

A Test of Hypotheses about Random Mutation

Using Classic Experiments To Teach Experimental Design

Douglas S. Green Donna M. Bozzone

Introduction

Many of the laboratory exercises performed in an undergraduate biology course have the important goal of teaching students to use modern techniques or to illustrate new discoveries. It is also true, however, that students can learn many valuable principles from studying and repeating "classic" experiments.

In 1943 Luria and Delbruck performed an experiment that we think is a good candidate for consideration as a classic. Their experiment addressed the question of whether mutations (in this case *E. coli* resistance to bacteriophage) occur at random or are directed by the environment. The answer to this question has a strong influence on our understanding of evolution and adaptation. If the environment can direct the production of beneficial mutations, this mechanism could account for much of the adaptation we see. If the effects of mutations are independent of the environment, another mechanism (e.g. natural selection) is required to explain adaptive change. The majority of evolutionary biologists today accept the random nature of mutation. However, directed mutation continues to hold an allure, since it seems at first glance to be a much more efficacious process. In the conclusion section, we discuss this point and review briefly some of the more recent attempts to find a mechanism for directed mutations.

In this paper we describe how we have used Luria and Delbruck's experiment in our evolution course to illustrate several important concepts, such as the random nature of mutations,

experimental design, and the meaning of scientific proof. First, we present an overview of their experiment.

Luria and Delbruck began their paper by describing a readily observable phenomenon that was well known at the time. When a turbid culture of bacteria is exposed to a virus, the culture clears quickly as bacteria that are sensitive to the virus lyse. However some bacteria survive and reproduce, and the culture eventually becomes turbid again.

Luria and Delbruck present two alternative hypotheses that could account for this observation:

1. **The mutation hypothesis.** A bacterial cell has a small but finite probability of mutating from sensitive to resistant during its life, regardless of whether viruses are present in the environment.

2. **The acquired immunity hypothesis.** A bacterial cell has a small but finite probability of surviving an attack by a virus, and if it survives it passes on its resistance to its daughter cells.

Distinguishing between these two hypotheses presented a challenge. If resistant bacteria could be found in the absence of virus, the mutation hypothesis would be supported. However, the only known assay for resistance was to expose the bacteria to the virus and identify the surviving cells. But if virus is present in the bacterial culture, the existence of surviving cells by itself is not sufficient to rule out either hypothesis.

Luria and Delbruck found a clever way around this problem, and it provides the foundation for their work: the two hypotheses predict different times for the occurrence of the first mutation. The mutation hypothesis predicts that a mutation to resistance can occur at any time during the growth phase of a bacterial culture. The acquired immunity hypothesis, on the other hand, predicts that mutations will occur only *after* the introduction of virus.

Suppose that a large number of parallel cultures with equal concentrations of bacteria are placed on petri plates inoculated with virus. The acquired immunity hypothesis predicts that each plate will have approximately the same number of resistant colonies. This is because with respect to resistance, all the cultures were identical at the time of plating. None contained any resistant bacteria, and since the chance of mutating to a resistant form is the same for all bacteria, the number of such mutants should be about the same on each plate. Indeed, the only variation expected comes from statistical variation in the number of mutations that occurred and variation that results from the plating technique.

The mutation hypothesis predicts a very different result. The parallel cultures are not the same at the time of plating. In some of these cultures, mutation to resistance may have occurred early in the culture's growth phase. Such cultures will contain many resistant cells, since the original mutants will have had time to produce many resistant daughter cells. Other cultures can produce many fewer resistant cells if the mutation to resistance occurs at a later time. The number of resistant colonies will fluctuate greatly from one plate to another, and the variance in number of colonies will be correspondingly greater than that predicted by the acquired immunity hypothesis.

These differences form the basis of Luria and Delbruck's "fluctuation test." They grew up a large number of parallel cultures of *E. coli* and put aliquots from each on petri plates infected with virus. If the number of resistant cells fluctuated greatly from plate to plate (i.e. if the variance in number was high), the mutation hypothesis would be supported. If the numbers were similar among the plates, this would favor the acquired immunity hypothesis.

Douglas S. Green and Donna M. Bozzone are Associate Professors in the Department of Biology at Saint Michael's College, Colchester, VT 05439. Their e-mails are: dgreen@smcvf.edu and dbozzone@smcvf.edu, respectively.

Luria and Delbruck recognized one last problem with their experimental design. A large fluctuation in resistant colonies could also result from sampling error when the bacterial cultures were plated. To control for this possibility, they also made a set of replicate plates, with aliquots of equal volume taken all from the same culture. A low variance in number of resistant colonies on these plates rules out the possibility that fluctuations found among the parallel plates is due to experimental error.

Not surprisingly given our current understanding of mutation, Luria and Delbruck found that plates from the same culture showed a low variance in number of resistant colonies, but plates from different cultures showed a much larger variance. They concluded that mutations (in this case, at least), are not directed by environmental stimuli, but instead occur randomly. The key to their success was an elegant experimental design, motivated by clever reasoning and a good analysis of the problem, and backed up by well-designed controls. As such, their experiment is an excellent model, and we feel that students benefit greatly by studying it.

We don't wish to oversimplify Luria and Delbruck's work, for they did more than we have described above. For example, using probability theory they were able to derive the frequency distributions for the number of resistant colonies expected according to both hypotheses. They were also able to compute the variance of both distributions, and were able to show that the expected variance of the distribution of resistant colonies was indeed much greater for the mutation hypothesis. Students would undoubtedly profit from studying the theoretical underpinnings of Luria and Delbruck's work. We have focused mainly on its empirical aspects in our undergraduate courses.

We feel that this experiment provides three important benefits in an undergraduate course:

1. It provides an elegant demonstration of experimental design, and gives a clear-cut illustration of how good experiments provide a clear distinction among competing hypotheses.
2. It helps students better understand a concept that many of them find difficult: that mutations occur randomly and that genetic variation is *selected* by natural selection, but not *caused* by it, no

matter how attractive that prospect seems.

3. It provides good insight into some interesting aspects of the nature of science because (Point 2 notwithstanding), the notions of directed mutation and the inheritance of acquired characteristics (in particular acquired immunity) have both recurred as recent topics of study, even though Luria and Delbruck's work "proved" that these notions have no validity.

We have addressed the first point already, and we will address the other points in our discussion. Here we provide a brief overview of the experimental protocol. Complete details and preparation instructions are available upon request.

The Experiment

The experiment we perform is reasonably close to Luria and Delbruck's original experiment. Instructors for the genetics course at Vassar College developed the actual protocol for this lab during the early 1980s. We have augmented the basic protocol to include the computation of mutation frequency, an interesting (if optional) step.

Figure 1 outlines the student protocol. Students take about 3 to 4 hours to perform this experiment, distributed over three days as shown in Figure 1. Pre- or co-requisite knowledge for the students includes a facility with sterile technique, serial dilutions and bacterial culture, and the ability to understand and compute variances. Table 1 presents two data sets that show student results. Figure 2 outlines how the results are analyzed.

The materials needed for this experiment include a stock culture of *E. coli* B, a high-titer suspension of T4 phage, dilution buffer, nutrient broth, and nutrient agar. In addition the experiment requires a fairly large number of sterile test tubes, sterile pipettes, and petri plates. We have found that the preparation and expense is manageable for small class sizes: we typically have 20 students in a class, who work in groups of two or three. We have one or two undergraduates who work under our direction to prepare the materials and set up the lab.

We spend considerable time discussing with students the design of this experiment. Although our students are adept at following the instructions and obtaining results, a casual reading of

the laboratory exercise leaves them unclear on two main points: 1) the nature of the controls used and 2) the interpretation of the results.

It is important that students realize that the 10 replicate plates (those from a single culture) serve as a control for their plating technique. In principle, each of the 10 replicate plates should have the same number of resistant colonies, since each was inoculated from a single culture. The variance in resistant colonies on the replicates estimates the variance due to sampling error that results from taking an aliquot and spreading it on the plate.

It is also important for students to understand the predictions made by the acquired immunity hypothesis and the mutation hypothesis. First, neither hypothesis makes predictions about the *mean* number of colonies on the replicate plates vs. the parallel plates. Students sometimes expect, mistakenly, that what they are looking for is a difference in mean, and in particular that a larger mean for the parallel cultures supports the mutation hypothesis.

The key result, as Luria and Delbruck point out and as we discussed in the Introduction, is whether the *variance* in colony number is the same for the replicate and parallel plates. If it is, the correct interpretation is that all 11 cultures contained the same number of mutant cells at the time of plating, and that the only factor contributing to the variance is sampling error during plating. The most likely explanation for this is that the number of mutant cells at the time of plating is zero. None of the tubes had any resistant mutants, and mutants resistant to the phage appeared only after exposure to the phage. This result supports the acquired immunity hypothesis.

On the other hand, the mutation hypothesis predicts that the variance in the parallel plates will be higher than that of the replicates. Both sets get the same contribution in variance from plating technique. However, the 10 cultures used for the parallel plates will exhibit additional variance due to variance in the times when mutations occurred. In cultures where the mutation occurred early, large numbers of resistant daughter cells lead to many resistant colonies, while cultures with later mutations will contain correspondingly fewer resistant cells. Thus a larger variance for the parallel plates supports the mutation hypothesis. This also explains why a difference in mean number of colonies between replicate and parallel plates is not informative. The culture chosen for the replicate

Day 1. Set up cultures.

1. Dilute a log phase culture of *E. coli*, to a final concentration of 10^{-5} of the original.
2. Set up 11 test tubes with 3.0 ml of *E. coli* culture medium.
3. Inoculate each tube with 0.3 ml of the final dilution from Step 1.
4. Incubate the 11 test tubes overnight at 36° .

Day 2. Set up petri plates.

1. Get 20 petri plates with nutrient agar. Label 10 plates "replicate" and the other 10 "parallel."
2. Inoculate each plate with 0.2 ml of a T₄ phage suspension, and spread evenly over entire plate. Allow to dry.
3. Select at random one of the 11 *E. coli* cultures from Day 1. From this culture, put a 0.1 ml aliquot on each of the 10 plates labeled replicate. Spread evenly, allow to dry, and invert.
4. For each of the remaining 10 cultures, take a 0.1 ml aliquot, and spread it evenly over one of the petri plates labeled parallel. Allow to dry, and invert.
5. Perform a serial dilution on the culture selected in Step 3 to achieve final concentrations of 10^{-6} and 10^{-7} .
6. Obtain four additional petri plates. Label two of them " 10^{-6} " and the other two " 10^{-7} ." Do not put phage on these plates.
7. Plate a 0.1 ml aliquot from the 10^{-6} tube (Step 6) onto each of the petri plates labeled 10^{-6} . Plate a 0.1 ml aliquot from the 10^{-7} tube onto each of the petri plates labeled 10^{-7} . Allow to dry, and invert.
8. Incubate all 24 petri plates overnight at 36° .

Day 3. Record the number of colonies that appear on each petri plate.

Figure 1. Outline of experimental protocol.

Table 1. Representative student results.

Group 1			Group 2		
Plate	Replicates	Parallel	Plate	Replicates	Parallel
1	113	67	1	47	36
2	111	195	2	55	49
3	101	420	3	70	35
4	85	150	4	46	65
5	93	146	5	71	61
6	143	112	6	58	47
7	91	148	7	47	68
8	171	198	8	60	37
9	198	192	9	58	82
10	85	58	10	63	44
mean:	119	169	mean:	58	52
variance:	1524	10261	variance:	82	252
Plate	10^{-6}	10^{-7}	Plate	10^{-6}	10^{-7}
1	65	0	1	1	0
2	0	0	2	4	0
mutant frequency:	3.7×10^{-6}		mutant frequency:	2.3×10^{-5}	

plates may have had an early (or late) mutation, and as a result the mean number of colonies could be larger or smaller than that of the parallel plates.

Discussion

This experiment is relatively inexpensive to perform and relatively easy

to set up (at least for smaller classes). It doesn't require specialized equipment. It draws upon techniques and skills that most undergraduate biology majors will (or should) have. Barring major mistakes during the procedures, this experiment always produces interpretable and "correct" results. Thus the experiment meets some of the min-

imal requirements that make it acceptable to students and instructors.

In our experience the experiment provides a good deal of additional pedagogical benefit. It challenges students to think about experimental design; the conclusions drawn link to a number of disciplines like evolution, cell biology and genetics; and there are interesting tie-ins to recent research and the philosophy of science.

Once they understand the experimental design, many students come to appreciate the elegance with which it distinguishes between the two hypotheses. Assuming only that all 11 *E. coli* B cultures contained approximately equal concentrations of cells and that the probability of mutation is equal for all cells, the larger variance of resistant colonies on the parallel plates completely rules out the acquired immunity hypothesis. Conversely, had the results been otherwise and the variance of resistant colonies was found to be the same for both sets of plates, the mutation hypothesis would have been completely ruled out. This experiment reinforces the point that hypothesis testing is one of the major functions of an experiment, and also provides a good example of how experimental design promotes success in this endeavor. Less tangible, though still important, is our belief that presenting students with examples of good experimental design will help them when they begin to design their own independent experiments.

We perform this experiment in our Evolutionary Biology course. Although it would be appropriate in other courses as well, in an evolution course this experiment helps illustrate a particularly thorny point. As we study examples of adaptations and describe the mechanisms by which they arise, it is inevitable that some students find it difficult to accept that adaptations evolved by means of selection acting on what initially was random genetic variation. The possibility of some mechanism that can direct mutations along environmentally favorable avenues seems to have such explanatory power and seems so effective at producing adaptation that it is difficult to abandon. The results of Luria and Delbruck's experiment don't rule out this possibility for all cases, of course. They do, however, demonstrate conclusively (although see below) that in this case environmental factors like phage cannot have caused the mutation to resistance, since the mutation occurred before exposure to the phage.

The last question we discuss with our students is: What exactly is meant

1. Compute the mean and variance in number of colonies for the parallel plates.
 - a. *To compute the mean:* add the number of colonies on each plate, and divide by the number of plates.
 - b. *To compute the variance:* for each plate, subtract the mean from the number of colonies on that plate, and square the result. Add up all the squares, and divide by the number of plates $- 1$.
2. Compute the mean and variance in number of colonies for the replicate plates.
3. Compute the mutation frequency, as follows:
 - a. Use the four plates from Day 2, Step 6 to estimate the concentration of bacteria in the original culture from which the replicate plates were made.
 - i. Average the number of colonies on the 10^{-6} plates.
 - ii. If there are no colonies on the 10^{-7} plates, ignore them. If there are colonies on the 10^{-7} plates, compute the average number of colonies. Multiply the average by 10 to make the results from these plates equivalent to the results on the 10^{-6} plates. Then average these two averages to get the overall estimate of number of colonies at a dilution factor of 10^{-6} .
 - iii. To find the concentration in the original culture, you divide the average number of colonies by the dilution factor. The dilution factor is 10^{-6} , but an additional "dilution" occurred since only 0.1 ml of the final dilution tube was plated, making the total dilution factor 10^{-7} . Divide the result of Step 3.a.i (or 3.a.ii) by 10^{-7} to get the estimate of bacterial concentration in the original replicate culture.
 - b. Use the average number of colonies on the replicate plates to estimate the frequency of mutations in the replicate culture. Each plate was produced from a 0.1 ml aliquot of the replicate culture, resulting in a 10^{-1} "dilution." The estimated frequency of mutations is therefore the average number of colonies observed on the replicate plates divided by 10^{-1} .
 - c. Divide the result of 3.b by the result of 3.a.iii to get the mutation frequency.

Figure 2. Analysis of results.

by a "conclusive demonstration" in science? In spite of results like those of Luria and Delbruck, some scientists find it difficult to accept that an apparently "random" or mechanistic process can bring about the adaptations we see all around us. Dating back at least to the early 1980s, a number of researchers have reported evidence of environmentally directed (and therefore adaptive) mutations. Each report has been followed almost instantaneously by a flurry of rebuttal or alternative interpretations that preserve the traditional view that mutations are random. The discussion has been lively, at times vitriolic, and it is a good example of what scientific discourse can be.

Gorczynski and Steele (1981) published results suggesting that in mice an acquired resistance to certain antigens could be passed on to offspring. This hypothesis is similar in some respects to one of the hypotheses Luria and Delbruck proposed to account for the mutation to phage resistance. Two months later, Brent et al. (1981) challenged these results when they were unable to repeat them, which led in turn to a counter-challenge by Steele (1981) suggesting that Brent's results did indeed support his own.

Cairns et al. (1988) addressed the issue directly by repeating Luria and Delbruck's fluctuation test in a different context. They argued that in the original experiment the lethality of the phage precluded the possibility that given more time, environmentally directed mutants might have been produced. In Cairn's version of the test, *E. coli* that were unable to metabolize lactose were plated on media with lactose as the only energy source. Some colonies appeared on the media immediately, presumably spontaneous mutants of the type Luria and Delbruck observed that were able to metabolize lactose. Several days later, additional mutant colonies formed. Cairns et al. concluded that these colonies were the result of environmentally directed mutations to cells that were lactose deficient when plated, but the presence of lactose in the medium induced the appropriate mutation that allowed these colonies to grow. Foster and Cairns (1992) went on to propose mechanisms by which such mutations could be produced.

As with the results of Gorczynski and Steele (1981), a number of researchers questioned these results or offered alternative explanations (see, for example, Lenski & Mittler 1993),

and a moderately sized controversy ensued. Goodman (1992) provides a good review of the recent history of directed mutation research.

When we discuss this research with our students, we don't present it as evidence that Luria and Delbruck were "wrong." Our point is that in actual practice science doesn't always proceed in an orderly fashion, where an experiment, even a well designed one, once and for all puts to rest hypotheses about the most fundamental concepts of a discipline. Luria and Delbruck's work (and of course a host of others) shows conclusively that environmental influences played no role in establishing a mutation for resistance to phage. Generalizing this, we come to the well-established conclusion that mutations occur randomly and are not directed by environmental influences into specifically favorable forms. Even so, the search for a more directed, and perhaps more efficient, mechanism for generating favorable genetic variation continues.

In conclusion, we have found Luria and Delbruck's experiment and the many issues that surround it to be a rich topic for discussion. In addition to questions about the experiment itself, students can analyze questions about the nature of controls, experimental design, and reasons why some themes in science keep recurring. While current techniques and modern perspectives are important, students can derive a great deal of benefit from studying the classics as well.

Acknowledgment

We wish to thank the students in our evolution course for their hard work on this experiment, and for their thoughtful discussion. The insights gleaned from these discussions provided the motivation and much of the content for this paper.

References

- Brent, L., Rayfield, L.S., Chandler, P., Fierz, W., Medawar, P.B. & Simpson, E. (1981). Supposed Lamarckian inheritance of immunological tolerance. *Nature*, 290, 508-512.
- Cairns, J., Overbaugh, J. & Miller, S. (1988). The origin of mutants. *Nature*, 335, 142-145.
- Foster, P.L. & Cairns, J. (1992). Mechanisms of directed mutation. *Genetics*, 131, 783-789.
- Goodman, B. (1992). Heredity made to order. *MOSIAC*, 23(1), 24-33.

Gorczyński, R.M. & Steele, E.J. (1981). Simultaneous yet independent inheritance of somatically acquired tolerance to two distinct H-2 antigenic haplotype determinants in mice. *Nature*, 289, 678–681.

Lenski, R.E. & Mittler, J.E. (1993). The directed mutation controversy and Neo-Darwinism. *Science*, 259, 188–194.

Luria, S.E. & Delbruck, M. (1943). Mutations of bacteria from virus sen-

sitivity to virus resistance. *Genetics*, 28, 491–511.

Steele, E. (1981). Lamarck and immunity: a conflict resolved. *New Scientist*, (7 May), 360–361.

Vote Now

Make *your* voice heard!

NABT is conducting a major member needs analysis and biology teacher survey online. We need everyone to participate and complete our surveys. Please visit our web site, www.nabt.org, and click on one of the surveys:

- NABT Program Analysis
- *The American Biology Teacher* Readership Survey
- Technology 2001—Needs and Capacities
- Conventions—What Works and What Doesn't?
- Publications Today and Tomorrow
- Biology and Education—Where Do We Go from Here?

Better yet, fill out all six surveys. You can complete each in less than five minutes. We need maximum participation, so make a difference in the future of *your* association and *your* profession. Thanks for helping to make NABT *The Leader in Life Science Education*.

NABT Affiliate Members

The National Association of Biology Teachers thanks its affiliate organizations for their support & for their efforts to further biology & life science education.

Biology Association of Teachers of St. Louis

Biology Teachers Association of New Jersey

California Biology Education Association

Cleveland Regional Association of Biologists

Colorado Biology Teachers Association

Empire State Association of Two-Year College Biologists

Illinois Association of Biology Teachers

Illinois Association of Community College Biologists

Indiana Association of Biology Teachers

Kansas Association of Biology Teachers

Louisiana Association of Biology Educators

Maryland Association of Biology Teachers

Massachusetts Association of Biology Teachers

Michigan Association of Biology Teachers

Mississippi Association of Biology Educators

New York Biology Teachers Association

South Carolina Association of Biology Teachers

Texas Association of Biology Teachers

Virginia Association of Biology Teachers

Western Pennsylvania Biology Teachers Association