THE PRODUCTION OF MUTATIONS IN STAPHYLOCOCCUS AUREUS BY CHEMICAL TREATMENT OF THE SUBSTRATE

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The literature on the induction of mutations in microorganisms by irradiation might well begin with the report by Henri (1914) on the appearance of two new forms of the anthrax bacillus subsequent to exposing these organisms to ultraviolet light. That Henri appreciated the significance of his finding is evident from his conclusion, "La lumière apparaît donc ainsi comme un agent fondamental de l'évolution intervenant par l'attaque plus ou moins profonde des fonctions nutritives intimes de la cellule." However, since many of the implications of studies on bacterial mutations are apparent only by liberal use of analogy to the genetics of higher forms, similar studies were necessarily retarded awaiting the development of the modern concepts of gene mutations. The recent interest in bacterial genetics has resulted in excellent reviews on this subject (Luria, 1947; Braun, 1947). One observes that in addition to radiations a variety of other methods are available for the production of mutations. Auerbach (1945) demonstrated that mustard gas would produce mutations in *Drosophila melanogaster*, and a similar treatment of microorganisms with the nitrogen or sulfur mustards, acenaphthene, and other chemical agents has resulted in the enhancement of the mutation rate.

Many of the reports on mutations in microorganisms are concerned with selection of naturally occurring mutants from the population. For example, Pinner and Voldrich (1932) observed the production of occasional nonpigmented colonies of *Staphylococcus aureus*, when a strain of that culture developed from a single cell and grown in nutrient broth was streaked on nutrient agar. If the organisms were transferred routinely in nutrient broth containing 5 per cent pleural fluid with a high agglutinin titer for the *S. aureus*, the culture would finally appear to be almost a pure *Staphylococcus albus*. In some cases it is difficult to decide to what extent the factors of selection are operative. The mutations in aspergilli reported by Thom and Steinberg (1939) and Steinberg and Thom (1940) may involve selections, or they may be due entirely to induced mutation. By the addition of a wide variety of agents to the medium these authors consistently found mutations with aspergilli. Nitrite, in the acid medium used for molds, was particularly active in producing large numbers of

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mutant forms. These mutants were stable over a long series of transfers on normal media. The types of mutations observed were similar to those occurring spontaneously; the number of mutants, however, was increased tremendously by the addition of various substances to the medium. Most of the substances employed were selected because they reacted with the amino groups on proteins. The authors suggested that the chemicals reacted with the protein components of the genetic mechanism and showed that some reversion occurred when the mutants were grown in a medium containing excess *d*-lysine.

Using another approach, Stone, Wyss, and Haas (1947) increased the resistance of *S. aureus* to penicillin and streptomycin by irradiation of the substrate prior to inoculation. Several lines of evidence were presented in an attempt to rule out selection as the determining factor in these experiments. They suggest that modified substrate molecules may be assimilated by the organism and built into inexact replications of the genetic mechanism. To test this theory we have treated the substrate with a number of chemical agents and have measured the effect of such treatment on the mutation rate of *S. aureus*. In most

TABLE 1

Increase in mutation to penicillin and streptomycin resistance by treatment of broth with hydrogen peroxide one hour prior to inoculation

TREATMENT OF BROTH	TOTAL COUNT	PENICILLIN 0.05 UNITS/ML		STREPTOMYCIN 3 UNITS/ML	
		Colonies	Mutants/million	Colonies	Mutants/million
Control	470,000,000	2,300	4.9	8,800	18.7
6 ppm H_2O_2	330,000,000	50,000	151	230,000	696

cases unstable chemicals were used to treat the substrate so that at the time of inoculation no residuum remained to obscure the result by possible direct action upon the organism.

EXPERIMENTAL

The methods employed involved the detection of penicillin- and streptomycin-resistant mutants in *S. aureus*. They are essentially identical with those reported by Stone *et al.* (1947). The substrate, usually nutrient broth, was treated with the chemical agent and permitted to stand at room temperature for 1 hour. If tests indicated the disappearance of the agent, the medium was inoculated with about a million cells per ml from a young broth culture. Residual hydrogen peroxide was checked qualitatively by starch-iodine, by catalase, and by the titanium sulfate method of Bonet-Maury (1944). The latter method was employed with the Coleman spectrophotometer; it permitted quantitative measurements to 0.1 ppm, but in the presence of broth it was somewhat less sensitive. After incubation the assay for resistant mutants was made. The experiment reported in table 1 shows the plate counts when organisms are grown in untreated broth and in broth treated with 6 ppm hydrogen peroxide 1 hour before inoculation.

On agar containing 0.05 units of penicillin per ml 2,300 organisms out of the initial population of 470 million per ml of untreated broth were able to form colonies; of the organisms grown on the broth treated with hydrogen peroxide 50,000 out of a total population of 330 million per ml were able to grow. Thus the number of mutants per million was increased over 30 times. A similar situation exists with the streptomycin mutants. A number of colonies were picked from the plates made from the treated broth and the drug resistance was shown to persist even after serial transfer on plain nutrient agar. These organisms were no more resistant to peroxide, nor did they grow faster in the peroxide-treated broth, than the control organisms.

TABLE 2

Effect of concentration of hydrogen peroxide and other oxidizing agents on the mutation rate

TREATMENT OF BROTH	MUTANTS PER MILLION	
	Penicillin	Streptomycin
	0.05 units/ml	*3 units/ml*
Control	8.4	26.7
H_2O_2 9 ppm	no growth	no growth
H_2O_2 3	400.0	1334.0
H_2O_2 1	26.7	84.0
H_2O_2 .3	10.0	23.4
Cl_2 125	no growth	no growth
Cl_2 75	10.0	30.0
Cl_2 6	12.0	23.0
I_2 6	2.5	10.5
$NaNO_2$ 500	—	25.0
$KMnO_4$ 10	20.0	36.0
Control	8.0	33.4
Aerated with oxygen	7.0	30.0
Irradiated 15 minutes (ultraviolet light)	180.0	700.0

Although at the time of inoculation no peroxide could be detected in the broth treated with 6 ppm H_2O_2, experiments on concentration were instituted to observe whether or not mutations would be induced by treatment of the broth with concentrations well below the amount inhibiting growth. As shown in table 2 the bacteria failed to make visible turbidity in 18 hours in broth treated with 9 ppm H_2O_2. However, even the treatment with 3 and 1 ppm peroxide resulted in a definite increase in the mutation rate.

The addition of 6 ppm Cl_2 or I_2 failed to produce any increase in the mutation rate. In fact, when the concentration of Cl_2 added to the broth was increased to a value just short of that which gave a free chlorine residual and thus prevented growth, the mutation rate to streptomycin and penicillin resistance still remained essentially that of the control. High concentrations of $NaNO_2$ failed to increase the mutation rate to streptomycin resistance (penicillin not tested), although in this case much of the nitrite remained in the broth at the time of inoculation. The nutrient broth used was of a neutral pH, so the reaction with

amino acids suggested by Steinberg and Thom could not be expected to occur in this experiment. Potassium permanganate added at a level which reacted completely with the broth failed to affect the mutation rate. Bubbling pure oxygen gas through the medium for 1 hour prior to inoculation did not have any effect on the mutation rate of organisms subsequently inoculated therein. These experiments suggest that the effect of hydrogen peroxide on the mutation rate is fairly specific and not merely the result of growing organisms in a medium with a high oxidation-reduction potential.

The time elapsing between the addition of 6 ppm of peroxide to the broth and inoculation was varied from 15 minutes to 22 hours without markedly affecting the result (table 3). Within experimental error the mutation rate was increased about 5- to 10-fold in the case of penicillin and 10- to 20-fold in the case of streptomycin. From these data it appears reasonable that the effect of the hydrogen peroxide is due to its reaction with some component in the medium.

TABLE 3

The effect of time elapsed between treatment of the broth with 6 ppm hydrogen peroxide and inoculation

TIME BEFORE INOCULATION	MUTANTS PER MILLION	
	Penicillin	Streptomycin
	0.05 units/ml	*3 units/ml*
15 min	73.5	541.0
40 min	97.5	795.0
2 hours	74.8	621.0
3 hours	64.0	848.0
5 hours	60.3	578.0
22 hours	42.7	331.0
Control (no H_2O_2)	9.4	46.4

In order to determine whether or not a selective action of the treated broth was responsible for the result the rate of appearance of the mutants in the young culture was studied. Platings made at 0, 3, 6, and 24 hours after inoculation indicated that in the peroxide-treated broth the mutants appeared at a rate that could best be explained by assuming that the mutations were induced by treated substrate. Very careful measurements on growth rates of mutant subcultures, of the parent strain, and of mixtures of the latter with mutant cultures indicate that in neither normal broth nor peroxide-treated broth did population changes occur which would permit attributing the results to a selective action.

Several chemical substances have been treated with hydrogen peroxide and then added to normal broth. For example, 100 mg of phenyl alanine were dissolved in 100 ml of water to which 100 ppm hydrogen peroxide were added. After an hour one ml of this mixture was added to 50 ml of broth and inoculated with *S. aureus*. After a suitable growth period the resistant mutants were determined and compared with results obtained with control cultures. The results show a considerable enhancement of the mutation rate. Much of the

hydrogen peroxide had disappeared from the amino acid solution before the latter was added to the broth, so it appears unlikely that the effect was due to the residual peroxide acting on the broth components.

Other substances giving the increased mutation rate when treated with peroxide are tryptophane, tyrosine, adenine, uracil, and guanine. Tryptophane is reported to be converted to indole acetic acid by the action of hydrogen peroxide or ultraviolet light, but the addition of indole acetic acid to the medium had no effect on the mutation rate. A number of reducing agents such as thioglycolic acid, sodium sulfite, and sodium sulfide had no effect on the rate of mutation.

Stahmann and Stauffer (1947) treated fungous spores with methyl-*bis*-(β-chloroethyl)-amine and obtained a high mutation rate measured by colonial variation. The concentrations used (0.01 M) killed a large percentage of the mold spores in 30 minutes and showed a pronounced increase, not only in the fraction of the survivors which were mutants, but in the total number of mutants in the smaller surviving population. We employed *tris*-(β-chloroethyl)-amine, which at equivalent molar concentrations showed about the same killing rate with *S. aureus* cells as the methyl derivative used by Stahmann and Stauffer exhibited with the mold spores. When 90 per cent of the *S. aureus* cells were killed, the rate of occurrence of penicillin-resistant cells was found to have increased 20-fold. When this substance was added to the broth at several concentration levels and permitted to react for 4 hours before inoculation, it resulted in a pronounced increase in the number of penicillin-resistant and streptomycin-resistant cells in the resulting population. It is believed from lack of odor and inhibitory action of the broth at the time of inoculation that the mustard had completely hydrolyzed before the cells were added. The action here, also, appears to be one of mutation induced by action upon the substrate.

The correlation between the action of hydrogen peroxide and ultraviolet light is difficult to determine. Irradiation of water by ultraviolet under the conditions of our experiments produces a considerable amount of peroxide. Similar irradiation of the broth produces no detectable residual peroxide since it appears to react quickly with broth constituents. Experiments in which catalase was added to the broth during irradiation and after treatment with hydrogen peroxide gave conflicting results.

SUMMARY AND CONCLUSIONS

These data indicate that treatment of broth with ultraviolet light is not a unique indirect method of inducing mutations. Hydrogen peroxide reacts with some broth components, and an increase in mutant forms appears when organisms are grown in their treated medium, although no peroxide remains at the time of inoculation. A similar action occurs with a nitrogen mustard.

REFERENCES

AUERBACH, C. 1945 Chemically induced mosaicism in *Drosophila melanogaster*. Proc. Roy. Soc. Edinburgh, **62**, 211–222.

BONET-MAURY, P. 1944 Photocolorimetric method for determining small quantities of hydrogen peroxide. Compt. rend., **218**, 117–119.

BRAUN, W. 1947 Bacterial dissociation. Bact. Revs., **11**, 75–114.

HENRI, V. 1914 Studies on the "metabioteque" action of ultraviolet rays. Compt. rend., **159**, 413–414.

LURIA, S. E. 1947 Recent advances in bacterial genetics. Bact. Revs, **11**, 1–40.

PINNER, M., AND VOLDRICH, M. 1932 Derivation of *Staphylococcus albus*, *citreus*, and *roseus* from *Staphylococcus aureus*. J. Infectious Diseases, **50**, 185–193.

STAHMANN, M. A., AND STAUFFER, J. F. 1947 Induction of mutants in *Penicillium notatum* by methyl-*bis*(β-chloroethyl)amine. Science, **106**, 35–36.

STEINBERG, R. A., AND THOM, C. 1940 Mutations and reversions in reproducibility of aspergilli with nitrite, colchicine, and *d*-lysine. Proc. Natl. Acad. Sci. U. S., **26**, 363–368.

STONE, W. S., WYSS, O., AND HAAS, F. 1947 The production of mutations in *Staphylococcus* by irradiation of the substrate. Proc. Natl. Acad. Sci. U. S., **33**, 59–66.

THOM, C., AND STEINBERG, R. A. 1939 The chemical induction of genetic changes in fungi. Proc. Natl. Acad. Sci. U. S., **25**, 329–333.