FACTORS INFLUENCING THE RECOVERY OF BIOCHEMICAL MUTANTS IN LUMINOUS BACTERIA

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In an effort to obtain a clearer understanding of the relationship between luminescence in bacteria and the metabolic processes concerned with growth, attempts have been made to induce and recover biochemical mutants that would affect these two systems differentially. Since Beadle and Tatum (1945) have clearly shown in their investigations on the mold Neurospora crassa that it is possible by mutation to block at specific points the synthesis of factors required for growth, it seemed likely that such an approach to a study of the luminescent reaction would yield fruitful results. Attempts have been made, therefore, to obtain specific biochemical mutants in the luminous bacterium, Achromobacter fischeri. However, it was found that the rate of induction of biochemical mutants was very low in these bacteria, even after treatment with such mutagenic agents as X-rays, ultraviolet, and nitrogen mustard. Consequently the search for specific biochemical mutants was greatly impeded. Results that have recently been obtained throw light on the various factors involved in the recovery of biochemical mutants in these organisms and appear to be of general interest.

EXPERIMENTAL PROCEDURES AND RESULTS

The salt water bacterium Achromobacter fischeri was grown on a complete agar medium for 18 hours at 23 C. Prior to the exposure of the cells to a mutagenic agent they were removed from the agar surface by a platinum loop and suspended in a minimal medium that has been previously described (McElroy and Farghaly, 1948). Exposure of the cells to ultraviolet light was made by placing 5 to 10 ml of the suspension in a sterile quartz flask at a distance of 24 inches from a 15-watt Westinghouse “sterilamp,” operating at 110 volts. The quartz flask, suspended at a 45° angle, was rotated at 100 revolutions per minute to ensure an even distribution of the irradiation. Sterile samples of the irradiated suspension were removed at definite intervals, diluted, and streaked immediately onto complete agar plates. Preliminary experiments had indicated that it was necessary to dilute the treated sample immediately, since longer contact with the irradiated minimal medium rapidly killed all cells. The agar plates on which the bacteria had been streaked were incubated for 48 hours at 23 C. Subsequently, single colony isolations were made on complete agar slants and were tested for their ability to grow in the minimal medium. The specific growth requirements of those that failed to grow were then determined in the usual manner (see Beadle and Tatum, 1945). The requirements for growth of the wild-type organism at 23 C are relatively simple and are met by supplying an inorganic nitrogen source

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505
(NH₄NO₃), a carbon source (glycerol), 3 per cent NaCl, and the usual inorganic elements (Doudoroff, 1942; McElroy and Farghaly, 1948). At higher temperatures, however, supplementation with hydrolyzed casein or a mixture of amino acids is necessary for growth (Anderson, 1948). Growth as well as luminescence is normal on the medium described.

For treatment with the nitrogen mustard bis-beta-chloroethylmethylamine, the latter was added to the bacterial suspension described above. At the appropriate times samples were removed, diluted, and streaked on complete agar plates. In the experiments reported, the usual time of treatment of the bacterial cells was 15 minutes; however, in some cases, with low concentrations of the nitrogen mustard, the time was extended as long as 30 minutes. Several different concentrations of the nitrogen mustard ranging from 0.01 to 0.1 per cent have been employed. With these concentrations and for a 15-minute exposure, the number of survivors varies from approximately 15 per cent to 0.01 per cent. However, there was very little difference in the rate of induction of biochemical mutants for the various concentrations of the nitrogen mustard. In addition to streaking out the bacteria immediately after treatment with the mutagenic agent, a liquid complete medium was inoculated with the treated suspension and incubated at 23 C for various periods of time (6 to 18 hours). After growth the bacteria were streaked on complete agar plates, and the colonies that developed were isolated and tested. A summary of the results of several experiments is presented in table 1. Although there was an increase in the number of recoverable biochemical mutants in the treated series, it was evident that the rate is quite low when compared with those found by other investigators using the same agents on other organisms. In the experiments summarized in table 1 the survivals amounted to 1 per cent or less of the original population. A much higher frequency of biochemical mutants usually occurs in this range of killing in both molds and bacteria (see Tatum, 1946) and we have routinely obtained 2 per cent of biochemical mutants in Neurospora. It was also apparent that neither varying the intensity of the mutagenic agent nor incubation in the complete medium increased the number of recoverable mutants in a given population.

From the studies reported above, it was evident that the increase in the mutation rate was so low that the search for specific mutants affecting both growth and luminescence would be a tedious process. The layer technique reported by Lederberg and Tatum (1945) was not feasible, since under the prevailing cultural conditions the mutant cells are selectively killed. A modification of this method, similar to the cup assay for penicillin, proved to be more successful and allowed large numbers of colonies to be rapidly screened for mutants. However, it was apparent that, even with this method, rigorous control of the bacterial density on the plate was necessary for the complete recovery of all mutants. In this technique the treated bacterial suspension was streaked upon a minimal medium agar plate (150 mm by 15 mm; 40 ml medium) and after 48 hours of incubation the colonies (wild type) that had developed were marked with a sterile glass needle. Sterile glass cups were then placed on the surface of the agar plates to which 2 ml of a concentrated (2X) complete medium were added. Presumably
all colonies that appeared subsequently should be biochemical mutants. Preliminary tests, in which known mutants were streaked on the minimal medium agar plates, indicated that such mutants develop following the addition of the complete medium, even after 7 days of incubation. The results from experiments designed to test the recovery of mutants from a known mixture indicated

Descriptions of the various techniques are given in the text. The biochemical mutants identified include those that require the following substances for growth: tryptophan (3), glutamic acid (4), aspartic acid (2), arginine (2), proline, histidine (4), guanylic acid, leucine, phenylalanine plus tyrosine, tyrosine, p-aminobensonic acid, lysine, essential amino acid mixture, and hydrolyzed casein. The requirements of the mutants indicated in parentheses in the last row have not been completely identified. "Immediate" and "delayed" refer to the time of streaking of the treated bacteria. When "delayed," the treated bacteria were grown in a complete medium for 18 hours. See text for details.

A suspension of the wild type and an arginineless mutant was made in the minimal medium so that the ratio of wild type to mutant form was 1 to 1. The mixture was streaked immediately and the frequency of the two types recovered was determined. The relationship between the number of colonies per plate and the recovery of mutants is shown that the density of colonies on the plate markedly influenced the growth and viability of both mutant and wild-type organisms.

In the experiments reported in table 2, suspensions of the wild type and an arginineless mutant, in the minimal medium, were mixed so that the ratio of the wild type to mutant form was 1 to 1. The mixture was streaked immediately, and the frequency of the two types recovered was determined as described above. All colonies were subsequently tested on the minimal liquid medium. Only those plates with well-isolated colonies were included in the experiment. The
identity of the apparent mutants recovered was confirmed by testing the ability to grow on the minimal medium supplemented with arginine. Complete recovery of the mutant type was obtained only if there were 10 or fewer well-isolated colonies per plate. With increasing colony density the ratio of wild type to mutant increased rapidly. By adding complete medium immediately after plating the mixture, it was possible to recover the expected number of mutants, a fact indicating that metabolic products of the wild-type growth were killing the mutants when little or no cell multiplication was taking place in the latter. A change of initial pH, or of the phosphate buffer concentration, or the addition of CaCO₃ in varying concentrations failed to influence in any marked degree the ratio of recovery on the more densely populated plates (25 to 75 colonies per plate).

In an effort to determine whether wild-type cells selectively inactivate mutants under conditions where relatively little cell multiplication occurs, a mixture of an aspartic acid mutant with the wild type in minimal medium was stored at 8 C. At the end of 6 days of cold incubation, the ratio of wild type to mutant was determined. Under these conditions it appeared that the mutant possessed a greater survival value than did the wild type, since now the ratio of wild type to mutant was decreased. Since Fries (1948) had already described a selection for Ophiostoma mutants in “starving cultures,” it was of some importance to repeat the experiment just described, to determine whether or not a similar selection was occurring under these conditions of cold. A mixture of an aspartic acid mutant and the wild type was made in the minimal medium, so that 20 per cent of the viable cells were mutants. This suspension was placed at 8 C, and samples were removed at 3-day intervals, diluted, and streaked on complete medium agar plates. Three hundred isolations were made for each 3-day interval. The ratio of mutants to wild type was determined, the mutants being tested not only for their inability to grow on minimal medium, but also for their ability to use aspartic acid as the sole growth requirement. The results of this particular experiment are shown in figure 1. Also shown on the graph are the results obtained by incubating the mixture on complete medium at 8 C. On complete medium there was relatively little change in the original ratio of mutant to wild type, whereas in minimal medium the mutant exhibited a marked selective advantage. Plate counts made during the interval indicated that the number of viable organisms decreased rapidly with incubation time in both minimal and complete media.

An interesting observation was made during the identification of mutants in the foregoing experiments. After 3 or more days of cold incubation in the minimal medium, a certain percentage of the isolates failed to grow when the minimal medium was supplemented with aspartic acid. These were therefore initially classified as biochemical mutants. However, these mutants fail to remain stable but revert to the wild type after 2 to 3 transfers on the complete medium. In figure 2 the results of a similar selection experiment using a tryptophan-requiring mutant are recorded, giving the results of the apparent number of mutants obtained when the initial isolations were made and tested. When the
bacteria were streaked out immediately after mixing, those isolates that failed to grow on the minimal medium invariably turned out to be stable mutations of the original type used, i.e., they required tryptophan for growth. However, with subsequent incubation in the cold there appeared, upon isolation and testing,

![Graph 1](image-url)

*Figure 1.* Cold treatment and recovery of biochemical mutants. The wild type and an aspartic acid mutant were mixed in a minimal medium and the suspension was placed at 8°C. At various intervals of time samples were removed, and the ratio of mutant to wild-type cells was determined, as well as the number of viable cells. The solid curves illustrate the change in recovery with time on the two types of medium. The dotted curve illustrates the change in the number of viable organisms. See text for detail.

![Graph 2](image-url)

*Figure 2.* Recovery of apparent and true mutants after cold treatment. See figure 1 and text for details. A tryptophan mutant was used in this experiment.

a large number of organisms that failed to grow not only on the minimal medium when first transferred, but also on the minimal medium supplemented with tryptophan. After a few transfers (usually 2 to 3) on the complete medium, these particular organisms regained their wild-type growth characteristics.
This loss of the ability of the wild-type organisms to grow on the minimal medium could be shown by incubating a suspension in the minimal medium, for 6 to 10 days at 8 C, and then streaking on minimal medium agar plates as described previously. On most plates a few colonies then developed; however, upon adding complete medium by means of the cup method an additional large number of organisms appeared. The majority of the latter isolates grew on the minimal medium when subsequently tested; however, a small percentage (2 to 5 per cent) did not revert to the wild-type requirements until after 2 to 3 transfers on the complete medium. These results suggest that the wild-type organism under these conditions loses certain essential growth components from the cells by diffusion. This view is supported by the fact that the higher the density of the suspension, the less rapid is the conversion of the wild-type cells to the "apparent mutant" state. The ability to synthesize these diffusible components is regained only by transfer to a complete medium. Several stages of instability and finally irreversible inactivation of metabolic function are indicated by the results. The small percentage of strains that require several transfers on the complete medium before regaining the ability to grow on the minimal medium are usually slow in developing even on the complete medium. Apparently the factors in the complete medium that allow growth are rapidly lost when the organisms are inoculated in the minimal medium. It is also interesting that the luminescence of these cultures is lower during the slow developmental period; however, upon complete reversion to the wild-type growth characteristics, the light intensity becomes normal again.

In an effort to identify the components in the complete medium that are necessary for the growth of the "unstable mutants," wild-type cells that have been incubated at 8 C were streaked on minimal medium plates. After 2 days of incubation at 23 C, various components were then added to the plates by means of sterile cups. A few colonies usually developed on plates to which a synthetic vitamin mixture or hydrolyzed yeast nucleic acid was added. However, the largest proportions of colonies developed on plates supplemented with hydrolyzed casein or the complete medium. In a few isolated cases it was possible to obtain growth with only one component, but in the majority of cases a very complex mixture was required; it was not possible therefore to determine the frequency of cells with a specific requirement.

The foregoing results seem to explain partially why the mutant cells have a selective advantage over the wild-type organisms. Apparently wild-type cells lose essential growth components much more rapidly than do mutant types and eventually reach a stage in which they have not only lost the ability to synthesize certain diffusible components present in the complete medium but have also become unable to regain the ability to rebuild nondiffusible components within the cell when placed on a complete medium. This irreversible loss of synthetic ability results in the death of the cell.

Since it is possible to increase the percentage of mutants in a given population by the cold incubation technique, this procedure has been used in several different experiments. After treating the wild-type organisms suspended in the minimal
medium with 0.025 per cent nitrogen mustard for 15 minutes, the bacteria were inoculated into complete medium and allowed to grow for 18 hours. They were then centrifuged and resuspended in the minimal medium and placed at 8 C. After 9 days of incubation the bacteria were streaked on complete medium, and single colony isolations were made. Subsequently these cultures were tested for their ability to grow on the minimal medium, and those that failed to grow were retested in the usual manner to determine their biochemical requirements. Under the conditions described above, it has been possible to increase the apparent mutation rate over 20 times above that of the untreated control. The results are summarized in table 1. From 1,193 isolations, 27 strains with different growth requirements from those of the wild type have been obtained. By using the cold incubation technique it has been possible to obtain biochemical mutants that have not been previously observed in this organism.

DISCUSSION

The results of the present investigation demonstrate that under nutritional conditions just sufficient to support the normal, wild-type strain and at temperatures at which metabolic activity is low, there is a selective advantage favoring strains that have more exacting growth requirements. Such selection may be explained as due to a greater loss of essential nutrients from the wild-type cells, resulting ultimately in their death. There is an interval during the selection process in which the wild-type cells, although still viable (as shown by their ability to grow on complete medium), fail to grow on the minimal medium. Usually, however, after one transfer on complete medium they regain their original wild-type growth characteristics. Although the wild-type organism can normally make those components in the complete medium necessary to this recovery, certain systems are apparently unstable in the absence of their specific substrates, which are presumably lost by diffusion during the cold incubation in the minimal medium. The fact that the frequency of loss of the synthetic ability of the cells is inversely proportional to the suspension density supports the idea that certain stabilizing components are lost. The results are in some respects analogous to those that have been reported by Sonneborn (1946) for certain cytoplasmic factors in Paramecium, and by Monod (1947), Spiegelman (1946), and others for adaptive enzymes. Sonneborn, for example, has shown that, although certain cytoplasmic factors depend upon specific genes for their maintenance and increase, the genes cannot initiate the synthesis unless the cytoplasmic factors are present. He has considered these cytoplasmic factors as primers that are necessary to start the gene system functioning. But unlike the systems postulated here, the cytoplasmic factors (kappa and antigens) thus far studied by Sonneborn and his coworkers are of such a nature that they presumably fail to diffuse through the cell membrane. On the other hand, the studies on adaptive enzymes by Spiegelman and others have shown that a diffusible substrate is essential for the maintenance of certain enzyme systems. In these studies, however, consideration has been given only to what have been termed adaptive enzymes. Since several investigators (see Monod, 1947) have pre-
sented arguments in favor of the concept that the synthesis and stabilization of constitutive and adaptive enzymes are fundamentally the same, it is not altogether unlikely that deficiencies of constitutive enzymes might be obtained by the loss of naturally occurring substrates. The results reported in the present experiments support such an interpretation and therefore suggest that the resynthesis or stabilization of constitutive enzymes may require the presence of a substrate. Obviously, it is to be expected that losses from the cell might occur that could not be reconstituted by diffusible components in the complete medium. Under these conditions the cells would become inviable. Analogous to the latter is the conversion of killers to sensitives in Paramecium by the loss of kappa.

SUMMARY

Treatment of the luminous bacterium Achromobacter fischeri with either ultraviolet irradiation or nitrogen mustard gave a relatively low biochemical mutation rate. Several factors were shown to be important in recovering mutants. With a mixture of the wild type and known mutant cells it was shown that plate density, cold incubation, and the nutritional environment influenced greatly the recovery of the mutant cells. Under nutritional conditions just sufficient to support the normal wild-type strain, and at temperatures at which metabolic activity is low (8 C), there is a selective advantage favoring strains that have a more exacting growth requirement. Such selection appears to be due to a loss of essential nutrients from the wild-type cells. With the cold incubation technique it was possible to increase the apparent mutation rate over 20 times.

REFERENCES


