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ADAPTIVE MUTATION: The Uses of Adversity[1]

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Abstract

When populations of microorganisms are subjected to certain nonlethal selections, useful mutants arise among the nongrowing cells whereas useless mutants do not. This phenomenon, known as adaptive, directed, or selection-induced mutation, challenges the long-held belief that mutations only arise at random and without regard for utility. In recent years a growing number of studies have examined adaptive mutation in both bacteria and yeast. Although conflicts and controversies remain, the weight of the evidence indicates that adaptive mutation cannot be explained by trivial artifacts and that nondividing cells accumulate mutations in the absence of genomic replication. Because this process tends to produce only useful mutations, the cells appear to have a mech- anism for preventing useless genetic changes from occurring or for eliminating them after they occur. The model that most readily explains the evidence is that cells under stress produce genetic variants continuously and at random, but these variants are immortalized as mutations only if they allow the cell to grow.

Keywords

directed mutation; spontaneous mutation; selection; evolution

* * *

Sweet are the uses of adversity,

Which like the toad, ugly and venomous,

Wears yet a precious jewel in his head.

W. Shakespeare, ca 1600 (112)

INTRODUCTION

In a paper published in 1988 entitled “The Origin of Mutants,” Cairns et al (16) suggested that “populations of bacteria, in stationary phase, have some way of producing (or selectively retaining) only the most appropriate mutations” (p. 144). This conclusion was based on experiments showing that when *Escherichia coli* was subjected to prolonged, nonlethal selection, advantageous mutations arose in the population, whereas nonadvantageous mutations did not. Furthermore, the mutations did not arise in the absence of specific selection for them. This phenomenon has been variously called directed, adaptive, Cairnsian, selection-induced, and stationary-phase mutation. The controversy that followed the publication of this paper has been depicted as a renewal of the battle between neo-Darwinism and Lamarckism. Even in peer-reviewed articles, scientists have exhibited a surprising fervor, verging on the religious, in debating this issue. The historical and sociological reasons for the intensity of the debate have been discussed elsewhere (57,106). In this review, I give a brief historical

[1]Dedicated to John Cairns on the occasion of his seventieth birthday.

perspective, attempt to evaluate the evidence, and discuss various mechanisms that could result in the production of adaptive mutations. The focus is mainly on *E. coli*, which has been the subject of the majority of studies (and is my area of expertise), but relevant results with other bacteria are included. Similar experiments with yeast are discussed in a separate section.

The scientific community has had difficulty reaching a consensus about what to name this phenomenon. "Directed mutation" implies a mechanism, whereas "stationary-phase mutation" obscures the apparent directedness of the mutations. "Selection-induced" implies that selection is inducing a process, but the role of selection is more likely to stop an ongoing process. After trying various names (15,36,37), I now favor "adaptive mutation," which has historical precedence (27) and is preferred by the phenomenon's discoverer (J. Cairns, personal communication). I define as adaptive those mutations that occur in nondividing cells during selection and are specific to the selection. Mutants that arise in nondividing cells but that either are not adaptive, or have not yet been shown to be adaptive, I refer to as stationary-phase mutations, again with historical precedence (101).

A HISTORICAL PERSPECTIVE

"The Last Stronghold of Lamarckism" (72)

It is difficult today to imagine the confusion and controversy that reigned in the first part of this century about the nature and variability of bacterial species. Even without the complication of impure cultures, distinguishing between genetic changes occurring among individuals in large populations and adaptation, which we now understand as enzyme induction, was nearly impossible for many bacteriologists (12,55). The experiments of Luria & Delbrück (73), Newcombe (84), the Lederbergs (67), and Cavalli-Sforza & Lederberg (18) were of paramount importance in finally establishing that bacteria had genes, that rare individuals were the source of heritable variation, and that populations of bacteria did not have the capacity to endlessly respond to and be modified by their environment. That is, bacteria were fundamentally the same as other organisms.

Because the Luria-Delbrück experiment (73) is at the heart of the adaptive-mutation controversy, it is worth a brief review here. A mutant arising by chance during the exponential growth of a culture will give rise to a clone of identical progeny. Luria's insight was that such a mutant arising early during growth will give rise to an exceedingly large number of mutant progeny—a jackpot. Thus, among parallel cultures grown from identical initial inocula, the distribution of the numbers of mutants will be characterized by a large variance. In contrast, if mutations only arise after exposure to the selective agent, every cell so exposed has an equal probability of mutating, and the numbers of mutants obtained among parallel cultures will have the narrow variance of the Poisson distribution.

When Luria & Delbrück did this experiment, selecting for resistance to bacteriophage T1, they obtained a high-variance distribution.[2] Thus, the case was made—mutations arise at random and without regard to utility. Given the importance and elegance of this experiment, criticizing it seems almost sacrilegious. But because they used a lethal selection, and dead cells cannot mutate, Luria & Delbrück's experiment did not prove that mutations cannot also arise after selection, or even in response to selection. Delbrück himself clearly understood this limitation. Commenting on a paper presented by Lwoff in 1946, Delbrück (27) drew a sharp distinction between selection for phage resistance and selection for carbohydrate utilization. He concluded: "In view of our ignorance of the causes and mechanisms of mutations, one should

[2]In fact, because expression of T1 resistance is subject to phenotypic lag (51), Luria & Delbrück obtained a variance much larger than would be predicted (4,73).

keep in mind the possible occurrence of specifically induced adaptive mutations" (27, p. 154).[3]

Establishment of the Mutation Paradigm

After the Luria-Delbrück experiment established that mutations can arise at random during exponential growth, it was, and still is, commonly assumed that all spontaneous mutations originate as mistakes made during genomic replication. Chemostat experiments established that spontaneous mutation rates were, in most cases, generation dependent and not time dependent (39,88). The one notable exception to this rule, bacteria growing under tryptophan limitation (62,87), was later shown be the result of a corresponding increase in the DNA content of the tryptophan-limited cells (61). The structure of DNA immediately suggested that spontaneous mutations arise as base mispairs, and several mispairing schemes involving rare tautomers and rotated bases were postulated (129,134). These specific schemes have been recently and dismissively reviewed in light of current structural studies (30). Nevertheless, mispaired bases must sometimes cause mutations, as do base analogues. Similarly, mispairing can also account for mutations induced by chemical mutagens (such as alkylating agents) that create base analogues in vivo and by a number of spontaneous lesions (e.g. base deamination, alkylation, and oxidation). In addition, misaligned but correctly paired bases may cause frameshifts, deletions, complex mutations, and even base substitutions (1,64,98).

An entirely different aspect of mutagenesis began to be discovered in the late 1940s. When *E. coli* is exposed to certain mutagens, such as UV light and X rays, a number of apparently unrelated responses are elicited. These include mutation, filamentation, and prophage induction, as well as the increased survival and mutagenesis of irradiated bacteriophage plated on irradiated cells. It took years to understand the underlying relationship among these diverse phenomena, which are today recognized as expressions of the SOS response (32,132,137). Blocks to DNA replication, such as DNA lesions, result in the activation of RecA, *E. coli's* major recombinational enzyme, to a state in which it facilitates the cleavage of several proteins. These include LexA (the common repressor of the SOS genes), repressors lamboid bacteriophage, and UmuD. The UmuC protein, the carboxy-terminal fragment of the UmuD protein, and RecA promote or allow the production of mutations by a still-unknown mechanism. The simplest model for the process is that the SOS proteins modify the replication enzyme, DNA polymerase III, to allow translesion synthesis. One of the important concepts of SOS mutagenesis is that mutations of this class, unlike replication errors, are not passive but are produced only with the active participation of the cell. Furthermore, SOS-dependent mutations, like SOS-independent mutations, are created by DNA replication. The mutation is neither the DNA lesion nor the base-lesion mispair, but is the erroneous but correctly paired base pair that results from the subsequent round of replication.

Some mutagenic events may not require require DNA synthesis. These include transposition of some classes of insertional (IS) elements, excision of IS elements, and inversions, translocations, and deletions due to homologous recombination (1,21,38,74,108). However, some of these rearrangements may occur via slipped mispairing during replication (1), and most models (nonreciprocal) recombination involve DNA synthesis (116). Clearly duplications and amplifications require at least limited DNA replication (2,128).

Given the variety of mutagenic mechanisms, which of these is the source of spontanteous mutations in nature? That mutation rates themselves are under selection has been persuasively argued by Drake (31), and most models of evolution assume a constant mutation rate per generation. Thus, polymerase inaccuracy is widely regarded to be the source of most, if not

[3]This comment was recently uncovered by R. Owen.

all, spontaneous mutations. Indeed, defects in the activities that correct replication errors are powerful mutators (20). This, of course, is not at all a conclusive argument because, in wild-type cells, these error-correcting pathways may be entirely adequate for the purpose. Nor can the SOS-response be said to be a major contributor to spontaneous mutation because *umuDC* mutations are, at most, only weak antimutators (105). Considering that studies of spontaneous mutation rates typically use exponentially growing cells, whereas in nature a cell probably spends only a small part of its life in exponential growth, the origin of most natural spontaneous mutations still awaits investigation.

Evidence Contradictory to the Mutation Paradigm

Although the current controversy is a direct result of the publication of Cairns et al (16), there had been previous rumblings of dissatisfaction with the mutation doctrine. While attempting to confirm the Luria-Delbück kinetics for reversion of a histidine auxotrophy, Ryan observed that His^+ revertants continued to arise for a period of 10 days after the cells were inoculated into medium without histidine (104). In a serious of papers published during the next nine years, he proposed and discarded many possible artifactual explanations for these results. With increasingly heroic experiments he showed that the delayed appearance of mutants did not result from slowly growing mutants, phenotypic lag, cell growth, or cell turnover (100,101, 103). Left with no other explanation than that DNA is synthesized in the absence of cell division, Ryan attempted, but failed, to document the requisite amount of DNA replication (102). His final conclusion was that a small amount of DNA must be synthesized, but by some process that increased the error rate (102). Ryan did not investigate whether neutral mutations were likewise occurring during stationary phase, nor did he ask if the late-arising mutations would have occurred in the absence of selection for them.

Twenty years later Shapiro (113) published a study of the peculiar characteristics of fusion formation during prolonged selection. His strain, which has figured prominently in the adaptive mutation controversy, carries the regulatory region of the arabinose operon and the carboxy-terminal region of the *lacZ* gene separated by a defective Mu bacteriophage. The strain is both Lac^- and Ara^-. Cells with deletions that fuse *lacZ* in frame with *araB* can utilize lactose if arabinose is present as an inducer [these have come to be called $Lac(Ara)^+$, and the prefusion strain $Lac(Ara)^-$]. Shapiro found that after plating $Lac(Ara)^-$ cells on lactose-arabinose minimum medium at 32°C, $Lac(Ara)^+$ colonies did not appear until 5–6 days later [although if a $Lac(Ara)^+$ fusion was transduced into cells, colonies appeared on this medium within 2 days]. After 5 days, the rate of appearance of new $Lac(Ara)^+$ colonies accelerated, reached a maximum at about 20 days, and then declined. The estimated frequency of fusion production before plating was $<3 \times 10^{-11}$ per cell, whereas after plating it was 1×10^{-8} per cell. Shapiro also found that preplating culture conditions, including prolonged (static) starvation in buffer, had little effect on fusion formation. He concluded that the classical studies had been biased by the use of lethal selections, and that mutational processes would have to be reexamined in cells under nonlethal selection.

As discussed by Cairns et al (16), certain bacteria are classified as late fermenters because they develop the ability to utilize unusual (for them) sugars only after long incubation in the presence of these sugars. We now know that *E. coli* and other bacteria contain cryptic genes that can be mutationally activated (44). In some cases, more than one mutation is required (17,42). Because the apparent frequency at which such multiple mutants arise vastly exceeds the frequency predicted if the mutations are independent, Hall (42) suggested that the mutations occur by some concerted mechanism.[4]

[4]Predating Cairns by a year, Opadia-Kadim (91) concluded that these multiple mutations resulted from a mechanism he called "postadaptive mutation."

Cairns et al (16) drew upon these discordant results and their own experiments to argue for "a non-random, possibly product-oriented form of mutation" (p. 142). First, however, they presented a critical discussion of the classical studies, emphasizing that, although these experiments proved that random mutations do occur, the use of lethal selections excluded the possibility of detecting any other sort. Thus, the occurrence of nonrandom mutations was not disproved.[5] Cairns et al presented new experimental evidence of three types. First, fluctuation analysis of reversion of a Lac^- strain to Lac^+ showed significant deviations from the Luria-Delbrück distribution toward the Poisson, suggesting that mutations were occurring after selection was applied. In addition, Lac^+ mutants continued to arise for several days after plating on lactose minimal medium. Second, mere starvation did not induce Lac^+ revertants or the Shapiro $Lac(Ara)^+$ fusion—the presence of the selective agent (lactose) was required. Third, a population accumulating Lac^+ mutants was not simultaneously accumulating mutants with a neutral phenotype (resistance to valine). These findings, together with the results of Shapiro (113) and Hall (42), led Cairns et al (16) to conclude that a process of trial and error was taking place. In the last part of the paper, they presented three molecular models that would allow cells to "subject a subset of their informational macromolecules to the forces of natural selection" (p. 145). Both the experimental evidence and the models are discussed below.

On a final historical note, Fitch, in a remarkable paper written in 1982 (34) on the occasion of the Darwinian centennial, made the theoretical argument that because, mutations are advantageous during stressful times, but genome-wide mutagenesis would be deleterious, organisms probably have evolved a mechanism for selectively mutating only the genes of relevance. He then proposed a model that was essentially the same as that suggested by Davis (25) (see below).

EXPERIMENTAL EVIDENCE FOR AND AGAINST ADAPTIVE MUTATION

Table 1 summarizes various studies reporting the occurrence of spontaneous mutations during nonlethal selection. Some of these tested whether the mutations were specific to the selection, whereas others did not. Below, I focus on the types of evidence that support the idea of adaptive mutation, drawing upon these studies as appropriate.

Do Mutations Occur After Selection?

DEVIATIONS FROM THE LURIA-DELBRUCK DISTRIBUTION—If a fluctuation test gives the high variance characteristic of the Luria-Delbrück distribution, then mutations arose during prior growth of the cultures. Both Ryan (100) and Cairns et al (16) argued the obverse —that obtaining a Poisson distribution is evidence, although not proof, that mutations occur after selection is applied. However, alternative explanations for reductions in the variance of a mutant distribution have been offered (69,106,123). Some of these are simply wrong, and none are particularly compelling: (*a*) With a nonlethal selection, a delay in the expression of the mutant phenotype would affect only the timing, not the distribution, of the mutants. (*b*) Phenotypic lag only applies to recessive phenotypes (51), and actually increases the variance of the mutant distribution.[6] (*c*) Reduced fitness of the mutant during nonselective growth can narrow the distribution, but one cannot argue that every mutant in every case has a poor fitness relative to the nonmutant;[7] in some cases the mutant is genotypically wild-type (37,47) or can reasonably be assumed to carry sequence change only in the relevant gene (15). (*d*) Poor plating

[5]As pointed out by Keller (57), this fact has not been disputed, but most people continue to believe that "Luria & Delbrück were 'right,' even if for the 'wrong' reasons" (57, p. 294).

[6]Phenotypic lag refers to the fact that certain traits are not expressed during the first few generations after the new mutant allele has appeared (51). During the unselected growth of population, mutants arising early will have ample generations to overcome this lag, whereas mutants that arise late will not. Thus, after selection is applied, cultures that actually contain low numbers of genotypic mutants will appear to contain none, whereas the size of jackpots will be unaffected. This will increase the variance.

efficiency of the mutant on the selective medium has only a slight effect on the distribution even when the efficiency is very low (123). (*e*) If the full mutant phenotype results only after sequential mutations, these must occur at unrealistically high rates to account for mutational events occurring at normal frequencies (69).

As stated by Sarkar (106), "any observation of a deviation from the Luria-Delbrück distribution can be explained by invoking subsidiary interactions...[but]...hypothesizing such subsidiary interactions consists of making additional empirical claims and these, in turn, require experimental support" (p. 255).

THE CONTINUED APPEARANCE OF MUTANT—With the exception of the sequential mutation hypothesis, the processes discussed above cannot account for the fact that, under selection, mutants continue to appear for days or even weeks (6, 15, 16, 45, 100, 113; B. Bridges, personal communication) and that the distribution of the late-arising mutants is Poisson (15,16).[8] In some cases, e.g. the Shapiro deletion, the mutants simply never arise during exponential growth (16,80,113). More typically, mutants arise both before and during selection, but most of the former are detected by 1–3 days after selection is applied (6, 15, 45, 100; B. Bridges, personal communication). Late-appearing mutants could simply be slow growers, but slowly growing mutants resulting from mutations that occurred before selection was applied will have a Luria-Delbrück distribution (100). Furthermore, although all the earliest arising mutants must, necessarily, be fast growers, fast-growing mutants also arise late (15,16,45,95).

Cell growth and turnover: Of all the possible trivial explanations for the appearance of mutants during selection, the most important is that the cells may be dividing, allowing mutations to arise by ordinary replication-dependent processes. No experiment should be taken seriously if this artifact has not been addressed. Growth could result from: (*a*) a nonstringent selection, (*b*) utilizable contaminants in the medium, (*c*) cross-feeding by preexisting mutants, and (*d*) cell turnover. All of these phenomena may occur, and the question, in every case, is whether the amount of resulting cell growth can account for the number of mutants that appear.

Table 2 compares the mutation rates before and during selection for experiments for which these data were available. I have calculated a parameter that I call "turnover," which can be thought of as the number of DNA replications that would have had to have taken place to account for the observed number of mutations, assuming that these mutations were replication dependent and arose at the same rate as the preselection mutations. For example, with the *lac133::lacZ* frameshift allele (15), 10^9 cells plated lactose minimal medium will give rise to 400 Lac^+ mutants during 5 days. If these resulted from generation-dependent mutations, then 96×10^9, or 10^{11}, Lac^- cells must have existed on that plate at some time during those five days. This is about the same amount of growth that Lac^+ cells can achieve (P. L. Foster, unpublished results), whereas Lac^- cells can, at most, double (15).

Clearly in most cases the magnitude of the required amount of cell growth is so great that the mutations cannot possibly be attributed to gross population increases. Indeed, under starvation for an amino acid, the viable cell population is often declining (45, 100; B. Bridges, personal communication). Also, in our experiments, cross-feeding could not account for late-arising mutants. When various dilutions of revertible Lac^- cells were plated with nonrevertible Lac^-

[7]Even when a mutation might be deleterious, such as those that create tRNA nonsense suppressors, the effect may be slight. For example, most amber suppressors (in contrast to ochre suppressors) have little effect on growth because UAG is infrequently used as a termination codon in *E. coil* (33).

[8]phenotypic lag cannot account for the late appearance of mutants during selection if the cells must divide to overcome the lag.

scavengers, the speed and frequency (per revertible cell) at which Lac^+ revertants arose was independent of the number of preexisting Lac^+ colonies (15).

Cryptic growth, i.e. the turnover of some of the population at the expense of the majority, is not so easy to dismiss. However, thermodynamic constraints apply—the number of cell deaths required to give rise to a new cell must be greater than one and has been estimated to be three to five (94). However, slow input of energy (i.e. from breakdown of components of the medium) plus nutrients from dead and dying cells could allow some cell turnover. In reconstruction experiments, both Ryan (101,103) and Hall (45) found penicillin did not increase death rates, which means that the cells were not, apparently, undergoing cell division. However, such experiments would not detect a small subpopulation of cells that might be metabolizing but not dividing, or dividing only slowly.

DNA synthesis: Cell turnover is perhaps irrelevant if what we are really interested in is DNA turnover. As discussed above, DNA synthesis is believed to be required to produce most types of mutations. If the preselection mutation rates apply, the amount of DNA synthesis needed to account for the mutations occurring during selection is the same as the turnover calculated in Table 2.

Only a few studies of DNA synthesis in cells in stationary phase have been done. After cells cease to divide, ongoing rounds of replication are completed, resulting in a 30–70% net increase in DNA, but this synthesis falls to zero within a few hours (83,102). Ryan (102) attempted to measure DNA synthesis in histidine-starved cells with a density-shift experiment. After ^{15}N-labeled cells were starved in the presence of ^{14}N for 18 days, <5% hybrid DNA was detected, although considering the number of His^+ mutants that appeared, 67% of the DNA should have been hybrid.[9] Using ^3H-thymidine incorporation, Boe (9) reported 5% turnover per genome per day in cells early into (glucose-limited) stationary phase, but this rapidly declined to 0.5%. Another estimate of DNA turnover in nondividing cells, although not cells in stationary phase, was; made by Grivell et al (41). After shifting a temperature-sensitive *dnaB* mutant to the nonpermissive temperature (to stop chromosomal replication), ^3H-thymine was incorporated into preexisting DNA at a rate equivalent to about 1% of the genome per day. However, Ryan was the only one to attempt to measure DNA turnover and mutation in the same experiment.

Taking these estimates at face value, the amount of DNA synthesis in nondividing cells, whether by genome-replication or turnover, would appear to be in the range of 0.5–5% per genome per day. Table 2 shows that these rates fail, in some cases by orders of magnitude, to account for the mutations that arise. We are left, therefore, with a limited number of possibilities: (*a*) the amount of DNA synthesis is being underestimated (e.g. because stationary cells might not use exogenous precursors); (*b*) the DNA turnover is restricted to a few regions of the genome that, somehow, always include the region where the mutation is being monitored; (*c*) the mutations that occur stationary-phase cells are, in every case, independent of DNA synthesis; or (*d*) the DNA synthesis that is taking place is far more error-prone than normal DNA synthesis. These possibilities are discussed further below.

Are Mutations Stimulated by Specific Selection for Them?

The radical conclusion reached by Cairns et al (16) was not simply that mutations occur in nongrowing cells, but that the mutations appear to be directed by selective pressure. The evidence presented was, first, that starvation per se did not result in the accumulation of mutants and, second, that only mutants with the selected phenotype appeared. For convenience, these two related aspects of the phenomenon are discussed separately.

[9]If only the *his* region was experiencing turnover, Ryan would surely not have detected it.

IS STRESS PER SE MUTAGENIC?

Point mutations: Cairns et al (16) introduced the delayed-overlay experiment to demonstrate that mutations did not accumulate when the cells were simply starving. Stationary-phase (glycerol-limited) Lac^- cells were plated in top agar on minimum medium without a carbon source, and at various times thereafter the plates were overlaid with top agar containing lactose. We have used this technique to study the reversion of several mutant *lacZ* alleles. In every case, no revertants accumulated during the time without lactose, and the rate at which mutant colonies appeared after the lactose was added was the same no matter how long the delay (15,16,37). We have also shown that Lac^+ mutants do not accumulate even in the presence of lactose if the cells are deprived of a required amino acid (15). Similar experiments, involving plating cells on filters that can then be transferred to different media, also have been used to show that starvation in the absence of specific selection is not mutagenic (45,127).

The delayed-overlay experiment has been criticized on the grounds that mutants appearing during the starvation period might die, whereas those appearing after lactose is added would not (68). This criticism ignores that fact that the numbers of Lac^- parents and preexisting Lac^+ revertants are perfectly stable during starvation (13,15,37). Another claim is that the potential artifact can apply even if mutants have the same death rate as nonmutants (68). However, in our experiments (15), this would mean during the first two days of starvation the total population on a plate would have had to decline 10-fold, and the remaining cells would have had to increase their mutation rate to exactly compensate. It seems more likely that the simple interpretation of the delayed-overlay experiments holds—starvation per se does not result in an increase in mutations.

It is true that if newly arisen mutants were somehow at a disadvantage in the absence of selection for them, the delayed-overlay experiments would fail to detect them. As stated by Cairns (13), “that, of course, is just another way of describing the very anomalies we are seeking to explain” (p. 527).

Movement of IS elements: Several studies of adaptive or stationary-phase mutation have involved mutational events mediated by IS elements (9,16,43,89,113,127). In these cases, nutritional conditions might play a role by nonspecifically triggering the movement of the elements. Complex patterns of Mu transpositions have been detected during the development of *E. coli* colonies (114), and several studies have reported increased movement of IS elements during stationary phase (3,93,130). In the case of the $Lac(Ara)^+$ fusion, both Shapiro (113) and Cairns et al (16) found that incubation stationary-phase cells in the absence of lactose did not induce fusion formation. Mittler & Lenski (80) subsequently reported that after incubation of the prefusion strain with aeration in liquid (glucose-limited) minimal medium for 9 days, $Lac(Ara)^+$ cells appeared at a frequency of 2×10^{-6} after plating on selective medium. Significantly, this frequency was unchanged after several generations of growth in nonselective medium, suggesting that the mutants preexisted and were able to give rise to clones of $Lac(Ara)^+$ progeny in the absence of selection. Both I (P. L. Foster, unpublished results) and Shapiro (79) confirmed these basic results using a medium comparable to that used by Mittler & Lenski. There are two interesting questions about this controversy. First, did Mittler & Lenski actually prove that the $Lac(Ara)^+$ fusion occurred in the absence of selection for it? The answer is clearly no. Mittler & Lenski did not confirm the preexistence of $Lac(Ara)^+$ mutants with any of the methods of classical genetics invented expressly for this purpose (namely, the fluctuation test, replica-plating and sib-selection). Thus, they did not rigorously exclude the possibility that the $Lac(Ara)^+$ cells were arising from events occurring after lactose selection among cells still predisposed to fusion formation. In addition, in Mittler & Lenski’s experiments, $Lac(Ara)^+$ cells had an eightfold greater survival than their $Lac(Ara)^-$ parents even in the absence of lactose. Thus, the fusion was being strongly selected whether or not lactose was present. In

contrast, Cairns et al (16) starved the cells in a moderately enriched (glycerol-limited) medium that did not select against the prefusion cells (14).

The second question is, why do different experimenters get different results? Clearly, uncontrolled factors are influencing the rate at which fusions occur. Fusion formation is under control of the Mu transposition functions (113), which are responsive to the nutritional status of the cell (J. Shapiro, personal communication). In addition, Shapiro & Leach (115) provided a model by which lactose could specifically stimulate the in-frame fusions that render the cell Lac(Ara)$^+$. During the recombinational events that are required for Mu transposition, a hybrid template could form, allowing transcription of an *araB::lacZ* fusion from the *ara* promoter before rearrangements are completed. In-frame hybrids would result in β-galactosidase production, which, in the presence of lactose, could stimulate completion of the process.

Hall (43) obtained an even more controversial result involving an IS element. He found that excision of an IS*103* element from the *bglF* gene, which codes for the β-glucoside transport protein, occurred in colonies on MacConkey medium only in the presence of salicin, the substrate of the *bgl* operon. However, Mittler & Lenski (81) have reported that a low frequency of excision events can occur in cells incubated in rich broth for 15 days in the absence of salicin, although once again they did not do a fluctuation test or any form of sib-selection. Hall (personal communication) has recently disputed this finding—in his hands, no Sal$^+$ mutants were found among approximately 3×10^{12} cells during four weeks of incubation on MacConkey medium without salicin. Hall also points out that Mittler & Lenski (81) failed to demonstrate that the Sal$^+$ mutants they obtained were excision mutants.

In conclusion, starvation appears not to be universally mutagenic, but can trigger IS elements to move, depending on the nutritional status of the cell. In addition, changes in DNA topology occur in starving cells (60), and supercoiling is known to influence transposition, site-specific inversions, and other types of chromosomal rearrangements (52,82,107,133). Such rearrangements may give rise to a variety of new phenotypes; for example, it is well known that cryptic genes can be activated both by insertion and by excision of IS elements (70,92, 97). Although mutations due to these kinds of events, if they occur regardless of utility, do not meet the narrow definition of adaptive mutation, they do fall into the more general category of stationary-phase mutation.

ARE THE MUTATIONS THAT ARISE SPECIFIC FOR THE SELECTION?—If mutations arise in response to selection for them, a population under selection would not accumulate mutants with a useless phenotype (16). To test this hypothesis, many cells under selection for one phenotype must be scored for a second, neutral phenotype. Because the cells are not growing, the second phenotype must be dominant and immediately expressed, making many easily scored phenotypes, like drug and phage resistances, inappropriate. Mutation to the second phenotype must be frequent enough so that the absence of mutants is meaningful, and these mutants should also be able to accumulate under selection for them. These constraints severely limit the number of mutational targets available.

Valine resistance (Val^R), which meets the above criteria, has been used as a neutral phenotype in several studies (6,16,43,45,56). All these experiments are consistent in showing that populations of cells under selection for carbohydrate utilization or reversion of an amino acid auxotrophy are not simultaneously accumulating Val^R mutants. However, it has been argued that Val^R may be a special case and its use should be viewed with caution (30,119). *E. coli* K-12 strains are sensitive to exogenous valine because one of the three acetohydroxy acid synthase isozymes, which catalyze the first step in the biosynthesis of valine and isoleucine, is inactivated by a two–base pair deletion in the *ilvG* gene (66). Because the other two isozymes are sensitive to feedback inhibition by valine, exogenous valine deprives *E. coli* K-12 of

isoleucine. The original mutation can be reverted by frameshifts, but mutations in at least eight other loci will also give a Val^R phenotype (26). Thus Val^R is a broad mutational target, but the expression of Val^R may be modified by genetic and physiological factors. Mutation rates to valine resistance vary widely (45, 56; R. Schaaper, personal communication), which suggests that under some conditions only subsets of Val^R mutants are expressed. In addition, excess valine slows the growth of even Val^R mutants (R. Schaaper, personal communication).

Hall (45) devised a reciprocal test. Colonies of a *trpB*$^-$ *cysB*$^-$ strain were grown for 11 days on filters placed on media limited for either tryptophan or cysteine. During that time, revertants appeared as papillae. Hall then switched the filters to media limited only for the other amino acid and incubated the plates for another 40 hours. Because no new papillae appeared, he argued that no Trp^+ mutants had occurred in the cysteine-limited colonies, nor had Cys^+ mutants accumulated in the tryptophan-limited colonies. One question about this experiment is whether 40 hours was sufficient time to allow a cell, starved for one amino acid but supplied with another, to switch its biosynthetic pathways in time to produce a visible papilla (36).

Thus the perfect second marker has not been found and, indeed, may never be. In addition to physiological constraints, any two mutational events may respond differently to the selective conditions (24,68). However, a persuasive experiment was done by Steele & Jinks-Robertson (121). Using Lys^- auxotrophs of *Saccharomyces cerevisiae* (see below), they found that while mutational events giving rise to LYS prototrophs did not occur during starvation for amino acids other than lysine, recombinational events that gave the same phenotype did. Because each result was obtained under the same conditions, this experiment eliminates most artifactual explanations.

In conclusion, the few examples showing that mutations that are not selected do not arise, and the many showing that mutations do not arise when they are not useful, are consistent with the idea that, in most cases, only useful mutations accumulate during selection.

The Specificity of Mutational Events

If the mutations that occur during selection are different from those that occur during exponential growth, the two processes are likely to be different. But there is a problem of selection bias. For example, a mutant appearing during exponential growth may have several generations to express its phenotype, whereas after selection is applied, only immediately expressed phenotypes allow growth. In addition, as the cells age, the selection pressure could change to favor certain subsets of phenotypes and disfavor others.

Benson (5) reported that, after plating *lamB*$^-$ cells (missing *E. coli's* major maltoporin) on maltodextrin medium, mutants that could use the maltodextrins, designated Dex^+, continued to appear for many days. All of the late-appearing mutants had mutations in *ompF*, which codes for a porin protein, although Dex^+ mutants in *ompC*, which codes for another porin, could be obtained if the starting strain was *ompF*$^-$. On subsequent investigation, Benson et al (6) demonstrated that OmpC(Dex^+) cells were at a disadvantage within a lawn of Dex^- cells whereas OmpF(Dex^+) cells were not. Thus, the apparent mutational bias was actually a selection bias.

Cupples and colleagues (22,23) have created a series of strains with constitutively expressed *lacZ* alleles carrying various mutations affecting the active site of β-galactosidase. Each of these can revert to Lac^+ by only a single, defined mutational event. In addition, all the alleles that detect base substitutions revert only to the wild-type DNA sequence; thus, all these revertants must have the same phenotype. Hall (47) compared the mutation rates of these strains during exponential growth (determined by fluctuation tests) to the frequency of Lac^+ papillation during lactose selection. The conclusion was that there was little correlation

between the exponential mutation rate and the ability of each strain to revert during selection. For example, among the base substitutions, the event with the highest rate during exponential growth was the A:T to T:A transversion, whereas during selection it was the G:C to T:A transversion. Similarly, among the frameshift events, +A did well during nonselective growth, but −G was the winner after selection.

Using *Salmonella typhimurium*, Prival & Cebula (95) identified the mutations carried by over 1300 His^+ revertants isolated early (2 days) and late (5–10 days) after cells were plated on minimal medium without histidine. The two *hisG* alleles used—a missense and an ochre mutation—can revert by a variety of intragenic events as well as by the creation of extragenic suppressors. To avoid selection bias, Prival & Cebula only considered mutants that were fast-growing after isolation. The results at the two loci were strikingly different. At the missense site, there was no difference in the spectra of mutational events obtained early and late; at the ochre site, the proportions of some events were changed whereas others were the same. In both cases, extragenic, suppressors were conspicuously absent among the late-arising, fast-growing revertants.

B. Bridges (personal communication) reported that the revertants of tyrosine auxotroph that arise during prolonged selection did not result from any of the well-characterized mutations that revert this auxotrophy during growth or after treatment with mutagens. In unpublished results, we have found that base substitutions are relatively poor whereas frameshift mutations are relatively good at reverting under selection. However, factors other than the mutational event are clearly important. For example, we generated random Lac^- and Ara^- mutants with a frameshift mutagen; out of 10 of each, half of the Lac^- and only one of the Ara^- mutants reverted under selection. Finally, in general not every mutational target that could, in principle, give rise to mutants under selection does in fact do so (47, 56; B. Bridges, personal communication; P. L. Foster & J. Cairns, unpublished results).

These results, particularly those obtained with the *lacZ* and *hisG* alleles that are relatively free of selection bias, strongly support the idea that the mutational processes that give rise to mutants during selection are different from those that give rise to mutants during exponential growth. Whether the results shed any light on the mechanisms by which the mutations arise is discussed below.

Multiple Mutational Events

As mentioned above, Lenski et al (69) argued that both the deviations from the Luria-Delbrück distribution and the continued appearance of mutants observed by Cairns et al (16) could be explained by the occurrence sequential mutations. They hypothesized that the final Lac^+ phenotype was due to two mutations. The first would give an intermediate phenotype, allowing the mutant to grow on lactose plates into an unobservable microcolony. The second mutation would then occur within this population, giving rise to a visible Lac^+ colony. This model has three parameters: the two mutation rates and the growth rate of the intermediate. To explain events occurring at frequencies of the order of 10^{-8} to 10^{-9}, as are typical of normal reversion events, and to prevent the population of the intermediate from being noticeable to the experimenter, the mutation rates must be set unreasonably high (S. Sarkar, personal communication). In fact, to model the results given in Cairns et al (16), the second mutation rate was set at 10^{-5} (69). Furthermore, the hypothesis requires additional assumptions about the nature of the mutational events. It does not seem likely that every single-step mutation that has been observed to occur during selection actually came about by a circuitous pathway. On the other hand, when two genetic events are known to be required for a particular phenotype, the frequency of the end result will be greatly increased if one of the genetic events confers some advantage. Hall (43,46) has reported two dramatic experiments in which double

mutations occurred at frequencies many orders of magnitude higher than expected; these are discussed in detail below.

THE BGL EXPERIMENT—Hall's first double-mutant experiment, mentioned above, was activation of a cryptic *bgl* operon (43) In the parental strain, the regulatory gene is noninducible (designated *bglR*^{0}) and the gene, *bglF,* that encodes the β-glucoside transport protein is inactivated by an *IS103* element. When this strain was plated on MacConkey salicin plates, Hall found that up to 10% of the cells in aged colonies were *bglF*$^+$. Because *bglR*^{0} *bglF*$^+$ cells cannot utilize salicin, he concluded that each mutant represented an independent excision event, somehow driven by an anticipated need for the *bglF*$^+$ gene product. The population of *bglF*$^+$ cells could then give rise to *bglR*$^+$ mutants with the full Sal$^+$ phenotype. However, as pointed out by Symonds (125), if *bglR*^{0} *bglF*$^+$ cells had even a slight ability to grow, then the frequency of excision events could be very much lower. Mittler & Lenski (81) tested this hypothesis and indeed found that *bglR*^{0} *bglF*$^+$ cells do have a selective advantage over *bglR*^{0} *bglF::*IS*103* cells on MacConkey salicin plates. This has been confirmed by Hall (personal communication), who agrees that growth of a few *bgIR*^{0} *bglF*$^+$ cells accounts for the high frequency of excision mutants that he observed. However, as mentioned above, Hall maintains that the excisions only occur when salicin is present.

THE DOUBLE-TRP EXPERIMENT—Hall's second double-mutation experiment used a *trpA*$^-$ *trpB*$^-$ strain (46). Both alleles (*trpA46* and *trpB9578*) carry missense mutations, and neither the *trpA*$^+$ *trpB*$^-$ nor the *trpA*$^-$ *trpB*$^+$ cells can grow without tryptophan. When plated on medium with limiting trypto-phan, which allowed colonies of about 5×10^7 cells to form, Trp$^+$ papillae began appearing on the colonies after 20 days of incubation. The frequency at which these papillae appeared was orders of magnitude greater than would be expected from the reversion rates of each single mutant. Hall then sequenced 11 revertants and found no changes other than the reverted bases themselves, a result that cannot be explained by any of the current models for adaptive mutation (46) (see below).

This, too, could be an example of growth of an intermediate. The *trpB9578* allele would allow *trpA*$^+$ *trpB*$^-$ revertants to accumulate and excrete indole, the product of the TrpA protein (C. Yanofsky, personal communication). *trpA*$^-$ *trpB*$^+$ mutants can convert indole to tryptophan (138). I observed that *trpA46 trpB*$^+$ cells could grow vigorously while being cross-fed indole by *trpA*$^+$ *trpB9578* cells, and I (36) and others (R. Kolter, personal communication) proposed that a subpopulation of *trpA*$^-$ *trpB*$^+$ cells could grow within the aging colonies and give rise to the Trp$^+$ papillae. If the time-dependent rate of reversion of *trpA46* to *trpA*$^+$ (45,46) is applied, about 105 *trpA46 trpB*$^+$ cells per colony would be required to produce the number of Trp$^+$ papillae observed. Khakoo (58) independently derived the same model, incorporating a more sophisticated mathematical treatment but reaching roughly the same conclusion. Importantly, Khakoo's model reproduces the time course of appearance of Trp$^+$ papillae that Hall observed.

Hall (personal communication) has now done a reconstruction experiment with artificial colonies of 10^8 *trpA*$^-$ *trpB*$^-$ cells each seeded with an average of one *trpA*$^-$ *trpB*$^+$ and one *trpA*$^+$ *trpB*$^-$ cell. He found that the population of *trpA*$^-$ *trpB*$^+$ cells indeed increased and reached an average level of 10^5 per colony. However, he disagrees that this amount of growth can account for the number of Trp$^+$ papillae that arise. The resolution of this disagreement awaits further experimentation; however, I feel we are not obliged to account for Hall's results when considering the mechanisms by which adaptive mutations may arise.

GENE AMPLIFICATION—Another type of sequential mutation can display all the attributes of adaptive mutation. If a gene has a partial activity, then successive amplifications may lead to a full phenotype. In eukaryotes, a classic example is the increased resistance to heavy metals

conferred by amplification of the metallothionein genes (35). In *E. coli*, certain leaky *lacZ* alleles can produce up to 200 copies during lactose selection (128). Such events may be interesting in themselves, and they may be manifestations of a general genomic instability induced by stress (75,76). However, because they are not stably inherited, whether amplifications per se should be considered as part of the phenomenon of adaptive mutation is questionable. Thus, it is important to check by prolonged cultivation under nonselective conditions that supposedly adaptive mutations are stable (75,128). For example, I easily screened 500 independent Lac^+ revertants of the *lac133::lacZ* frameshift allele and found that not one resulted from amplification (P. L. Foster, unpublished results).

ADAPTIVE MUTATION IN YEAST

In 1978, von Borstel reported that revertants of a tryptophan auxotroph(*trp1–1*) of *Saccharomyces cerevisiae* continued to appear for weeks during selection (131). Although von Borstel did, and still does (personal communication), attribute this to cell turnover, others have recently demonstrated adaptive mutation in *S. cerevisiae* (48,121). Repeating his previous experiments with *E. coli*, Hall (48) found that revertants of a histidine auxotroph (a missense mutation in *his4*), scored as papillae, arose after 5 days and continued to arise for an additional 25 days after cells were plated on histidine-limited medium. Because the histidine-starved cells did not accumulate revertants of a second defect (requirement for inositoll[10]), he concluded that reversion of *his4* was specific to the selection. [11] Steele & Jinks-Robertson (121) found that revertants of a lysine auxotroph (a frameshift mutation in *lys2*) continued to appear for 8 days during lysine selection, but did not accumulate when the cells were starved for tryptophan, leucine, or both lysine and tryptophan. Both studies included penicillin-type reconstruction experiments to show that the cells were not growing. Hall used medium without inositol and Steele & Jinks-Robertson used canavanine—in both cases growing cells would have been killed, and in neither case did death rates significantly increase. Both studies demonstrated that preexisting revertants could appear within 2-3 days, and Steele & Jinks-Robertson have subsequently confirmed this result with prototrophs that have been starved for various times (S. Jinks-Robertson, personal communication).

Although the production of mutations during selection was not as vigorous as observed with bacteria, these studies are convincing. As mentioned above, a subsequent study adds even more weight to the results with *lys2*. Steele & Jinks-Robertson (122) found that LYS prototrophs due to interchromosomal recombination events also continue to arise in nondividing cells, but in this case, the production of recombinants continued whether there was selection for them or not. Thus, mutation occurred in stationary phase only when it was adaptive, but recombination occurred whether it was adaptive or not.

Delayed appearance of mutants has also been reported for *Candida albicans* (75). With long exposure to sublethal concentrations of heavy metals, colonies of resistant cells began to appear after 5–10 days and continued to appear for 1–2 weeks thereafter. These resistances could have resulted from gene amplification, although the phenotypes were stable during a short period of nonselective growth. However, revertants of two auxotrophies also appeared with similar kinetics. None of these events in *Candida albicans* have, as yet, been shown to be specific to the selection imposed.

[10]Although this is a lethal selection, Hall (48) argued that “inositoless-death” is not exerted for one generation, and thus the INOL phenotype would have time to be expressed.

[11]Hall (48) also reported the delayed appearance of *his6*revertants, but did none of the required controls, nor did he demonstrate that the papillae were composed of HIS cells.

MECHANISMS OF ADAPTIVE MUTATION

> "Is there any other point to which you would wish to draw my attention?"
>
> "To the curious incident of the dog in the night-time."
>
> "The dog did nothing in the night-time."
>
> "That was the curious incident," remarked Sherlock Holmes.
>
> A. C. Doyle, 1892 (29)

Two aspects of adaptive mutation need explaining. The first is, what gives rise to the mutations? If, as seems likely, DNA synthesis is required to produce adaptive mutations, this question may be equivalent to asking what forms of DNA synthesis in nongrowing cells are important for the production of mutations. The second question is, why do only useful mutations appear under selection, or (the curious incident) why do mutations not appear when they are not useful? The answer to the second question will influence the answer to the first. Additional constraints are placed by the genetic evidence, meager though it is at present. Below, I present and discuss several possible mechanisms for adaptive mutation. I use the term variant to distinguish potentially transient changes in the cell's informational molecules from mutations, which are heritable sequence changes in the DNA.

Mutational Bias

Transcription is a readily available way that an organism under stress could target mutations to useful genes (16,25,34). Davis (25) and Fitch (34) proposed that because transcription results in regions of single-stranded DNA, and these are particularly vulnerable to the various DNA-damaging processes that give rise to mutations, transcription per se could be mutagenic. Because one difference between a cell starving in the presence of lactose and one starving in its absence is that the *lac* operon is induced in the former case, Davis (25) argued, and gave some evidence supporting his case, that the experiments reported by Cairns et al (16) needed no other explanation.

This model in its simple form has not stood up to the evidence. First, although the addition of isopropyl-β-D-thiogalactopyranoside (IPTG), a gratuitous inducer of the *lac* operon, increases the speed at which Lac^+ mutants appear after lactose is added, it does not change the numbers of Lac^+ mutants even if a second, metabolizable, carbon source is available (16,37). Second, constitutively expressed genes do not accumulate mutations in the absence of selection for them. This has been shown for suppressors of a *lacZ* amber mutation (16) and for two constitutively expressed *lacZ* alleles (15,37).[12] Thus, although the transcription model might explain why mutations arise when they are useful, it does not explain why mutations do not arise when they are not useful. If transcription is inherently mutagenic, it could be a source of variant DNA sequences in nongrowing cells, but some other mechanism must account for the selectivity of adaptive mutations.

Transcription might provide at least a partial solution to the problem of the missing DNA synthesis. As discussed above, the amount of DNA synthesis estimated to occur in nongrowing cells fails to account for the mutations that appear (Table 2). If DNA synthesis in stationary phase were confined transcribed genes, this gap would be closed by one or two orders of magnitude, depending on how many genes are being transcribed in a nongrowing cell. Both in *E. coli* and in mammalian cells, repair of UV damage is coupled to transcription and occurs

[12]Although it has been argued that a constitutively expressed gene might not be expressed in starving cells (120), the *lac133::lacZ* fusion is not likely to be inducible, and therefore a mutant could not declare itself Lac^+ in the presence of lactose if the gene were not being constitutively expressed.

preferentially on the transcribed strand (77,78).[13] Recently, this information was tied to a phenomenon called mutation-frequency decline (MFD) When *E. coli* strains are exposed to UV light and then held for a while under conditions that do not allow protein synthesis, mutations that would have arisen from DNA lesions in the transcribed strand are selectively lost (8,90,136). The *mfd* gene, which is required for MFD, encodes transcription-repair coupling factor (TRCF), which couples *E. coli's* UvrABC excision repair enzymes to the transcribed strand (63,111). Thus, TRCF supplies a possible mechanism by which DNA synthesis, prompted by randomly occurring DNA lesions, could be targeted to transcribed genes. Excision repair, of course, is designed to prevent errors, but it must also sometimes create errors and perhaps would be more likely to do so in starving cells.

B. Bridges (personal communication) investigated the effect of TRCF reversion of a tyrosine auxotrophy. As would be predicted by the above model, *a mfd*$^{-}$ strain could not revert during selection. However, this defect was not seen if the strain was also deficient in excision repair because of a mutation in *uvrA.* The simplest interpretation of these results is that the UvrA protein (or excision repair itself) prevents mutation in stationary phase cells unless TRCF is also present (B. Bridges, personal communication).

This hypothesis is consistent with other studies showing that adaptive mutation occurs perfectly well in cells that are deficient for the UvrABC repair pathway (16, 37, 95; P. L. Foster, unpublished results). However, Grigg & Stuckey (40) showed that caffeine, which inhibits excision repair, also inhibits stationary-phase mutation. We too have found a large decrease in the appearance of Lac^{+} revertants when caffeine is added to lactose plates; however, caffeine also decreased the number of colonies appearing on day 2, which are mostly due to mutations that occurred during growth before plating (P. L. Foster, unpublished results). Caffeine intercalates into the DNA and can cause the UvrA protein, which is the damage-recognition subunit of the UvrABC system, to bind nonspecifically to DNA (110). suggest that in nongrowing cells, intercalated caffeine may itself, or in conjunction with the UvrA protein, prevent gene expression (e.g. by inhibiting transcription).

Trial And Error

Cairns et al (16) concluded that cells under selection are engaged in some "reversible process of trial and error" (p. 145). That is, variants arise continuously, but they are immortalized as mutations only if the cell achieves success. Various models have been proposed that incorporated this idea; they differ in the level at which the variants arise and in the way that natural selection is postulated to exert its effect.

VARIANT RNAs—Cairns et al (16) proposed that a cell under selection might be producing highly variable mRNA molecules. If one such mutant transcript happened to code for a good protein, this might stimulate reverse transcription, immortalizing the mutation in the DNA. This process would be highly efficient if the cell had some way of associating a given transcript with its protein and targeting that specific transcript for reverse transcription (16).[14] However, the mechanism would work without this association if all transcripts that were in existence at the moment that the cell achieved success were reverse transcribed at random.

Of course this model depends on reverse transcriptase being present in *E. coli.* Subsequent to the publication of Cairns et al (16), retroviral-like elements encoding reverse transcriptase were discovered in many strains of *E. coli,* but not, so far, in K-12 strains (54). Bridges (personal communication) has shown that curing *E. coli* B of its retron has no effect on the production

[13]This is not true for all tested eukaryotic genes, possibly because the coupling factor is specific for RNA polymerase II (111).
[14]This was the most Lamarckian of the three models proposed by Cairns et al (16), although Sarkar points out that it only falls into a weak definition of neo-Lamarckism (106).

of mutations during selection. However, neither result precludes the possibility that reverse transcriptase from some other source might still be present in the cells.

The efficient version of this model predicts that the only sequence changes that occur in the successful cell are those that code for the good protein. Using reversion, of a *lacZ* amber allele, we found that extragenic suppressors continued to arise with time during lactose selection (37). The majority of the late-arising, suppressors were fast growing upon isolation, and their distribution was Poisson (P. L. Foster, unpublished results). Therefore, these suppressors had all the characteristics of adaptive mutations. Because in these suppressors the information that restored activity to the mutant protein was not present in its RNA or its DNA, there could have been no direct link of the kind proposed (37).

Although these experiments do not exclude the inefficient version of the reverse-transcription model, they do tend to discourage speculation in that direction.

VARIANT DNAs

Slow repair: Stahl (118) and subsequently Boe (9) started with the assumption that in stationary phase, cells are sporadically replicating or repairing their DNA. In a nutritionally deprived cell, the error-correction enzymes that monitor newly synthesized DNA might be slow to act. This delay might allow errors on the transcribed strand to persist long enough to encode a useful protein. The cell could then replicate its DNA before the mismatch was corrected. If an active protein were not encoded, then the error would eventually be corrected. To achieve specificity, this model requires that the slow repair pathway always corrects errors in favor of the template DNA. Stahl (118) and Boe (9) both nominated the methyl-directed mismatch repair system (MMR) as the relevant pathway.

Defects in MMR greatly enhance the frequency of mutations occurring during selection (9, 37,56). However, the critical prediction of the hypothesis is that, when MMR is not active, mutations should occur whether or not they are being selected. This proved not to be the case —when a *lac*$^{-}$ strain deficient in MMR was starved in the absence of lactose, no Lac^{+} mutants accumulated (37).

If *E. coli* is defective for both of its alkyltransferases, which remove alkyl groups from DNA bases, the frequency of mutations in stationary-phase cells (but not in growing cells) is greatly increased (96). Because this repair pathway restores the original DNA sequence, it could also, potentially, reverse transient variants. However, we obtained the same result with alkyltransferase-defective cells as with MMR-defective cells—mutations did not accumulate in the absence of selection (37).

Has the slow repair model been disproved? Not conclusively. Our experiments with the MMR-defective strain were complicated by the rapid loss of viability that these mutants display during starvation (9,37). Furthermore, DNA repair pathways are extremely abundant. Prival & Cebula (95) have postulated both increases and decreases in these various pathways to account for the differences in the mutational spectra obtained from early- and late-appearing His^{+} revertants. Of course, the problem with this kind of analysis is that, given enough pathways and the freedom to increase or decrease activities at will, anything could be explained. Steele & Jinks-Robertson (121) argued that the Stahl model is supported by their finding that UV-light irradiation increased the frequency of adaptive mutations. However, this result is consistent with any model that assumes variants can arise at the level of DNA; if the DNA contains lesions, the mutation frequency will increase, but this tells us little about the underlying mechanism.

Extra DNA copies: Adaptive mutation could be achieved if a cell made copies of its genes and allowed these to accumulate sequence changes, but kept a pristine master copy. If one of

the variant DNAs allowed the cell to divide, it would be retained in the cell's successful progeny. In the absence of a useful variant, the master copy would be retained and the useless variants destroyed.

Prompted by the finding that, in at least some cases, RecA function is required to produce adaptive mutations (15,56), we proposed a model involving gene amplification (37). Bacteria are known to duplicate regions their genomes at frequencies of 10^{-3} to 10^{-4} per cell (2). Duplications can initiate rounds of amplification owing to RecA-dependent events (e.g. unequal crossing over) (128). Furthermore, amplified DNA sequences appear in some cases to accumulate mutations at higher rates that single-copy sequences (11,99). Amplifications are inherently unstable, but if one DNA copy contained a useful sequence change, the cell would begin to grow, resolve the amplified region (again by a RecA-dependent process), and retain the useful copy. In the absence of success, the amplified region would also eventually be resolved, but because most copies retain the original DNA sequence, the cell would remain unmutated.

This model has been criticized by Stahl (120) on the grounds that, to achieve the required specificity, many tandem copies would have to accumulate. Otherwise:, when the amplification was resolved in the absence of success, the cell would have nearly as great a chance of retaining a variant as retaining the original DNA copy. In the absence of selection for it, Stahl does not believe that "such a monstrous aberrancy" (120, p. 866) would occur. have indeed found no evidence that amplification of the *lac133::lacZ* allele is, of itself, advantageous (P. L. Foster, unpublished results). However, mathematical models for the evolution of arrays of hundreds of repeats, as found in eukaryotic genomes, work without any assumption of selective pressure (49). The rate-limiting step is the initial duplication, and duplications can be stimulated by activated RecA in *E. coli* (28) and by loss of cell-cycle control in eukaryotes (50). Thus, we are not ready to abandon the amplification model on the basis of Stahl's objections.

Stahl has come up with a new model that he calls "The Toe in the Water Model" (120). Stable replication is a RecA-dependent form of DNA replication that occurs in the absence of protein synthesis (65). It can be induced DNA damage and by physiological imbalances such as shift-up from minimal to rich medium. It may also be error-prone (65). A related, but not identical, form of stable replication, which is also RecA-dependent, occurs in cells that have low RNase H levels because RNase H normally prevents random RNA transcripts from priming aberrant DNA replication (59). Thus one can imagine that stable replication could be active in starving cells and be a source of DNA variants (36,37,56). Stahl has proposed that in a starving cell, stable replication would initiate but not proceed past the D-loop stage and the new DNA would eventually be degraded. But if the DNA contained a useful sequence change, a full replication fork would form, the cell would replicate, and the mutation would be immortalized.

These models have clearly been great fun to devise, and they do suggest some obvious experimental approaches. However, although we (15) and Jayaraman (56) have found that the production of adaptive mutations depends on RecA function, others have not (see below). So, apparently more than one mechanism gives rise to adaptive mutations.

THE HYPERMUTABLE STATE—Hall (45) proposed the most extreme form the trial and error hypothesis. During selection, a subpopulation of cells would enter into a state of hypermutation, producing DNA-sequence changes at a high rate. If a cell achieved a successful mutation, it would exit and resume growth. If it did not succeed, it would die. Thus, unsuccessful variants would be eliminated from the population.

Recently Higgins (53) suggested a modification of this model, which might be called the altruistic model. Within a colony of *E. coli* carrying a Mu element, the majority of

transpositions occur within apparently "dead" cells. These cells cannot form colonies or grow in rich medium, but yet they appear to be able to make DNA, RNA, and protein. Drawing upon examples from other bacteria, such as *Neisseria* spp., which uses DNA from dead cells to change its antigenic determinants (109), Higgins suggested that, during starvation, a subpopulation of cells that are technically dead experience a mutation rate at least 1000-fold higher than normal. If a useful mutation is achieved, the dead cell obtains enough energy to transfer its DNA (by conjugation or transduction) into colony-forming cells. Recipient cells that capture the useful sequence change would then persevere. This model has the beauty of allowing some cells to benefit from an extremely high genomic mutation rate without suffering the lethal consequences. The model might also work if all hypermutated cells transferred their DNA into the colony-forming cells whether or not the DNA had a useful sequence change. The recipient of an useful piece of DNA would then grow whereas the rest of the population might incorporate, at most, a few useless mutations.

THE MISSING NEUTRAL MUTATIONS—All the trial-and-error models predict that neutral mutations should not accumulate in a population under selection. All (except the efficient reverse-transcription model) also predict that neutral mutations should be found at enhanced frequencies in the cells that achieve success. This is because, at the moment of success when the useful variant is immortalized as a mutation, other variants that existed in the cell would also be immortalized. To date, two cases of neutral mutations occurring at higher frequencies than expected among selected mutants have been published. Hall (45) reported that 2 out of 110 revertants of *trpB*$^{-}$, versus zero out of 4530 nonreverted cells, carried auxotrophies. Boe (9) found that 20 out of 2000 late-arising revertants of an asparagine auxotrophy, whereas <2 out of 2000 revertants isolated from exponentially growing cells, grew poorly on succinate (however, it is not clear that the control revertants were truly independent). These rather small numbers and can provide only tentative support for the trial-and-error models. Furthermore, if the mechanism by which variants are produced involves only small regions of the genome, as would be predicted by most of the models, then mutations should occur only within those regions. Screening for a second phenotype is unlikely to find clustered mutations.[15] Hall (46) sequenced 700 bases surrounding the *trpA* and *trpB* loci from 11 Trp^{+} double revertants and found no sequence changes other than the reverted bases themselves. He argued that this frequency was orders of magnitude lower than would be predicted by any model. However, for the reasons discussed above, this result will have to be reevaluated.

Other Possible Contributors

A few other phenomena may be relevant to the production of mutations in stationary-phase cells. None of these supply a mechanism by which mutations would be adaptive, because all would seem to result in a general increase in mutation rates. However, they are indicative of the kinds of things that may happen to cells during prolonged nonlethal selection.

Certain stresses, such as carbon starvation and iron deficiency, change the level of modification of some tRNAs. The product of the *miaA*gene is required for modification of the adenosine 3′ to the anticodon of tRNA species that read codons beginning with U (33). Defects in *miaA*, as well as iron deficiency, increase the spontaneous rates of some mutations by 10- to 100-fold (19). Interestingly, *mioA* is part of a complex operon with *mutL*, which codes for one of the MMR enzymes (19). The immediate effect undermodification of these tRNAs is to increase the accuracy and decrease the efficiency of translation, but the global physiological effect is

[15]Boe (9) argued that the succinate-utilization defect resulted from mutations in the *atp* operon, which is close, to *asnA;* thus, both mutations could have arisen in the same heteroduplex. However, he did not show that the mutations were in fact in *atp*, and it seems unlikely that a heteroduplex region would extend for the roughly 10 kb of DNA that separate the genes.

dramatic, especially on the biosynthesis of amino acids that are regulated by attenuation (7). Why this should increase mutation rates is unknown, but global changes in the levels of repair enzymes and nucleotide pools could result from decreased translational efficiency (19).

Ninio (85) and subsequently Boe (10) have postulated that transcriptional and translational errors could result in error-prone DNA polymerases and defects in DNA error-correcting functions. These would then act as transient mutators. Ninio calculated that the contribution of such mutators to single mutational events would be insignificant but could account for up to 95% of simultaneous double mutations. However, this hypothesis seems at odds with the tRNA modification results, in which increased translational accuracy increased mutation rates.

Finally, several studies of adaptive mutation have used mutational targets carded on F′ episomes (15,16,37,47). Male cells carrying F factors conjugate, and conjugation results in single-stranded DNA (135). Furthermore, conjugal transfer among F′ cells results in elevated RecA levels (126). Thus, the mutation rates on F′ factors may be higher than on chromosomes, or the mechanisms by which the mutations arise may differ between F′ factors and chromosomes. This might explain why we found that RecA is required for production of adaptive mutations (15). However, Jayaraman (56), used chromosomal alleles, also found that adaptive mutations (in a *mutS* background) depended on RecA function.

GENETIC CONTROLS

If the process by which mutations arise in stationary-phase cells is truly different from the process by which mutations arise during exponential growth, one should be able to find mutants altered in one pathway but not in the other. Because increases in mutation rates could result from aberrant processes that have nothing to do with the way mutations are produced in wild-type cells, the most informative genetic defects would be those that decrease mutation rates. Any defects that alter the physiology of cells in stationary phase also would be expected to affect adaptive mutation. As yet, no mutants with clear-cut phenotypes have been obtained, but the experiments that have been done at least eliminate some possible mechanisms for adaptive mutations.

The SOS response is not required to produce adaptive mutations. As mentioned above, we found that adaptive reversion of the *lac133::lacZ* frameshift required some activity of RecA +. However, the SOS functions of RecA are not necessary. Revertants arise during selection in the absence of UmuDC functions (which are required for SOS mutagenesis) and when all LexA-controlled genes other than *recA* are repressed (15). Similar results were found for Val^R mutations in a *mutS* background (56). Although we favor the hypothesis that it is RecA's recombination functions that are required, our results are also consistent with the hypotheses that RecA activity is necessary to process some protein other than LexA or UmuD, or to allow stable replication to occur (see above). At this point, it is a mystery why we found such a strong requirement for $RecA^+$, whereas others found that RecA is completely dispensable (B. Bridges, personal communication; B. Hall, personal communication; S. Benson, personal communication). These conflicting results are not due to differences in the *recA* alleles that were used; although a possible interpretation of our original experiments was that the *recA* allele we studied, *recA430*, was actually inhibiting the production of adaptive mutations (15), we have confirmed our results with a null allele *recA* (P. L. Foster, unpublished results). The simplest explanation is that RecA is required for some, but not all, mutational events occurring in stationary-phase cells.

The UvrABC excision repair pathway could be the source of the DNA synthesis that gives rise to mutations, or it could repair spontaneous DNA lesions that, if unrepaired, give rise to mutations. Elimination of excision repair by defects in *uvrB* (16,37,95) or *uvrA* (B. Bridges, personal communication; P. L. Foster, unpublished results) either has no effect or increases

the frequency of mutations during selection. Thus, the UvrABC pathway is certainly not required to produce adaptive mutations, but it may prevent some classes of mutations from occurring.

As discussed above, cells defective in MMR have a greatly increased mutation rate during selection (9,37,56), but the MMR pathway does not appear to he responsible for the reversal of useless variants (37). Nevertheless, in *mutS*$^{-}$ strains, the frequency of adaptive mutations increases and cell viability decreases during stationary phase (9,37). These results suggest that DNA synthesis is occurring in stationary-phase cells (because MMR normally corrects errors only in newly synthesized DNA) and that MMR activity is required to prevent some lethal outcome. It seems unlikely that the fairly dramatic loss in viability of MMR- cells is the result of the accumulation of lethal mutations unless mutations are more likely to occur in essential genes than in nonessential ones. As Boe pointed out (9), the lethality may be a result of events occurring when cells attempt to resume replication after being plated for viability.[16]

Because cells missing all alkyltransferase activity accumulate more mutations in stationary phase than do wild-type cells, DNA bases alkylated by some endogenous source can, if unrepaired, be a significant source of mutations in nondividing cells (96). However, our results indicate that the alkyltransferases are not responsible for the specificity of adaptive mutation, because in the absence of these enzymes, mutations do not arise in the absence of selection (37). The *lacZ* allele that we used to score mutations in alkyltransferase-defective strains reverts only by a specific G:C to A:T transition (23), the mutation induced by alkylguanine (71). As the guanine on the nontranscribed strand, variant transcripts could not arise by transcription, but could only be produced after a thymine was inserted opposite the alkylguanine. Thus, DNA synthesis would appear to be required to produce the variants,[17] but because mutants do not appear in the absence of selection, the newly synthesized DNA must be lost in cells that cannot benefit from it.

Finally, Cairns et al (16) demonstrated that a deletion of the *uvrB-bio* region increased adaptive reversion of a *lacZ* amber allele. Although the Δ (*uvrB-bio*) strain produces more Lac^{+} revertants than wild-type when plated on lactose minimal medium, it fails to papillate on MacConkey lactose plates even though Lac^{+} revertants are being produced. S. Miller & J. Cairns (personal communication) suggest that the defect interferes with entry and exit from stationary phase; thus the mutant cells may have an increased mutation rate during selection because they enter into stationary phase early but then have difficulty resuming growth in rich medium.

CONCLUSIONS

Perhaps it was inevitable that the subject of adaptive mutation would be contentious. Critics have postulated a variety of artifacts that they feel can explain the observed results. Proponents have published a variety of experiments that, in some cases, deal with those artifacts and, in some cases, do not. Thus the two sides may at times appear equivalent—an abundance of artifacts, an abundance of controls. Heated exchange may be useful to science, but nothing can ultimately be gained by a selective reading of the evidence (68) or by angry adherence to preformed beliefs (117). The experiments given in Table 1, and others that may not have been included, each have to be evaluated on their own merits.

[16]Death by assay, i.e. that cells may be viable until their viability is tested, could be a complication in many experiments with stationary-phase cells.

[17]The required mutation might be created without DNA synthesis by reciprocal recombination with a small homologous sequence somewhere else in the genome (J. Cairns, personal communication).

What would constitute absolutely convincing evidence that adaptive mutations occur? It has been argued that nothing will do so except the demonstration of a molecular mechanism (106,120). This is an entirely unwarranted burden to place upon any field of study. Traditionally, the reality of a phenomenon is established by observations of it, not necessarily by understanding its cause. Where would the science of genetics be now if classical genetics had had to wait for the discovery of DNA?

The preponderance of evidence indicates that, as Ryan observed (101), mutations can arise in stationary-phase cells. In some cases, stress and nutritional factors may trigger the movement of IS elements and other types of genomic rearrangements, but the mechanism by which point mutations arise in stationary-phase cells is entirely unclear. DNA synthesis would appear to be required for most mutational events, but there is a vast gap between the amount of DNA synthesis that has been measured in nondividing cells and the amount that would seem to be required to produce the mutations observed (Table 2). The DNA synthesis that takes place probably is targeted to only certain regions of the genome (e.g. transcribed genes) or is unusually error prone, or both. An increase in the error rate of DNA synthesis might be an unavoidable consequence of nutritional deprivation, or it could be the result of an induced response, analogous to the SOS system.

Regardless of how mutations arise, the real mystery is why they appear to do so only when they are useful. The simplest explanation is that the role of selection is not to direct a process, but to stop a process that is creating transient variants at random. However, we still do not know the nature of the transient variants or the identity of the editing mechanism.

“Sweet are the uses of adversity.” The importance of adaptive mutation is not that natural selection is being circumvented, but that natural selection is apparently being allowed to choose among a cell’s population of informational macromolecules (16). Thus, individual cells not only control their phenotypes by regulating the expression of their genes, but they also seem to have access to a multitude of potential genotypes, allowing the individual to increase its variability when it would be useful to do so, while maintaining its genome more or less intact. Understanding the mechanisms underlying this phenomenon in microorganisms may also shed some light on the way mutations arise in nondividing somatic cells in mammals, leading to the success (for the cells) that we call cancer. [18]

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[18]For example, Strauss (124) has argued that because generation-dependent mutation rates cannot account for the number of genetic changes found in tumor cells, they might result from a process of adaptive mutation.

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Table 1

Summary of studies of spontaneous mutations occurring during nonlethal selection

Organism	Reference	Selected phenotype	Mutational target	Probable mutational events	Evidence[a]
Adaptive mutations[b]					
Escherichia coli	16	Lactose utilization	*lacZ* amber	Base substitutions	A, B, C
	44	Methionine prototrophy	*metB*$^-$	Unknown	A, C
	45	Tryptophan prototrophy	*trpA* missense	Base substitutions	A, B
	45	Tryptophan prototrophy	*trpB* missense	Base substitutions	A, B, C, D
	45	Cysteine prototrophy	*cysB* missense	Base substitutions	B, C
	15	Lactose utilization	*lacI :: lacZ* frameshift	Frameshifts, deletions	A, B
	15	Tryptophan prototrophy	*trpE* frameshift	Frameshifts	A, B
	6	Maltodextrin uptake	*ompF*$^+$	Base substitutions	A, C
	56	Valine resistance	*ilv G* frameshift	Various	A, B
	37	Lactose utilization	*lacZ* missense	Base substitution	A, B
Pseudomonas putida plasmid	127	Halogenated alkanoic acid utilization	Poorly expressed *dehI*$^+$	Rearrangements	A, B[d]
Saccharomyces cerevisiae	48	Histidine prototrophy	*his4* missense	Base substitutions	A, C
	121	Lysine prototrophy	*lys2* frameshift	Frameshifts	A, B
Stationary-phase mutations[c]					
Escherichia coli	100	Histidine prototrophy	*his*$^-$	Unknown	A
	9	Isoleucine prototrophy	*ilv::*Tni	Excision	A
	9	Asparagine prototrophy	*asnA*$^-$	Not specified	A, D
	130	Survival in stationary phase	*surA :: miniTn10*	Excision	A
	47	Lactose utilization	*lacZ* missenses	Base substitutions	A
	47	Lactose utilization	*lacZ* frameshifts	Frameshifts	A
	B. Bridges (personal communication)	Tyrosine prototrophy	*tyrA* ochre	Unknown	A
Escherichia coli plasmid	11	Tetracycline resistance	*mnt*$^+$	Probably deletions	A, D
Salmonella typhimurium	95	Histidine prototrophy	*hisG* missense; ochre	Base substitutions; deletions	A

Organism	Reference	Selected phenotype	Mutational target	Probable mutational events	Evidence[a]
Clostridium thermocellum	86	Glucose, fructose utilization	Unknown	Unknown	A
Pseudomonas putida	127	Halogenated alkanoic acid resistance	*dehI*$^{+}$, *dehII*$^{+}$	Rearrangements	A
	127	Halogenated alkanoic acid utilization	*dehI*$^{-}$, *dehII*$^{-}$	Rearrangements	A, induced by starvation
Pseudomonas putida plasmid	89	Phenol utilization	Promoterless *pheAB*	Transpositions	A, D
Saccharomyces cerevisiae	48	Histidine prototrophy	*his6*	Not specified	A
Candida albicans	75	Heavy metal resistance	Unknown	Probably amplification	A
		Adenine prototrophy	*ade2/ade2*	Unknown	A
		Leucine prototrophy	*leu/leu*	Unknown	A
Disputed cases					
Escherichia coli	16,113	Lactose utilization	Lac(Ara)$^{-}$	Mu excision, transposition	A, B
	80	Lactose utilization	Lac(Ara)$^{-}$	Mu excision, transposition	A, induced by starvation
	43	Salicin utilization	*bglF* :: IS*103*	Excision	A[d], B, C
	81	Salicin utilization	*bglF* :: IS*103*	Excision	A, induced by starvation
	46	Tryptophan prototrophy	*trpA trpB* missenses	Base substitutions	A[d], D

[a] The evidence that adaptive or stationary phase mutation was occurring: A. The mutants appeared late during nonlethal selection and/or the numbers of mutants in a fluctuation test had a Poisson distribution. B. The mutants did not accumulate during nonselective starvation or other appropriate nonspecific stress. C. Mutants with a neutral phenotype did not accumulate among the population during selection. D. Possible incidence of multiple mutation.

[b] The evidence supports the hypothesis that the mutations were specific for the selection imposed.

[c] The evidence supports the hypothesis that the mutations occurred in nongrowing cells during selection. Whether the mutations were also adaptive was not shown or, where indicated, the mutations were shown to be induced by nonspecific stress.

[d] The population increased under selection but possibly not enough to account for the mutations.

Table 2

Comparison of spontaneous mutation rates during exponential growth and during nonlethal selection

		Mutation rates		Turnover required		
Organism	**Mutational target**	**Mutations per cell per generation during nonselected growth[a]**	**Mutations per cell per hour during selection[b]**	**Total for experiment[c]**	**Per day[d]**	**Reference**
Escherichia coli	*lacI:: lacZ* frameshift	1.4×10^{-9}	1.1×10^{-9}	96	19	15
	lacZ amber	1.7×10^{-10}	1.1×10^{-10}	75	25	16
	trpE frameshift	3.0×10^{-10}	3.1×10^{-10}	50	25	15, ud[e]
	lacZ missense	3.1×10^{-10}	7.9×10^{-11}	43	6.1	47
	trpB missense	1.5×10^{-10}	1.4×10^{-11}	26	2.2	45
	trpA missense	9.4×10^{-11}	4.5×10^{-12}	14	1.2	45
	his$^-$	3.0×10^{-8}	4.1×10^{-10}	2	0.33	100
Saccharomyces cerevisiae	*lys2* frameshift	4.9×10^{-9}	3.0×10^{-10}	7	1.5	121

[a] Calculated as $m/(2N)$ where m = average number of mutations per culture, N = cells per culture. These rates are slightly different from published rates calculated as [mln(2)]/N (45,47).

[b] Calculated as $M/(N_0 h)$ where M = total number of mutants appearing under selection, N_0 = cells originally plated, and h = hours the experiment lasted after preexisting mutations appeared. These rates are different from published rates that took into account cell death (45,100).

[c] Calculated as M/N_0 divided by the exponential-phase mutation rate.

[d] Total turnover divided by the days the experiment lasted.

[e] J. Cairns & P. L. Foster, unpublished data.