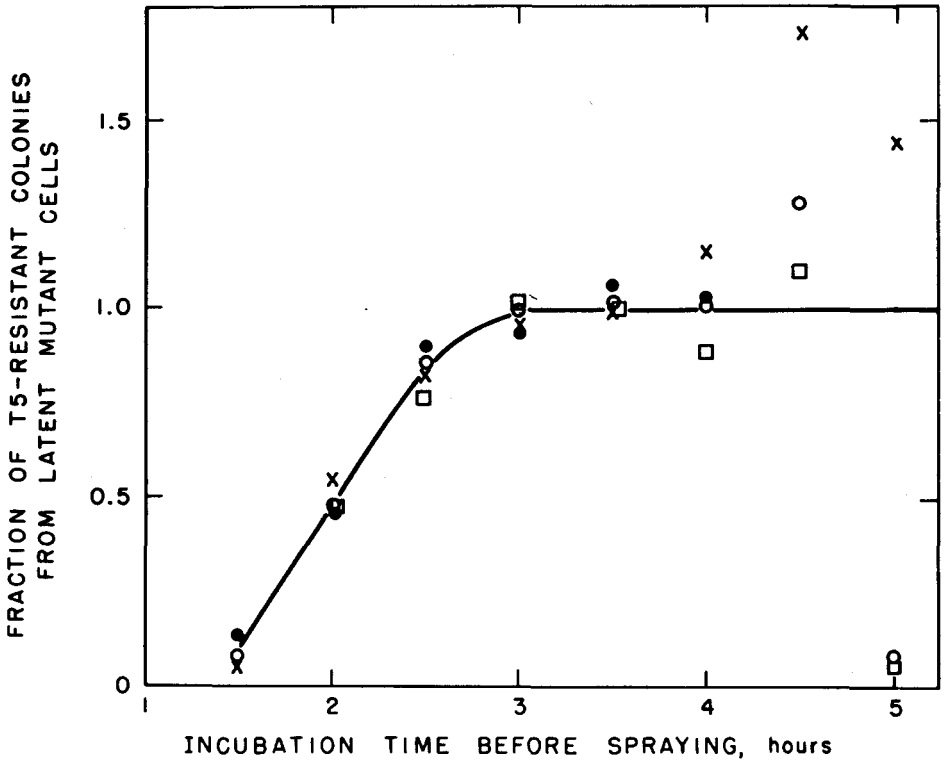

FIGURE 2.—Expression of T5 resistance for latent mutants from tryptophan-limited cultures. Strain B/l, *try*⁻ limited with L-tryptophan, 0.5 mg/l, at 37°C and plated upon iron-supplemented nutrient agar. Different symbols represent parallel experiments with different chemostats. The data were corrected by subtracting the contribution of mutants expressed initially and of mutants induced upon plates.

of the mean delay period for phenotypic expression at various growth rates (KUBITSCHEK and BENDIGKEIT 1958).

If the upper accumulation rate in Figure 3 is constant at all times after the addition of mutagen and, therefore, if it is representative of Case I, two conclusions follow. First, latent mutants have a growth rate equivalent to that of the parent culture. Second, every latent mutant gives rise to a completely resistant cell line, without segregation. If either selection or segregation were present the rate of accumulation of mutagen-induced latent mutants could not remain constant.

A criterion for the constancy of accumulation of the sum of latent and expressed mutants is given by the point of intersection of the upper straight-line segments (asymptotes $M + L_\infty$) in Figure 3. In the absence of segregation or selection this intersection should occur precisely at the time of application of the mutagen. Our results with tryptophan-limited chemostats give estimated intersections of 0, 0, ½, and −½ generations, making it unlikely that the true intersection deviates

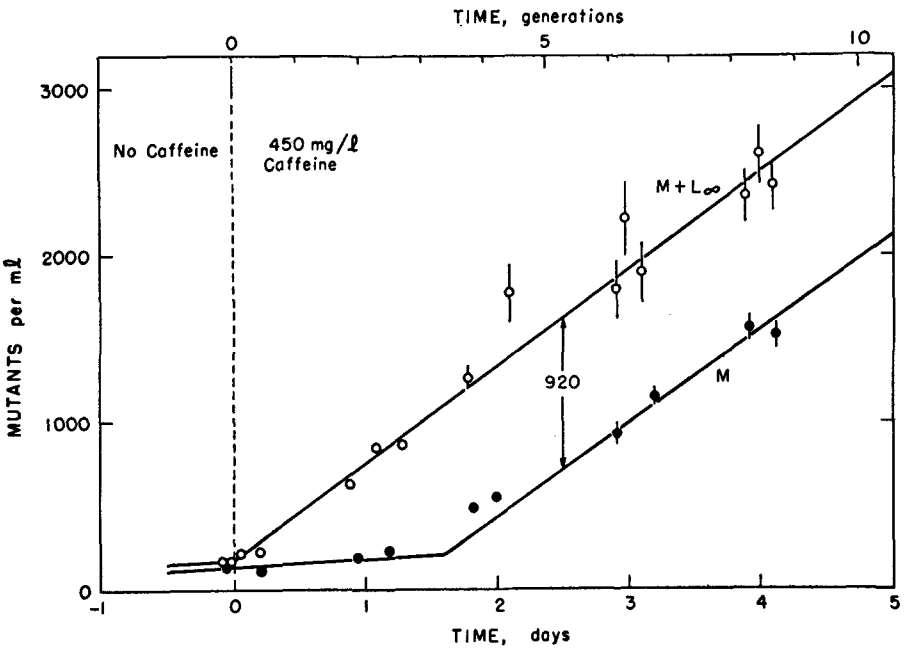

FIGURE 3.—Accumulation of caffeine-induced T5-resistant mutants in a tryptophan-limited chemostat. Strain B/l,*try*⁻ limited with L-tryptophan, 0.5 mg/l, at 37°C, generation time 11.4 hr, cell concentration about 1.5×10^8/ml. Exposed to 0.45 gm/l caffeine from time 0. ●, concentration M determined for expressed mutants; o, concentration sum $M + L$ of expressed and latent mutants. Vertical bars above and below the points as in Figure 1.

as much as half a generation, and therefore, unlikely that segregation occurs. It should be noted, however, that we have not ruled out the possibility that the latent mutant is born in a segregational division.

Constancy of caffeine-induced mutation rates in tryptophan-limited cultures: Since all latent mutants appear to be expressed at each growth rate, the accumulation of caffeine-induced mutants should be independent of growth rate if their rate of induction is constant. Figure 4 shows the rate of accumulation of a T5-resistant substrain designated E (KUBITSCHEK and BENDIGKEIT 1958) when mutations were induced with caffeine in strain B/l,*try*⁻. The values shown are rates calculated from measurements over periods of 3 to 4 days of frequencies of phenotypic mutants from experiments like that represented by the lower curve in Figure 3; the spontaneous rate of mutation, $1.4 \times 10^{-8}\text{hr}^{-1}\text{bact}^{-1}$, has been subtracted. These results are in agreement with constancy of mutation rate.

Caffeine-induced mutation in glucose-limited and lactate-limited cultures: When cultures of *E. coli* B or B/l,*try*⁻ are limited with glucose or lactate, the mean delay period does not appear changed (Table 1). However, the rate of accumulation of mutants becomes proportional to growth rate with lactate, as had been previously observed with glucose (KUBITSCHEK 1960); Figure 5 compares these results. Mutant accumulation rates appear to be about 40 percent greater for strain B/l,*try*⁻ than for B.

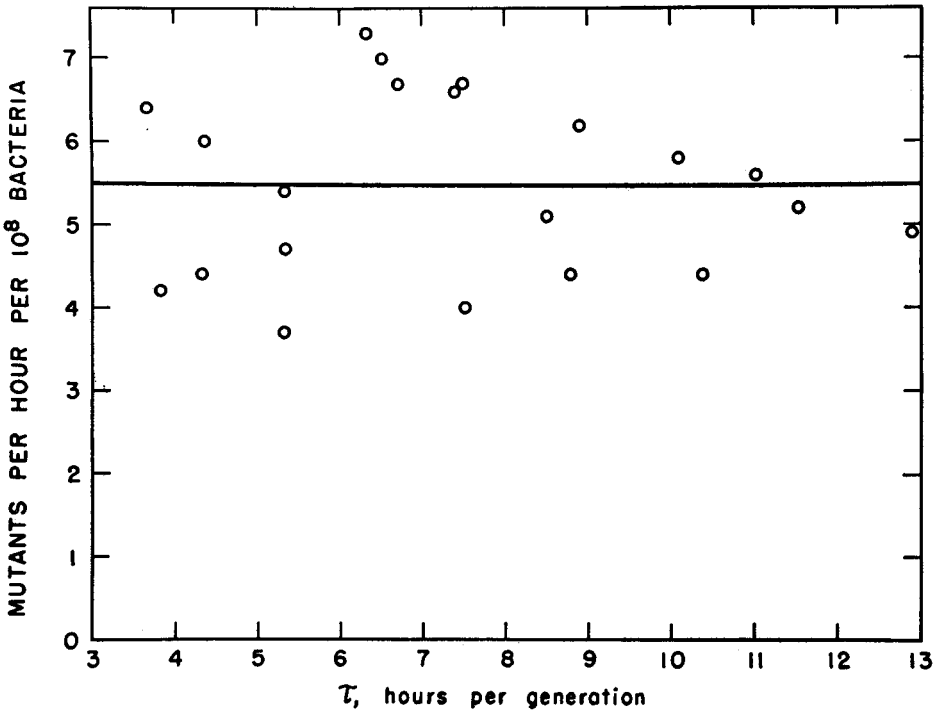

FIGURE 4.—Rates of accumulation of T5-resistant mutants in tryptophan-limited cultures exposed to caffeine. Measurements of *E. coli* strain B/l,*try*$^-$/5E (KUBITSCHEK and BENDIGKEIT 1958) at 37°C, growth limited with 0.5 mg/l L-tryptophan, exposed to 0.45 gm/l caffeine and plated upon nutrient agar without iron supplement. The average mutation rate is 5.5×10^{-8} hr^{-1} bact^{-1} with a standard error of 0.7×10^{-8} hr^{-1} bact^{-1} and has been corrected for the spontaneous rate of 1.4×10^{-8} hr^{-1} bact^{-1}.

In addition, when the proportional response is observed there is an associated change in the kinetics of accumulation of mutants at different growth rates. At rapid growth rates mutant accumulations are similar to those in tryptophan-limited chemostats (Figure 6). At slow growth rates, however, a deviation from simple linearity occurs in the accumulation $M + L$ which is best illustrated by the data shown in Figure 7: after the addition of caffeine latent mutants first accumulate rapidly, later at a more moderate rate. A relatively decreased growth rate of latent mutants in more slowly growing cultures would give a similar result since latent mutants would have a greater chance of being washed out of the culture than would more rapidly dividing cells. However, we would expect such a relatively decreased growth rate to be reflected in a longer delay in the appearance of the mutant phenotype of these more slowly metabolizing cells. Since the mean delay appears rather constant, we interpret these results as the failure of expression of some latent mutants, Case III. In view of the difficulty of obtaining more accurate data in continuous cultures, confirmation for this loss of

TABLE 1

Mean delay periods for glucose-limited and for lactate-limited chemostats

Strain	Limiting nutrient	Growth rate, gen./day	Mean delay, generations
B	glucose	2.1	3.2
		2.1	3.5
		3.6	2.8
		5.6	3.6
	lactate	2.0	3.5
		4.2	4.0
		6.0	3.3
B/l, *try*⁻	glucose	2.5	3.7
		4.5	3.6
		6.9	3.4
	lactate	4.4	3.4
Average of all determinations			3.46 (S.E., 0.30)
Slow growth rates:			
< 3 gen./day			3.5 (S.E., 0.1)
Rapid growth rates:			
> 5 gen./day			3.4 (S.E., 0.1)

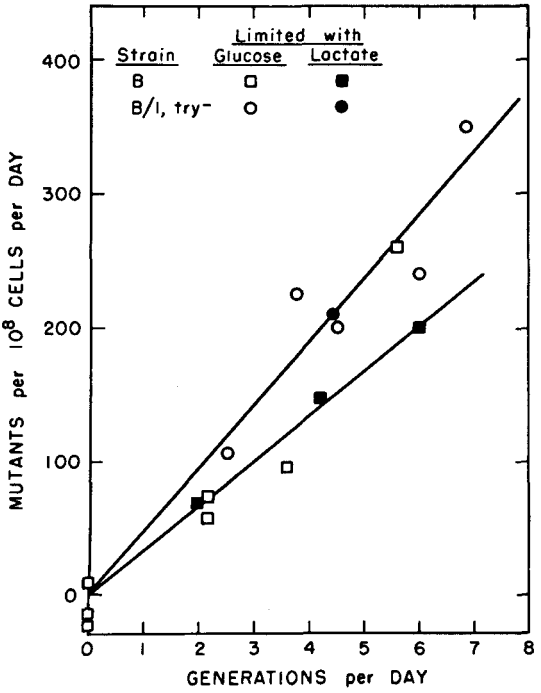

FIGURE 5.—Comparative rates of accumulation of caffeine-induced T5-resistant mutants of *E. coli* B and B/l,*try*⁻ in glucose-limited or lactate-limited cultures 37°C, 0.45 gm/l caffeine.

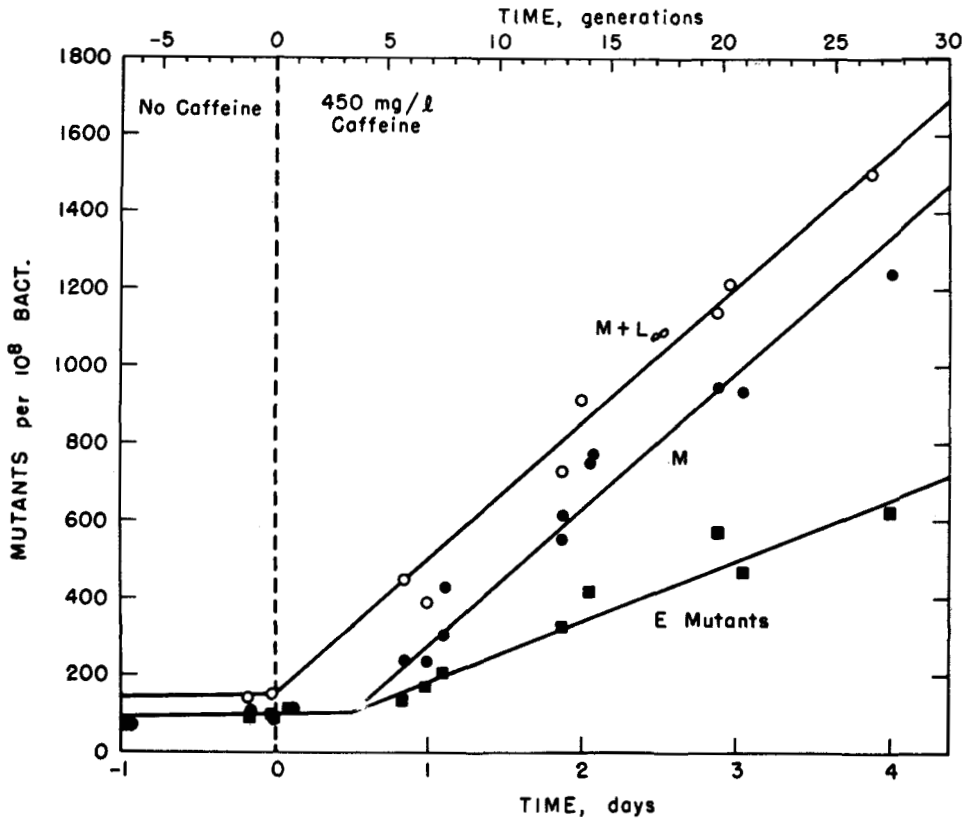

FIGURE 6.—Accumulation of T5-resistant mutants in a glucose-limited culture exposed to caffeine. Strain B/l,*try*⁻, limited with glucose, 100 mg/l, at 37°C; generation time 3.5 hrs, cell concentration about 10^8/ml; caffeine added from time 0. ●, expressed mutant frequencies M; ○, sum $M + L$ of expressed and latent mutant frequencies; ■, frequencies for expressed E mutants (nutrient agar not supplemented with iron).

latent mutants was sought and found, using ultraviolet light (UV) as the mutagen for cultures in which growth had been interrupted (semicontinuous cultures).

UV-induced mutation in semicontinuous glucose-limited cultures: Figure 8 shows the accumulation of mutants of strain B in two glucose-limited chemostats with initially identical histories. A parent culture was grown in a chemostat (10.8 hr/gen) in which growth rate was limited with glucose (150 mg/l). Nutrient flow was blocked starting one half hour before irradiation with UV. The source was a 2.5 cm section of a GE Germicidal Lamp (15 W) at a distance of 117 cm from a quartz port (3 cm diam.). The aerated culture (250 ml, 3×10^8 bact/ml) was irradiated for 2.5 hr in order to provide a uniform dose. Cell growth was again permitted after the culture was used to fill the growth tubes of two smaller chemostats having washout times corresponding to steady state generation times of 3.9 hr and 9.7 hr. About half the cells were viable.

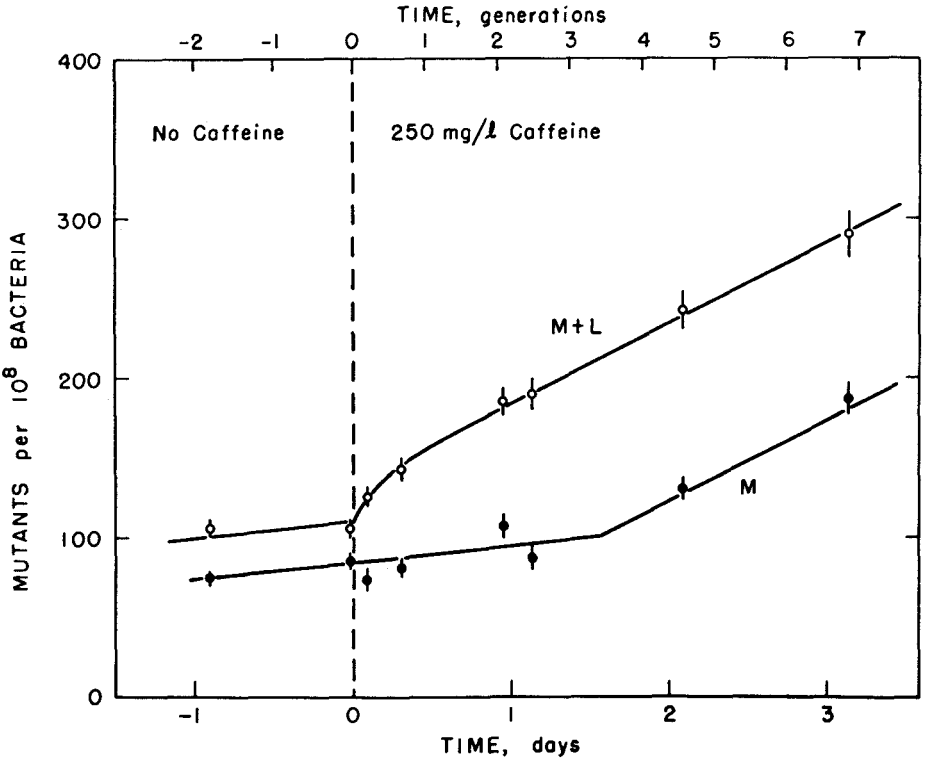

FIGURE 7.—Nonlinear accumulation of mutants at a slow growth rate in a glucose-limited culture. Strain B, glucose, 150 mg/l, at 27°C; generation time 12.0 hrs, cell concentration 2.3×10^8/ml. ●, expressed mutant frequency M; ○, the sum $M + L$ of expressed and latent mutant frequency.

When nutrients were supplied, cell concentrations first dropped exponentially with time as would have been expected for nondividing particles washing out of the chemostats. We interpret this decrease as due primarily to a temporary cessation of cell division, but not growth, since cell concentrations soon surpassed the original concentration within the first mass doubling of these cultures. Even approximately steady state kinetics were not reached within the time required to wash out one culture volume. We find that the formation of filamentous cells, commonly produced in continuously growing chemostat cultures given the same dose of UV, is greatly inhibited when resting cultures are irradiated.

It may be seen from Figure 8 that the loss of latent mutants is displayed clearly at the generation time of 9.7 hours; here, at least 30 percent of the latent mutants did not give rise to mutant progeny. At the generation time of 3.9 hours, however, the loss was much less. Values of the mean delay periods are not accurate enough to rule out increased selection against latent mutants at the longer generation time: after correcting for the population increase, the mean delay in generations was 3.5 ± 0.5 at the more rapid growth rate and 4.1 ± 0.5 for the slower. However, differential selection is made improbable by the observation that the latent

mutant frequencies in the two chemostats are quite similar at all times after the first few hours.

In these and other experiments upon the effect of UV in cultures limited with glucose, a further parallel to the results with caffeine is the crude proportionality which is found between mutant yield and growth rate, Figure 9. The mutant yield is defined, customarily, as the increase in expressed mutants attributable to UV and is directly comparable to the rate of expression of mutation in experiments in which mutagen is applied continuously.

DISCUSSION

The results presented here provide strong evidence that some latent mutants fail to reach expression in slowly growing cultures limited with glucose, and moreover, that in these cultures the rate of expression is proportional to growth rate. The linearity of accumulation of latent mutants during rapid growth in glucose-limited cultures (Figure 6) implies the absence, here, of both segregation and selection. The nonlinear accumulations at slow growth rates would not be expected to be due to the onset of segregation from filamentous bacteria, since those become increasingly rare as growth rate is reduced, nor, as noted earlier, to the onset of selection, since the mean delay period appears unchanged. It was

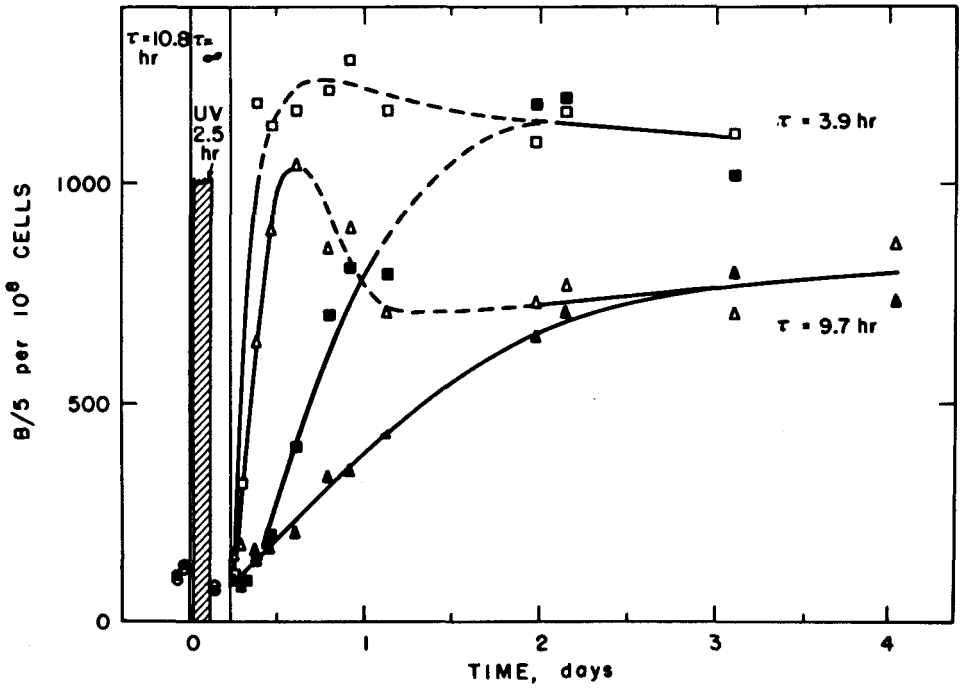

FIGURE 8.—Ultraviolet induced mutants at two growth rates. *E. coli* B at 37°C, limited with glucose, 150 mg/l, and plated upon iron supplemented salts-lactate-glucose agar. ▲, ■, expressed mutant frequency for T5 resistance; △, □, sum of expressed plus latent mutant frequency.

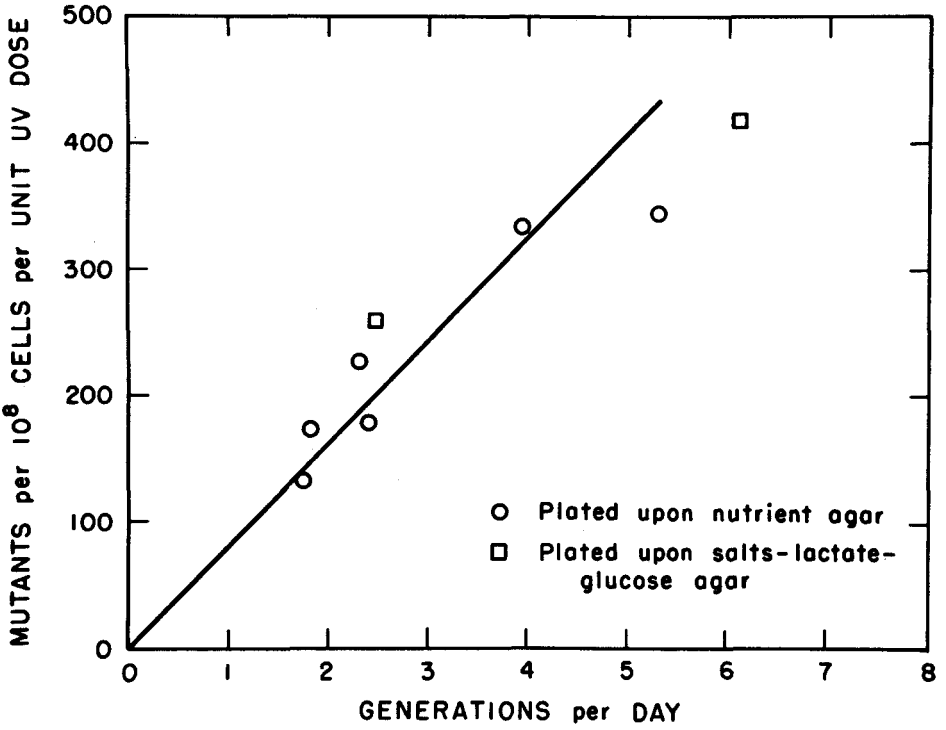

FIGURE 9.—The yield of T5-resistant mutants as a function of growth rate in glucose-limited cultures exposed to ultraviolet light. *E. coli* B at 37°C. Data corrected to a period of one hour of irradiation under conditions described in text.

noted also that the loss of UV-induced latent mutants is unlikely to be due to differential selection because of the similarity in latent mutant frequencies. Furthermore, another possibility for the loss of latent mutants is that it arises from a growth-rate-dependent artifact in plating. However, such an artifact would not be expected to occur, as has been observed, (a) upon two markedly different plating media, (b) when cultures are limited with glucose or lactate, but not when they are limited with tryptophan in otherwise identical media, and (c) without disturbing the time of onset of the latent mutant plateau in experiments of the kind shown in Figure 1. These characteristics show that mutants expressed after plating carry little information about their earlier physiological history.

The most likely interpretation for the loss of latent mutants is that some of these cells do not express the mutant phenotype in the chemostat. This loss occurs because of one or more of the following: interruption of the chain of events which would normally lead to establishing mutation (premutational damage), reversion of the genetically-established mutation, or death of the latent mutant. These are the usual interpretations which have been advanced to explain the decrease in mutant frequency obtained after secondary treatments of bacteria that had been exposed to UV (see WITKIN 1958). The hypothesis of premutational damage and

its repair has also been proposed to explain results with paramecia exposed to one of several kinds of radiation (KIMBALL, GAITHER and PERDUE 1960). The earlier postulate of premutational damage (WITKIN 1956, 1958; DOUDNEY and HAAS 1958) had a requirement of metabolism to induce mutation.

A clear distinction between these and other hypotheses is not yet available, partly because previous studies have not distinguished between concentrations of latent mutants and those already phenotypically expressed. Attempts to determine latent mutant concentrations by "chloramphenicol challenge" or by inference from the time-course of expression of mutations upon various media are ever subject to the questions of the presence of selection and segregation, compounded by the accretion of plate mutants. That these procedures, by now more or less customary, can give rise to ambiguous interpretations may be seen clearly by a consideration of the results presented in Figure 8. In the absence of a knowledge of *either* expressed mutant concentration M or the sum of mutant concentrations $M + L$ it would be impossible to determine from these measurements whether yields were changed as a result of premutational or postmutational processes.

The correspondence demonstrated in our experiments between UV-induced and caffeine-induced mutation confirms and extends the similar finding by KIMBALL, GAITHER and WILSON (1959) of a common "reversible" step (death is not ruled out) in the mutational process induced with UV, X-rays, or alpha particles. In addition we have observed loss of x-ray-induced latent mutants in glucose-limited cultures. The similarity of the results with widely different mutagens may well arise because the mutations studied have in common rather lengthy terminal stages for the development of the newly-forming mutant. Early stages of induction would be expected to be rather different for mutagens as dissimilar as UV and caffeine, but changes in growth rate apparently have little effect upon them.

For our results the copy-error hypothesis of mutation is untenable in its original form. Without the addition of further assumptions the error hypothesis would predict at most the finding for glucose-limited and for lactate-limited cultures that mutation rate (or mutant yield) is proportional to growth rate. The error hypothesis fails to provide satisfactory explanation for the following observations.

Induction of mutation is independent of growth rate in tryptophan-limited cultures: Unmodified, the copy-error hypothesis predicts mutation to be induced at a rate proportional to growth rate, and in the absence of loss or reversion, the rate of accumulation of phenotypic mutants would also be proportional to growth rate. Instead, the constancy of the rate of expression observed in tryptophan-limited cultures of B/l,*try*$^-$, along with the equivalence between the rate of induction and the rate of mutation, gives evidence for the constancy of the rate of induction of latent mutants.

Induction of mutation in glucose-limited cultures is not proportional to growth rate: The ratio of the rate of accumulation of latent mutants just after adding caffeine to the rate of accumulation of phenotypic mutants (i.e., rate of induction/

rate of expression) is largest for the most slowly growing cultures. Since the rate of expression of mutation is proportional to growth rate, the rate of induction cannot be proportional but might well be constant. Thus the prediction of the error hypothesis fits accidentally: the prediction would be for increased expression resulting from increased induction at the more rapid rates of replication.

Our results support a unitary hypothesis previously given specifically for cultures limited with tryptophan or with glucose (KUBITSCHEK 1960): latent mutant frequencies are independent of growth rate in continuously growing cultures; when loss of latent mutants occurs the probability of expression of a latent mutant is proportional to growth rate.

In tryptophan-limited cultures this hypothesis is consistent with the constancy of the rate of accumulation of caffeine-induced mutants at different growth rates (unit probability of expression) and with the linear accumulation of mutants after addition of caffeine to the growth medium. In glucose-limited cultures it is consistent with the growth-rate proportionality of expression of mutation found with caffeine and with UV, and with the loss of UV-induced latent mutants and the kinetics of accumulation of caffeine-induced latent mutants.

If this unitary hypothesis be accepted, then the primary problem for kinetic models of mutation is the nature of the growth rate independence of induction of latent mutants. The most direct explanation would be that genetic material is modified after its formation rather than during the replication process. Constancy of rate of induction would then follow from constancy of amount of functional genetic material. A possibility might be base pair changes in deoxyribonucleic acid (DNA) during a time (replication?) when it is especially accessible to base analogs resulting from the application of the mutagen. Conversely, if mutagen could be shown to affect DNA directly then the constancy of induction would require the constancy of functional genetic material. If, on the other hand, functional genetic content were shown to vary with growth rate despite constancy of delay and of mutation rate, then a more sophisticated kinetic model would be required. In this case mutation might be due to an error during the production of new DNA but not the single-stamping error of template DNA upon replication.

Whether genetic content remains constant or varies, there is no need to assume a kinetic model in which the probability of mutation remains constant over a division cycle. Constancy of mutation would result provided only that the probability of mutation were always the same function of the stage of the cycle which could be expanded or contracted uniformly to fit different growth rates (i.e., mutation rate is describable at all growth rates by the same Fourier function applied between birth and death of the cell by fission). The probability of inducing a mutation during a division cycle would then be proportional to growth rate, agreeing with the constancy of induction rate in tryptophan-limited cultures.

Finally, we noted earlier the absence of segregation of the mutational potential or character, but the possibility that the latent mutant arises during an initial segregational division cannot be ruled out. Mutational heterozygotes of this kind have been demonstrated clearly in bacteriophage (see, for example, PRATT and STENT 1959). Although our studies permit a bipartite replication structure,

absence of segregation in latent mutants rules out greater complexity: there can be no more than two groups of nuclear strands capable of independent mutation if nuclear polyteny occurs. Thus our data provide kinetic evidence, quite apart from cytological considerations of structure, that *E. coli* B and B/l,*try*$^-$ inherit single functional nucleoids during exponential growth in the chemostat.

SUMMARY

A method of delayed selection of cells resistant to bacteriophage T5 has been applied to chemostat cultures of *Escherichia coli*, strains B and B/l,*try*$^-$, as an assay for *latent mutants*, cells phenotypically wild, but in which the mutational process has been initiated. The kinetics of expression of mutation are dependent upon the nutrient used to limit growth rate, but appear rather independent of the mutagen.

With tryptophan-limited cultures, no selection or segregation was observed in latent or expressed mutants, and the rate of accumulation of caffeine-induced mutants appears independent of growth rate. Furthermore, every latent mutant appears to give rise to completely mutant progeny lines at a later time, providing kinetic evidence that cells of *E. coli* B/l,*try*$^-$ inherit single functional nucleoids. However, when glucose or lactate is used to limit growth rate, the fraction of latent mutants which becomes phenotypically expressed appears proportional to growth rate. This proportionality results from the growth-rate dependence of expression of mutation and not from a growth-rate dependence of induction. Corresponding results are obtained for glucose-limited cultures when UV is the mutagen.

The kinetics of accumulation of latent mutants and expressed mutants make the copy-error hypothesis improbable (in the sense that mutation is an error in template replication) or would require its modification. These kinetics are summarized in a unitary hypothesis: In continuous cultures induction of mutation is not dependent upon the growth rate of the culture. Expression can also be independent of growth rate (tryptophan-limited cultures), but when it is not, expression is proportional to growth rate (glucose-limited or lactate-limited cultures).

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