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*THE RÔLE OF ORGANIC PEROXIDES IN THE INDUCTION OF MUTATIONS**

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Communicated by G. W. Beadle, August 15, 1949

The discovery by Wyss, Stone, and Clark[1] that bacteria grown on a substrate recently exposed to ultra-violet light are subject to high mutation rates shows clearly that some meta-stable chemical substance, probably of no great complexity, is an intermediate in at least a part of the mutagenic action of ultra-violet light. It was supposed that hydrogen peroxide might be responsible for these results, but subsequent work has shown that this cannot be the whole explanation.[2] However, organic peroxides are known to be formed by the action of ultra-violet light on many compounds and such peroxides might very well be the intermediate agents producing the substrate irradiation effect.

The process by which organic compounds, especially ethers, olefins and aldehydes, form peroxides simply on contact with molecular oxygen is not wholly understood. In many simple cases, however, a chain reaction of the sort pictured below appears to be involved.

$$\begin{aligned} \text{R—H} &\longrightarrow \text{R—} + \text{H} \\ \text{R—} + \text{O}_2 &\longrightarrow \text{R—OO—} \\ \text{R—OO—} + \text{R—H} &\longrightarrow \text{R—OO—H} + \text{R—} \end{aligned}$$

The peroxide-forming process is catalyzed by ultra-violet light[3] which, presumably, supplies the energy for breaking a carbon-hydrogen bond in the first step.

The hypothesis that organic peroxides play an essential rôle in the mutagenic action of ultra-violet light has been under investigation in this laboratory for some time. One result of this work, and the subject of the

present writing, is the discovery that many simple organic peroxides increase mutation rates.

Testing Procedure.—The organism used for detecting and comparing mutagenic agents has been an adenineless, colonial strain of *Neurospora crassa*, the double mutant 70007-38701. The adenineless character in this strain is subject to a low spontaneous rate of reversion to adenine independence. These occurrences are probably back mutations at the adenineless locus, but this has not been demonstrated in the present study. The colonial character, introduced into the strain to permit plate counts, appears to be quite stable. Following essentially a method described by Giles and Lederberg[4] and by Westergaard and Mitchell,[5] conidial suspensions were exposed to various peroxides and the effects calculated from the fraction of these spores that gave rise to colonies on adenine-free medium. Since as many as 2×10^8 spores may be conveniently treated in a single experiment, this method is capable of detecting very weak mutagenic activity.

In a typical experiment a thoroughly mixed suspension of two-day old spores was divided into four portions, centrifuged and decanted. Two portions were retained as controls and the other two were re-suspended in an aqueous solution of the peroxide and allowed to stand at room temperature for 30 minutes. After washing to remove the treating solution, the concentration of conidia in each centrifuge tube was determined with a hemocytometer, and dilution platings on adenine-supplemented medium were made from each sample to indicate conidial viability and percentage mortality. Finally, the suspensions were spread on a series of adenine-free plates and incubated at 25°C. Mutants formed distinct colonies that could be counted during the third or fourth day after treatment.

Mutation rates were calculated by dividing the number of mutants counted by the number of spores plated. By subtracting from the mutation rate shown by spores subjected to a particular treatment the spontaneous rate shown by untreated spores from the same spore batch there was obtained the quantity termed the "induced mutation rate." It is not practical to determine an average value for the fraction of untreated spores that produce adenine-independent colonies and to use such a fixed figure for correcting the observed mutation rates. Although the spontaneous rate is lower than 0.8×10^{-7} in more than 75% of the spore batches, occasionally this rate is very high (e.g., 13×10^{-7}), presumably as a result of an early mutation in the culture from which the spores were obtained. The same problem has been described by Delbrück[6] in connection with mutations in bacteria.

It appears that the largest numbers of mutants are obtained with treatments that kill 60 to 80% of the spores. Mutation rates based on the numbers of spores surviving the treatment might increase up to very high

mortalities but in the present investigation more consistent values have been obtained for mutation rates based on the number of spores treated. Accordingly, treatments producing the greatest actual numbers of viable mutants have been sought.

A danger in working with very high mortalities is the occasional appearance of numbers of adenineless colonies, apparently sustained by adenine released to the medium from dead spores. Such false mutants can usually be distinguished by their frail appearance and their failure to develop beyond an early stage, and always by their inability to grow when transferred to minimal medium.

Demonstration of the Mutagenic Action of Peroxides.—Table 1 illustrates the mutagenic action of *tert*-butyl hydroperoxide at various concentrations. The data are taken from typical experiments selected from an extensive study of this material. The table is intended to show the effect of concen-

TABLE 1

MUTAGENIC ACTION OF *tert*-BUTYL HYDROPEROXIDE

(Treatment: 30 minutes exposure to an aqueous solution of *tert*-butyl hydroperoxide at the indicated concentration)

CONCENTRATION, MOLES PER LITER	SPORES TREATED, MILLIONS	MUTANTS	MUTATION OBSERVED	RATES × 10^7 INDUCED	MORTALITY, %
0.004	48	6	1.2	0.5	5
0.010	96	51	5.3	2.5	42
0.089	70	106	15.1	15.0	29
0.089	72	140	19.3	19.2	38
0.089	40	51	12.8	10.5	80
0.089	29	21	7.2	7.1	91
0.11	32	6	1.9	1.8	85
0.27	38	0	0.0	0.0	100

tration of the agent and to give a rough idea of the reproducibility of the results at a particular concentration. The first objective of this work has been to show that peroxides induce mutations and the values obtained for induced mutation rates are only approximate. Variations in the conditions of treatment, especially temperature, may account in part for the irregularities in the results.

In Table 2 averages of the results of many experiments with six different peroxides are presented. The concentrations of the respective agents, shown in this table, are the ones which have given the highest mutation rates. For comparison the effects of four established mutagenic agents have been included and also the results of experiments with four mildly toxic substances which had no definite effect on mutation rates. It might be noted that two of the last named group, phenol and formaldehyde, have been reported to have a weak mutagenic action in experiments with Drosophila.[7, 8]

The active principles in the mixtures of hydrogen peroxide with formaldehyde and acetone may be, respectively, HO—CH_2—OO—CH_2—OH and HO—$C(CH_3)_2$—OO—H. Hydroxymethyl *tert*-butyl peroxide, derived similarly from the interaction of *tert*-butyl hydroperoxide and formaldehyde, is a well-defined compound.[9] The peroxide derived from diisopropyl ether was obtained by extracting old samples of the ether with water and freeing the extract of volatile material by passing an air stream through it.

TABLE 2

MUTAGENIC ACTION OF VARIOUS AGENTS

(Treatment: 30 minutes exposure to indicated aqueous solutions)

AGENT	CONCENTRATION, MOLES PER LITER	TOTAL SPORES, MILLIONS	INDUCED MUTATION RATE × 10^7	MORTALITY, %
Hydrogen peroxide	0.21	1200	1.8	40
tert-Butyl hydroperoxide	0.089	211	14.5	50
Hydroxymethyl *tert*-butyl peroxide	0.089	270	16.3	68
Peroxide derived from diisopropyl ether	0.15	146	23.0	61
Hydrogen peroxide and formaldehyde	0.022 (0.033 in CH_2O)	464	8.8	77
Hydrogen peroxide and acetone	0.21 (1.36 in $(CH_3)_2CO$)	96	10.4	32
X-rays	*	61	15.8	37
Ultra-violet light	†	418	46.0	32
Bis(β-chloroethyl)sulfide	0.0002	103	4.0	70
Bis(β-chloroethyl)methylamine	0.004	279	3.6	85
Phenol	0.077	465	−0.4	87
Formaldehyde	0.0244	251	0.5	73
Potassium permanganate	0.00052	383	0.0	35
tert-Butyl alcohol	1.75	275	−0.3	84

* Approximately 30,000 r units.

† Exposure of an aqueous suspension with agitation for 75 seconds to 30-watt General Electric germicidal lamp at a distance of 10 cm. The incident ultra-violet energy, nearly all in the vicinity of 2537 A. U., was 6000 ergs/mm.²/min.

The peroxide concentration in the resulting solution was determined iodometrically.

Mustard gas, *bis*(β-chloroethyl)sulfide, and the nitrogen mustard, *bis*(β-chloroethyl)methylamine, were used in borate (pH 8) and acetate (pH 5) buffers, respectively. The solution of the sulfur compound contained, in addition, 1.36 moles per liter of acetone to increase its solubility.

In presenting this demonstration of the mutagenic action of peroxides it is assumed that the adenine-independent colonies appear as the result of true mutations. The action of the peroxides certainly produces a heritable change since adenine independence of the reverted strains persists through repeated subcultures on an adenine-free medium. It has been the general experience of other workers that non-genetic reversions of Neurospora mutants, which occur spontaneously in some strains, do not persist through subcultures. This, together with the observation that known mutagenic agents (radiations, mustard gas) produce effects similar to those of the peroxides, constitutes presumptive evidence for the view that the action of the peroxides is mutational. It is recognized, however, that the results of genetic crosses will constitute the only proof that gene mutations are involved. A genetic study directed toward this end is now in progress and will be reported in a later communication.

There can be little doubt that the peroxide treatment *induces* the presumed mutations. Selection cannot account for the results since the spores are not growing during the treatment and since it is an increase in the actual number of adenine-independent colonies that is observed and not merely a higher ratio of the number of such colonies to the number of surviving spores.

Conceivably, certain nuclei (the conidiospores are multinucleate) possess a latent capacity for adenine synthesis that is only made manifest by the peroxide treatment. However, there is no precedent for such a phenomenon. This question as well as the question of the mutational nature of the peroxide-induced changes will be resolved by the current genetic studies mentioned above.

Finally, it should be noted that the experimental method used here is of a rather special sort. In all probability, only the back mutation of a single gene is involved. It will be important to confirm these findings by different techniques and in different organisms.

Conclusions.—The foregoing evidence that organic peroxides are capable of producing mutations lends substantial support to the idea that ultraviolet light produces mutations by forming such compounds in irradiated media and in cells. A demonstration that this is the mechanism of the substrate-irradiation effect may eventually be obtained by correlating the mutagenic action of irradiated material with peroxide content.

The facts that so few mutation-inducing agents are known and that the effects of those that are known are in large measure similar suggest that they have a common mode of action. Accordingly, the possibility that peroxides are in some way involved in the action of x-rays and of the mustards is currently under investigation. Possibly compounds of the mustard group or certain products of their interaction with organic compounds are especially liable to peroxide formation.

By determining just what feature of the chemistry of peroxides is responsible for their mutagenic action one might hope to shed light on the nature of the mutation process. It seems unlikely that this action is simply related to oxidizing power, since oxidizing agents are common and organic peroxides are not especially effective ones. Of more interest is the characteristic decomposition of peroxides by which free radicals are produced. If this is the essence of peroxide action non-peroxidic free radical sources (e.g., diazomethane) should show similar effects. It should be noted that irradiation of a cell could produce free radicals directly as well as by peroxide formation.

Besides affording a basis for speculation on the nature of the mutation process, the discovery of the mutation-inducing power of organic peroxides substantially increases the number of known mutagenic agents. Organic peroxides of widely varied structure can be prepared. It will be of interest to compare the action of these various agents on different genes and to search for agents having pronounced effects on particular genes.

* This investigation was supported in part by a research grant from Merck and Co.

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‡ The authors wish to thank Professor Norman H. Horowitz for valuable assistance and advice.

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CROSSING-OVER BETWEEN ALLELES AT THE LOZENGE LOCUS IN DROSOPHILA MELANOGASTER

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Communicated by L. J. Stadler, July 22, 1949

It has been reported that females of *Drosophila melanogaster* having one *X*-chromosome containing the lozenge allele glossy (lz^g) and the other *X*-chromosome the lozenge allele spectacle (lz^s), crossed to either lz^g or lz^s males, occasionally produce individuals wild type (lz^+) in appearance.[1,2]