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Research: The Luria-Delbrück experiment

Are mutations spontaneous or directed?

Where do mutations come from, and what do they have to do with mathematics? A lot, as this article will demonstrate. Indeed, it was a historical milestone of mathematical biology that revealed some fundamental insight into the nature of mutations during the 1940's. An unresolved question in those days was: 'Are mutations due to directed adaptation to environmental change, or do they occur spontaneously, in a random way?' Today, the answer is basic knowledge in genetics, and many textbooks briefly describe the crucial experiment, along with some plausibility arguments. It is hardly known, however, that it was a fascinating interaction of theory and experiment that made this breakthrough possible. Ellen Baake, professor in bio-mathematics at the university of Bielefeld in Germany, and 'Kloosterman-hoogleraar' 2006–2007 at the University of Leiden gives an account of this history.

Before we formulate the problem in a precise way, let us describe the experimental observation that solicited the question in the early 1940's (see Figure 1).

The experimental observation

The microbiologist S. Luria analyzed how resistance against certain bacteriophages originated in *E. coli* bacteria. Bacteriophages are viruses that attack bacteria, multiply within their cells and eventually destroy them — provided the bacterium is *sensitive* to this phage, that is, if it is not resistant. To this end, Luria raised *E. coli* cultures in a suitable medium, starting from a single sensitive cell every time (or very few of them, but let us idealize a bit here). The growth of the bacterial popula-

tion is visible through the increasing turbidity of the medium, caused by the scattering of light by bacteria (which distribute themselves all over the medium). After a few days, when the population is fairly dense, one adds a certain amount of phages. As a consequence, the bacteria are destroyed, the dead cell fragments sediment, and the culture clears. But after a few days, the medium turns turbid again, meaning that a new bacterial population is growing. What has happened?

Two hypotheses

Clearly, a few cells have survived that are happy in the presence of the phage, so they must be *resistant*. This resistance persists over many further generations in culture, so it must

be *heritable*. But all our bacteria go back to a single sensitive cell, so a *mutation* must have happened under way. The crucial question is: when? Figure 2 shows the two principal possibilities.

- (SM) A few bacteria are already resistant before the phage arrives; mutations thus happen *spontaneously* while the culture is growing, independently of the selection to be applied later by the phage.
- (DM) A few bacteria acquire resistance when they are exposed to the phage; that is, mutations are *directed* (in the sense of a specific response to the selection applied; they would not occur in the absence of the phage).

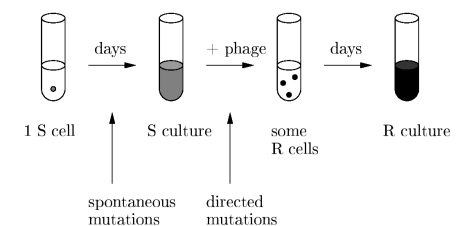


Figure 1 The origin of resistance in a bacterial culture. S stands for sensitive, R for resistant

Today, it is basic biological knowledge that (SM) is true: mutations occur in a spontaneous way. Beneficial mutations (these tend to be very rare!) may later be filtered out (selected) by the environment (in our case, only resistant cells survive the phage), but they are not directed by selection.

Luria and Delbrück managed to prove this in 1943 [1], at a time where one could not even think of the powerful genetic methods that are standard in modern labs. The molecular basis of inheritance, and even more so that of mutations, was still in the dark. (It had to wait until 1952, when Hershey and Chase discovered that genetic information is carried by DNA; one year later, Watson and Crick unravelled its famous double helix structure.) Indeed, Luria and Delbrück's decisive experiment boiled down to nothing more than counting cells; and the evaluation of their data required only elementary probability. However, the story is fascinating due to exactly this simplicity, in combination with the underlying idea and the stringency of the argument.

The model

In what follows, we will even simplify the original considerations a bit further. This will make the essential idea still clearer; we hope that specialists will forgive us. The hypotheses (SM) and (DM) lead to the following fundamental consideration (see Figure 2).

After T rounds of division (or generations) starting from the initial cell, the bacteria come in contact with the phage. In case (SM), the mutants have originated before this; one will

assume that every sensitive (S) bacterium becomes resistant (R) in every generation $t < T$ with some small probability p . Resistance is heritable, that is, it is passed on to the offspring. In generation T , we will therefore have resistant clones, the offspring of the primary mutants.

In case (DM), resistance solely originates in generation T . Now, every bacterium mutates independently of all others, with probability \tilde{p} , and survives; the others fall prey to the phage. It is actually irrelevant whether every individual cell truly has the same chance \tilde{p} ; it may also be conceived that the result is determined by small physiological differences between the cells (e.g., size or nutritional status, which may vary within certain limits). In this case, one would assume that cells with certain favourable properties are predisposed in the sense that they will become (stably) resistant if they meet a phage. As long as these physiological dispositions themselves are not heritable, i.e., as long as they are not passed on to the offspring over several generations, the situation still boils down to the described random experiment (provided the experimentalist cannot, or does not, distinguish between the different cell variants).

The essential property of (DM), relative to (SM), lies in the fact that physiological dispositions are defined by their temporary occurrence — if they were heritable, we would be in the setting (SM). This is because it is irrelevant for the result whether the cell is already resistant before T , or whether it has a heritable disposition that leads to resistance as soon as it comes across the phage.

How could Luria and Delbrück distinguish between these possibilities? The only method available to them was counting resistant cells in generation T (we will describe below how exactly this is done). The question therefore reads: How can we decide, by counting resistant cells, whether they are clones or independent individuals (in the sense of a random sample from the population)?

Clearly, this is impossible to decide on the basis of the number of R cells in a single experiment (cf. Figure 2), and the average over many experiments is equally uninformative. However, the stochastic fluctuations between experiments yield the desired distinction. To analyze these, we will now formulate an idealization (a model) of the scenarios (SM) and (DM), and make our assumptions more precise.

The model must describe both cell divi-

sion (i.e., population growth) and the mutation process. *Population growth* will be modelled *deterministically*, owing to the fact that it only takes a few generations until the population is very large. For simplicity, we assume that cells divide into two daughter cells in a synchronous way (that is, time is discrete). Starting with a single cell then results in a population of $n(t) = 2^t$ cells in generation t . We set

$$N := n(T) = 2^T,$$

the number of cells in generation T .

In contrast, the *mutation process* must be described in a stochastic manner, for mutations are rare events ($p, \tilde{p} \ll 1$). The quantity to be described is Z , the number of resistant cells in generation T ; it is a random variable. In this section, we will calculate the expectation $\mathbb{E}(Z)$ and the variance $\mathbb{V}(Z)$ under the hypotheses (SM) and (DM); on the basis of these quantities, Luria and Delbrück solved the problem in 1943. In the next section, we will characterize the distribution of Z in more detail.

In the case (DM), no further assumptions are required. Each of the N cells in generation T turns resistant with probability \tilde{p} , hence Z has a binomial distribution with parameters N and \tilde{p} ; we write $Z \sim B(N, \tilde{p})$. Therefore,

$$\mathbb{E}(Z) = N\tilde{p}, \quad \mathbb{V}(Z) = N\tilde{p}(1 - \tilde{p}). \quad (1)$$

In experiments, $\tilde{p} \approx 10^{-8}$ and N lies between 10^8 and 10^{10} ; hence, expectation and variance are equal up to a tiny error term. (This error term is just the deviation that occurs if the binomial is approximated by a Poisson distribution with parameter $\lambda = N\tilde{p}$.)

To treat case (SM), we must first make our assumption about the mutation mechanism more precise. We will use the following *idealization*.

- (A0) The initial cell is sensitive.
- (A1) Mutations (S \rightarrow R) occur on the occasion of cell divisions. With probability p , one of the daughter cells is a mutant. (Here we have in mind a mechanism of division where the 'original' remains intact, whereas the 'new copy' may contain an error.)
- (A2) As long as the phage is absent, S and R cells divide in the same way, i.e., in every generation.
- (A3) The number of resistant cells is, at any time, negligible relative to $n(t)$ (this is justified since $p \ll 1$), so that the number of S cells may be described by $n(t)$.
- (A4) Back mutations (R \rightarrow S) are negligible. (The probability for any single R cell to

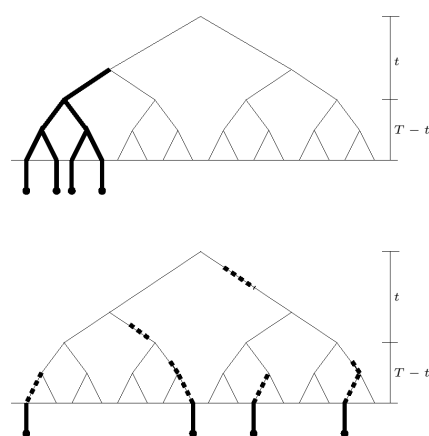


Figure 2 Genealogy (discrete generations) of a bacterial culture with spontaneous (top) and directed (bottom) mutations. Thin lines indicate sensitive bacteria, fat lines mean resistant ones. Dashed lines indicate cells that are predisposed to become resistant when they come in contact with the phage. The phage arrives in generation T ; only resistant, or predisposed, cells survive it. If mutations arise spontaneously, we observe resistant clones; if they are directed, the resistant cells are independent.

mutate back is of a similar magnitude as p , wherefore — given (A3) — the number of events is tiny indeed.)

In order to calculate $\mathbb{E}(Z)$ and $\mathbb{V}(Z)$, we will now proceed generation by generation. We will denote by $X(t)$ the number of *mutation events* that occur at the t 'th cell division (that is, the division that leads from generation $t-1$ to generation t , where the initial cell is generation 0). Let $Y_T(t)$ then be the number of *mutants* in generation T that go back to a *mutation event* in generation t . With a *mutation event*, we mean every transition $S \rightarrow R$, whereas a *mutant* is any R cell, whether it just originated by a 'primary' mutation event, or is an offspring of an already resistant cell.

Due to (A2), every mutation event that occurred in generation t will produce a resistant clone of size 2^{T-t} until generation T . Therefore,

$$Y_T(t) = 2^{T-t} X(t), \quad 1 \leq t \leq T. \quad (2)$$

For ease of notation, we will, in what follows, abbreviate $Y_T(t)$ by $Y(t)$; but keep in mind the dependence on T .

Finally, the number of resistant cells in generation T is

$$Z = \sum_{t=1}^T Y(t). \quad (3)$$

Let us now calculate expectation and variance for $X(t)$, $Y(t)$ and Z . Due to (A1), (A3), and (A4), we have $X(t) \sim B(n(t), p)$ and therefore

$$\begin{aligned} \mathbb{E}(X(t)) &= n(t)p, \\ \mathbb{V}(X(t)) &= n(t)p(1-p) \\ &= (1-p)\mathbb{E}(X(t)). \end{aligned} \quad (4)$$

In contrast to $X(t)$, $Y(t)$ is not binomially distributed (the members of a clone are not independent!). Actually, it has none of the standard distributions, but its expectation and variance follow directly from (2) and (4) (as well as $2^T = N$):

$$\begin{aligned} \mathbb{E}(Y(t)) &= 2^{T-t} \mathbb{E}(X(t)) = Np \\ \mathbb{V}(Y(t)) &= 2^{2(T-t)} \mathbb{V}(X(t)). \end{aligned} \quad (5)$$

These relationships are simple but illuminating. Eq. (5) shows that, on average, every generation eventually produces the same number of mutants: for small t , there are few mutation events, but those that do occur have large offspring, and vice versa, and these effects just compensate each other. Equation (5) contains an important hint towards the idea of

the Luria-Delbrück experiment: the variance of $Y(t)$ is increased by a factor of $2^{T-t}(1-p)$ (i.e., close to clone size) relative to the expectation.

Finally, (3) and (5) jointly give

$$\mathbb{E}(Z) = \sum_{t=1}^T \mathbb{E}(Y(t)) = TNp \quad (6)$$

and

$$\begin{aligned} \mathbb{V}(Z) &= \sum_{t=1}^T \mathbb{V}(Y(t)) \\ &= 2^T Np(1-p) \sum_{t=1}^T \left(\frac{1}{2}\right)^t \\ &= 2^T \left(1 - \frac{1}{2^T}\right) Np(1-p). \end{aligned} \quad (7)$$

In the first step of (7), we have used the independency of $Y(t)$ (one has dependence *within* clones, but none *between* clones — the latter being due to (A3).)

Comparing (1), (6) and (7) now yields the crucial difference between spontaneous and directed mutations, which we summarize as

Fact 1. *Under assumptions (Ao)–(A4), the ratio of variance to expectation is*

$$\frac{\mathbb{V}(Z)}{\mathbb{E}(Z)} = \begin{cases} 1 - \tilde{p} \approx 1 & \text{for directed mutations,} \\ (2^T - 1)(1-p)/T \gg 1 & \text{for spontaneous mutations.} \end{cases} \quad (8)$$

Experiment and control

Equation (8) suggests how to distinguish between (SM) and (DM). Grow a large number of parallel cultures (each from a single cell), add the phage, count the surviving cells, and compare the mean m_z to the empirical variance s_z^2 (as estimates of $\mathbb{E}(Z)$ and $\mathbb{V}(Z)$). What is still missing is the counting method. This is illustrated in Figure 3 and works as follows.

Every culture is transferred to a separate agar plate covered amply with a suspension of phages (filled arrowheads in Figure 3). The sensitive cells die, the (few) resistant ones continue to divide. Every single one of them forms a colony, which may be discerned with the unarmored eye. Proceeding this way with 50–100 parallel cultures and counting the resistant cells in each of them, Luria and Delbrück [1] obtained values for s_z^2/m_z in the range of 4 to 620, with a typical value of 225 as in the example in Figure 4. This figure shows the empirical distribution of R in one particular set of parallel cultures, and compares it with the corresponding distribution that would be expected under the direct-

ed mutation hypothesis. The observed histogram deviates from the expected one in a striking way, clearly demonstrating the 'jackpot effect' that results from the rare early mutations with their large progeny.

Although the results look very convincing, it must be mentioned that a factor as large as $(2^T - 1)(1-p)/T$ (as predicted by (8) for spontaneous mutations) is never observed in any such set of experiments. Indeed, it is not expected to be observed in any real experiment due to a sampling effect. By (5), the largest contribution to the variance comes from the very early generations; here, however, cells are so few, and mutation events so rare, that they are practically never observed in any given (*finite*) number of parallel experiments. To correct for this, Luria and Delbrück replaced T by \hat{T} , where \hat{T} is (essentially) the expected age of the oldest mutation, taken over the given number of parallel experiments. With this (heuristic) correction for sampling, the predicted factor is even smaller than the observed one in all but one set of experiments [1].

Clearly, therefore, the observations do point towards spontaneous rather than directed mutations. As we have seen, this has been decided on the basis of the underlying source(s) of randomness, for which there are two possibilities. The fluctuations caused by the fact that some cells become resistant while others don't is present in either case; it leads to a binomial distribution. The additional (and much larger) fluctuations caused by the variability of the *time* at which a mutation event occurs are only present if mutations are spontaneous. Since early mutations with very large progeny are rare, they cause the jackpot distribution with its abnormally high variance.

This latter insight occurred to Delbrück (the theoretician in the team) while watching a

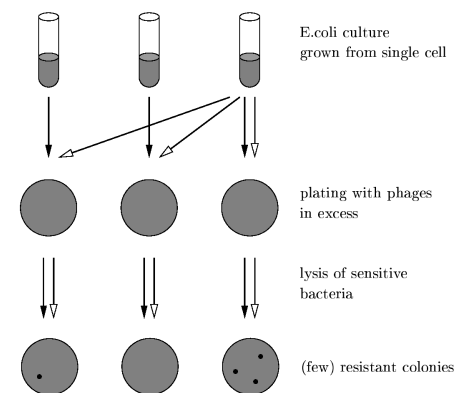


Figure 3 The Luria-Delbrück experiment (filled arrowheads) and its control experiment (hollow arrowheads)

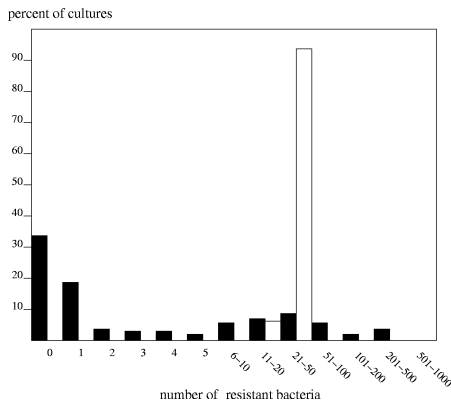


Figure 4 Histogram of the number of resistant bacteria, as observed in 87 parallel cultures (black bars; set of experiments no. 23 in [1]), and corresponding distribution expected under directed mutation (white bars). The latter is a binomial distribution determined by the observed total number of cells per culture, $N = 2.4 \times 10^8$, and the observed mean number of resistant cells, $N\bar{p} = 28.6$; this is indistinguishable from the Poisson distribution with mean 28.6, $P(28.6)$. Note the increasing class sizes on the horizontal axis. In this set of experiments, $s_z^2/m_z = 225$.

(maybe illegal) slot machine in a country club in Bloomington, which, in true slot machine manner, spits out a little money fairly frequently, whereas only very rarely, one receives a large return.

Luria (the experimentalist among the two) suspected something different. Performing a large number of experiments over an extended period of time, he worried about the large fluctuations of his cell counts from day to day, and first blamed the counting method (incubation on selective medium and colony formation) to be unreliable and bring about the fluctuations — until he performed the decisive *control* experiment, which we will now describe.

Here, a large number of plates (again with phage suspension) is inoculated with samples from *the same* bacterial culture (Figure 3, hollow arrowheads). If the counting method

works correctly, the number of bacteria per plate should now be distributed according to $B(m, \bar{p})$, if m is the number of bacteria per plate and \bar{p} the proportion of resistant cells in the particular culture used, so one would expect $s_z^2/m_z = 1 - \bar{p}$, i.e., a value very close to one. This is indeed what is observed: the average and empirical variance of Z now turn out approximately equal. This proves that the extra fluctuations observed in the experiment proper are inherent in the original cultures, rather than being an artifact of plating and counting.

Afterthoughts

The insight gained by Luria and Delbrück was the beginning of our understanding of mutations. Of course, the analysis may be (and has been) improved (statistically and otherwise), but the essence remains unchanged: Large fluctuations point to spontaneous mutations. It should be noted that Luria and Delbrück did not ‘only’ *answer* the question about the nature of mutations — in fact, they were the first to clearly *pose* it, formulate the alternatives, and put up the correct conceptual framework. Their original paper is illuminating to read because of the clarity of the argument.

Their historical experiment has lost nothing of its relevance until today. Known under the name of ‘fluctuation test’, it belongs to the standard repertoire of many genetics practicals, for the simple reason that it always works, in a foolproof way, independently of the selection pressure applied. In particular, resistance to antibiotics is readily bred by applying antibiotics — an ardent problem these days.

For Luria and Delbrück, their 1943 paper was only a start into further fundamental research in genetics of both bacteria and phages; to-

gether with A. Hershey, they received the Nobel Prize for physiology and medicine in 1969.

To avoid misunderstandings, let us briefly revisit the notions concerning the mutation mechanism. The attentive reader may have noticed that, following standard terminology, we have formulated the alternative mutation mechanisms as ‘spontaneous’ versus ‘directed’, in the sense of ‘arising independently of selection’ versus ‘arising as a response to selection’.

We have, so far, not considered the alternative that mutations may be *induced* by the environment, but in an undirected way. Of course, it is common knowledge today that UV radiation, mutagenic substances etc. can drastically increase an organism’s mutation rate; and these mutations are independent of whether they are advantageous or deleterious for the organism under the given circumstances. In the conceptual framework used here, they would simply be spontaneous mutations. Luckily, the T1 phages used by Luria and Delbrück were not mutagenic (phages are rarely mutagenic). If the phage had been (highly) mutagenic, the counts of R cells would have been dominated by the mutations induced by the phage at the moment of its appearance, which would have pointed to directed mutations. Indeed, as Luria and Delbrück correctly note, their experiment tells us *when* the mutations arise, but not *why*.

Acknowledgement

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References

- 1 S. Luria and M. Delbrück, ‘Mutations of bacteria from virus sensitivity to virus resistance’, *Genetics* **28** (1943), pp. 491–511.