SOME NEW CHALLENGES IN MUTAGENESIS

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Revision of the theory of the delayed action of alkylating agents, the wavy kinetics of mutagenesis on the strength of long-lived potential changes, enzymatic repair systems and replicating instability, cutting of the double-stranded DNA molecules by the chromosomal "vesting" (encircling) pattern and possibility of elimination of potential injuries have not only their own intrinsic interest but are of principal significance.

These findings reveal the fact that after advances which brought about the classical molecular theory, the modern theory of mutations is again faced with grave difficulties.

The new phenomena detected in recent studies are exceedingly difficult for interpretation. Among them are potential changes, the wavy kinetics of mutagenesis during the storage of cells after a single mutagenic treatment, replicating instabilities, etc. All the segenetic phenomena are found to be related to the work of intracellular enzymic systems and cell metabolism. It may be inferred that the phenomenon of mutation is not confined to physico-chemical interaction between the genic or chromosomal molecular structure and the energy of mutagen. The fate and peculiarities of initial changes, their transfer to a potential state and fixation as mutations all are utterly dependent on the cellular biology. Thus, the modern notions of mutagenesis are arrived at not just in terms of the classical molecular theory but at a molecular biological level. This latest stage is significant, for it offers now possibilities of control over one of the most essential biological phenomenon.

INTRODUCTION

The theory of mutations is an outstanding development of the last two decades (Watson and Crick 1953). As a result of speculative and operational research, the nature of the initial damage in the DNA molecules induced by radiation or chemicals or caused by the environmental factors has been understood. It has been acknowledged that either the damages themselves or new bases arising from errors in the synthesis turn into real mutations (Freese 1971).

Though the classical molecular theory of mutations is on the whole successful, to date the approach to the problem is becoming essentially different presenting a new stage in the development of the theory of mutations. For, the classical concept did not reckon with the complex machinery of interaction of initial lesions in the DNA molecule with the enzymic systems conferring a biological significance of these molecules, nor with the cell metabolism. The new stage presents endeavour at understanding the reasons, conditions and essentials of mutation initiation involving emergence of initial damage, and of arisal of mutations as a physical, chemical, biochemical and biological complex. In the literature, the new approach is sometimes stated by the phrase "Mutations as a cellular process" (ed. Wolstenhoms and O'Conor 1969).

New Challenges in the Mutation Problem

Several new lines of the current enquiries into the mutations suggest that the problem is being approached in a new way. I shall dwell upon the studies undertaken at the Institute of General Genetics, USSR Academy of Sciences.

It should be noted that a crucial issue as regards the mutation arisal could not be explained in terms of the classical theory. I mean the phenomenon of complete mutations, that is to say when on exposure of the resting DNA molecule to a mutagen, initial lesion first emerges in one strand and consequent to it complementary alteration occurs in the other polynucleotide strand.

Many mutagens would generally cause local injury of a single DNA strand. UV irradiation would largely induce pyrimidine dimers, ionising radiation—strand breakage and photochemical adenine reactions, chemical mutagens—deamination, alkylation, etc. From the viewpoint of the old molecular theory, such lesions will lead to changes in both DNA strands during the replicative synthesis, the injured strand serving as a template. It entails mosaicism in the progeny of the injured molecule. However, it has been shown by numerous studies of phages, bacteria and higher organisms that mutations may be induced by mutagen treatment without replication of DNA, while it has a double-stranded structure. In this case, all progeny of the altered molecule carry the mutation.

Then the problem popped up how the injury of a single strand in a resting DNA molecule can be transferred to the other strand? In 1966 this transfer was termed "resonance mutagenesis" (Dubinin 1966).

The discovery of the repair enzymes was an important landmark in the development of the mutation theory. The cell was shown to contain a set of enzymes repairing initial lesions of DNA. In 1949 it was found (Kelner 1949) that by splitting thymine dimers induced by UV-radiation, visible light caused photoreactivation of the DNA native structure. Great importance is attached to the "dark repair" (Setlow and Carrier 1964 ; Boyce and Howard-Flanders 1964) in which without action of visible light the injured region is excised from the DNA molecule by endo- and exsonucleases, the ensuing gap is built up in the course of non-replicative synthesis by the DNA-polymerase with the opposite region serving as a normal template and is finally linked by ligases. These findings cast a new light upon the question of heredity conservation. It was previously held that the stability of the genetic material resulted from its non-involvement in the metabolism. It is obvious to date that stable as they are, DNA molecules synthesized anew in every cell division and partly while at rest between the syntheses, are deeply involved in the metabolic processes. The very stability of the genetic material and its impairing both are the results of the metabolism. Protection of DNA molecules from damage is provided by the expedient co-function of repair enzymes.

In this regard, it was essential that several workers attempted to investigate the relationship between the work of repair enzymes and the mechanism by which initial lesions in the DNA molecule turn into mutations during the non-replicative synthesis and recombination events. Strains of microorganisms and mammalian cells mutant for genes of repair enzymes or for those of recombination displayed varied mutability. According to one hypothesis, the phenomenon of complete mutation with

cutting the double-stranded DNA molecule is treated as the basis for appearance of chromosomal structural mutations (Dubinin 1968; Dubinin and Soyfer 1969). There is no doubt some kind of relationship between repair systems and mutagenesis, the knowhow of which is to be acquired from further investigations.

There can be little doubt that the work of polymerase and other syntheses is connected with the mutational process since impairing the synthesis of DNA molecules brings about alterations in the genetic code (Speyer 1965; Drake and Greening 1970; Drake 1970; Bernstein *et al.* 1972).

The whole bulk of evidence regarding the role of enzymes in the mutational process explicitly indicates that the cellular processes perform a part of regulators of the events leading to the arisal of mutations.

New questions were posed by the discovery of the phenomenon of potential lesions (Swanson and Schwartz 1953; Kimball 1961; Dubinin 1961) and prolonged mutagenesis (Auerbach 1947; Dubinin and Saprykina 1964; Dubinin and Dubinina 1968). It was established that contrary to the earlier views, mutation was not a rapid singular response of DNA to mutagen but a complex many-staged process. Potential changes related to the initial damage on the DNA molecule can persist for minutes, hours or months ready to either turn into a mutation or be repaired to the normal structure. It is evident that the discovery of the stage of a potential lesion offers immense possibilities for intrusion upon the mutational process.

The study of potential lesions has permitted a distinction between three major types (Dubinin 1969 *a*, *b*), namely, short-lived changes realized within one particular phase of the cellular cycle; long-lived changes realized in subsequent phases of one and the same cellular cycle; extra-long-lived with many changes persisting through a number of DNA syntheses. The genetic and molecular nature of prolonged mutagenesis is not yet clear. There are two very intricate questions here. The first is how can ordinarily short-lived changes realized within a pre-synthetic phase turn into extra-long-lived ones persisting for months or years in seeds under the condition of storage? The second question concerns the yet unknown mechanism of persistence of potential changes through several DNA syntheses (replicating instability). In recent experiments with yeast (Dubinin *et al.* 1972) it was shown that after 270 cell generations DNA molecules were still carrying the mutagenic information obtained on exposure of a founder cell to UV-radiation. It was proved (Dubinin *et al.* 1971) that replicating instabilities passed over to the DNA molecules made up of newly synthesized strands. It suggests that the mutagenic information of replicating instability is registered in the genetic code, thereof permanent arisal of mutations in a number of cell generations.

The above problems were investigated at the Institute of General Genetics, USSR Academy of Sciences, Moscow. The major results are revision of the theory of the delayed action of alkylating agents, findings of the wavy kinetics of mutagenesis and new systems of natural protection, the molecular interpretation of the phenomenon of "chromosome vesting" (a ring chromosome encircling a rod-shaped one), penetration into the role of non-replicative systems in the mutation arisal and of repair enzymes in the emergence of replicating instabilities. Some of these will be discussed below.

Revision of the Theory of the Delayed Action of Alkylating Agents in the Light of the Concept of Potential Mutations

During the last decade, the theory of the delayed mutagenic action of alkylating agents on a cell played an important part in the general theory of mutations.

The phenomenon was discovered by Ford (1949) on horse bean rootlets treated with HN_2. He observed only rearrangements of a chromatid type no sooner than 8–10 hours after the mutagenic treatment. Kihlman (1961) proposed that the mutagens of the delayed action were effective only in the S-phase and held the disability of alkylating agents to induce mutations within G_1 and G_2 phases and this was their fundamental distinction from radiation (Kihlman 1966).

It looked as if there was specific relationship between mutagen effect and the replicative synthesis of the DNA molecules. As to the stage of the double-stranded structure of the DNA molecule, it did not respond to alkylating agents.

This performance of alkylating agents could hardly be explained in terms of the concept of the alkylating agent action on DNA molecules (Brookes *et al.* 1969) and in view of the evidence for an unscheduled non-replicative synthesis of DNA at a stage of the double-stranded DNA. This inconsistency led us to undertake cytogenetic studies of *Crepis capillaris* germ cells in which the nuclei are naturally synchronized in the pre-synthetic phase (Nemtseva 1965). In these studies, the mutagenic effect of the treatment of dry seeds was observed only on resting double-stranded DNA molecules (G_1).

The delayed effect is generally manifested by the treatment of proliferating tissues. In our experiments, the result of the treatment of *Crepis capillaris* dry seeds with ethylenimine solution was quite different. (Dubinina and Dubinin 1967). That is, not only chromatid but also chromosomal rearrangements occurred. The frequency of the chromosomal type rearrangements was demonstrative of the complex mutagen dose dependence which suggested the existence of the dose threshold. The mutation rate was 0.17 per cent at an EI concentration of 2.3×10^{-3} M, it increased 40 times being equal to 6.26 per cent at a dose 2.3×10^{-2} M. The frequency of mutations of a chromosomal type was shown to be dependent upon the time passed from administration of mutagen to mitosis (Dubinina 1969). For example, after treatment of seeds with a 2.3×10^{-2} M EI solution and fixation of the rootlets after 40–70 hr of growth, chromosomal type mutations amounted to 5.39 per cent, after 100–150 hr of growth to 12.21 per cent.

However, in the total rate of chromosome mutations induced by EI in G_1, chromatid rearrangements prevailed. Ethylenimine is known to be short-lived mutagen, its molecules could not last till S-phase initiated 10 hr after soaking of seeds. Lack of these molecules in the early hours after the mutagenic treatment of seeds was confirmed by special tests with washing of *Crepis* rootlet cells. (Dubinina and Chernikova 1972). Two principally important inferences were drawn from the evidence obtained in these studies. Firstly, under certain conditions and doses, alkylating agents are capable of causing complementary changes in the double-stranded DNA molecule, besides acting on a synthetic phase, as proposed by the theory of the delayed action. The former action is expressed in the arisal of complete mutations manifested by rearrangements of a chromosomal type.

The occurrence, under the same condition, of chromatid rearrangements realized in the pre-synthetic phase, i.e. when there are no molecules of mutagen left in the cell enabled the second inference to be arrived at, namely, potential lesions persist to the synthetic phase at which they turn into chromatid rearrangements.

It is expedient to mention here that as proved by the experiments on the storage of seeds treated with mutagen, complete mutations are initially presented by potential changes in the DNA molecule turning into mutations only in the germinating seeds.

Wavy Kinetics of Mutagenesis as Based on the Long-Lived Potential Changes

The assay of the mutation rate in the cells of seeds treated with mutagen and stored dry for a different time up to germination is a handsome model for the enquiries into the life-time of potential changes. Such an assay was carried out with *Crepis capillaris* seeds treated with ethylenimine solution, dried, stored in desiccators over granular KOH and germinated at 24 different terms after 3-133 day storage (Dubinin and Dubinina 1968*a*, *b*).

For all different storage periods the rootlet meristematic cells displayed structural mutations of both chromosomal and chromatid types. The possibility of persistence of the mutagen molecules under the described condition is excluded, hence mutations occurring at all storage periods arose from potential changes. Mutations were not accumulated during the storage but emerged in germinating seeds, which is supported by the constant chromosome/chromatid mutation ratio for all storage terms. For example, this ratio was 1.2 after treatment with a 9.3×10^{-3} EI solution, 1.1 on the 9th day, 1.5 on the 15th day, etc. It was 1.2 on the 45th, 1.1 on the 62nd, 1.3 on the 97th, and 1.5 on the 126th days. If mutations went on arising during the storage, the amount of chromosomal mutations must have been expected to grow with time.

The wavy kinetics of the mutation frequencies discovered in these studies came as a surprise. In the seeds treated with mutagen the level of mutations showed wavy fluctuations, rising or falling at different terms of storage. The wavy kinetics of the mutation frequencies was concentration specific but held true for all. The experiments on the 554-day storage of seeds treated with EI revealed the same wavy kinetics of the mutation frequencies after this prolonged storage.

The nature of the phenomenon remains unexplored, yet it is obvious that the wavy fluctuations in the mutagenesis are related to some changes in the metabolism affecting the ratio of repair to mutation fixing processes. The phenomenon itself was reported by several workers. The wavy kinetics during the storage of barley seeds was observed by Garina and Romanova (1970) and Garina and Korytova (1972) of *Crepis* seeds—by Korytova *et al.* (1971) and of maize by Korobko and Krasnobaev (1972).

Garina reported parallelism between the wavy kinetics of the frequencies of chromosomal structural changes and that of the chlorophyll mutation frequencies in barley. Her study also showed that prolonged storage created different possibilities of arisal for structural and chlorophyll mutations.

The dependence of the wavy kinetics upon the intracellular condition was proved by the examination of a temperature effect on the mutation rate during the storage of seeds treated with EI. Administration of a EI solution at a concentration of

2.3×10^{-2} M without storage induced 37.8 per cent structural mutations, of which 5.1 per cent were chromosomal mutations. After a 3-day storage at room temperature (22—25°C the seeds entirely lost germination capacity. The research for a year confirmed that the germination capacity was lost irreversibly. At the same time, the toxic effect was removed if the seeds were stored at about 5°C. The seeds germinated, and the wavy kinetics of mutagenesis at various periods was explicitly expressed.

Of much interest is the finding (Korytova *et al.* 1971) that chromosomal injuries caused by irradiation in G_1 were partly removed by a subsequent ethylenimine treatment while the rest contributed to the wavy kinetics of mutations characteristic of ethylenimine.

Enzymatic Repair Systems and Replicating Instability

The phenomenon of replicating instability is of principal significance for further development of the mutation theory. Replicating instability means that after a single mutagenic treatment the DNA molecule receives some mutagenic information persisting throughout a number of DNA syntheses. Both genic (Auerbach 1947; Loprieno *et al.* 1968; Mathew 1964) and chromosomal (Buiatti and Ronchi 1964; Dubinin and Saprykina 1964; Evans and Scott 1964; Dubinin and Tarasov 1968) mutations were found in dozens and even hundreds of successive cell generations.

It is essential that strains of yeast *Sch. pombe* manifesting both replicating instability and deficiency in dark repair genes yield more mosaic colonies than normal strains do (Nasim 1969; Dubinin *et al.* 1972*a*). It suggests involvement of the enzyme of dark repair in the arisal of complete mutations.

Our study of yeast cells detected that instability induced by a single treatment of a founder generation replicated in all 270 examined cell generations, the mutation frequency going up.

Of interest is the evidence obtained from the study of the caffeine effect on cells with replicating instability. Caffeine is known to suppress dark repair processes. Yeasts have two dark repair systems with or without response to caffeine(Falre 1971; Loprieno and Schphbach, 1971). In our experiments, a sharp fall in the mutability after administration of caffeine was observed in all the generations with realized replicating instability, replication of the instability as such remained unaffected.

This piece of evidence also suggests that realization into mutation of such a complex thing as replicating instability is related to the work of repair systems.

Cutting of Double-Stranded DNA Molecules in the Chromosome "Vesting" (Encircling) Pattern

Examination of a peculiar pattern of chromosomal aberrations made up of a ring chromosome encircling a common rod-shaped one brought us nearer to the understanding of the mechanism of this rearrangement (Dubinin and Nemtseva, 1969, 1970). There is a possibility that such a pattern arises after irradiation on the strength of the formation of a rigid loop by means of intramolecular cross-linkages. In their crossing point the double-stranded DNA molecule is cut into fragments by repair enzymes. As a result of their joining, the ring can surround a rod-shaped

chromosome. At the point of cutting after the DNA synthesis the inner strands may slip out of the encircling ring yielding 12 major configurations of the encircling pattern.

Enquiries into the dose response revealed that exposure to a double dose of X-irradiation brought about a 30-fold enhancement in the amount of the encircling rings. The implication is that the high radiation dose affects the work of the repair system (Dubinin *et al.* 1971*a*).

The study of a transfer of the encircling pattern from diploid to tetraploid *Crepis* cells at C-mitosis detected some new configurations. It is a proof of the cutting of the DNA double-stranded molecules in the somatic crossing-over. The inner chromatids of rod-shaped chromosomes slip, in various combinations, out of the surrounding ring through the gaps formed (Dubinin *et al.* 1972*b*). These molecules are cut by endonucleases. Polymerases and lygases are involved in the joining of the fragments.

New Evidence for the Removal of the Mutagenic Effect of Alkylating Agents

Dependence of the mutagenic effect of alkylating agents upon the intracellular condition is explicitly expressed in the possibility of its removal with a change of the condition.

In *Crepis capillaris*, initial injuries giving rise to potential changes appear as a result of ethylenimine treatment. The evidence for the wavy kinetics of mutagenesis during storage of the seeds treated with mutagen indicate a varying probability of mutation fixation for different storage terms. Moreover, under certain conditions the mutagenic effect of ethylenimine may almost entirely be removed. It was shown (Dubinin *et al.* 1972*a*) that subject to the protracted synthetic phase the injured chromosome could almost recover. In these studies, 5-aminouracil was used as an inhibitor of the phase.

That potential changes do arise with protraction of the S-phase and that generally losing their ability to turn into mutations they pass over to G_2 is warranted by the following observation. When cells were repeatedly treated with amino uracil in G_2 after protraction of the S-phase, mutations occurred in them at a higher rate.

The evidence for a natural cellular system of protection against alkylating agents bears on the understanding of the mutations as a cellular process. It was shown with *Crepis capillaris* (Shevchenko *et al.* 1971) that upon administration of ethylenimine to ground seed coats drastic sensitization of its mutagenic properties was observed. Yet, upon addition to homogenate prepared from cells of seedlings released from the coat, ethylenimine entirely lost its mutagenic properties. When introduced into the homogenate made up of seed coat, ethylenimine caused 41.13 per cent mutations, in a combination with the homogenate consisting of ground seedlings, it caused 0.55 per cent mutation (Dubinina and Dubinin 1972). It suggests that cellular protoplasm of ground seedlings contains certain substances which immediately neutralize many molecules of the mutagen or eliminate many initial injuries in the DNA molecules. The tests for the action of ethylenimine in combination with the homogenates made up of seedling cells and seed coats were of a particular interest. The mixture must

presumably contain activated protective substances and activated mutagenesis sensitizing substances. Only 1.05 per cent mutations were manifest in this condition, that is to say, the above combination induced very few, if any, mutations. Therefore, the tremendous potential of sensitized mutagenicity arising in the combination of ethylenimine and ground seed coats was neutralized by the reaction with the activated protective substances in the ground seedling cells.

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