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On a logical difficulty in the directed mutation debate

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Summary

This paper calls attention to an overlooked logical difficulty that has impeded the directed mutation debate for over half a century. It further suggests that the random mutation hypothesis be regarded at present as a null hypothesis in evolutionary biology.

A logical difficulty has been hampering the directed mutation debate for over half a century, and it has so far gained little recognition. The lack of a clear understanding of this logical difficulty is responsible for much of the confusion surrounding the directed mutation debate. The nature of this logical difficulty is most readily understood by examining a recent investigation into the mutation that confers on microalgea resistance to Rio Tinto water (RTW), which was too acidic for wild-type microalgea to survive. Adapting the classic Luria–Delbrück experimental protocol (Luria & Delbrück, 1943), Costas *et al.* (2007) isolated wild-type *Dictyosphaerium chlorelloides* cells from a pristine pond of non-acidic water and used the cells to seed culture flasks containing non-acidic water. After a period of incubation, the investigators added RTW to each of the flasks. The number of RTW-resistant cells in each flask was determined 25 days later. Because the investigators observed a large fluctuation in the number of RTW-resistant cells across the flasks, they argued as follows:

> The large fluctuation in the number of RTW-resistant cells observed … unequivocally demonstrates that [RTW]-resistant cells arose by rare spontaneous mutations and not through direct and specific adaptation in response to RTW. RTW did not stimulate the appearance of resistant cells at all … it seems very unlikely that adaptive mutations occurred in our cultures … The rapid lethal effect of RTW seems unlikely to allow the appearance of adaptive mutations.

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Two questions arise. First, if RTW is too lethal to allow cells to mutate, why is it necessary to perform the experiment? The mere existence of RTW-resistant cells in Rio Tinto would already have answered the purpose of the experiment. Second, what is the relevance of their observation that adaptive mutations did not occur in this particular experiment? Of direct interest is whether such mutations occur in nature.

To answer these questions, let us first articulate the defining feature of the adaptive mutation mentioned above. This kind of mutation has been variously called adaptive mutation or directed mutation. To avoid semantic difficulties encountered in the past, here we use these two terms to designate the adaptive mutation defined by Shapiro (1997). To be precise, in the present context, directed or adaptive mutations are 'mutational events that occur more frequently under selective conditions, when the resulting genetic changes are adaptively useful, than during normal growth'. Let us now place the two questions in historical perspective. In the context of Luria and Delbrück's original fluctuation experiments, the mutation in question confers resistance to T1 phage on *Escherichia coli* cells. Wild-type cells were first allowed to grow in test tubes containing liquid culture and then the contents of each tube were transferred (a procedure called plating) onto a solid culture coated with phage. Finally, the number of phage-resistant colonies formed on each solid culture was determined. The following hypotheses are basic:

A. All the observed mutants were due to mutations that occurred independently of the phage.

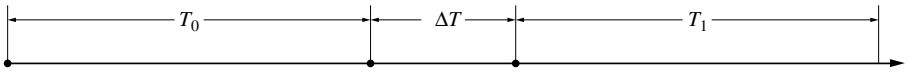

Fig. 1. Three conceptually distinct time periods in a typical fluctuation test.

A′. Some of the observed mutants were due to mutations that occurred independently of the phage.
B. All the observed mutants were due to mutations caused by the stimulation of the phage.
B′. Some of the observed mutants were due to mutations caused by stimulation of the phage.

However, these hypotheses are not testable in the laboratory. Instead, experimentalists considered the following surrogate hypotheses:

a. All the observed mutants came into being prior to plating.
a′. Some of the observed mutants came into being prior to plating.
b. All the observed mutants were due to mutations that occurred after plating.
b′. Some observed mutants were due to mutations that occurred after plating.

Because of the large fluctuation in the number of phage-resistant colonies across the solid cultures, Luria and Delbrück's fluctuation test successfully proved hypothesis a′ (which implies hypothesis A′), and which hence refuted hypothesis B. In the early 1940s, hypothesis B was still prevalent. Luria and Delbrück's experiment is thus a major milestone in understanding the origin of bacterial mutations. However, this accomplishment neither proves hypothesis a nor refutes hypothesis b′, leaving open the possibility for hypothesis B′. Because hypothesis a contradicts hypothesis B′, it is tempting to generalize hypothesis a′ to hypothesis a; but this has been attempted in a logically loose manner. Luria and Delbrück's original goal was to refute hypothesis B by proving hypothesis a′, but in his later years Luria appeared to believe that the fluctuation test established the validity of hypothesis a, not merely hypothesis a′ (Luria, 1984: p. 75).

> How could I try to decide which of the two mutually exclusive hypotheses was correct? What I need was some way of establishing whether or not the exceptional, phage-resistant bacteria were already there in a culture of sensitive bacteria before they came in contact with phage. If they were, then clearly it was not the phage that made them resistant.

The last statement of the above quote is the negation of hypothesis B′. While hypothesis A implies the negation of hypothesis B′, hypothesis A′ does not. Because the fluctuation test proved hypothesis A′, but not hypothesis A, this statement is stronger than the logic allows. In other words, the outcome of Luria and Delbrück's experiments does not unequivocally preclude the possibility that phage could further stimulate this type of mutation. Delbrück thought similarly 3 years after the celebrated fluctuation experiments. At that time, Lwoff (1946) reported experiments in which wild-type bacteria incapable of using succinate were exposed to an environment where succinate was the only source of carbon and energy. After a period of 3–20 days, mutants capable of using succinate began to appear. Intrigued by the non-lethal nature of selection in Lwoff's experiment, Delbrück (1946) was among the first to ponder an overlooked possibility.

> In the case of mutations of bacteria from phage-sensitivity to phage-resistance, the selective environmental factor consists in the addition of phage, which eliminates the wild type and permits the resistant mutant to multiply. In this case it could be shown that the phage does not cause the mutations.

There seems to be no evidence suggesting that Luria or Delbrück conducted additional experiments to show that the phage does not cause the mutations. With the information available today, this author conjectures that Delbrück's claim was based on the rapid lethality of the phage, to which the thinking of Costas *et al.* (2007) bears resemblance. Shapiro (1984, 1995) is among the first to call attention to this kind of limitation of the classic Luria–Delbrück experiment; he discourages thinking about the origin of mutants in terms of the issues current in the 1940s and 1950s. Similar views were voiced later by Cairns *et al.* (1988) and others. With the benefit of hindsight, we see clearly the difficulty in bridging the gap between hypothesis a′ and hypothesis a. However, even if hypothesis A were proved via hypothesis a, it still does not provide the last word. Of vital interest is the following hypothesis:

A*. All phage-resistant bacteria in nature resulted from mutations that occurred independently of stimulation of the phage.

Clearly, hypothesis A does not imply hypothesis A*, even though the generalization is tempting.

To provide a different perspective, let us conceptually divide a typical fluctuation test into three time periods, T_0, ΔT and T_1, respectively (see Fig. 1). In the T_0 period, wild-type cells divide and possibly mutate under non-selective conditions. In the ΔT period, cells may continue to divide and mutate, but wild-type cells eventually die if they fail to mutate in the ΔT period. The final period T_1 is for mutants to form visible colonies. Let $m[T_0]$ and $m[\Delta T]$ denote the numbers of mutations occurring in the T_0 and ΔT periods, respectively. Luria and Delbrück successfully

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demonstrated that $m[T_0]>0$, which implies hypothesis a′. Luria and Delbrück's assertion that $m[T_0]>0$ was based on the distributional pattern of the number of mutant colonies appearing on solid cultures. Whether $m[\Delta T]>0$ they did not address, although the possibility that $m[\Delta T]>0$ was integral to their 'second hypothesis' (Luria & Delbrück, 1943).

> There is a small finite probability for any bacterium to survive an attack by the virus. Survival of an infection confers immunity not only to the individual but also to its offspring.

The length of ΔT may be small, but the directed mutation must conceptually be allowed to occur in this time period. However lethal the selective agent might be, ΔT is not a zero quantity; and hence the probability in the above quote, i.e. the probability that $m[\Delta T]>0$, might not be zero either. The quantity $m[\Delta T]$ is not as amenable to existing experimental approaches as the quantity $m[T_0]$. Moreover, even if $m[\Delta T]=0$ is proved in a particular experiment, no definitive conclusions can be drawn regarding directed mutation in nature. If the probability is minute that a wild-type cell withstands a hit by phage by means of mutation, the experiment that nature performs could in theory still yield phage-resistant mutants due to the vastly larger number of wild-type cells involved. Luria & Delbrück (1943) were vaguely aware of the difficulty in interpreting the possibility $m[\Delta T]>0$ and hence were cautious in drawing conclusions from their experimental data.

> We consider the above results as proof that in our case the resistance to virus is due to a heritable change of the bacterial cell which occurs independently of the action of the virus.

This conclusion is implicitly predicated on the important assumption that $m[\Delta T]=0$ in the reported experiments. Luria and Delbrück thus essentially claimed the truth of hypothesis a, which leads to hypothesis A. Delbrück's comments in 1946 seem to reflect his uncertainty about the claim. With lethal selection as in the original fluctuation test, $m[\Delta T]=0$ is difficult to prove, but without this assumption, the experimental data can lead only to hypothesis a′, and hence the strongest conclusion that can logically be drawn from the experimental data is hypothesis A′. With non-lethal selection as in Lwoff's experiments, $m[\Delta T]>0$ can be experimentally established with relative ease, but mutations occurring during ΔT are not necessarily directed (Zheng, 2003). In other words, even if hypothesis b′ is proved, little can be said about hypothesis B′.

All experiments conducted to date to support the random mutation hypothesis were designed to prove assertions similar to hypothesis a′ (and hence hypothesis A′). No assertions similar to hypothesis A or hypothesis A* were strictly based on experimental evidence, as such assertions are beyond the capability of the Luria–Delbrück protocol. On the other hand, most experiments in support of the directed mutation hypothesis succeeded only in proving assertions similar to hypothesis b′, which is logically not the same as hypothesis B′. Despite the vast amount of data accumulated in the past 60 years or so, the random mutation hypothesis in the spirit of hypothesis A* still remains a null hypothesis in evolutionary biology. It was this null hypothesis that generated thought-provoking predictions and inspired further novel experiments. Nonetheless, the logical difficulty encountered in the past in proving hypothesis A* or hypothesis B′ suggests that a breakthrough in resolving the controversy will perhaps be achieved by approaches other than the fluctuation test.

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References

Cairns, J., Overbaugh, J. & Miller, S. (1988). The origin of mutants. *Nature* **335**, 142–145.

Costas, E., Flores-Moya, A., Perdigones, N., Maneiro, E., Blanco, J. L., Garciá, M. E. & López-Rodas, V. (2007). How eukaryotic algae can adapt to the Spain's Rio Tinto: a neo-Darwinian proposal for rapid adaptation to an extremely hostile ecosystem. *New Phytologist* **175**, 334–339.

Delbrück, M. (1946). Discussion of Lwoff's 1946 paper. *Cold Spring Harbor Symposia of Quantitative Biology* **11**, 154.

Luria, S. E. & Delbrück, M. (1943). Mutations of bacteria from virus sensitivity to virus resistance. *Genetics* **28**, 491–511.

Luria, S. E. (1984). *A Slot Machine, A Broken Test Tube: An Autobiography*. New York: Harper and Row.

Lwoff, A. (1946). Some problems connected with spontaneous biochemical mutations in bacteria. *Cold Spring Harbor Symposia of Quantitative Biology* **11**, 139–153.

Shapiro, J. A. (1984). Observations on the formation of clones containing *araB–lacZ* cistron fusions. *Molecular and General Genetics* **194**, 79–90.

Shapiro, J. A. (1995). Adaptive mutation: Who's really in the garden. *Science* **268**, 373–374.

Shapiro, J. A. (1997). Genome organization, natural genetic engineering and adaptive mutation. *Trends in Genetics* **13**, 98–104.

Zheng, Q. (2003). Mathematical issues arising from the directed mutation controversy. *Genetics* **164**, 373–379.