The Directed Mutation Controversy and Neo-Darwinism

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According to neo-Darwinian theory, random mutation produces genetic differences among organisms whereas natural selection tends to increase the frequency of advantageous alleles. However, several recent papers claim that certain mutations in bacteria and yeast occur at much higher rates specifically when the mutant phenotypes are advantageous. Various molecular models have been proposed that might explain these directed mutations, but the models have not been confirmed. Critics contend that studies purporting to demonstrate directed mutation lack certain controls and fail to account adequately for population dynamics. Further experiments that address these criticisms do not support the existence of directed mutations.

A fundamental tenet of evolutionary biology is that mutations are random events. This tenet does not mean that mutation rates are unaffected by environmental factors or that all portions of the genome are equally susceptible to mutation. Indeed, enzymes that catalyze certain DNA repair processes are regulated by environmental factors, and many mutations are mediated by mobile elements that are not uniformly distributed in the genome (*1*, *2*). Rather, the randomness of mutation refers to the supposition that the likelihood of any particular mutational event is independent of its specific value to the organism (*3*).

Darwin did not know the material basis of heredity or the role of mutation in generating differences among individual organisms. What he realized, however, was that the causes of heritable differences could be logically separated from their consequences for survival and reproduction (*4*). This insight was critical to Darwin's formulation of the theory of adaptation by natural selection because it freed him from teleological constraints that had bound earlier natural philosophers (*5*). The discovery of mutation, the rediscovery of Mendelian segregation, and other developments in genetics and the mathematics of populations were subsequently incorporated into a synthetic theory of population genetics and evolution. According to this neo-Darwinian framework (6), random mutation produces heritable differences among organisms whereas natural selection tends to increase the frequency of advantageous alleles in a population.

The tenet of random mutation has recently been challenged. In 1988, Cairns *et al.* (*7*, p. 142) described "some experiments suggesting that cells may have mechanisms for choosing which mutations will occur," which provoked vigorous discussion among biologists and philosophers of science (*8*–*13*). Subsequent studies (*14*–*22*) have also suggested that certain mutations occur more often when the resulting phenotype is advantageous, and such mutations have been variously described as directed, Cairnsian, adaptive, or selection-induced.

In this paper, we review the history and current status of the controversy, including key experimental findings, alternative explanations for these findings, and their relationship to neo-Darwinism. The hypothesis of directed mutation supposes a very specific relationship between environments and mutations (*23*). We define as directed a mutation that occurs at a higher rate specifically when (and even because) it is advantageous to the organism, whereas comparable increases in rate do not occur either (i) in the same environment for similar mutations that are not advantageous or (ii) for the same mutation in similar environments where it is not advantageous (*24*). But we emphasize that the reality of directed mutation is disputed and its mechanistic basis, if real, is unknown. Consequently, details of experimental design, analysis, and inference (and possible limitations thereof) are critical to this discussion.

Early Experiments on Mutations in Bacteria

Long after neo-Darwinism had gained nearly universal acceptance among zoologists and botanists, debate continued over the causes of variation in bacteria (*25*). Zoologists and botanists could observe mutations that affected visible phenotypes, and they could discern that these occurred even though mutants often had impaired fitness. But bacteriologists could neither see individual mutants nor demonstrate their existence except by imposing selection for the mutant phenotype. Consequently, it was unclear whether selection had caused the mass conversion of cells from one state to another or whether selection had increased the proportion of mutant cells in a population by differential survival and growth.

Strong support that neo-Darwinism could be extended to bacteria came in 1943, when Luria and Delbrück devised the fluctuation test (*26*). They formulated two alternative hypotheses to account for the appearance of bacteria resistant to infection by viruses. Under the hypothesis of random mutation, each bacterium has some probability of spontaneously mutating from a viral sensitive to a viral resistant state, even in the absence of virus. Under the hypothesis of directed mutation (which Luria and Delbrück called "acquired hereditary immunity"), each bacterium has some chance of surviving and becoming resistant to viral attack. Under both hypotheses, resistance is inherited. The critical distinction between these models is that, in bacterial populations grown from small inocula in the absence of virus, resistant cells should tend to be clustered within a subset of the populations under the hypothesis of random mutation, whereas they should be distributed more evenly among populations under the hypothesis of directed mutation (Fig. 1). Luria and Delbrück observed a high variance-to-mean ratio in the numbers of mutants in replicate cultures, supporting the hypothesis of random mutation.

The fluctuation test and two later experiments (*27*) that also supported random mutation rested on quantitative analyses, and they did not enable an investigator to isolate a mutant without exposing cells to the appropriate selective conditions. However, in 1952 Lederberg and Lederberg (*28*) overcame these limitations with the replica-plating experiment. Thousands of bacteria were grown from single cells into confluent lawns of microcolonies on agar plates. The bacteria on these master plates were sampled by making an impression with a piece of velvet, which was then used to stamp the bacteria onto several replica plates containing virus. The Lederbergs saw a striking correspondence among replica plates in the location of resistant colonies, indicating that clones of resistant bacteria

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were already present on the master plate. Moreover, the Lederbergs isolated pure cultures of resistant cells, without ever exposing their progenitors to virus, through a series of master and replica plates (*29–31*).

These experiments proved that certain mutants existed before bacterial populations were exposed to selective agents, and hence that the mutations could not have been caused by that exposure. Still, experiments could only show that some mutations were random; they could not exclude the possibility that others might occur at higher rates when they were advantageous. Indeed, had Luria and Delbrück, or the Lederbergs, used a temperate virus rather than a virulent one, they would have obtained evidence for acquired hereditary immunity. Some fraction of infections with temperate viruses convert bacteria into lysogens, wherein the virus is integrated into the host's chromosome (*32*). In this state, the virus synthesizes a protein that confers immunity to further infections, and this immunity is inherited. Thus, we have an example of the inheritance of an acquired characteristic (*2, 33*) and, hence, an exception to strict neo-Darwinism (*6*). However, bacteria do not acquire this immunity by the "sustained use of any organ" (*34*, p. 113) or other structure, as Lamarck proposed; nor can bacteria be said to choose or direct the change, which is part of the virus' life cycle and presumably evolved by random mutation and natural selection (*2, 35*).

Recent Evidence for Directed Mutation

Cairns *et al.* (*7*) suggested that these early experiments may have overlooked mutations that occur specifically when they are advantageous. They pointed out that virus resistance is not expressed until several generations after the mutation has occurred because receptors to which viruses adsorb must be diluted out by cell growth. Therefore, experiments that use lethal selective agents, such as viruses, may not detect mutations that occur as a specific response to an organism's needs. Cairns *et al.* argued that cells exposed to nonlethal selective conditions would have the opportunity for "a nonrandom and possibly product-oriented form of mutation" (*7*, p. 142).

Cairns *et al.* (*7*) presented two types of evidence to support the existence of directed mutations in bacteria. (i) Fluctuation tests with *Escherichia coli* deficient in lactose utilization (Lac^-) gave distributions of Lac^+ mutants that fit a hybrid model, in which some mutations occur randomly prior to selection whereas others occur only after selection. (ii) Cairns *et al.* found that Lac^+ mutants accumulated over time when Lac^- cells were incubated on medium containing lactose. They found no comparable increase in mutants at an unselected locus nor any accumulation of Lac^+ mutants on medium without lactose. In addition, Cairns *et al.* (*7*) summarized earlier work by Shapiro (*36*) and Hall (*37*), which Cairns (*38*) thought provided even stronger evidence for directed mutation.

Shortly thereafter, Hall (*14*) published the first in a series of papers presenting his own new evidence for directed mutation, in which certain double mutants were observed only under selective conditions. Evidence for directed mutation has now been reported in *E. coli* under a variety of nonlethal, selective conditions (*7, 14–21*), and recent reports indicate similar phenomena occur in yeast (*22*). These cases include single and double mutations as well as point mutations, suppressors, frame shifts, and excisions. The selected mutants have acquired the ability to utilize specific sugars, to synthesize particular amino acids, and to resist certain bacteriostatic agents. In some cases, the reported difference in mutation rates between selective and nonselective conditions is many orders of magnitude.

Alternative interpretations. The most extreme interpretation of the evidence for directed mutation is that a cell can somehow monitor or anticipate the consequences of potential genetic changes for its fitness and then choose or direct the specific change that would be most advantageous under its present circumstances. This view was expressed most forcefully by Cairns *et al.* (*7*). If such a mechanism were found, it would provide an important exception to the neo-Darwinian tenet of random mutation, even if it operated only in certain microorganisms (*39*).

An alternative interpretation is that some mutations occur more often when they are advantageous, but the underlying molecular mechanism does not imply any choice on the part of a cell. Several plausible mechanisms have been proposed that might allow for such selection-induced (but not truly directed) mutation in microorganisms (*8, 17, 40*). Even if one of these mechanisms was demonstrated, however, it seems doubtful to us that it would substantially alter the neo-Darwinian view of mutation.

According to a third interpretation, the apparently directed mutations are, in fact, completely random with respect to the selective value of the resulting phenotype. This view acknowledges that mutation rates are affected by the environment and by a cell's physiological state, and it accepts that mutations may occur in both growing and nongrowing cell populations (*41*). This view also recognizes that mutation rates vary within and between genomes, providing the potential for characteristic mutation rates to evolve in different genomes and even in different regions of a particular genome (*42*). But according to this view, the studies purporting to show that certain mutations happen at a higher rate specifically when they are advantageous are flawed by a lack of important controls and by inappropriate bookkeeping to back-calculate mutation rates from numbers of mutants (*9–11*).

Possible Molecular Mechanisms for Directed Mutation

Truly directed mutation. Cairns *et al.* (*7*, p. 145) suggested that "the cell could produce a highly variable set of mRNA molecules and then reverse-transcribe the one that made the best protein." It is a fact that a single allele yields variable mRNA molecules as a result of transcriptional errors, which upon translation may also yield variable protein molecules. Also, reverse transcriptase has been found in certain strains of *E. coli*, although not in the K12 strain used in studies of directed mutation (*43*). Discussion of this model has subsided, however, in the absence of any known structure capable of monitoring the performance of

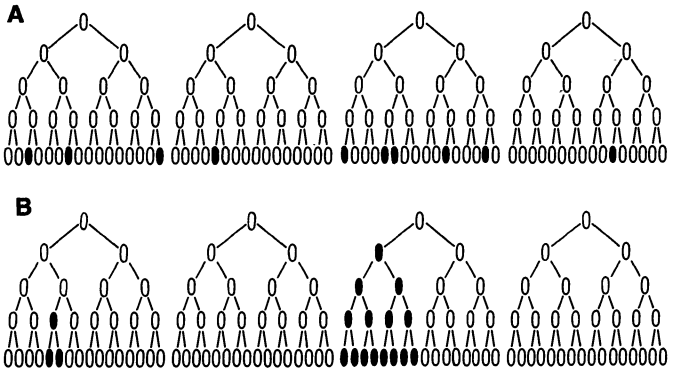

Fig. 1. Schematic representation of the Luria-Delbrück fluctuation test. Typical distributions of mutants (filled symbols) across four replicate populations, each founded from a single progenitor cell, expected under the hypotheses of (**A**) directed and (**B**) random mutation. The final row represents the cell generation that is challenged with the selective conditions. [From *Molecular Genetics* by Gunther S. Stent and Richard Calendar. Copyright 1971 and 1978 by W. H. Freeman and Company. Reprinted with permission]

protein variants and sequestering the mRNA molecule that produced the best protein. Moreover, suppressor mutations that occur outside the transcribed gene also accumulate when Lac$^-$ cells are incubated on media containing lactose (*7, 20*), which is inconsistent with a reverse flow of information from protein to DNA by way of the transcribed message. Foster and Cairns (*20*) have recently concluded that "selective condition does not play an 'instructional' role in determining which DNA sequence changes arise."

Selection-induced mutation mediated by noninstructional mechanisms. Stahl (*8*) proposed a bias in the conversion of DNA mismatches into full-blown mutations under conditions that permit mutant cells, but not their progenitors, to grow. Mutation is a two-step process. DNA damage and subsequent repair of the lesion may generate a mismatch between the two strands, which may be corrected by methyl-directed mismatch repair (*44, 45*). If a mismatch is not corrected before the DNA is replicated, this incipient mutation is rendered permanent. Stahl hypothesized that an incipient mutation encoding a newly functional transcript (one that facilitated growth) might not be repaired before the growing cell had replicated its DNA, whereas an incipient mutation that did not permit replication would eventually be corrected by mismatch repair. This process might thereby increase the mutation rate when the resulting phenotype is advantageous, even though the DNA mismatches occur randomly.

Boe (*46*) observed that strains deficient in methyl-directed mismatch repair show elevated mutation rates when cells are maintained under selective conditions. However, such strains also have elevated mutation rates during growth under nonselective conditions. A key prediction of Stahl's model is that strains deficient in mismatch repair should accumulate incipient mutations (uncorrected mismatches) under nongrowth, nonselective conditions at a rate similar to the mutation rate under selective conditions. Incipient mutations that occur under nonselective conditions may be detected by subsequently imposing selection. But Foster and Cairns (*20*) observed no discernible accumulation of incipient Lac$^+$ mutants for repair-deficient strains in the absence of lactose, whereas mutants accumulated on selective medium. Stahl's model also cannot easily explain double mutants that occur at dramatically increased frequencies under selective conditions (*14, 18*) because in these studies the individual mutations are presumed not to permit cell replication.

Another molecular model to account for directed mutation was presented by Davis (*40*), who hypothesized that transcription itself might be mutagenic. According to this model, bacteria defective in *lac*Z, for example, should revert to Lac$^+$ at higher rates in the presence of lactose or other inducers of the *lac* operon than in the absence of induction. A corollary prediction is that Lac$^+$ cells should mutate to Lac$^-$ states at a higher rate when lactose is present than when it is absent. Clearly, this model does not imply choice or instruction by a cell because the mechanism causes a systematic increase in disadvantageous (misdirected) mutations for already functional genotypes.

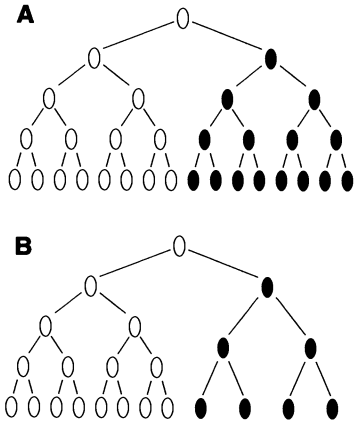

Fig. 2. Effect of differential growth of mutants and progenitors on the size of a mutational jackpot in the Luria-Delbrück fluctuation test. The number of mutants (filled symbols) resulting from an early spontaneous mutation is greater (**A**) when the mutants grow at the same rate as the progenitor than (**B**) when the mutants grow more slowly. Slow growth of mutants yields distributions similar to those expected under the hypothesis of directed mutation (Fig. 1A).

Davis (*40*) detected no increase in Lac$^+$ mutants when he added the gratuitous (not growth-supporting) inducer IPTG to starving populations of an inducible Lac$^-$ strain, although he reported a slight effect (less than twofold) of IPTG on the corresponding mutation rate in growing populations. But Foster and Cairns (*20*) found that even this slight effect is artifactual because the same response is seen for preexisting mutants seeded into the populations. Evidently, induction merely accelerates the growth response of mutants when they are provided with lactose. Moreover, Cairns and Foster (*19*) reported more Lac$^+$ mutants in the presence of lactose than in its absence for a strain that expressed a defective *lac* operon constitutively, even though the *lac* genes should be transcribed equally under both conditions.

Finally, Hall (*17*) has proposed that some starving cells might enter a hypermutable state. In this physiological state, a cell generates multiple mutations, at random, seemingly in a desperate attempt to get one that allows it to replicate. If the cell is unsuccessful, it soon succumbs to a lethal mutation. This model implies a specific relationship between mutation and selection only to the extent that cells die within a short time if they do not come up with the correct mutation. This model predicts a high frequency of secondary mutations at unselected loci.

To account for the observed frequency of double mutants in the *trp* operon (*18*) by this model, Hall (*47*) had to invoke an extremely high mutation rate for cells in the hypermutable state. Because such a high rate seemed incompatible with the survival of any cells, he made the ad hoc assumption that hypermutability was confined to small stretches of DNA. Several thousand base pairs were sequenced around the reversion sites in several *trp* double mutants, but no other substitutions were found, contrary to the prediction of the hypermutable state model (*47*).

Neo-Darwinian Explanations Based on Random Mutation

Fluctuation tests. Cairns *et al.* (*7*) observed lower than expected variance-to-mean ratios in fluctuation tests, which they interpreted as evidence supporting directed mutation. However, several groups quickly pointed out that such deviations can be produced by random mutation if certain assumptions of the test are not met (*9–11*). For example, some mutants favored under selective conditions grow more slowly than their progenitors under so-called "nonselective" conditions. Any slower growing mutants that appear early in the growth of a replicate population will leave fewer progeny, reducing the resulting jackpot and concomitantly the variance-to-mean ratio (Fig. 2). In fact, many of the Lac$^+$ mutants counted by Cairns *et al.* (*7*) were nonsense suppressors, which are likely to grow more slowly than their progenitors (*10, 12*). Stewart *et al.* (*48*) demonstrated several other plausible departures from the assumptions of Luria and Delbrück that also yield distributions similar to those taken as evidence for directed mutation.

These analyses indicate that deviations in fluctuation tests cannot provide strong evidence for directed mutation. If care is taken to ensure that the assumptions of this test have been met (or if any violations are taken into account quantitatively), then deviations may be used to show that mutations occur after the imposition of selection. Even so, it does not follow that any such post-selection mutations are caused by the selective conditions. Rather, to establish that some post-selection mutations are directed would require a demonstration that the mutation rate is higher under selective

than nonselective conditions, all else being equal.

Nonspecific increases in mutation rate. Directed mutation requires that advantageous mutations occur as a specific response to particular selective conditions. There are two ways that an increased rate of advantageous mutation under selective conditions could be nonspecific and, therefore, nondirected. (i) The selective conditions might be generally mutagenic. For example, mutations conferring resistance to ultraviolet (UV) radiation occur at an elevated rate when such mutations are advantageous, not because they are directed but rather because UV radiation is mutagenic (*49*). (ii) Selection and control treatments might differ in aspects other than the specific selective conditions, and these other differences may affect mutation rates. For example, it is inappropriate to compare mutation rates for cells growing in the absence of a selective agent and starving in its presence because starvation may be mutagenic. Several studies have scored mutations at another, unselected locus in an effort to rule out nonspecific increases in mutation rate. Implicit in this type of control is the troubling assumption that all DNA sequences respond equally to the mutagenic effects of such factors as starvation (*9, 12, 50*).

For example, Hall (*16*) observed that methionine-independent mutants of a strain believed to contain a deletion in *metB* appeared at a very low rate during growth, whereas such mutants became quite common during 9 days of methionine starvation. As a control, he also examined mutations to valine resistance, which were common during growth but showed no comparable increase during methionine starvation. However, mutations to methionine independence may occur by different molecular mechanisms than mutations to valine resistance. In particular, the events causing valine resistance mutations evidently occur primarily during replication and growth, whereas the events causing Met^+ revertants may depend on some mechanism whose rate is time-dependent rather than growth-dependent. Time-dependent mutational events may be caused by DNA damage and repair, or by rearrangements, that do not require a growing cell population. An important additional control for this study would be to measure the rate of reversion of *metB* cells that are starving as a result of some limitation other than methionine. If mutations to Met^+ occur in a time-dependent manner, then the increase in mutation rate under selective conditions may be spurious.

Population dynamics under selective and control conditions. Natural selection generates correspondence between genotypes and environments. Therefore, it is not at all surprising that specific mutants should be more common (and hence detected more easily) when they have an advantage. The issue is whether specific mutational events occur at a higher rate when the resulting phenotype has an advantage. Unfortunately, these events cannot be directly counted. Instead, the number of mutations that occurred in the past must be inferred indirectly from the number of mutants observed at present. Calculating a mutation rate further requires knowledge of the number of progenitors at risk for mutation, whose dynamics may depend on subtle features of the environment. Therefore, one must be cautious in interpreting experiments in which the evidence for directed mutation consists largely of finding more mutants when they have an advantage.

For example, several studies purporting to show directed mutation compare the number of mutants on selective plates (obtained by counting colonies) with the number of mutants on control plates (obtained by imposing selection, such as by adding lactose, and counting the colonies that appear soon thereafter). A bias arises because a mutant cell on a control plate may die before it is detected by imposing selection whereas a mutant on a selective plate grows into a colony that remains indefinitely (Fig. 3). This explanation does not require differential death rates for mutants and their progenitors, although the bias is greater if mutants die at a higher rate. Failure to account for the death of mutants prior to the imposition of selection was a criticism (*9, 10*) of the lactose-overlay experiments presented by Cairns *et al.* (*7*).

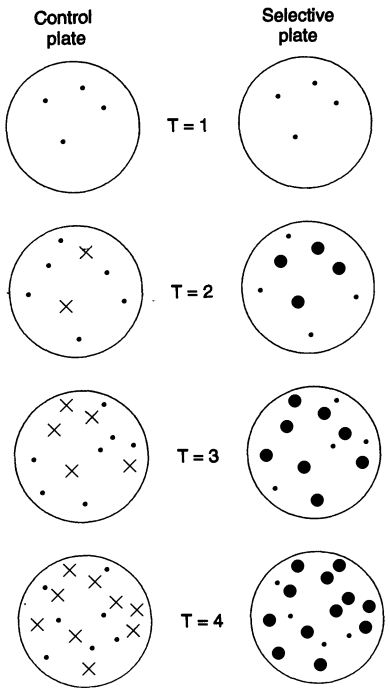

Fig. 3. Schematic representation of the effect of cell death on the number of mutations detected on control and selective plates. • = viable mutant cell; × = dead mutant cell; ● = mutant colony. Four new mutations occur each day on both types of media. When the selective substrate is present, each mutant cell produces a colony within 1 day. On the control medium, however, half the mutant cells die each day. If the selective substrate is applied to the control medium on day 4, then seven colonies will be observed the next day, whereas 16 colonies will be seen on the selective medium at that time.

Another difficulty presented by such experiments is the decision of when to count colonies that appear on control plates after imposing selection. If the time after selection is too long, post-selection mutations may be included; but if the time after selection is not long enough, mutations occurring prior to selection will be underestimated. Hall (*17*) observed that doubly auxotrophic *trp cys* populations limited for tryptophan accumulated Trp^+ revertants, but when these populations were transferred to a medium in which cysteine was limiting, no Cys^+ revertants were found within a 40-hour window. Similarly, Cys^+ mutants accumulated in populations starved for cysteine, but when these cells were transferred to a medium in which tryptophan was limiting, no Trp^+ revertants were found within 40 hours. Hall based his decision to count only those revertants observed within 40 hours on the dynamics of reconstruction experiments, in which revertants were seeded in progenitor populations under amino acid limitation. However, his reconstruction experiments used freshly grown cells, whereas cells in the experiments purporting to show directed mutation had been starving for ten days. In fact, starved cells sometimes take longer to form colonies when placed under permissive conditions (*20, 51*), and this bias could explain the failure to detect mutants in the reciprocal control experiments (*52*).

Comparisons of mutation rates between selective and control conditions also depend critically on the number of progenitors at risk for mutation on each type of medium. There are at least three mechanisms that may increase the number of progenitors under selective conditions. (i) Certain selective agents may be contaminated by growth-supporting substrates. For example, lactose preparations often contain small amounts of glucose, which may permit a small inoculum to increase manyfold. (ii) Many strains described as growth-negative for a particular substrate are, in fact, phenotypically leaky. Certain Lac^- strains, for example, can grow slowly on lactose, illustrating the danger of using a plus-or-minus nomenclature to describe quantitative characters. (iii) The progenitor may use metabolites excreted by mutants grow-

ing on the selective substrate. Such cross-feeding cannot occur on control medium because there is no substrate to support growth of the mutants.

Growth of a progenitor population on a selective medium is especially problematic in studies of directed mutation involving the unexpected accumulation of double mutants. The accumulation of advantageous double mutants on a selective medium is only surprising if the intermediate genotypes cannot increase in frequency under the selective conditions; double mutants accumulate rapidly if either intermediate has an advantage (*11, 53*). For example, Hall (*18*) observed unexpectedly frequent reversion of a *trpA trpB* strain to Trp^+ during starvation for tryptophan. But even if neither intermediate can grow in isolation without tryptophan, *trpA* $trpB^+$ mutants can grow on indole (a precursor to tryptophan), which is excreted by $trpA^+$ *trpB* mutants (*52*). Indole may also be produced by the spontaneous breakdown of indoleglycerol phosphate, which is excreted by the progenitor population (*52*). Such growth may allow a population of intermediates to reach a density at which secondary mutations become common.

Experimental Evidence for Explanations Based on Random Mutation

Mu excisions. E. coli MCS2 is a genetically manipulated strain in which part of the *ara* operon (including a regulatory gene) has been placed upstream of the structural genes from the *lac* operon, but the *ara* and *lac* sequences are separated by Mu bacteriophage DNA. This strain can grow on lactose if arabinose is present as an inducer and the Mu element has been excised. Although Mu excisions generating *ara-lac* fusions occur at an extremely low rate during growth on glucose, Shapiro (*36*) reported that excision mutants appear at high frequency after cells are starved for several days on medium containing lactose and arabinose. On the basis of additional experiments in liquid media with and without these sugars, Cairns *et al.* (*7*) claimed that excision mutations occur only in the presence of these sugars. However, such experiments do not permit rigorous estimation of mutation rates as a result of the lack of correspondence between numbers of mutants and mutational events (*54*).

We (*51*) confirmed that excision mutations are quite common in populations starving in the presence of lactose plus arabinose and quite rare in populations growing on glucose. However, we found that mutations also occurred when populations were starving in the absence of the sugars that confer an advantage on the mutant phenotype. The numbers of mutational events under selective conditions were still somewhat greater than under the control starvation conditions, but this apparent discrepancy was due to growth of the progenitor population on the selective medium. Growth of progenitors resulted mostly from cross-feeding by mutants, and it led to a 100-fold difference in the numbers of cells at risk for mutation on selective and control media. Once these effects are taken into account, the rate of excision mutation increases by at least four orders of magnitude as cells starve for several days (Fig. 4), whether or not the selective substrates are present (*55–57*).

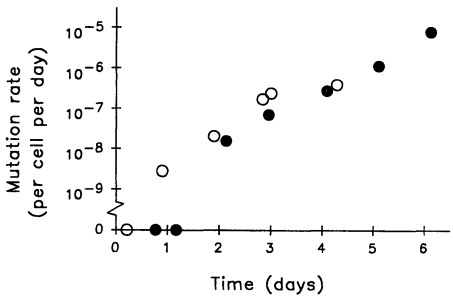

Fig. 4. Rates of Mu excision on selective (○) and control (●) media, as a function of time that cells have starved. This mutation is rare in growing cells, but during several days of starvation the rate increases by several orders of magnitude, whether or not the substrates that permit growth of the mutants are present (*51*). [Reprinted with permission from *Nature* **344**, 175 (1990). Copyright 1990 Macmillan Magazines Limited]

Biased recovery of Dex^+ mutants. Benson (*15*) has studied an *E. coli* strain that lacks the LamB outer membrane protein and cannot grow on large maltodextrins (Dex^-). Dex^+ phenotypes can occur by point mutations in genes encoding either of two other proteins, OmpC and OmpF. In populations starved on medium containing maltodextrins, $OmpF^+$ mutants are observed at a much greater frequency than $OmpC^+$ mutants, and Benson suggested this bias might be due to directed mutation promoting the $OmpF^+$ mutations under selective conditions. Benson *et al.* (*58*), however, reexamined in greater detail the dynamics of mutation and growth on selective medium, including the relative competitive abilities of the two classes of Dex^+ mutants. They found that $OmpF^+$ mutants quickly overgrow their Dex^- progenitors whereas $OmpC^+$ mutants do so more slowly, if at all. Benson *et al.* (*58*, p. 647) concluded that "the biased recovery of mutants observed in this selection does not result from adaptive or directed mutagenesis."

Double mutants in bgl. Hall (*14*) studied a strain of *E. coli* that requires two mutations in the *bgl* operon to grow well on salicin. One of these mutations is an excision of IS*150* from a structural gene, and the other mutation is in a regulatory gene. Sal^+ double mutants became abundant after prolonged incubation on a medium supplemented with salicin, but not on medium without this substrate. Prior to the accumulation of Sal^+ double mutants, excision mutants were found at high frequency on medium with salicin, but not on control medium. Reconstruction experiments pur-

Table 1. Processes that may contribute to spurious increases in mutation rates under selective conditions. The approximate magnitude of potential bias values are intended to provide conservative estimates for maximum effects. The effects of several biases are usually multiplicative. For example, Mittler and Lenski (*51*) observed a 10,000-fold nonspecific increase in Mu excisions due to starvation, a tenfold potential underestimation of mutations on control medium due to failure of starved mutant cells to form colonies within 1 day after applying selective substrates, and a 100-fold excess of cells at risk for mutation on selective medium due to growth on contaminating substrates and cross-feeding. Together, these effects can account for a spurious 10,000,000-fold increase in the mutation rate under selective conditions. Of course, not all biases are relevant to every situation, and actual biases may be much smaller than the potential effects listed here. The possibility of even larger biases in extreme cases cannot be excluded.

Process	Approximate magnitude of potential bias
Nonspecific increase in mutation rate due to uncontrolled environmental covariables (effect of starvation)	10,000-fold
Overestimation of number of mutational events on selective medium due to growth of mutants (or intermediate types, in the case of double mutants)	1,000,000-fold
Underestimation of number of mutational events on control medium due to (i) death of mutants prior to imposing selection or (ii) failure of mutants to form colonies within allotted time after imposing selection	100-fold
Greater numbers of progenitor cells at risk for mutation on selective medium due to (i) contaminating substrates, (ii) leaky phenotype, or (iii) cross-feeding	1,000-fold

portedly showed that excision mutants cannot grow on the salicin in the selective medium, and thus their accumulation could not be explained simply by selective enrichment. Hall (*14*, p. 887) concluded that "Excision [mutation] occurs only on medium containing salicin, despite the fact that the excision itself confers no detectable selective advantage and serves only to create the potential for a secondary selectively advantageous mutation."

On physiological grounds, however, Symonds (*50*) expected the excision mutants to have leaky phenotypes, which would allow some growth. Moreover, the critical reconstruction experiments were flawed in certain respects (*59*). Therefore, we (*60*) performed additional experiments to test whether enrichment of intermediates could explain the accumulation of these double mutants under selective conditions. We found that most excision-like mutants overgrew their progenitors on medium with salicin, but not on medium without salicin. This growth undermines the inference that each excision mutant is the result of an independent mutation, and it increases the expected number of double mutants on the selective medium by many orders of magnitude. We also showed that excision mutations occur frequently as the progenitor population is starved in medium without salicin.

Conclusions

The directed mutation hypothesis has received a great deal of attention for several reasons. (i) This hypothesis seems, at least in its original form (*7*), to promote a Lamarckian and even teleological view of organic evolution, which is antithetical to the core of neo-Darwinism (*5*). In fact, the entire body of formal population genetic theory, which seeks to describe the dynamics of biological evolution, presumes that the rate parameters governing mutation and selection are independent (*61*). (ii) Evidence for directed mutation has been published in major journals by several respected biologists and for many different experimental systems. Moreover, the effect is often reported to be very large, and the inference that certain mutational events occur at much higher rates specifically when they are advantageous is said to be unambiguous. (iii) The nonlethal selective conditions under which directed mutations are hypothesized to occur suggest how earlier experiments might have been biased against directed mutation and, thus, the phenomenon could have been overlooked.

But there has been no demonstration of any mechanism that would allow truly directed mutation, and even the most ardent proponents seem to have retreated from this view. Nor have the several noninstructional molecular models of selection-induced mutation been shown to account for any case of directed mutation. Still, the view that certain mutations happen much more often when they are advantageous has been accepted by some observers (*62*).

In three cases of directed mutation that have been carefully reexamined, however, the purported increases in mutation rate under selective conditions disappear when additional controls and more precise accounting of population dynamics are performed (*51*, *58*, *60*). During the 1940s and 1950s, similar analyses were shown to explain several apparent cases of environmentally induced heritable changes in bacteria (*63*), which had also challenged the generality of the findings of Luria and Delbrück and the Lederbergs. Of course, there are other reports of directed mutation that have not been shown to be flawed. But in our opinion, none of the claims for directed mutation has compellingly excluded all of the alternative hypotheses based solely on random mutation. These alternative hypotheses are numerous, the processes invoked are general, their individual effects span many orders of magnitude, and their effects are multiplicative (Table 1).

Even so, the directed mutation controversy has stimulated interest in other questions concerning mutation and its evolutionary causes and consequences. Do rates of mutation in bacteria and yeast generally increase during starvation, even if mutations are random with respect to the specific effects of the resulting phenotypes? If so, what are the mechanisms for this response, and is it adaptive, in the sense of having evolved by natural selection for alleles that specifically promote increased genetic variation under stress? Or might such a response merely be an unavoidable consequence of either physiological deterioration or the induction of mechanisms to repair damage to the DNA? More generally, how do mutation rates reflect the evolutionary trade off between the load of deleterious mutations and the potential benefits of occasional favorable mutations?

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Atmospheric Lifetimes of Long-Lived Halogenated Species

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The atmospheric lifetimes of the fluorinated gases CF_4, C_2F_6, c-C_4F_8, $(CF_3)_2$c-C_4F_6, C_5F_{12}, C_6F_{14}, C_2F_5Cl, $C_2F_4Cl_2$, CF_3Cl, and SF_6 are of concern because of the effects that these long-lived compounds acting as greenhouse gases can have on global climate. The possible atmospheric loss processes of these gases were assessed by determining the rate coefficients for the reactions of these gases with $O(^1D)$, H, and OH and the absorption cross sections at 121.6 nanometers in the laboratory and using these data as input to a two-dimensional atmospheric model. The lifetimes of all the studied perfluoro compounds are >2000 years, and those of CF_3Cl, CF_3CF_2Cl, and CF_2ClCF_2Cl are >300 years. If released into the atmosphere, these molecules will accumulate and their effects will persist for centuries or millennia.

Most of the chemicals released into the atmosphere as a result of natural processes or human activities are converted to other forms or are completely removed from the atmosphere within a few years. This happens because most of these molecules react with the major oxidants in the atmosphere or are photolyzed at wavelengths greater than 190 nm. A few species, however, exhibit very low reactivities. We might ask: What happens to molecules that do not react with most of the oxidants in the stratosphere and the troposphere? The answer to this question is not only of fundamental interest but also of practical significance. If an industrially produced chemical has an atmospheric lifetime of a few thousand years or more, it would accumulate in the atmosphere for times longer than human civilization.

Fully fluorinated organic compounds are chemically very inert and fall into the above class of unusually long-lived atmospheric constituents. Even when some of the fluorine is replaced by chlorine, the stability remains high. These gases will be efficient greenhouse gases and, because of their stability, they will persist in the atmosphere. The atmospheric lifetime of each of these species, a measure of its accumulation tendency, is of particular importance because it is used in the calculation of the global warming potential (GWP), an index of global climate impacts used in policy decisions. The GWP depends on the infrared absorption spectrum and the lifetime of atmospheric gases [see (*1*)]. The GWP of CF_4, for example, is estimated (*2*) to be at least as large as that for chlorofluorocarbon-11 (CFC-11 or $CFCl_3$).

Because of their stability and thermophysical properties, these long-lived molecules have been and are being considered for various industrial applications, including fire extinguishing, foam blowing, and refrigeration. The simplest molecule in this class, CF_4, is also a by-product of aluminum production. Three such compounds, CF_4 (*3–5*), C_2F_6 (*3*, *5*), and SF_6 (*6*), have been observed in the atmosphere. The observed vertical profiles of these molecules are consistent with atmospheric lifetimes of hundreds of years. All these molecules are solely anthropogenic (human-made).

Cicerone (*7*) was the first to consider what could happen to CF_4 in the atmosphere. He concluded that this molecule is nearly inert in the stratosphere and troposphere and has a lifetime of more than 10,000 years. He suggested that a likely process that could destroy this molecule would be photolysis by solar Lyman-α radiation at 121.6 nm. A window in oxygen absorption at this wavelength coupled with a larger incoming flux make absorption of Lyman-α radiation a potentially important mesospheric loss process for tightly bound molecules [such as H_2O (*8*)]. As far as we know, the atmospheric fate and lifetimes of the other fully fluorinated compounds have not been investigated.

We have examined the possible paths by

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