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INDUCTION OF r- AND h^c-MUTATIONS DURING THE TREATMENT
OF EXTRACELLULAR T-2 BACTERIOPHAGE WITH HYDROXYLAMINE AND O-METHYL-
HYDROXYLAMINE

Following is the translation of an article by V. F. Chubukov and S. G. Tatarinova, Institute of Epidemiology and Microbiology imeni Gamaleya, AMN USSR, published in the Russian-language periodical Zhurnal Mikrobiologii, Epidemiologii i Immunobiologii (Journal of Microbiology, Epidemiology, and Immunobiology), 42: 80-83, No 4, 1965. It was submitted on 3 Jun 1964.]

In bacterial viruses mutations in the majority of cases are connected with small changes in the sequence of nitrogen bases in specific sectors of DNA (Freese, 1962). For obtaining such mutations various chemical compounds are used which cause changes in nucleic acid, but with a small exception the spectrum of their action is extremely wide, i.e., is connected not with a change of any one specific base, but several, which complicates a detailed understanding of the mutation process.

Almost simultaneously with the report of the mutagenic activity of one of the carbonyl compounds - hydroxylamine - works appeared in which the chemical specificity of this compound was analyzed. It was shown (Franklin and Wecker, 1959, and others) that treatment of nucleic acid with hydroxylamine is not accompanied by a decrease of molecular weight. From here the assumption was made that biological effects - inactivation and induction of mutations in various viruses - are the result of a specific interaction of hydroxylamine with nitrogen bases. In model experiments for investigating the products of the reaction of hydroxylamine with RNA and DNA from various biological sources, and also from processed native viruses (i.e., in reactions in vivo), it was noted that the reacting components of nucleic acids were only the pyrimidine bases - uracil and cytosine in RNA and cytosine or (in the case of T-even numbered phages) its derivative hydroxymethylcytosine in DNA (Brown and Schell, 1961; Freese and associates, 1961; Schuster, 1961; Verwoerd and associates, 1961; Kochetkov and associates, 1962; Morozova and Salganik, 1964).

Thus the induction of mutations during treatment of T-2 bacteriophage with hydroxylamine should be conditioned by a change of any one of the four bases of DNA, and namely - hydroxymethylcytosine.

The reaction of hydroxylamine with cytosine and certain of its derivatives is accomplished in several phases with the formation of a number of intermediate reaction products.

The number of r-mutants in T-4 and T-2 phages increased linearly with an increase in the duration of treatment with hydroxylamine (Freese and associates, 1961a; Schuster and Vielmetter, 1961; Goldfarb, 1963). Consequently induction represents a single-stroke process, probably connected with a change of a very small number (possible even one) of hydroxymethylcytosine groups, leading to minute mutations caused by substitution of the primordial pairs of bases guanine - hydroxymethylcytosine (G - HMC) by the pairs adenine - thymine (G - HMC \rightarrow A - T). The minute nature of mutation during treatment with hydroxylamine was then confirmed by various genetic tests (Freese and associates, 1961; Champe and Benzer, 1962).

The development of a minute mutation can possibly be explained by the fact that each of the intermediate products of the reaction can be found in a tautomeric form, most closely calling to mind thymine in its capacity to be combined in a pair with adenine in place of guanine. The formation of some pair at the mutating point is always carried out only through several replications of treated nucleic acid (Freese, 1964).

Since the process of mutagenesis under the action of hydroxylamine is connected with the formation of several intermediate products of the reaction of hydroxylamine with hydroxymethylcytosine, it is possible to attempt by various methods to disrupt the course of this process and, consequently, change the mutagenic activity of hydroxylamine.

For this purpose attention was turned to various derivatives of hydroxylamine. One of such compounds is O-methylhydroxylamine ($\text{NH}_2\text{---O---CH}_3$). Kochetkov and associates (1963) showed that O-methylhydroxylamine, just as hydroxylamine, reacts only with cytosine at the greatest reaction rate \approx pH 6.0. A noticeable reaction with thymine, uracil, and the purines was not noted.

We undertook the mission to check the mutagenic activity of O-methylhydroxylamine. We followed the dynamics of induction of two types of mutations r and additionally h^c after treatment of extracellular T-2 phage with hydroxylamine and O-methylhydroxylamine. It is known that r-mutations cause a change in the morphology of a sterile plaque, and h-mutations affect the spectrum of lytic action also on the indicator strain (*E. coli* B/2), on which the wild type does not grow; phages containing this mutation form clear negative colonies.

In connection with the necessity of a clear differentiation of mutant types during a calculation of them, we selected special bacterial strains of *E. coli* and also the corresponding media for titration of phage in the method of Gratsiya. For obtaining the initial concentrate of T-2 phage ($\approx 1.5 \times 10^{12}$), control titrations, preadsorption in tests on the induction of h^c -mutations, and for indication of r-mutants we used the bacterial line *E. coli* B (benzer strain), and also the modified medium of Hershey and Rotman (1948) for preparation of the lower 1.5% agar layer, in which in place of Difco agar we used Soviet far-eastern agar and in place of Difco bacto-peptone - Czechoslovakian peptone. In the experiments on induction of h-mutations we used the line of *E. coli* B/2 (Hershey strain) and Hershey-Rotman medium; 0.7% agar for the upper layer was also prepared by the Hershey-Rotman prescription.

The method for treating phage with hydroxylamine (O-methylhydroxylamine) differed somewhat from the method of Freese (1961). A suspension of hydroxylamine hydrochloride was dissolved in bidistilled water and then the solution was alkalized with NaHCO_3 to a pH of 6.0. The working concentration of hydroxylamine comprised 1 M (+1M NaCl). A suspension of free O-methylhydroxylamine was also dissolved in bidistilled water with the addition of sodium chloride. The end concentration comprised 1 M (+1 M NaCl), pH 6.0. To 9.8 ml of solution (37°) we added 0.2 ml of phage T-2 concentrate, after which we immediately took a sample and carried out dilution in Difco broth. With this was achieved the simultaneous cessation of action of each of the mutagens. This probe corresponded to the initial time of treatment. A test tube with a working solution of mutagen and phage was then placed in an incubator (37°) and after specific intervals of time samples were taken from it for an analysis of both inactivation and induction of r- and h^c -mutations. An additional control for the initial time of treatment was titration of the phage concentrate and determination of both the number of active phage particles and the level of spontaneous r- and h^c -mutations.

An analysis of the results showed that inactivation following treatment with hydroxylamine (or O-methylhydroxylamine) set in only in an hour after the onset of treatment: after this the number of phage particles decreased exponentially. Within the limits of an hour the survival rate comprised around 100% and variations of results were within the limits of permissible error, connected with the accuracy of titration of phage by the method of Gratsiya. Differences in the rate of inactivation following treatment both with hydroxylamine and O-methylhydroxylamine were not observed, because graphically the process can be expressed only by one common curve.

Since within the first hour of treatment inactivation did not set in, and the absolute number of mutants of both types increased linearly, it was possible to compare the mutagenic activity of hydroxylamine and O-methylhydroxylamine by relating the number of mutants of each type to the dose of mutagen. Here the time, equal to one hour of treatment, made up the conditional unit of dose. O-methylhydroxylamine conditioned a weaker induction both of r (3.6 times) and h^c (2.1 times) mutations than hydroxylamine.

A calculation of mutagenic activity of two chemical mutagens as a function of dose of mutagen with 100% survival rate is probably most accurate in a study of their peculiarities of interaction on DNA. It is known that during treatment with hydroxylamine some of the lethal damages, apart from damages in DNA, are conditioned by the action of this mutagen on the fibrils of the phage tail (Kozlov, 1959). This same process, leading to mutation, is often affected by other structures of phage DNA, in contrast to the process of inactivation (Brookes and Lawley, 1963).

It is necessary to note that induction of h^c-mutations was connected with replication of DNA of the treated phage, which confirmed the hypothesis of the mutation process advanced by Freese (1959); no induction of h-mutations was noted if the treated phage was initially preadsorbed on a bacterial culture of *E. coli* B which was sensitive to phage. This also testified against the possible hypothesis that induction of h^c-mutations could be in actuality connected with the activation of premutant forms under the influence of hydroxylamine (or O-methylhydroxylamine).

The findings obtained by us indicate the weaker mutagenic activity of O-methylhydroxylamine in comparison with hydroxylamine. This may be explained either by the different rate of change of hydroxymethylcytosine under the action of each of the mutagens, or by the complementary activity of the compounds formed.

From the data cited it follows that from the treatment both with hydroxylamine and with O-methylhydroxylamine a greater induction is observed of r-mutations in comparison with h^c-mutations. The greater mutability of r-loci in comparison with h-loci, which probably are minute mutations (Shtayzinger and Franklin, 1956), is connected mainly with the difference in their extension. It is also possible to admit that there is a sharp heterogeneity of r- and h-loci on pairs of nitrogen bases analogous to that which is revealed, for example, in various sectors of bacterial DNA (Guild, 1962).

Conclusions

1. An analysis of the induction of r- and h-mutations during treatment of extracellular T-2 bacteriophage with hydroxylamine and O-methylhydroxylamine made it possible to reveal the diverse mutagenic activity of these compounds.
2. The lower induction of mutations during treatment of phage with O-methylhydroxylamine is probably connected either with the lesser rate of change of hydroxymethylcytosine in the DNA of T-2 phage or with the diverse complementary activity of the derivatives of hydroxymethylcytosine which are formed in both cases.
3. The diverse rate of induction of r- and h-mutations during treatment of T-2 phage with hydroxylamine and O-methylhydroxylamine may be explained both by the various extent and by the heterogeneity on pairs of nitrogen bases of genetic segments which control the stated properties.

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