The Origin of Mutants Revisited

The orthodox Darwinian position that evolution occurs by natural selection of pre-exisiting random variations has recently been the subject of much debate. In this Darwinian view, mutations occur spontaneously, regardless of the presence or absence of selection pressure, with subsequent selection of "fitter" variants. A challenge to this concept has surfaced in two papers, in which the the authors suggest that some mutations arise as a direct and specific response to environmental conditions. The papers, one by John Cairns, Julie Overbaugh, and Stephan Miller in Nature[1], and the other by Barry Hall in Genetics[2], both examine the distribution and timing of *E. coli* colony formation under conditions in which mutations from the standard type are needed for colony formation to occur. Their results suggest that, while some mutations are pre-existing, others seem to arise only after placing the *E. coli* in the selective conditions. In this paper I will discuss the experiments by Cairns *et al*, and Hall, focusing on the former due to the large amount of published debate concerning their paper.

In 1943 Luria and Delbruck[3], conducted an experiment designed to quantify the debate between Lamarckian (directed) and Darwinian (spontaneous) origin of mutations. In their now classical "fluctuation analysis", duplicate *E. coli* cultures of approximately 10^9 cells were grown from a very small inoculum of cells. The cultures were then exposed to bacteriophage T1, a killer of all standard type cells. If mutations arose in response to the presence of T1 (directed), then the number of resistant colonies would follow a Poisson distribution across a large number of cultures. However, if mutations arose spontaneously, during the formation of the cultures from the inoculum, then the number of resistant colonies would vary greatly from culture to culture, with early mutations giving rise to many mutants, and late mutations to very few. Their results showed variation above two orders of magnitude, which led them to conclude that only spontaneous mutations were occurring in their system. Based on these results, and support from a limited number of other experiments, the idea of directed mutagenesis was rejected.

However, Cairns *et al* point out that Luria and Delbruck did not allow for directed mutagenesis to occur, because T1 would kill any non-resistant bacteria immediately, and only pre-existing mutants could survive. In addition the expression of phage resistance is delayed, so that even if directed mutants could arise they would not be counted for a few generations. Both Cairns *et al* and Hall designed experiments in conditions in which non-mutant cells could

survive the selection process, existing in "stationary phase", allowing for the possibility of directed mutations. Mutant cells would be identified by the formation of red papillae amidst the white, stationary phase colonies.

The first set of experiments by Cairns *et al* , based on Ryan[4], were designed in much the same was as the Luria and Delbruck fluctuation test, except the selection was done on lactose-minimal plates, with a mutation from $lacZ_{am}$ to $lacZ^+$ needed for growth. They observed papillae with a wide ranging, clonal pattern of distribution arising soon after plating on selective conditions, as expected if mutations were pre-existing. However, they also observed papillae showing up well after selective conditions were imposed, which followed a Poisson distribution. Since these mutants appeared after a delay, they concluded that somehow, mutations were occurring in stationary phase $lacZ^-$ cells. Supplementary experiments indicated that the occurrence of the late-mutants was dependent on the presence of lactose and the uvrB gene. To determine lactose-dependence, cells were grown to culture in non-selective conditions, and in some plating on lactose was delayed for 1-3 days. The distribution and timing of the appearance of late-mutants followed the same pattern with a delay than without. They concluded that these mutations were not occurring until lactose was added to the cultures.

Many problems have been pointed out in the conclusions reached by Cairns *et al* based on the above experiments. First of all, the expected patterns of distribution of mutants (wide range for pre-exisiting mutation, and Poisson for directed) may be based on false or misleading assumptions. It is assumed that the $lacZ^-$ phenotype was not leaky - that such cells were completely unable to utilize lactose. If the $lacZ^-$ phenotype was leaky, then these cells could continue to grow even in selective (lactose-minimal) conditions, spontaneously producing new lac^+ mutants. The pattern of such mutants would follow a Poisson distribution, just as the late-occurring papillae did. Halliday and Rosenberger[5] suggest a mechanism to explain the growth and possibly the replication of the $lacZ^-$ cells. Suppose a cell were deprived of energy, unable to metabolize the only sugar around, in this case lactose. After a period of starvation, trancription, translation, and DNA replication may become increasingly error prone due to changing substrate concentrations, decreasing proofreading efficiency, and cannibalism of important enzymes. These mutations might begin a cascade of errors known as the general error theory,[6] and eventually lead to the appearance of a lactose utilizing mutant. Although

Halliday and Rosenberger do not specify how mutations would be passed on to future generation, perhaps the new ability to utilize lactose would allow DNA synthesis, and if error producing mechanisms have not been completely corrected, then some $lacZ^+$ mutations at the DNA level might occur.

Another criticism of Cairns *et al.* lies in their assumption that, prior to plating on selective conditions, mutants and non-mutants have the same growth rate. However, if $lacZ^+$ mutants grow more slowly or have decreased survivorhip under conditions prior to selective plating, then the total number of mutant per culture will be lower than estimates, and will be weighted proportionally to ones that have spent less time competing with the standard cells (ones more recently evolved).[7,8,9,10] Charlesworth *et al.* [8] suggest that, since mutations from $lacZ_{am}$ to $lacZ^+$ could include amber tRNA suppressors (the likelihood of tRNA suppressor mutation would depend in part on the importance of the amino acid at the position of the mutation - and Cairns *et al.* fail to specify what the position is), some $lacZ^+$ cells would grow at a slower rate.[8] The decrease in $lacZ^+$ over time, as shown by the Cairns *et al.* lactose dependence experiment is seen as an indication of the inferiority of $lacZ^+$. This lower level of fitness of revertants under non-selective conditions is supported by unpublished studies by S. K. Liu and I. S. Hwang[9] in which half of the gal^+ revertants in gal^- colonies grew more slowly than the standard cells. $LacZ^+$ mutations that occurred near the end of growth on non-selective conditions might be "saved" by the addition of lactose before their lower levels of fitness harmed them, while some of the earlier occurring $lacZ^+$ mutations might die or stop growing by remaining in non-selective conditions for too long. This natural selection before the selection that Cairns *et al.* induced would affect the distibution of papillae, and thus change any conclusions made.This Cairns[12] attempts to wave off this criticism by suggesting that most of the revertants in his experiment behaved as wild types, and grew just as well as the standard-type cells. This is both hard to believe and undocumented in any way.

Cairns *et al.* did receive some support for their first series of experiements. S. A. Benson[13] also examined *E. coli* revertants in non-lethal conditions. In his system, the carbon source was too big to cross the outer membrane, and, after a few days in selective conditions, colonies formed from cells with mutations in the ompF (outer membrane porin protein) gene. However, if ompF was deleted, colonies appeared with mutations in the ompC

gene. OmpC mutations did not occur when ompF was present, and the two types appeared to be equally viable. Ostensibly this is some type of directed selection, but it seems more likely that ompC is structurally protected from mutation by the presence of ompF. Only if the omp genes had higher rates of mutation than other genes (which was not shown), would this example be incompatible with the theory of spontaneous mutation.

It is exactly this type of higher mutation rate that Cairns *et al.* attempted to document in regard to the lac gene in lactose-minimal agar, which would imply that *E. coli* preferentially mutate the gene in which a mutation is needed. To do this they compared mutation rates in the lac and val genes, by using replica plating techniques. They found that while late $lacZ^+$ revertants were occurring at a high rate, late-occurring val^R (valine resistant) mutants were not showing up. They concluded that lacZ was preferentially mutated in the presence of lactose, and that the $lacZ^-$ was not leaky (otherwise some late-val^R cells should have arisen). This conclusion has also generated much debate. Critics say that since val^R is a frameshift mutation and $lacZ^+$ is a amber mutation, that comparing the two can be at best inconclusive.[14] Cairns admits using a mutation similar to $lacZ_{am}$ as a control would help determine whether any mutations are being directed at the lac gene.[12] This experiment with val^R actually served to highlight the aforementioned criticism that $lacZ^+$ cells are at a disadvantage before selection, because the number of val^R cells also decreased with more time spent in non-selective conditions.[8,10] Cairns refers to unpublished experiments that showed that the decline in val^R is not caused by cell death but due to a delay in colony formation when cells are starved for a few days[12]. If this is correct then some of the criticism would be less valid. Expecting much criticism of their first experiment Cairns *et al.* also studied more complex system of mutations because directed mutagenesis in such systems would be more impressive, and would be less weighted down by the noise of spontaneous mutations.[12]

In one experiment Cairns *et al.* studied selection in *E. coli* in which a short piece of foreign DNA (from phage-Mu) containing transcription termination factors is inserted between the lac and ara (arabinose) operons., which disrupts the function of boith operons[15]. Deletion of Mu allows growth on plates with both lactose and arabinose. Cells with this phenotype are termed $lac(ara)^+$. Many separate tests suggested that the excision of Mu was directed occurring only in the presence of the selective condition. One criticism of this

experiment is identical to previous criticism of the lac experiment, for lac(ara)$^+$ may also be at a relative disadvantage on non-selective conditions. Other criticism is based in part on the "unnatural" properties of the foreign Mu DNA. Since there was no control experiment to examine the excision of Mu in other parts of the genome, critics suggest that the excision of Mu may be due to starvation conditions, and not specific to the lac(ara) region of the *E. coli* genome.[7]

In another experiment Cairns *et al.* studied the expression of a cryptic gene, ebgA, which is used to hydrolyze lactose when the lac gene is non-functional. Hall studied the expression of another cryptic gene, bgl, which is used to metabolyze β-glucoside sugars such as salicin. Hall's study went into much greater depth, but both were very similar. Each experiment is based on naturally occurring events in *E. coli* and in each case two separate mutations are needed for expression of the cryptic genes. In Hall's system, a point mutation in R^0 and the excision of a transposon IS103 must occur if the cell is to utilize salicin. The expected frequency of these events occurring together is around 10^{-20}. However, within two weeks after placing colonies of 10^9 cells under selctive conditions (just as in the Cairns *et al* and Luria and Delbruck experiments), over 60% of the colonies showed sal$^+$ mutants. The appearance of sal$^+$ was determined to be dependent on the presence of salicin, suggesting that directed selection was occurring. Furthur experiments indicated that the excision of IS103 precedes the R^0 to R$^+$ mutation, and that this excision was occurring at a very high rate, while the R$^+$ mutation was occurring at almost normal frequencies. However, it appeared that cells in which the excision occurred had no selective advantage over their IS103 containing precursors, suggesting not only directed selection, but a mutation that anticipates the need for another. However, Hall has been criticized on two main fronts. Transposon activity throughout the cell was not studied. If such activity were systemically increased with the addition of salicin, or focused at any IS103 throughout the cell, then neither directed nor anticipatory mutagenesis would be occurring. In addition Lenski *et al.* [10] have proposed a theory that would explain the results of Hall and Cairns *et al.* This Darwinian explanation suggests that mutations may be occurring in two steps. The first mutation would produce a cell that could grow in selective conditions, but only slowly. This would somehow set the stage for a second mutation yielding a cell which could grow very fast. This could account for the delay seen by Cairns

et al. in expression of "late" mutants, and the ostensibly "anticipatory" nature of Hall's results. The presence of two step mutations theory has previously been documented in *E. coli* but only in relation to phage resistance, therefore plagued by the same problems as Luria and Delbruck's experiment. Van Halen (just kidding - Van Valen) suggests that what may be occurring is "periodic selection" - natural selection due to competition between revertants under selective conditions [14]. This would give similar results as a two-step mutation system. However, Cairns disregards this because it has only been shown to occur when cultures are maintained for a long period of time.

Cairns *et al* propose their own theory to explain their results. They suggest that due to sloppy transcription, bacteria may be constantly testing a few "extra" proteins for each gene. If one of these were deemed useful, through reverse transcription, it could be incorporated into the genome. How the protein would be deemed useful is difficult, but if the cell reverse transcribed any RNA present when growth begins anew after a pause (for example, just after starvation), then beneficial mRNA may be incorporated into the genome, even if it was not a direct complement of the original DNA[15].

Finally, Stahl[16] has presented a model that seems to fit all of the data. If, under starvation conditions, parts of the *E. coli* genome are replicated, but complete replication is prevented by the lack of macromolecules, nucleotides, and energy. This partial DNA replication would result in "bubbles" of diploid DNA. Suppose the only sugar around is lactose and the cell is lac^-. Suppose furthur that the lac gene is one of the small regions of DNA replicated, and an error occurs that creates lac^+ on one of the new helices. If this part of the gene was then transcribed and translated, the subsequent utilization of lactose might allow the rest of the genome to be replicated. This would result in one $lacZ^-$ and one $lacZ^+$ cell, apparently (at the phenotypic level) creating lac^+ cells from one cellular division. Since the parent cells would have acquired a functional lac gene, this is along the lines of Lamarckian evolution. In the absence of lactose, the entire genome would not be replicated and thus "bubbles" with lac^+ mutations would confer no selctive advantage, and mutations would not be "directed" at the lac gene. This theory seems to fit in with both Darwinian and Lamarckian evolution. Mutations would not be directed at the lac gene, but only those that were would be passed on to future generations.

The fundamental debate on which all of these theories are based

concerns the origin of mutations. The evolution of this debate is outlined very well in a recent article by Lederberg in Genetics.[17] In the traditional view, selection and variation are temporally separated, with variation occurring first. However, Cairns *et al.* and Hall suggest that sometimes variation and selection may be temporally ~~copuled~~ coupled, with selection acting as both a cause and filter of variation. Although both of the new experiments have been shown to have many potential flaws, they do emphasize many problems with the Darwinian theory on the origin of mutations. Little information has been gathered concerning mutation in starvation, or any other sub-optimal conditions. Collection of such information will not only facilitate debate on the origin of such mutations, but may also help better understand natural as opposed to laboratory evolution.

Jonathan Eisen '90
Harvard College
Biology 99hf - The Origin of Life
4/12/89

[1] Cairns, J., Overbaugh, J., & Miller,S. Nature **335**, 142-145 (1988).
[2] Hall, B. Genetics **120**, 887-897 (1988).
[3] Luria, S. E., & Delbruck, M., Genetics **28**, 491-511 (1943).
[4] Ryan, F.J. Nature **169**, 882-883 (1952).
[5] Holliday, R. & Rosenberger, R. F. Nature **336**, 526 (1988).
[6] Kirkwood, T. B. L., Holliday, R., & Rosenberger, R. F. Int. Rev. Cytol. **92**, 93-132 (1984).
[7] Partridge, L. & Morgan, M. J., Nature **336**, 22 (1988).
[8] Charlesworth, D., & Charlesworth, B., Nature **336**, 525 (1988).
[9] Tessman, I. Nature **336**, 527 (1988).
[10] Lenski, R. E., Slatkin, M., & Ayala, F.J. Nature **337**, 123-124 (1988).
[12] Cairns, J. Nature **336**, 527-528 (1988).
[13] Benson, S. A., Nature **336**, 21-22 (1988).
[14] Van Valen, L. M., Nature **336**, 526 (1988).
[15] Cherfas, J. New Scientist **119**, 34-35 (1985).
[16] Stahl, F. W., Nature **335**, 112-113 (1988).
[17] Lederberger, J., Genetics **121**, 395-399 (1989).

See over →

Jon

pre-existing vs. directed mutn's.

Luria + Delbrück —

This is a nice review. You have clearly thought about the experiments presented, and understood the gains and pitfalls of the work. It seems that no one has (yet) done an experiment with all necessary controls — hopefully these are forthcoming.

Class participation: A

Papers / final presentation: A

Course grade (A)

Good luck next year - hope to see you around!