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REVIEW

ADAPTIVE STATE OF MAMMALIAN CELLS AND ITS NONSEPARABILITY SUGGESTIVE OF A QUANTUM SYSTEM

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A b s t r a c t

Established mammalian cells were assayed for their resistance to different selection conditions which had not been used against these cells before, including exposure to thioguanine, ethionine, high temperature and a protein-free, chemically defined culture medium. Single assays were negative, showing that the cell lines contained no spontaneous mutants, or that these were present in a number below detectable limits. To obtain such mutants, we designed experiments of mutant isolation by serial assays. The cells were kept growing without selection and, at each passage, cell samples were withdrawn and assayed for resistance in separate cultures. As a result, we found no mutants at the beginning, then a few and, finally, a great number. This was in conflict with the postulate of random occurrence of mutants and, furthermore, with their spontaneousness. On the contrary, the results provided evidence that mutants occurred as an appropriate response to selection pressure. The most amazing feature was that this response could be detected in cells growing without selection and never exposed to selection pressure before. If one tried to explain the adaptive response in terms of signals, the signals would have to travel from the exposed to the unexposed cultures. The results are instead discussed in terms of adaptive states and the nonseparability of cellular states due to quantum entanglement of cells, in particular daughter cells, distributed between the exposed and unexposed cultures. Whatever the mechanism, we concluded that the finding of resistant cells in growing unexposed cultures, as a response to selective pressure on cells in physically separated cultures, tends to render meaningless any theory based on the spontaneous origin of mutants.

K e y   w o r d s

mutation, drug resistance, adaptation, quantum state, quantum entanglement

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PRELIMINARY OBSERVATIONS

This story begins in the early eighties. In one of our study of retroviral integration, the question was raised as to whether the provirus can be amplified and, if so, translocated to different chromosomes. We designed experiments using Rous sarcoma virus (RSV) -transformed Chinese hamster cells and their somatic hybrids with mouse cells *(1).* This brought us to explant newborn Chinese hamster cells and establish fibroblastic cell lines. One of these lines, designated CH1, was then used to isolate mutants resistant to thioguanine (TG). The TG-resistance selection method employed was originally developed to quantify mutations at the hypoxanthine-guanine phosphoribosyl-transferase (HGPRT) locus *(2).* We assumed, based on the postulate of *Luria and Delbrück (3),* that such mutants arose at random in the absence of selection. Their frequency, expressed as mutants per clonable cell *(2),* would be about 1×10^{-6} to 5×10^{-6}, as determined in Chinese hamster ovary (CHO) cells *(4),* i. e., cells of the same animal species as CH1. Thus, by growing our CH1 cells to a density of 10^7 or more per culture, and applying selection, we could expect to obtain a detectable amount of TG-resistant (TG^r) colonies. The CH1 cells grew as an established cell line and displayed growth kinetics and plating efficiencies similar to those of CHO cells. At that time we did not think of taking into account that CH1 cells had not been exposed to TG before and, therefore, differed from CHO cells. To consider this difference would have challenged the hypothesis that mutants arise independently of selection pressure *(3),* which is generally taken for granted. Hence, we carried out a series of single assays. Samples (>10^7each) of wild-type (wt) CH1 cells were seeded into culture flasks with a TG-containing medium and further cultured. The wt cells died and, in spite of our expectation, no TG^r colonies appeared. This was odd. It seemed that either something was wrong with our experimental system, or that the mutants we were looking for arose at a much lower frequency than in CHO cells or did not arise at all.

At this point, we were not much interested in examining these possibilities. Instead we concentrated on the problem of how to isolate TG^r mutants under these conditions and, at the same time, how to avoid the usage of chemical mutagens. Finally, we settled on a pragmatic approach. We tentatively assumed that there was no intrinsic reason for CH1 cells not to mutate to TG resistance and we adopted an old laboratory technique known from hearsay, rather than from cell culture manuals, and often used by technicians to save time and equipment. Let us call it „a serial assay“. Briefly, cells in the logarithmic phase of growth are, at each passage, divided into two unequal samples and subcultured by seeding the large sample into a selection medium and by growing the small one in a parallel culture without selection. The cell monolayer in the selection medium, when depleted, is periodically replenished by the addition of fresh cells from the parallel culture. In accordance with this non-conventional selection protocol, we seeded CH1 cells into a TG-containing medium and periodically replenished the monolayer of dying cells with fresh cells. About three weeks later we could discern the first TG^r colonies. Cells from TG^r colonies were unable to grow in the hypoxanthine-aminopterin-thymidine (HAT) medium, as expected for $HGPRT^-$ cells *(2).* To further ascertain the genetic origin of TG resistance, we carried out Southern blot analyses, using a HGPRT cDNA probe and found that, in TG^r cells, the HGPRT gene was grossly rearranged (unpublished data).

In order not to omit a trivial explanation of the serial assay, we tested and eliminated the feeder effect of wt cells on both the colony-forming capacity and the growth rate of TG^r mutants. Furthermore, we compared the input numbers of cells and found that series of single assays, though consistently negative, were based on a much larger number of cells than was the total input number of cells for each serial assay. There is a deep difference between these two assays: a serial assay extends across several cell passages, while a series of single assays spans not more than one passage. However, this difference could not be taken into account unless considering the serial assay as a whole rather than a series of separate, although successive, single assays. Here we were at the edge of the neo-Darwinian framework since only the latter, reductionist, alternative could conform to the random origin of mutants *(3).* If refuted in experiments, we would have to do with the former alternative, the wholness of serial assays, and any explanation of this phenomenon would disagree with neo-Darwinian doctrine.

STEPPING BEYOND INTUITIVE WISDOM

We could not go farther without taking into account also counter-intuitive possibilities. In particular, we asked whether the classical concept of cell identity sufficed to explain what we had observed in our experiments. We did not believe that, in our serial assays, the cells of the first sample (assayed for resistance) were the same in all properties as the cells of each one of the subsequent samples that had been added to replenish the monolayer of dying cells. This led us to the hypothesis that in the time span of a serial assay, and because of the repeated exposure of cells to TG, the whole cell population, i. e., including the subsets growing without selection, evolved towards TG resistance. This hypothesis, though counter-intuitive and in conflict with Mendelian genetics, could be directly tested. To measure the occurrence of mutants in a growing cell line, the serial assay was redesigned as follows. A cell line was grown from small inocula in a culture medium containing no selection agent. At each passage, one or more cell samples were withdrawn and seeded into separate flasks (instead on a depleted monolayer) containing the selection medium. In these flasks, because of selection pressure, the wt cells died while the drug-resistant cells gave rise to visible colonies. Mutation frequency at each passage level of the cell line was then calculated from the total number of colonies divided by the number of cells assayed for resistance and corrected for cloning efficiency.

We have noticed that, for a given cell line, the more one selects for resistance to a given substance, the more mutants one finds. For instance, the CH1 cell line, though free of TG^r mutants in the very first experiment (reported above), evolved towards TG resistance at later passage levels so that TG^r mutants could be scored in single assays *(5)*. Hence, there was no necessity to use the protocol of serial assay for their isolation. As we were interested in determining how many mutants were „spontaneous“ (arisen in the absence of selection), in further experiments we replaced TG with a selection agent not yet used in mutant selection experiments of any kind.

NON-RANDOM ACQUISITION OF ETHIONINE RESISTANCE

Ethionine *(6)* is an artificial analogue of methionine, an essential amino acid. It has not been found in animal cells and has only been used on rare occasions in studies involving mammalian cells *in vivo* or *in vitro*. The biochemical basis for ethionine effects on tissues and its action as a carcinogenic agent have been reviewed *(7, 8)*.

We chose ethionine because of its high toxicity; until then no ethionine resistant (Eth^r) mammalian cells had been isolated. Again, we avoided the use of chemical mutagens. The mutants we were looking for would arise either spontaneously or as a specific response to selection pressure. Since the time of *Luria and Delbrück (3),* spontaneous mutations (arisen in the absence of selection) have been considered to occur at random. On this basis, if some or all of our mutations to Eth resistance were found to be, in fact, non-random, they would be best described by the term „adaptive“.

Experiments to isolate Eth^r mutants were carried out by using normal Chinese hamster cells, line CH1, and their RSV-transformed derivative, clone CHR1-3 *(6)*. In the case of CH1 cells, serial assays for Eth resistance were carried out at passage levels 179 through 241, i. e., over 31 weeks. However, in spite of a large cumulative number of cells being exposed to ethionine, no single colony of Eth^r cells could be found. This only showed that mutation to ethionine resistance, if it existed, would be a very rare event. There was still a small hope that the adaptive process was slow in cells whose genetic stability was as high as that of normal CH1 cells. Therefore, to accelerate this process in further experiments, we used cells with a transformed phenotype, the CHR1–3 cells, which were supposed to be genetically unstable. These cells were assayed for Eth resistance in two separate and overlapping experiments based on serial Eth resistance assays at passage levels 304 through 384. The results were more convincing than expected. The clone contained no Eth^r cells from the start through 24 passages. The subsequent passages were characterised by a sudden appearance of mutants. These were more frequent from passage to passage. They disappeared when the seeding of cells into parallel ethionine-containing cultures was omitted and reappeared when it was resumed. Thus, in both experiments, Eth^r mutants occurred in cultures growing without selection and arose, in these growing cultures, in response to an ethionine attack on cells in parallel, physically separated cultures.

To test the stability of the Eth^r phenotype, we collected cells from Eth^r colonies, generated clonal lines, and split one into sublines further grown both in the presence and absence of Eth. Three months later, the subline growing without selection was returned into the Eth-containing medium and both sublines were tested for growth kinetics and plating efficiency. In these tests, no sign of reversion of the Eth^r phenotype could be detected. The Eth^r phenotype could therefore be considered to be a fairly stable cell property. Genetic analysis of Eth^r cells was not carried out.

We could now draw the following conclusions: (i) The cells are not *a priori* able to generate a drug-resistant phenotype, in this case resistance to ethionine. (ii) Such a capacity is developed in a cell population when exposed to the drug. Typically, drug attacks are made upon the subsets rather than upon the whole cell population. (iii) Most surprisingly, the cells exposed to the drug shared information for the adaptive changes with the unexposed cells in physically separated cultures. As a consequence, these so far unexposed cells, unlike wt cells, survived when exposed to the drug.

Additional remarks refer to the protocol of mutant isolation by serial assays. The assays are, in fact, subcultures of the cell line growing without selection. Usually, at each passage, the number of assayed cells is much larger than the size of growth inoculum. Under these conditions, we can expect that the pairs of daughter cells arising from mitoses before passage will be found either nonsplit in the assay sample and, less frequently, in the inoculum, or split between the assay sample and the inoculum. From each split pair, one cell will then grow exposed to the drug, while the other will grow without selection. We think that the relationship between exposed and unexposed cells is best described as „closely related, other than descendants“. On this occasion, we may quote from *Luria and Delbrück (3):* „If we find that a bacterium survives an attack, we cannot from this information infer that close relatives of it other than descendants, are likely to survive the attack.“ Obviously, in light of our results with ethionine, this postulate is no longer tenable.

We may add some more general considerations. Let us assume that the protocol of mutant isolation by serial assays is working even on a much larger scale. Then, the object, herein named the cell, is any living being and the protocol would take the form of adaptation of objects to environmental pressure. However, to continue let us stay with cells rather than with objects. We may assume that adaptation starts when wt cells are distributed in different, physically separated compartments. Some compartments are more compatible with cell survival than others because of drugs, abnormal temperature, lack of essential components or, generally speaking, harmful environmental agents. In some compartments, all cells die because no single cell is able to resist the harmful conditions unless it is adapted. Let us assume that there are one or more compartments which succeed in remaining free from harm. If a continuous flow of cells takes place from compartments with harmless conditions to those with harmfull conditions, fresh cells would systematically replace dying cells. After a certain lapse of time, also called the adaptation period, the cells growing free from harm will not be identical with the original wt cells and, unlike wt cells, will be able to resist exposure to harm. They will not die when exposed to harmful conditions and will grow as adapted cells. To recapitulate: at the heart of the adaptation process, there are adaptive changes which occur both in cells exposed to harmful conditions and in their close relatives growing unexposed. These adaptive changes are cumulative, leading to an adaptive state which is, let us say, measured when the cells are exposed to harm. If the adaptive state succeeds in ensuring cell survival, the resulting phenotype will be genetically fixed. The other possibility is that, in agreement with the central dogma, the adaptive state is generated first in the genotype and then in the phenotype. However, in the context of adaptive mutations, such a possibility seems rather unlikely. We have previously reported a case of induced phenotypic changes which had occurred before an appropriate rearrangement of the genotype *(9).*

ACQUISITION OF THERMORESISTANCE

To extend our adaptation experiments we left the field of drug-resistant mutants and looked for more complex systems. One of these systems is resistance to high temperature. We assumed that the establishment of cells at high temperature required alterations of many cellular proteins. This implied that multiple genetic changes were required for the cell to overcome the temperature barrier. At the time of our experiments, no thermoresistant mammalian cells were known. What had been known was the development of transient heat resistance (thermotolerance) and the selection/induction of stable resistance to heat challenge *(10–12).* Only more recently, one cell line was found to be able to proliferate at 41.1°C *(13).*

Our experiments *(6)* were designed essentially according to the cell adaptation protocol described above. CH1 cells were grown at 37°C and, at each passage, a cell sample was withdrawn and assayed for growth at 40.6°C. Cells in the first sample died within 3 days, in the second they survived a profound crisis and gave rise to 11 colonies, and in the third they became established after a barely noticeable crisis. These cells then grew continuously as a cell line at 40.6°C. To proceed towards higher temperatures, this cell line was kept growing at 40.6°C and the cell samples withdrawn at each passage were assayed for growth at 41.3°C. The expected colonies of temperature resistant cells were not found in the span of 31 passages. Subsequently, they occurred, first in small numbers, then more numerously and, finally, in great numbers so that they outgrew into semiconfluent monolayers. These monolayers could be cultured indefinitely as established cell lines growing at 41.3°C. In further adaptation experiments, we successfully established RSV-transformed CHR1–3 cells at a temperature as high as 42.0°C, but not exceeding 42°C, at least not in the time span of our experiments. Hence, unless found otherwise, 42°C may be the highest temperature at which mammalian cells can ever grow.

ADAPTATION OF MAMMALIAN CELLS TO GROWTH IN CHEMICALLY DEFINED MEDIA

Encouraged by these results, and ignoring our intuition, we considered the protocol of cell adaptation as a tool for creating new cell phenotypes with properties *ad libitum*. We thought that the protocol, if valid, should enable us to overcome the barriers so far considered unbreakable. This could actually be done as shown in our next experiments.

We intended to develop a cell phenotype that would render the cells independent of exogenously provided regulatory factors of any kind. We assumed that cell mutants expressing such a phenotype would proliferate in culture medium in the absence of serum and serum-replacement factors. Also, from the practical point of view, they would grow free of contaminants such as PPLO (pleuropneumomialike organism), viruses and prions which otherwise came from serum or various macromolcular supplements to the culture medium. This point is of great importance in relation to biotechnology; contaminants, if present, may co-purify into the final product. Unfortunately, this has already happened in the following two cases: human immunodeficiency virus (HIV) was present in serum Factor VIII concentrates for haemophiliacs and prions were found in growth hormone preparations from human pituitary glands. At present, there is a large choice of commercially available serum-free media. However, each of these still contains serum-replacement factors and additional macromolecular supplements, including hypophyseal extracts. In experiments below we show that all these supplements are superfluous when cells become adapted for growing in a protein-free, chemically defined medium.

The adaptation experiments presented here started with a temperature-resistant subline of Chinese hamster fibroblasts, clone CHR1–3. This original clonal line was believed to be unstable, because of its capacity to grow as an established line at 42°C and, at this temperature, segregate flat and round cell variants. These were subcloned and three subclones of each morphology (2 x 10^6 cells each) were seeded into serum-free cultures *(14)*. The cells survived as confluent monolayers, and then as sparse foci of proliferating cells surviving for several months. Cultures containing a great number of foci could be passaged. Eventually, only one subclone was maintained because of its flat morphology, which allowed firm attachment to the support and did not affect the v-src mitogenicity. This subclone then grew for a number of years in a chemically defined medium. We further showed that the serum-free phenotype was a stable cell property *(14),* independent of autocrine stimulation or, in CHR1–3 cells, mitogenic activity of v-src, the transforming gene of RSV *(15).*

Further experiments were carried out on a much larger scale. For instance we seeded five subclones of normal Chinese hamster cells CH1 into a serum-free medium and showed that, after the crisis period, each of them became established as a permanent line that grew attached to polystyrene and had no need for growth and attachment factors *(15).* Similarly, we established serum-free cultures of murine NIH3T3 cells *(14, 16),* human HeLa cells *(16)* and human HaCaT keratinocytes *(17).* The adaptation protocol for these different cell lines included preselection in a low-serum-containing medium and selection in a serum-free medium lasting up to 48 days *(16).* The ratio of isolates to trials varied from 1:3 to 10:10 *(16),* thus showing that adaptation of certain cell lines required several trials. However, it never failed completely. It also happened that certain cell lines needed to undergo an additional adaptation step in order to become attached to bare polystyrene in the absence of any attachment factor *(14, 16, 17).* When adapted, these cells adhered to a support better than wt cells, and could not be detached unless we used a modified trypsinisation procedure *(18).* The composition of our protein-free, chemically defined media has been described elsewhere(18). The cells capable of growing in these media are referred to as „autotrophs“ *(15)* as distinct from wt „heterotrophs“ which require macromolecular substances, such as proteins from specialised cells, for their growth.

CONCLUDING REMARKS

The postulate that genetic information flows solely from parents to descendants is central to Mendelian genetics. *Luria and Delbrück (3)* stated, in agreement with Mendelian genetics, that if a cell survived an attack, we cannot infer from this information that its close relatives, other than descendants, were likely to survive such an attack. We have shown that this is no longer tenable and should be restated as follows: Cells are more likely to survive an attack if their close relatives have already been exposed to such an attack. Our statement, even when assuming nonseparability of cellular states, does not contradict the mechanistic basis of molecular genetics. Rather, it goes beyond its orthodoxy by showing that there is an additional flow of information, not mediated by DNA, which may be referred to as adaptive information (i. e., in the absence of a more general term). We have shown for the first time the non-mechanistic nature of adaptive information which makes it distinguishable from genetic information. The fundamental unit of adaptive information is postulated as an abstract (quantum) state of molecule(s), whereas the fundamental unit of genetic information is a concrete chemical molecule, the DNA nucleotide. We may say that the flow of adaptive information is rather weak, as compared with the flow of genetic information. However, in spite of its (supposed) weakness, this flow of adaptive information could be detected in our experiments. Its most intriguing feature is that it travels between physically separated cells.

In his new biological theory, *Elsasser (19)* uses the term „formal science“ for a description that reduces phenomena to special applications of a fundamental set of equations or to a description by other forms of purely abstract logico-mathematical symbolism. He asks: „How much of biology can be converted into formal science?“. He then shows that, for living organisms, we have just one set of formal laws, namely, the laws of quantum mechanics. Returning to the problem of adaptation, it may be taken for granted that the fundamental unit of adaptive information, unlike genetic information, is not a concrete chemical molecule. It seems, therefore, that the problem is to be treated as one of formal science, or brought as close as possible to formal science. This, however, is far beyond the scope of our work.

To provide a clue: the pairs of daughter cells in our experiments behaved as if they were entangled quantum systems. The quantum states of entangled systems violate state separability. We demonstrated nonseparability of (entangled) cellular states by measuring resistance to a harmful agent. Let us assume that, on its own, each cell occurred randomly as either resistant or nonresistant. When we measured its resistance we switched random cellular processes to a non-random resistant state. Because of the nonseparability of cellular states, the entangled partner, i. e., the daughter cell of the same pair growing in a separate culture without selection, instantly switched to the resistant state and became more likely to survive when, in turn, it was exposed to a harmful agent. A magic feature of quantum entanglement is that it works across a distance and, if explained in terms of traveling signals, is faster than the speed of light. Naturally, the quantum rules make this impossible. In recent experiments with photons, a team of physicists have discovered that entanglement can be used to circumvent the limitations imposed by Heisenberg's uncertainty principle, without violating it, and therefore opened a way to teleportation *(20).*

We do not want to enter into a lengthy discussion of our experiments in terms of neo-Darwinism and Lamarckism. As put by *Symonds (21),* the central idea of neo-Darwinism – that organisms evolve by a combination of random genetic change and natural selection – has become tantamount to a dogma. Therefore, experiments not in agreement with the dogma provoke endless discussions about their faithfulness and possible flaws. Also the authors of such experiments are accused of heresy and may be suspected of Lamarckism. In 1988 *Cairns et al. (22)* published an article about adaptive mutations in bacteria. These authors partly repeated and extended already published studies *(23–26)* and concluded that „cells may have mechanisms for choosing which mutations will occur“. Their idea of adaptive mutations was later supported by even more surprising findings in different bacterial systems *(27–30).* However, in all these studies, the obvious question of what mechanism might be at the basis of the adaptive process remained unanswered. Perhaps for this reason, and also in defence of neo-Darwinian dogma, the publication of Cairns' article opened an extensive and rather controversial debate *(31–33).* The critics offered alternative interpretations of the data *(31, 34, 35)* as well as different models of molecular mechanisms *(21, 36–39)* which, in their opinion, could accommodate non-random processes within the neo-Darwinian orthodoxy. The reason why some of Cairns' mutations could not be reproduced *(32, 35, 40, 41),* a topic also raised in the debate, has not yet been elucidated.

In my opinion, the authors studying adaptive mutations in bacteria missed the point that, with all probability, these mutations could also be found in parallel, physically separated cultures. Therefore, they could not propose but mechanistic interprations that, of course, could not explain the phenomenon. Our advantage was that we were not burdened with the intent to find a molecular explanation. We realised from the beginning that our experiments were beyond the scope of classical molecular biology and, furthermore, that any experiment aiming at energetic information transfer between separate cultures would be meaningless. Instead, we focused our attention on a description of the phenomenon and its reproducibility. We provided evidence that what we observed were adaptive mutants and we showed that this kind of mutants could be obtained in very different experimental systems. No doubt these results and, in particular, the quantum entanglement of cells in separate cultures, too unusual and unexpected, will require further discussions and experiments to understand how the adaptive process functions. Formal studies of biological objects will be most rewarding.

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Hill M.

ADAPTAČNÍ STAV SAVČÍCH BUNĚK A JEHO NESEPAROVATELNOST PŘIPOMÍNAJÍCÍ KVANTOVÝ SYSTÉM

S o u h r n

Permanentní linie savčích buněk byly testovány na rezistenci vůči selekčním podmínkám, se kterými se nikdy předtím nesetkaly. Jednalo se o thioguanin, ethionin, vysokou teplotu a chemicky definované kultivační medium neobsahující žádné proteiny. Testy rezistence, jež byly zpočátku prováděny na výsevech buněk z jedné pasáže, byly všechny negativní. Tyto testy prokázaly, že použité linie buněk neobsahovaly žádné spontánní mutanty, alespoň ne v detekovatelném množství. Z toho důvodu jsme vypracovali pro selekci rezistentních mutantů zvláštní seriový test rezistence. Spočívá v tom, že buňky jsou kultivovány bez selekčního tlaku, avšak při každé pasáži je zbylá část buněk vyseta odděleně a v této oddělené kultuře testována na rezistenci. V takto uspořádaném pokusu jsme v prvních pasážích nenašli žádné mutanty, v dalších se objevily v malém množství a pak s přibývajícími pasážemi se jejich počet dále zvětšoval. Tento nález byl v konfliktu s postulátem náhodného vzniku mutantů a dále s jejich spontánním původem. Naopak výsledky byly důkazem toho, že mutanti vznikají jako adekvátní odpověď buněk na selekční tlak. Nejzajímavější bylo, že tato odpověď byla nalezena právě u buněk, které dosud rostly ve fyzikálně separovaných lahvích, kde nebyly vystaveny žádnému selekčnímu tlaku. Jestliže bychom se pokusili vysvětlit adaptační odpověď těchto buněk pomocí signálů, pak by tyto signály musely proběhnout mezi kulturami, vystavenými selekčnímu tlaku a těmi, které rostly v mediu bez selekčního tlaku. Je proto lépe si představit, že šlo o adaptivní stavy a připustit jejich neseparovatelnost jako výsledek jejich kvantového spřažení (entaglement) a to zvláště v případě párů dceřiných buněk z jedné mitozy, jež byly při pasáži rozděleny do dvou kultur, z nichž jedna byla vystavena selekčnímu tlaku a druhá ne. Ze skutečnosti, že k adaptační odpovědi dochází nejen u buněk exponovaných ale i u těch, které rostou odděleně bez selekčního tlaku, můžeme uzavřít, že všechny teorie založené na spontánním vzniku mutantů jsou neopodstatněné.

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