Spontaneous Point Mutations That Occur More Often When Advantageous Than When Neutral

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Manuscript received January 5, 1990
Accepted for publication May 8, 1990

ABSTRACT

Recent reports have called into question the widespread belief "that mutations arise continuously and without any consideration for their utility" (in the words of J. Cairns) and have suggested that some mutations (which Cairns called "directed" mutations) may occur as specific responses to environmental challenges, *i.e.*, they may occur more often when advantageous than when neutral. In this paper it is shown that point mutations in the *trp* operon reverted to trp^+ more frequently under conditions of prolonged tryptophan deprivation when the reversions were advantageous, than in the presence of tryptophan when the reversions were neutral. The overall mutation rate, as determined from the rates of mutation to valine resistance and to constitutive expression of the *lac* operon, did not increase during tryptophan starvation. The *trp* reversion rate did not increase when the cells were starved for cysteine for a similar period, indicating that the increased reversion rate was specific to conditions where the reversions were advantageous. Two artifactual explanations for the observations, delayed growth of some preexisting revertants and cryptic growth by some cells at the expense of dying cells within aged colonies, were tested and rejected as unlikely. The trp^+ reversions that occurred while trp^- colonies aged in the absence of tryptophan were shown to be time-dependent rather than replication-dependent, and it is suggested that they occur by mechanisms different from those that have been studied in growing cells. A heuristic model for the molecular basis of such mutations is proposed and evidence consistent with that model is discussed. It is suggested that the results in this and previous studies can be explained on the basis of underlying random mechanisms that act during prolonged periods of physiological stress, and that "directed" mutations are not necessarily the basis of those observations.

IT is a fundamental premise of biology that mutations occur randomly, continuously, and without respect to any advantage that they might offer in the prevailing environmental conditions. The corollary to that premise is that selection acts only to affect the relative frequency of a mutant allele in subsequent generations, not to alter the probability that a particular mutation will actually occur. These understandings underlie all of theoretical population biology, molecular evolution, and molecular methods of constructing phylogenetic trees. Thus, mutation and selection are considered to be entirely separate processes. Some recent studies of *Escherichia coli* have cast doubt on these beliefs by showing that some mutations appear to be induced by selection (CAIRNS, OVERBAUGH and MILLER 1988; HALL 1988, 1989). All three studies showed that some mutations occur more frequently when they are advantageous to the cell than when they confer no advantage. While we generally speak of spontaneous mutations being random, we are also aware that they are not truly distributed at random. There are hot spots for mutations, and some kinds of substitutions occur more often than others; see, for example, SCHAAPER, DANFORTH and GLICKMAN (1986) and SCHAAPER and DUNN (1987). Here we are concerned not with randomness with respect to sites (mutational spectra) but with respect to normal environmental fluctuations and to effects on the gene product, and thus on fitness. The nonrandom mutations have been called "directed" mutations by CAIRNS, OVERBAUGH and MILLER (1988), who suggested that "populations of bacteria, in stationary phase, have some way of producing (or selectively retaining) only the most appropriate mutations." That view was supported by the observation that some mutations, which were mediated by mobile genetic elements, only occurred when they were advantageous to the cell (HALL 1988; SHAPIRO 1984). The view espoused by CAIRNS *et al.* played an important role in pointing out the paucity of evidence to support our nearly universal view of the randomness of mutation and in stimulating considerable discussion of this issue in the literature (BENSON, PARTRIDGE and MORGAN 1988; CHARLESWORTH *et al.* 1988; LENSKI 1989; LENSKI, SLATKIN and AYALA 1989). The issue has been controversial and (as is often the case) a part of that controversy derives from the debate having been cast in terms of two extreme models: the classical

model, in which the probability that a particular mutation will occur is a constant property of the organism and is unresponsive to the environment or to any benefit that the mutation may confer, and the Cairnsian model, in which the probability that a particular mutation will occur depends both upon the external environment (*i.e.*, nutritional conditions leading to stationary phase) and upon the likelihood that the mutation will be useful. The Cairnsian view appears to require machinery for choosing "the most appropriate mutations" and the necessity for such apparent perception by bacteria has generated a good deal of skepticism. In considering this issue, it may be useful to ask whether there are mutations that occur more often when they are advantageous than when they are neutral. If so, does the existence of such mutations require that cells be able to choose either which mutations can occur or which mutations to retain?

The answer to the first question is clearly "yes." Some mutations occur at detectable frequencies only when they are useful (HALL 1988; SHAPIRO 1984). To call such mutations "directed" prejudges a mechanism for their occurrence. For the purpose of discussion let us call such mutations "Cairnsian" and define Cairnsian mutations as those that occur with a higher probability when they are advantageous than when they are neutral.

One purpose of this paper is to explore the second question, whether Cairnsian mutations require cells to choose which mutations can occur.

Cairnsian mutations appear to occur preferentially in cells that have resided in nutritionally depleted environments, *i.e.*, under conditions of cellular stress (HALL 1988, 1989). The increased frequencies of the advantageous mutations were not accompanied by an increased mutation rate at other loci (CAIRNS, OVERBAUGH and MILLER 1988; HALL 1988, 1989); thus, the phenomenon does not simply involve a general increase in mutation rate under conditions of cellular stress.

There are at least three reports of Cairnsian mutations involving mobile genetic elements (SHAPIRO 1984; SLATER, WEIGHTMAN and HALL 1985; HALL 1988). In two of these (HALL 1988; SHAPIRO 1984), the mutations occurred only when advantageous to the cell. In one case, the frequency of excision of an insertion sequence from within a gene increased from $<2 \times 10^{-12}$ to $>10^{-2}$ per cell division (HALL 1988). In another case, in which the target gene was located on a mobile element, the frequency of mutation to resistance to a toxic analog of a carbon source increased from about 10^{-6} under nonselective conditions to 1.0 under selective conditions (SLATER, WEIGHTMAN and HALL 1985). In that case the mutation was believed to involve loss of the mobile-element-borne gene for transporting the toxic analog.

Based primarily upon the distributions of mutants in replicate cultures, CAIRNS, OVERBAUGH and MILLER (1988) showed that a significant proportion of the reversions of a *lacZ* amber mutation that were detected would not have occurred in the absence of selection.

The above studies all involved mutations in catabolic genes. However, it was recently shown that a mutation of unknown nature in the *metB* gene reverted 60-to 80-fold more frequently during long-term methionine starvation than in growing cultures (HALL 1989).

If Cairnsian mutations are of evolutionary importance, then they must occur in a variety of oganisms and include mutations mediated by base-pair substitutions in both catabolic and anabolic genes. This study was undertaken to determine, by direct observation, whether the reversion frequency of a known missense mutation increased under selective conditions, and to examine some possible artifactual explanations that had been advanced as criticisms of the CAIRNS, OVERBAUGH and MILLER (1988) experiments. In this study I show that two known missense mutations in the *trp* operon of *E. coli* exhibit "Cairnsian" behavior; *i.e.*, reversions of these mutations occur much more frequently when advantageous than when neutral.

MATERIALS AND METHODS

Strains: All bacterial strains were *Escherichia coli* K12. Strains FCY2 (*trpA47*), FCY7 (*trpB9578 cysB*) and FCY6 (*trpA47 trpB9578*) are isogenic derivatives of the wild-type F^- strain W3110 (C. YANOFSKY, personal communication). Strain FCY21 (*trpB9578*) is a derivative of FCY7 that was created by transducing the $cysB^+$ allele from strain W3110 into strain FCY7. The derivatives designated FCY2B, FCY6B and FCY21B carry Δ*bgl-pho* and *tna*::Tn*10* which were introduced by P1 cotransduction from strain CSH62T (KRICKER and HALL 1984).

Media and growth conditions: Minimal medium consisted of 423 mg sodium citrate, 100 mg $MgSO_4 \cdot 7H_2O$, 1 g $(NH_4)_2SO_4$, 540 μg $FeCl_3$, 1 mg thiamine, 3 g KH_2PO_4, 7 g K_2HPO_4 and 2 g carbon source per liter. When required, tryptophan was added to a final concentration of 100 μg/ml. When glucose was the carbon source, minimal medium was designated PD medium.

TAD medium contained, in addition to the above ingredients, 5 g vitamin-free casamino acids (Difco), 20 mg adenosine, 30 mg cytosine, 20 mg guanosine, 20 mg uridine, 30 mg thymidine, 20 mg $MnSO_4$, 200 μg pantothenic acid, 400 μg pyridoxine, 400 μg riboflavin, 200 μg *p*-aminobenzoic acid, 200 μg niacin and 1 μg biotin per liter. CAD medium is identical to TAD medium except that it contains 20 μg/ml of each amino acid except cysteine, and contains no casamino acids. Plates for selecting trp^+ revertants were incubated at 30° in a humidified chamber. For all other purposes plates were incubated, and liquid cultures were shaken, at 37°.

The rich, complete medium was LB (Luria broth) (MILLER 1972).

Estimation of mutation rates in growing cells: The

TABLE 1

Reversion rates of *trp* mutations under selective and nonselective conditions

Under nonselective conditions[a]	
Mutation	Mutation rate
trpA46→*trpA*$^+$	$(1.3 \pm 0.24) \times 10^{-10}$
trpB9578→*trpB*$^+$	$(2.1 \pm 0.38) \times 10^{-10}$

Under selective conditions[b]			
Mutation	Mutation rate[c] on day 4	Increase of revertants[d] at end of experiment	Number of colonies
trpA46→*trpA*$^+$	$(7.6 \pm 1.7) \times 10^{-10}$	7.2-fold on day 12	727
	$(2.1 \pm 1.6) \times 10^{-11}$	13-fold on day 14	500
	$(1.2 \pm 0.6) \times 10^{-10}$	14.6-fold on day 14	1109
	$(1.7 \pm 0.4) \times 10^{-10}$	3.4-fold on day 12	1313
trpB9578→*trpB*$^+$	$(6.7 \pm 3.2) \times 10^{-10}$	12.3-fold on day 12	234
	$(1.7 \pm 0.7) \times 10^{-10}$	34.8-fold on day 14	1508
	$(4.8 \pm 1.0) \times 10^{-10}$	7.8-fold on day 12	1212

[a] By LURIA-DELBRÜCK fluctuation tests. The 60 *trpA* cultures contained $(3.7 \pm 0.4) \times 10^9$ cells/culture, the 60 *trpB* cultures $(3.1 \pm 0.8) \times 10^9$ cells/culture.

[b] Colonies were grown on TAD plates containing 5 μM tryptophan. The number of viable cells per colony was determined by resuspending colonies from parallel plates, diluting, and plating on LB medium. The mutation rate was estimated from the proportion of colonies with no revertant papillae.

[c] Mutation rates are per cell division ± standard deviations. Each line is a separate experiment.

[d] The increase is the ratio of the number of papillae present at the end of the experiment to the number present on day 4.

fluctuation test (LURIA and DELBRÜCK 1943) measures mutation rates in growing cultures where the mutation is neutral. To measure the reversion rates of the two *trp* mutations, 60 independent 10-ml cultures were grown from innocula of about 100 cells to a final density of about 5×10^8 cells/ml in minimal medium (PD) containing excess tryptophan. The cultures were concentrated and washed, and each was plated on a TAD plate to select *trp*$^+$ revertants. Dilutions of several parallel cultures were plated on LB plates to estimate the number of viable cells.

The average number of mutations per culture, M, was estimated as $M = -\ln(P_0)$ where P_0 is the proportion of cultures with no mutants. The mutation rate was calculated as $\mu = MC^{-1} \ln 2$ where C is the number of cells per culture (LURIA and DELBRÜCK 1943). The variance of μ is estimated according to LEA and COULSON (1949) as Var $\mu = (\ln 2)^2(e^M - 1)N^{-1}C^{-2}$ where N is the number of cultures tested.

Instant colonies: Cultures were washed and concentrated to ~5×10^{10} cells/ml and 1-μl drops were deposited in a grid on the surface of well dried plates. Under those conditions the suspension forms a "colony" that is normal in appearance except for a slightly dimpled center.

RESULTS

Reversion of point mutations in the *trp* operon: The *trpA* and *trpB* genes encode the α and β subunits of tryptophan synthetase, respectively, and functional alleles of both loci are required for *E. coli* to grow on medium lacking tryptophan. Strains FCY2 and FCY21 carry base pair substitution mutations in *trpA* and *trpB*, respectively. The *tnaA*::Tn*10* marker was introduced into those strains because tryptophanase, the product of the *tnaA* gene, can substitute for the β-subunit activity of tryptophan synthetase (YANOFSKY and CRAWFORD 1987). Because its synthesis is inducible by tryptophan, *tnaA*$^+$ can permit *trpB* mutants to grow for several generations after the exhaustion of tryptophan from the medium.

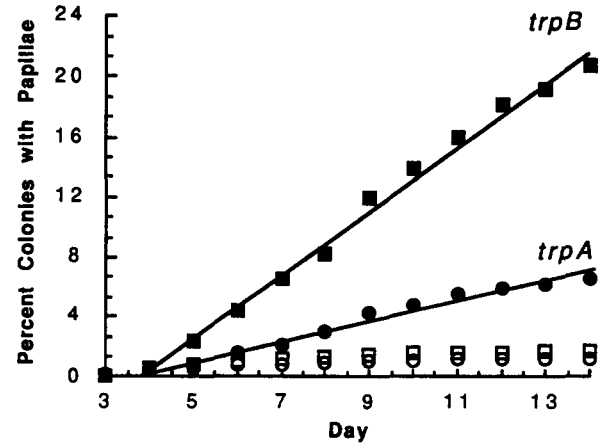

FIGURE 1.—Kinetics of appearance of *trp*$^+$ revertants as papillae on *trp*$^-$ colonies. Circles = FCY2B revertants. Squares = FCY21B revertants. Closed figures show the number of papillae observed. Open figures show the number of papillae that could be explained by cell turnover (see text).

The reversion rates of the *trpA47* and *trpB9578* mutations were estimated in growing cultures of strains FCY2B and FCY21B by the fluctuation test (LURIA and DELBRÜCK 1943) as described in MATERIALS AND METHODS. The values (Table 1) are in good agreement with the average total mutation rate per base pair in *E. coli* (DRAKE 1969).To estimate the mutation rate under selective conditions, where the reversion was strongly advantageous, about 30–50 cells of strains FCY2B and FCY21B were spread on TAD plates containing 5 μM tryptophan, which limits colonies to about 5×10^7 cells. Such colonies are very thin and any *trp*$^+$ revertants that arise within them form thick, dark papillae that are easily detected when the colonies are viewed through a dissecting microscope with substage lighting. The plates were incubated at 30° in humidified chambers and observed each day, and any new papillae were marked on the back of the plate. Figure 1 shows the results of one such experiment. Under these conditions, colonies reach a maximum size and number of viable cells by day 3. A reconstruction experiment, discussed in detail in a later section, showed that a *trp*$^+$ cell forms a visible papilla within 24 hr of its appearance within a colony; thus, papillae present on day 4 were probably the result of mutations that occurred during the growth of the colony prior to the exhaustion of tryptophan from the medium. Each colony can be treated as though it were an individual culture in a fluctuation test and the mutation rate can be calculated from the

proportion of colonies that have no papillae. In several experiments, mutation rates calculated on day 4 were in good agreement with rates estimated in growing cultures (Table 1). Thus, all of the trp^+ revertants that were expected were present on day 4.

Revertants continued to appear over the course of the following 8–10 days (Figure 1, Table 1). The distribution of papillae on colonies appeared to be purely random, in that the number of papillae per colony was indistinguishable from that predicted by the Poisson distribution and the proportion of colonies with trp^+ papillae was indistinguishable from plate to plate.

Mutations are time-dependent, not replication-dependent: The most obvious explanation for the continued appearance of trp^+ revertants after cessation of colony growth is that there is cryptic growth of cells within the colonies and that the additional mutants are the result of replication errors. Although the number of viable cells per colony declines as the colonies age (Figure 2), the observed residual viability might be the result of extensive cell death balanced by a similar amount of growth at the expense of the dead cells, in which case the mutants that appeared continuously would be ordinary, random, replication-dependent mutants that had been powerfully selected as they arose among the dividing cells.To measure cell death and turnover, the change in the number of viable cells per colony was measured in the presence and absence of the protein synthesis inhibitor chloramphenicol (Cam). The rationale was that no new cell divisions could occur in the presence of chloramphenicol but cell death could still occur. "Instant colonies" of strain FCY6 containing about 5×10^7 cells were deposited on filters on TAD or on TAD + Cam plates. Filters were resuspended daily, diluted, and plated on LB medium to estimate N, the number of viable cells (Figure 2A). Loss of viability is a first-order process in which $dN = -mNdt$; thus, $N_t = N_0e^{-mt}$ and the death rate, m, is estimated as the slope of $\ln(N)$ *vs.* t. The death rate observed in the absence of Cam is $m = 0.242$ per day in this experiment (mean in three experiments 0.242 ± 0.022 per day) and the death rate in the presence of Cam is $k = 0.732$ per day. Although viability declined more rapidly in the presence of Cam than in its absence, the difference was not sufficient to account for the continuous appearance of new *trp* revertants as the consequence of cryptic growth. If α is the proportion of dead cells that give rise to new cells, then the observed change in the number of viable cells per colony in the absence of Cam (dN) is the result of the death of some cells ($-kN$) plus the replication of cells at the expense of the dead cells (αkN). Thus, in the absence of Cam, $dN = (-kN + \alpha kN)\,dt$. Because no new cells can arise in the presence of Cam, k is estimated directly from the

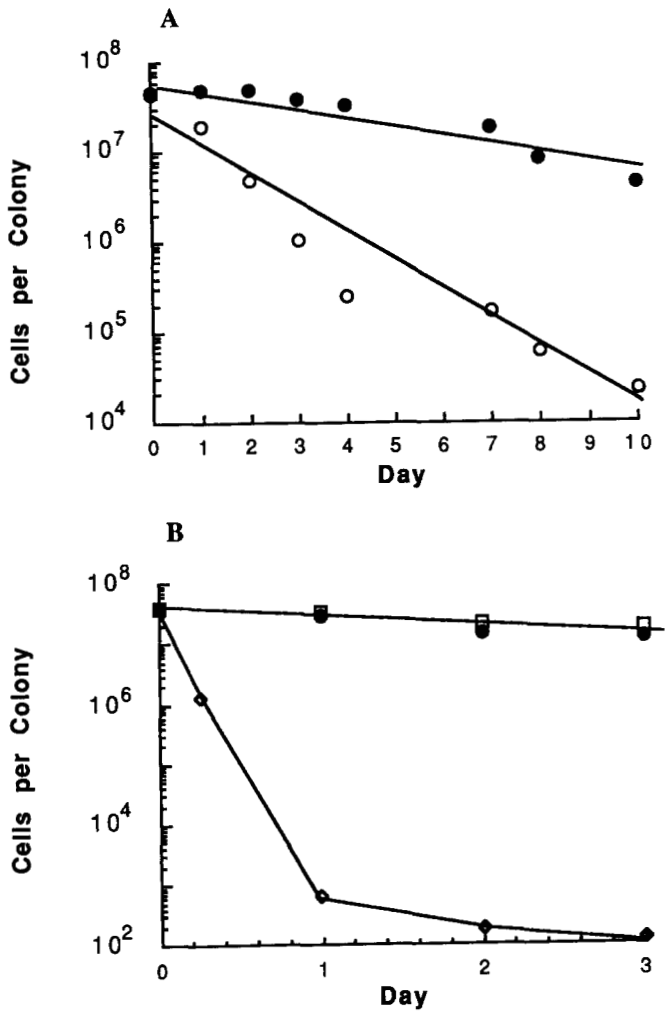

FIGURE 2.—Death rates of cells in colonies on TAD medium in the presence and absence of drugs. Twenty 1-μl drops, each containing about 5×10^7 FCY6 (*trpA46 trpB9578*) cells were distributed onto sterile Millipore HAWP filters on the surface of TAD plates with or without drugs. Filters were resuspended daily, diluted, and plated on rich medium to estimate the number of viable cells per colony. Panel A: filled circles = TAD medium, open circles = TAD medium containing 20 μg/ml Cam. Panel B: closed circles = TAD medium, open squares = filters transferred on day 3 from TAD medium to TAD medium containing 100 μg/ml Amp. (Day 0 of panel B thus corresponds to day 3 of panel A.) Open diamonds = filters transferred on day 3 from TAD medium to TAD medium plus tryptophan plus 100 μg/ml Amp.

death rate in the presence of Cam as 0.732 per day. Because m, the observed death rate in the absence of Cam, is actually a composite of births plus deaths, $-mN = -kN + \alpha kN$. Thus, $\alpha = 1 - (m/k)$ and, from the experiment in Figure 2A, $\alpha = 0.67$ on TAD medium. The number of new cells produced within a colony, β, is estimated as $\beta_t = \int_0^t \alpha kN\,dt$. Thus, $\beta = \alpha N_0(\alpha - 1)^{-1}[e^{k(\alpha-1)t} - 1]$ where N_0 is the number of viable cells in the colony at the beginning of the decay period. Figure 1 (open symbols) shows the number of additional trp^+ papillae that could have appeared in that experiment based on the measured mutation rate in growing cells (Table 1) and on the estimated num-

ber of cell divisions that could have occurred on that day from the data in Figure 2A (*i.e.*, the mutants that would be expected if they arose only from cryptic cell divisions). Clearly, the observed number of mutants that appeared each day greatly exceeded the number that could be explained by cryptic growth. The above experiment is based on the assumption that Cam itself does not affect cell viability. It is reasonable to expect that prolonged inhibition of protein synthesis by Cam would increase the death rate because it would prevent *de novo* synthesis of proteins from internal protein turnover. If, on the other hand, Cam reduced the death rate (perhaps by increasing the level of charged tRNA molecules), then there might be *more* cryptic growth than would be calculated on the basis of the data in Figure 2B. To test that possibility, the experiment was repeated using ampicillin (Amp) instead of Cam. Amp kills growing cells by interfering with cell wall synthesis, resulting in lysis. Thus, in the presence of Amp, a cell that grew at the expense of dead cells would itself die and the death rate in the presence of Amp should exceed that observed in the presence of Cam. In contrast to that expectation, the death rate on TAD + Amp was indistinguishable from that on TAD alone (Figure 2B). These results suggest that there was essentially no cryptic growth during prolonged incubation on TAD medium and that the effect of Cam was to increase the death rate.

Together, these experiments indicate that the continued accumulation of trp^+ revertants by colonies on TAD plates could not be explained by cryptic growth. That conclusion, however, is based upon the assumptions that the doubly mutant strain FCY6 exhibits the same death kinetics as the isogenic singly mutant strains and that there is no substantial difference between the death rate on TAD medium, which completely lacks tryptophan, and on TAD medium, in which the initial 5 μM tryptophan has been depleted by colony growth. In a separate experiment, strain FCY6 exhibited the same death rate on TAD medium that initially contained 5 μM tryptophan as it did in the above experiments. Measuring death rates in single mutant strains can be complicated by the presence of revertants as the colonies age. To avoid that complication, individual colonies of strain FCY21B growing on TAD medium containing 5 μM tryptophan, and containing no visible papillae, were resuspended. The death rate of that *trpB* strain, 0.22 ± 0.03 per day, was indistinguishable from that of the doubly mutant *trpA-trpB* strain. It thus appears likely that both of the above assumptions are valid.

If no cell divisions occurred during tryptophan starvation, then the papillae that appeared during that period must have arisen from time-dependent rather than replication-dependent mutations and the mutation rate should be expressed as mutations per cell

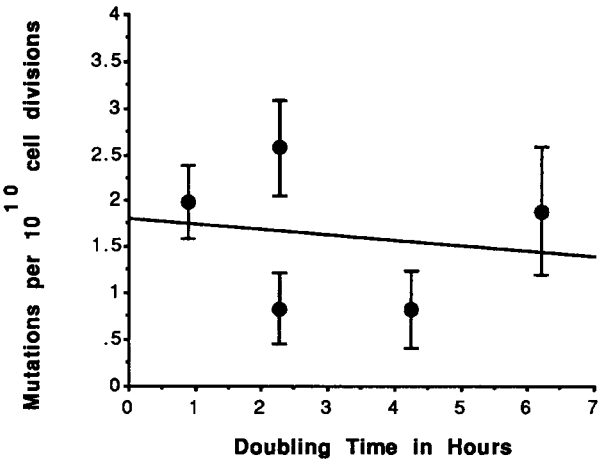

FIGURE 3.—Mutation rates in growing cells as a function of growth rate. Mutation rates of strain FCY2B from $trpA^-$ to $trpA^+$ were estimated from fluctuation tests. Growth rates at 30° were varied by varying the growth medium. From lowest to highest doubling times: LB medium, glucose minimal medium, glycerol minimal medium, acetate minimal medium.

per unit time. (DNA replication, which yields two molecules from one, is here distinguished from DNA repair synthesis, which produces no net increase in the number of DNA molecules present.) Clearly, replication-dependent and time-dependent mutations reflect different processes. The replication-dependent mutation rate during tryptophan starvation is essentially zero: no replications means no replication-dependent rate. In order to determine whether the mutation rate increased during tryptophan starvation, we need to know the time-dependent mutation rate in growing cells.

If there is a time-dependent component of the mutation rates measured by the fluctuation test, then the mutation rate per cell division should increase in slowly growing cells. To measure the time-dependent component of mutation rates in growing cells, the $trpA \rightarrow trpA^+$ reversion rate was measured when the cells were growing in media (LB, glucose minimal, glycerol minimal or acetate minimal) which established a range of growth rates. The slope of the mutation rate versus culture doubling time is -5.7×10^{-12} mutations per cell division per hour (Figure 3); however, given the uncertainties associated with these estimates, it can only be concluded that there is no detectable time-dependent component of the mutation rates measured in growing cells. It is reasonable to conclude that, rather than observing an increased mutation rate in old colonies during tryptophan starvation, we are observing a different mutational process.

An alternative interpretation of these observations is that all of the papillae that appeared over a period of 14 days on colonies grown on TAD-limited tryptophan plates were the result of mutations that occurred during the first 3 days of colony growth. This

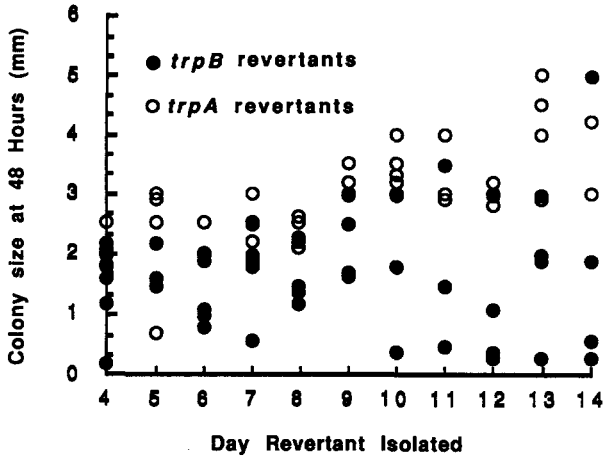

FIGURE 4.—Revertant colony sizes versus day of isolation. Revertants isolated in the course of the experiment described in Figure 1 were diluted, plated on TAD medium and incubated at 30° for 48 hr. Colony sizes were measured with an ocular micrometer using a dissecting microscope.

requires either that the growth of some revertants was very slow or that growth was sometimes delayed by the majority trp^- population. That explanation seemed unlikely on two grounds. First, when reversion rates in growing cultures were measured by the fluctuation test, continued incubation of the selective plates produced no additional revertants. Second, even the most slowly growing revertants produced visible colonies on TAD medium within 48 hr of plating, and the sizes of the revertant colonies were essentially unrelated to the day on which they were isolated (Figure 4; see discussion below). Reconstruction experiments were performed to rigorously test the hypothesis of delayed appearance. Strain FCY6 carries both the *trpA46* and the *trpB9578* mutations and has not yet been observed to revert to trp^+. Instant colonies (see MATERIALS AND METHODS) were prepared that contained either about 3×10^7 FCY6 cells or both 3×10^7 FCY6 cells and ~1 trp^+ revertant cell in the same colony (Table 2). TAD plates were prepared with 150 instant colonies of each kind and examined after 24 hr at 30°. As a control, 150 1-μl drops, each containing ~1 trp^+ revertant cell, were also placed on TAD medium. At 24 hr none of the pure-FCY6 instant colonies had papillae, and none appeared over the course of the next 2 days. In four such experiments, using two phenotypically normal and two unusually slowly growing revertants (see below), the number and distribution of papillae on the surfaces of instant colonies that contained ~1 revertant cell were indistinguishable from the number and distribution of colonies derived from microdrops each containing ~1 revertant cell (Table 2). Even the unusual revertants that grew slowly on TAD medium produced both colonies and papillae within 48 hr. The hypothesis that the delayed appearance of papillae results from the delayed growth of preexisting revertants can thus be rejected.

TABLE 2

Reconstruction experiments

trp^+ revertant	FCY6 cells per instant colony	Papillae per instant colony[a]	trp^+ revertant colonies per microdrop[a]
trpA-R10[b]	5.7×10^7	$N_0 = 95$	$N_0 = 96$
		$N_1 = 47$	$N_1 = 44$
		$N_2 = 8$	$N_2 = 8$
		$N_3 = 0$	$N_3 = 2$
trpA-R12[c]	3.1×10^7	$N_0 = 1$	$N_0 = 2$
		$N_1 = 9$	$N_1 = 15$
		$N_2 = 26$	$N_2 = 25$
		$N_3 = 31$	$N_3 = 33$
		$N_4 = 38$	$N_4 = 27$
		$N_5 = 27$	$N_5 = 19$
		$N_6 = 12$	$N_6 = 12$
		$N_7 = 5$	$N_7 = 14$
		$N_8 = 1$	$N_8 = 2$
		$N_9 = 0$	$N_9 = 1$
trpB-R14[b]	5.7×10^7	$N_0 = 88$	$N_0 = 99$
		$N_1 = 51$	$N_1 = 43$
		$N_2 = 11$	$N_2 = 8$
trpB-R38[d]	2.7×10^7	$N_0 = 23$	$N_0 = 24$
		$N_1 = 44$	$N_1 = 42$
		$N_2 = 39$	$N_2 = 40$
		$N_3 = 27$	$N_3 = 25$
		$N_4 = 13$	$N_4 = 11$
		$N_5 = 3$	$N_5 = 4$
		$N_6 = 1$	$N_6 = 2$
		$N_7 = 0$	$N_7 = 0$
		$N_8 = 0$	$N_8 = 2$

[a] N_i = Number of colonies with i papillae or of microdrops with i colonies.
[b] Papillae and colonies counted 20 hr after plating.
[c] Papillae and colonies counted 39 hr after plating.
[d] Papillae and colonies counted 48 hr after plating.

Increased production of *trp+* reversions depends on tryptophan starvation: In experiments such as that shown in Figure 1, the revertants that appeared between days 4 and 14 were the result of mutations that occurred during tryptophan starvation when the mutations were advantageous. Would those same mutations have occurred in nongrowing colonies if the mutations were not advantageous? FCY7 (*trpB cysB*) colonies were grown on sterile Millipore filters on either TAD plates supplemented with 5 μM tryptophan (259 colonies) or on CAD plates supplemented with 10 μM cysteine (197 colonies). On day 4 the colonies were examined for the presence of Trp^+ papillae on the TAD plates or Cys^+ papillae on the CAD plates. No papillae were detected; indeed, based upon earlier experiments, none were expected on the TAD plates. Several colonies from each medium were resuspended to estimate viable cells per colony. The plates were then incubated for another 7 days at 30° and again examined for papillae, and the number of viable cells per colony was determined from colonies

without papillae. On day 11 there were 33 papillae on the remaining 241 colonies on TAD medium, and 11 papillae on the remaining 184 colonies on CAD medium. On TAD medium the average survival of viable cells per colony was 22.7%, corresponding to a death rate of 0.212 per day, and on CAD the survival was 42.6%, corresponding to a death rate of 0.122 per day. The filters from the TAD plates were then transferred to CAD medium lacking cysteine, and those from CAD plates to TAD medium lacking tryptophan, and were incubated for an additional 40 hr. If *trp*$^+$ reversion mutations occurred at the same rate on cysteine-limited CAD medium as they did on tryptophan-limited TAD medium, then *trp*$^+$ revertants should have accumulated within the colonies held for 11 days on CAD medium. Similarly, *cys*$^+$ mutants should have accumulated in the colonies maintained on TAD medium. Because revertants accumulate linearly with time, during the 7-day interval the average time since the occurrence of a mutation would be 3.5 days. Based on the estimated death rates on the respective media and on the number of colonies tested, I would expect to have seen 16–17 additional papillae on the colonies shifted from CAD to TAD medium and 6–7 new papillae on the colonies shifted from TAD to CAD medium. However, I saw no additional papillae. Because the results of the reconstruction experiments reported above showed that papillae appear within 24 hr of the appearance of a revertant within a colony, I conclude that *trp*$^+$ revertants do not occur during prolonged cysteine starvation and that *cys*$^+$ revertants do not occur during prolonged tryptophan starvation.

Because colonies starved for amino acids continued to produce *trp*$^+$ revertants only in the absence of tryptophan, I conclude that about 95% of the *trp*$^+$ mutants present after 10 days of selection resulted from mutations that occurred as a consequence of the selection; *i.e.*, they were specific to the particular environmental challenge, they were not simply time- or starvation-dependent, and they would not have occurred in the absence of selection.

The selective conditions are not generally mutagenic: It is possible that long-term tryptophan starvation is nonspecifically mutagenic and that the continued accumulation of revertants simply reflected an increased overall mutation rate. To test this notion, 62 FCY6B colonies that had been on TAD plates for 14 days were resuspended and used to estimate the rate of mutation to valine resistance (Val^R).

Growth of *E. coli* K12 is inhibited by valine, which acts as a false feedback inhibitor of isoleucine biosynthesis, and mutations in several *ilv* genes can overcome this inhibition (VINOPAL 1987). When the resuspended colonies, containing an average of 2.4×10^8 cells, were plated onto high valine medium to select ValR mutants, 24 of the 62 produced no mutants, leading to an estimated mutation rate of $(2.8 \pm 0.5) \times 10^{-9}$ per cell in aged, tryptophan-starved colonies. The mutation rate of growing FCY6B cultures, as determined from the fluctuation test, was $(2.1 \pm 0.5) \times 10^{-9}$ per cell division. These rates are about 100-fold lower than observed in a different *E. coli* strain (HALL 1988) but the basis for this difference is presently unknown.

It is possible that long-starved Val^R mutants (*i.e.*, those arising in aged colonies) might have a lower plating efficiency on Val^R selective medium than would Val^R mutants in growing cultures. Were this the case, then a generally increased mutation rate in old colonies might fail to be detected. To test this possibility, four different Val^R mutants of strain FCY6B carrying Tn*10* and therefore tetracycline-resistant (Tet^R) were mixed with strain FCY6 (Val^S Tet^S) at ratios of ~1:100. The mixtures were concentrated to $\sim 10^7$ cells/μl and dilutions were plated on glucose minimal medium to determine the total number of cells, on high valine medium (HALL 1988) to determine the number of Val^R cells, and on LB + Tet to determine the number of Tet^R cells. In fact, the plating efficiency of the Val^R mutants on high valine medium was 0.697 ± 0.083 relative to plating on LB + Tet. Drops (1 μl) of the mixtures were dispensed to form instant colonies on filters placed onto TAD plates and the plates were incubated for 13 days. Colonies were resuspended, diluted, and plated onto the same three media. At that time the number of viable cells per colony had declined 50-fold but the ratio of Val^R to Val^S cells remained the same and the plating efficiency on high valine medium was 70% of that on LB + Tet. I conclude that an increased rate of mutations to Val^R would have been detected in these experiments had it occurred.

Because valine resistance has been used in three previous studies to determine whether mutation rates were generally increased at loci not under selection (CAIRNS, OVERBAUGH and MILLER 1988; HALL 1988, 1989), it seemed important to examine the mutation rate at another locus. Mutations to *lacI*$^-$ can be selected by plating on medium where phenyl-β-galactoside is the sole carbon and energy source (MILLER 1972; SCHAAPER, DANFORTH and GLICKMAN 1986). The mutation rate to *lacI*$^-$ in growing FCY6B cells was estimated from a fluctuation test as $(1.3 \pm 0.4) \times 10^{-6}$ per cell division. Thirty-six 13-day-old colonies of FCY6B from TAD plates supplemented with 5 μM tryptophan were used to estimate that the mutation rate in aged colonies was $(7.5 \pm 1.9) \times 10^{-7}$.

Based on these experiments, the hypothesis that the selective conditions were nonspecifically mutagenic can be rejected.

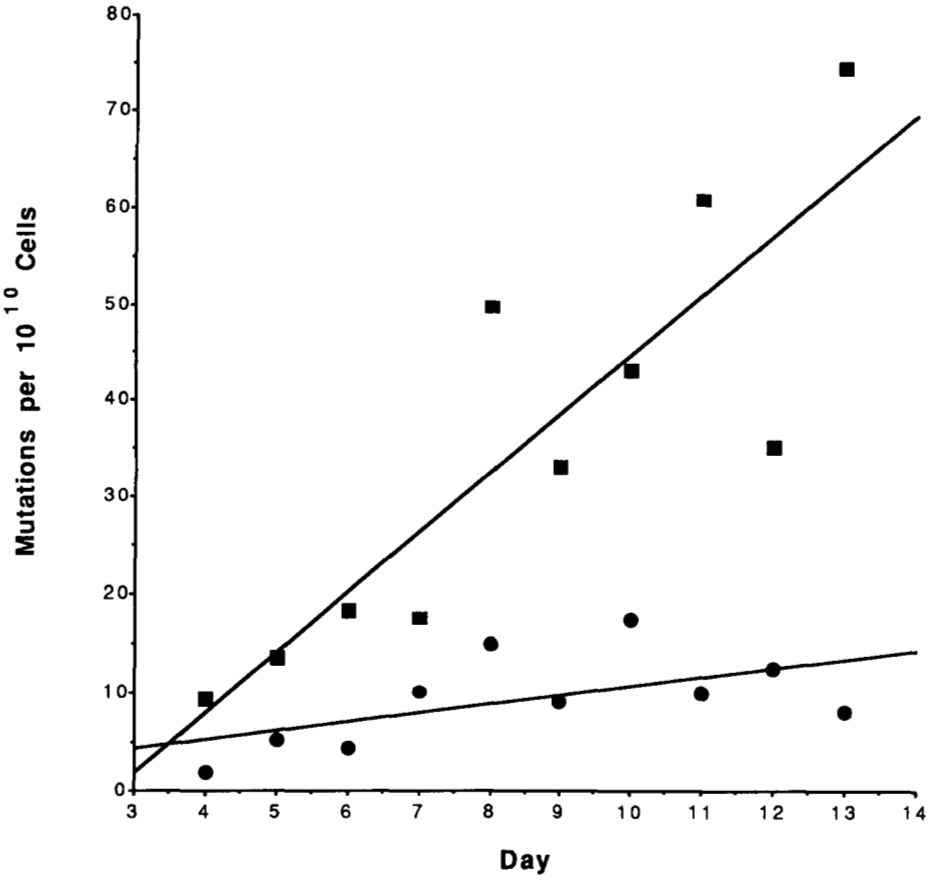

FIGURE 5.—Mutation rates in colonies on TAD medium as a function of the age of the colonies. Mutation rates were estimated from the proportion of colonies with no papillae and from the number of viable cells per colony assuming a death rate of 0.242 per day. Circles = reversion of *trpA*$^-$ in strain FCY2B, squares = reversion of *trpB*$^-$ in strain FCY21B.

The time-dependent mutation rate increases as colonies age: The increase in the fraction of colonies with *trp*$^+$ papillae was approximately linear over the course of the experiments (Figure 1). This is unexpected because the number of viable cells per colony decreases exponentially (Figure 2). The mutation rate each day was calculated as the number of papillae that appeared on that day divided by the number of viable cells on the previous day (Figure 5). For *trpA*, the mutation rate (probability per cell of a *trp*$^+$ mutation) increased by 1.3×10^{-10} per day, and the probability that the rate remained constant (*i.e.*, that the slope was actually 0) was 0.026. For *trpB* the rate increased by 7.9×10^{-10} per day and the probability that the rate was constant was 0.0003.

Properties of Trp$^+$ revertants: Fifty-two *trpA46* revertants and 58 *trpB9578* revertants were isolated immediately upon being identified as papillae. They were purified and characterized by the size of 48-hr colonies grown on TAD medium at 30° (Figure 4). Colony size was independent of the day on which the papilla appeared. On TAD and on PD (glucose minimal) plates without tryptophan, all but one of the *trpA46* revertants formed colonies of the same size and appearance as colonies of the wild-type strain, W3110. The exception, revertant *trpA*-R12, formed small colonies on TAD plates and failed to grow on PD plates. Growth rates of three of the ordinary *trpA46* revertants and of the revertant designated *trpA*-R12 were measured by monitoring increases in culture turbidity over several hours (Table 3); the revertant *trpA*-R12 grew slowly on TAD medium, not at all on PD medium.

Twelve of the *trpB9578* revertants also produced unusually small colonies on TAD medium. In contrast to the unusual *trpA46* revertant, the unusual *trpB9578* revertants did grow on PD medium. The growth rates of four of the unusual *trpB9578* revertants and of three ordinary *trpB9578* revertants were determined (Table 3). The unusual *trpB* revertants grew faster on the simple PD medium than they did on the richer TAD medium.

To determine whether the unusual revertants were the result of mutations in the *trp* operon, they were used as donors in bacteriophage P1-mediated transductions into the *trpB9578-cysB* strain FCY7. *cysB*$^+$ transductants were selected on PD medium supplemented with tryptophan and replicated to PD medium without tryptophan to determine the frequency with which the *trp*$^+$ reversion cotransduced with *cysB*. The wild-type donor W3110 exhibited 52% cotransduction between *cysB* and *trp*, and the various *trpB* revertants exhibited between 40% and 70% cotransduction, indicating that the reversions were, indeed, the

TABLE 3

Growth rates of *trp*$^+$ revertants

Strain	TAD	PD
W3110	1.18	0.59
FCY2 (*trpA*)[a]	1.09	0.47
trpA-R10	1.14	0.60
trpA-R12	0.59	<0.05
trpA-R15	0.92	0.50
trpA-R46	0.94	0.52
FCY21 (*trpB*)[a]	1.02	0.40
trpB-R14	1.15	0.55
trpB-R17	1.00	0.44
trpB-R26	0.56	0.36
trpB-R38	0.05	0.16
trpB-R45	0.15	0.22
trpB-R54	0.17	0.23
trpB-R56	0.11	0.22
trpB-R57	1.04	0.44
trpA-R12T[b]	0.99	0.52
trpB-R17T	1.12	0.55
trpB-R38T	0.26	0.08
trpB-R45T	0.40	0.24
trpB-R54T	0.21	0.27
trpB-R56T	0.99	0.47

Growth rates are reported as first-order rate constants in hr^{-1}. Values are means of at least two independent determinations.

[a] Determined in media containing excess tryptophan.

[b] Strains designated T are transductants.

result of mutations in the *trp* operon. When *trpA*-R12 was the donor, 13 of 26 *cysB*$^+$ transductants were *trp*$^+$; whereas when *trpA46* was the donor, none of 41 *cysB*$^+$ transductants was *trp*$^+$. Thus, the *trp*A-R12 mutation also mapped to the *trp* operon.

The growth rates of several of the transductants were determined (Table 3). Although the *trpA*-R12 revertant did not grow on minimal medium and grew at only half of the wild-type rate on TAD medium, the *trpA*-R12T transductant from that strain grew normally on both media, indicating that the *trpA*-R12 revertant carried an additional mutation elsewhere in the genome. Similarly, although *trpB*-R56 was unusual, its transductant *trpB*-R56T was normal. The phenotype of the *trpB*-R38T transductant was very similar to that of the parent strain, *trpB*-R38, suggesting that there was probably a second-site mutation within *trpB*. The other unusual *trpB* revertants were more complex. The transductants *trpB*-45T and *trpB*-54T grew at about the same rate as their parents on PD medium but grew more rapidly than did their parents on TAD. Because neither of these transductants grew at the wild-type rate on either medium, it is likely that both were also second-site *trpB* revertants; but the possibility that they, too, had additional lesions is not excluded.

Among the 110 *trp*$^+$ revertants that were screened, two (*trpA*-RI2 and *trpB*-R56), or 1.8%, carried additional auxotrophic mutations. When 4530 cells from a 15-day-old *trpA47* colony were plated on LB medium and the resulting colonies were replicated to PD plates supplemented with tryptophan, none carried an auxotrophic mutation. There is, therefore, a 99% probability that the frequency of auxotrophic mutations is $<10^{-3}$ per cell under these conditions, and the frequency of auxotrophic mutations is thus at least 18 times higher among the *trp*$^+$ revertants than among other cells in the colony.

The failure to detect such auxotrophs among the *trp*$^-$ cells in an aged colony is not surprising. Because there is no increase in mutation rates at other loci in aged colonies, the auxotrophic mutants must have arisen during the growth of the colony. There are about 100 *E. coli* genes in which mutations might lead to auxotrophy. Assuming $\sim 10^3$ bp per gene, and assuming that ~20% of base substitutions would lead to loss of function (SCHAAPER, DANFORTH and GLICKMAN 1986; SCHAAPER and DUNN 1987), the target size for base pair substitutions leading to auxotrophy is about 2×10^4 bp. At a rate of $\sim 10^{-10}$ bp substitutions per cell division (DRAKE 1969; J. W. DRAKE, personal communication), we would expect base substitutions to generate auxotrophy at a rate of about 2×10^{-6} per cell division. The frequency of other kinds of mutations (frameshifts, deletions, insertions, duplications) together is about twice the frequency of detected base substitutions (SCHAAPER, DANFORTH and GLICKMAN 1986); thus, we expect auxotrophic mutations to occur at a rate of about 6×10^{-6} per cell division among *trp*$^+$ revertants, and the frequency of auxotrophs in a colony of about 2×10^7 cells to be about 3×10^{-5}. The observed frequency of 1.8×10^{-2} is thus about 600-fold higher than expected if the two mutations (*trp* reversion and an auxotrophic mutation) were independent events.

CONCLUSIONS

LURIA and DELBRÜCK (1943) explicitly assumed that there was a "fixed small chance per time unit . . . to undergo a mutation" and that the time unit should be division cycles of the bacteria; *i.e.*, a constant probability of mutation per cell division. However, they also pointed out that "The assumption of a fixed chance per time unit is reasonable only for bacteria in an identical state. Actually the chance may vary in some manner during the life cycle of each bacterium and may also vary when the physiological conditions of the culture vary, particularly when growth slows down on account of crowding of the culture." With this caveat presumably in mind, most studies of spontaneous mutation rates have been conducted in exponentially growing cultures in which the cells are well dispersed. Neither exponential growth nor physiological homogeneity are characteristic conditions in nature, and an accurate understanding of the role of mutation in evolution demands that we consider conditions both

less consistent and less convenient than exponential growth.

The results presented here provide additional support for the proposition that mutations can occur under some conditions more often when they are advantageous than when they are neutral. The results thus extend similar recent reports (SHAPIRO 1984; CAIRNS, OVERBAUGH and MILLER 1988; HALL 1988, 1989). The key observation is that *trp*$^+$ revertants continue to accumulate during prolonged incubation of *trpA* and *trpB* colonies on medium lacking tryptophan. During that prolonged incubation, the number of revertants increased 3–30-fold over the number that arose during the growth phase of the colonies. Reconstruction experiments with "instant colonies" showed that the continued appearance of revertants was not the result of delayed growth by some mutants that had arisen during the initial phase of colony growth. Because there was no evidence for cryptic growth of cells within the colonies, the revertants that appeared between days 4 and 14 were the result of replication-independent mutations. The observation that neither the mutation rate to valine resistance nor that to *lacI*$^-$ increased under the same conditions means that the selective conditions did not generally increase the rate of replication-independent mutation. The rate of reversion of the *trpA* and *trpB* point mutations was not simply increased by prolonged incubation during amino acid starvation, because no increase in the *trpB* reversion rate was observed when strain FCY7 colonies were maintained on cysteine-depleted medium containing an excess of tryptophan. Together, these observations are consistent with the concept of Cairnsian mutagenesis: selectively advantageous mutations occur at an elevated rate in targeted genes without an overall increase in the mutation rate.

A heuristic model for selection-induced mutations: The existence of a mechanism that would preferentially produce advantageous mutations under conditions of cellular stress, conditions such as those found during prolonged starvation for amino acids or carbon sources, would be enormously advantageous to an organism. The problem is to explain how selective conditions could increase the frequency of useful mutations without increasing the frequency of mutations at other loci, such as those responsible for valine resistance.

CAIRNS, OVERBAUGH and MILLER (1988) suggested, among other possibilities, that either the production of such mutations or the decision to retain them might be mediated by reverse transcription of mutant mRNAs (RNA synthesis is quite error prone) that had by chance encoded a useful protein product. The absence of a demonstrated reverse transcriptase activity in *E. coli* KI2 (although it has been demonstrated in other *E. coli* strains), and the difficulty of imagining a means by which a cell could identify the particular mRNA molecule that encoded the useful polypeptide, prompted a search for alternative models.

STAHL (1988) suggested an alternative in which nutritionally depleted cells repair mutations slowly. The consequence of slow repair would be that mutations in the coding strand might persist long enough to be transcribed and translated. If the resulting mutant gene product permitted the cell to solve its current problem, *i.e.*, to make an amino acid or to obtain energy from an available carbon source, then replication of the chromosome might proceed through the site of the mutation and the mutation would be fixed in the DNA. If, on the other hand, the mutation provided no advantage to the cell, then no DNA replication would ensue and the mutation would eventually be repaired by the slowly acting mismatch repair system. In order to account for a failure to observe a general increase the the mutation rate, the STAHL mechanism requires that the *mutant* base always be repaired to wild type. If cells begin to degrade some of their own DNA under conditions of stress, and if the repair of such damage were error-prone, then the mutations would tend to arise in the newly synthesized (*i.e.*, undermethylated) strand. Because mismatch repair systems tend to selectively repair the undermethylated strand, unidirectional repair is a reasonable possibility. The STAHL model, however, does not explicitly account for the auxotrophic mutations that occurred among the *trp*$^+$ revertants 600-fold more often than expected from independent events.

Several years ago I discovered that all spontaneous *ebg*$^+$ mutants (mutants of Δ*lacZ* strains that grow on lactose) were, in fact, double mutants (HALL and CLARKE 1977) and I later pointed out that these spontaneous double mutants appeared as papillae at rates that precluded their being the result of independent events (HALL 1982b). Those observations suggested that multiple mutations may result from some common triggering event or condition, and they generated a model that can serve as an alternative to the "instructional" models of CAIRNS, OVERBAUGH and MILLER (1988) and to the STAHL model. Consider that, at any instant during periods of prolonged starvation, some fraction of the cells in a colony enter into a hypermutable state in which extensive DNA damage and resulting error-prone repair synthesis occur, while the remaining cells in the colony remain essentially immutable. When cells are in the hypermutable state, mutations might occur at many sites in the same cell. If one of those mutations provided a solution to the current problem of blocked growth (by permitting synthesis of a required amino acid or by permitting metabolism of an alternative carbon source), then the cell could exit the hypermutable

state and be recovered (as a mutant). If, on the other hand, none of the mutations provided a solution, then the cell might soon die. As a consequence, when aged colonies are tested, mutations in loci not under selection (*e.g.*, Val^R or *lacI*$^-$) would not be recovered because the only viable cells would be those that had never entered the hypermutable state. This model predicts that although the frequency of mutations in unselected loci would not increase in most cells, it would increase dramatically among the selected mutants because every successful mutant (*trp*$^+$ revertants in these experiments) would have experienced the hypermutable state and survived. Minor variants of this model make different predictions about the locations of the mutations with respect to one another. The observed increase in the mutation rate over time could result from an increasing probability of entering the hypermutable state as the cells age, or from the slow accumulation of a product that was mutagenic only to cells in the hypermutable state.

Results of a simple Monte Carlo simulation in which the probability of entering the hypermutable state increased from 10^{-3}per cell per day to 10^{-2} per cell per day over the course of 10 days, the probability of a mutation increased 10^3-fold (*i.e.*, the probability of *trp*$^+$ reversion was taken as 2×10^{-7} and of an auxotrophic mutation as 1.2×10^{-2} in the hypermutable state) and the death rate was 0.242 per day, showed that in 10 days, 12.4% of the colonies that initially contained 5×10^7 cells would produce a *trp*$^+$ mutant and 1.9% of those *trp*$^+$ revertants would also contain an auxotrophic mutation.

There are other examples of spontaneous multiple mutations that occurred during prolonged intense selection. The failure to utilize citrate as a carbon source is one of the primary criteria for distinguishing *E. coli* from other members of the family Enterobacteriaceae. A citrate-utilizing mutant of *E. coli* K12 was isolated during prolonged carbon-source starvation in medium containing citrate. The citrate-utilization phenotype resulted from mutations in two widely separated genes (HALL 1982a). Additionally, DNA sequencing has shown that one spontaneous mutation in the *E. coli ebg* operon, selected as a papilla on the surface of an aged colony on MacConkey lactulose medium, consisted of three base-pair substitutions, only one of which was responsible for the selected phenotype (HALL, BETTS and WOOTTON 1989).

Both the STAHL model and the hypermutable-state model explain the apparent specificity of Cairnsian mutations on the basis of an underlying random mechanism. Because the randomness of spontaneous mutations forms such a basic part of our view of biological processes, most of us may be more comfortable with an underlying random mechanism than with a directed one. We should be cautious, however, about rejecting the notion of "directed" mutations simply because it makes us more comfortable to do so. It is possible to envision mechanisms that might preferentially direct mutations to target genes that are under selection. For instance, it has been suggested (DAVIS 1989) that transcription might be mutagenic under conditions of cellular stress, and a recent study (MELLON and HANAWALT 1989) has shown that transcription affects the probability of repair of UV-induced lesions. It is certainly the case that the *trp* operon is expressed during tryptophan starvation at the maximum level permitted by the availability of substrates for RNA synthesis; thus, some of the apparent specificity for *trp* mutations (when compared with Val^R or *lacI* mutations) might well derive from transcriptionally mediated targeting.

SYMONDS (1989) pointed out the imprudence of extrapolating from transposon-mediated mutations (HALL 1988; SHAPIRO 1984) to point mutations. The evidence presented here supports the generality of the phenomenon of Cairnsian mutations and demonstrates that it is not limited to transposon-mediated events. Although the two classes of mutations are basically distinct and have different molecular mechanisms, they may well share a common physiological trigger under conditions of cellular stress. Evidence for Cairnsian mutations has now been found in all cases where it has been sought. These include transposon-mediated *ara-lac* gene fusions (SHAPIRO 1984), excision of an insertion sequence from the *bglF* gene (HALL 1988), reversion of a nonsense mutation in the *lac* operon (CAIRNS, OVERBAUGH and MILLER 1988), reversion of an X-ray-induced mutation in the *metB* gene (HALL 1989) and, now, reversions of missense mutations in the *trpA* and *trpB* genes. To the extent that *E. coli* is typical of bacteria, it now seems reasonable to say that Cairnsian mutations can occur in both catabolic and anabolic bacterial genes. However, no evidence is yet available concerning Cairnsian mutations in even the simplest eukaryote.

The issue of Cairnsian mutations is of considerable importance to our understanding of evolutionary processes. If adaptive mutations occur not randomly but more often when they are useful, then many of our theoretical approaches to evolution and population biology will have to be reconsidered. For instance, the hypermutable-state model predicts that neutral mutations will occur simultaneously with Cairnsian mutations. Because there is little recombination within natural *E. coli* populations, such neutral mutations would enter the population in consort with advantageous mutations and their accumulation might be episodic rather than clocklike, reflecting the frequency of strongly selective environments. Under conditions that produce the hypermutable state, deleterious, mildly deleterious and neutral mutations

would only enter the population as hitchhikers accompanying strongly advantageous mutations. If it should turn out that Cairnsian mutations are common, we will face the challenge of trying to estimate how much adaptive variation in natural populations derives from random mutations and how much from selectively driven mutations.

I am grateful to P. W. BETTS, DONNA DOTY and R. O'DONNEL for skilled technical assistance, to C. YANOFSKY for providing *trp* mutant strains and helpful suggestions, to I. CRAWFORD for helpful information on *trpB*, and to J. CAIRNS and F. W. STAHL for their interest, suggestions and comments on earlier versions of this paper. This work was supported by U.S. Public Health Service grant GM 37110 and by National Science Foundation grant DMB 8903311.

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Communicating editor: J. W. DRAKE