THE ISOLATION OF BIOCHEMICALLY DEFICIENT MUTANTS OF BACTERIA BY MEANS OF PENICILLIN

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It is a simple matter to isolate bacterial mutants when the mutants can proliferate or survive in an environment which suppresses or eliminates the parent strain. There is consequently no difficulty in obtaining mutants, even of low frequency, which differ from the parent strain by being resistant to antibacterial chemicals or viruses, or by having decreased nutritional requirements. Mutants with increased nutritional requirements, however, have been much less convenient to isolate. Recently developed techniques \(^1,2\) permit a considerable improvement over the earlier practice of random selection, but still permit selection from only a few hundred colonies per agar plate. This paper is concerned with a method of obtaining biochemically deficient mutants from very much larger populations. The method is based on the unusual mode of action of penicillin, which sterilizes only growing bacteria. Mutants which are unable to grow in minimal medium therefore survive, while the predominant non-mutant population is sterilized.

Historical Introduction.—The systematic isolation of microbial mutants with increased nutritional requirements was initiated by Beadle and co-workers,\(^3,4,5\) using the mold *Neurospora crassa*, and has since been extended to others to *Escherichia coli* and other bacteria (cf. 6), and to other molds. With respect to the genetic interpretation of the mutations, *Neurospora* has the advantage of permitting either sexual fusion or asexual multiplication at will; with bacteria, on the other hand, recombination of genetic characters, which has been discovered only recently,\(^7,8\) is apparently a rare event and is demonstrable with few bacterial strains. From the point of view of their use as biochemical tools, however, mutants of bacteria have certain advantages, especially with respect to ease of handling. Their rapid multiplication leads to maximal growth in 24 hours...
or less, and their growth as discrete cells and isolated colonies, in contrast to the mycelial mat of Neurospora, permits precise quantitative treatment of populations of any size. Bacterial numbers below the visible level can be measured by plating out on solid media, while denser growth can be measured most simply by turbidimetry. The possibility of aerobic or anaerobic growth increases the variety of problems which can be attacked. In addition, the uniform production of colonies and streaks on solid media permits easy recognition of the production by one bacterial strain of a growth factor which diffuses through the agar to stimulate another strain streaked nearby. Finally, as will be described below, it has become possible to isolate bacterial mutants considerably more efficiently than has been possible with molds, although recent developments may also render the isolation of mold mutants quite efficient.

The analysis of the behavior of deficient mutants, so far studied mostly with Neurospora, represents one of the major developments in biochemistry of the past decade. It has permitted recognition of a number of distinct enzymic steps in the course of various biosyntheses—a method of exceptional interest as the attention of biochemistry is increasingly focused on anabolic reactions. This development may also be expected to have several important applications, including microbiological assay; the recognition of previously unknown metabolites which can serve as models for synthesis of chemotherapeutic analogs; and perhaps microbial production of rare biochemicals, since certain deficient mutants have been shown to accumulate the substrate of the absent enzyme.

In particular, illustrating the unity of cellular metabolism, the growth factors (nutrilites) of various microorganisms found in nature have almost without exception turned out to be essential participants in the intermediary metabolism of higher animals and plants. In a number of instances the microorganisms have provided the first means of recognizing and isolating vitamins (essential growth factors of animals). But since the growth requirements of a large proportion of "wild type" organisms have already been identified, it appears reasonable to suppose that in the future the discovery of unknown metabolites will increasingly depend on the production of mutants for which these metabolites are nutrilites.

Under these circumstances, it would be highly desirable to have a technique for isolating biochemical mutants of microorganisms more efficiently than has hitherto been possible. The work on Neurospora has been done with mutants obtained by testing large numbers of unselected individual spores. The total frequency of detectable biochemically deficient mutants following irradiation is reported to be about 2% with Neurospora; maximal values of 6% have been recorded with bacteria. The incidence of any given type of mutant is extremely small. With this method it would not be profitable to undertake to isolate specifically any single type
of mutant which might be wanted by a biochemist, nor could one expect to obtain, except by rare chance, those mutants whose frequency of appearance following irradiation is less than perhaps $10^{-4}$, and whose existence can at present only be postulated.

A considerable improvement in efficiency of isolating biochemically deficient bacterial mutants has been effected by Lederberg,\textsuperscript{1} based on the delayed production of colonies by the mutants as a result of delaying the enrichment of the minimal agar medium until the non-mutants have grown for a while. A related method has recently been described which involves the production of small colonies of mutants by limiting the amount of enrichment.\textsuperscript{2} Both these methods, however, still restrict the search to comparatively common mutants, since a maximum of approximately 500 bacterial colonies per plate can be studied.

\textit{Principle of the Penicillin Method}.—The possibility, with biochemically deficient mutants, of selectively eliminating a large non-mutant population suggested itself on the basis of the reports\textsuperscript{17, 18} that penicillin sterilizes only multiplying bacteria. The data on which these reports were based show only that penicillin failed to sterilize bacteria in a grossly deficient meniscum, such as physiological saline, or in an adequate medium kept in the refrigerator. In order to test whether penicillin would also fail to sterilize a deficient mutant in a medium which was adequate except for the specific deficiency, the present investigation was begun by isolating a tryptophane-requiring mutant of \textit{E. coli} by one of the earlier methods.\textsuperscript{2} This mutant was not sterilized by penicillin in a minimal medium lacking tryptophane, while the parent stock strain was rapidly sterilized by penicillin in this medium (and the mutant in a medium containing tryptophane). It therefore appeared that penicillin should be an ideal selective agent for sterilizing large non-mutant populations in a minimal medium, while permitting survival of those mutants which fail to grow on this medium. Accordingly, a search was made for biochemical mutants among the survivors of extensive but incomplete sterilization by penicillin.

Experiments carried out over a period of nearly a year with several bacterial species consistently failed to yield any mutants among the survivors of irradiated populations exposed to penicillin in minimal medium. Success was attained, however, when the bacteria were passed through an extra stage of cultivation in enriched medium after irradiation, followed by washing to eliminate the growth factors present in the enriched medium. They were then suspended in minimal medium containing penicillin and finally plated in enriched agar media. This modified procedure yielded a large number of survivors (up to a hundred per plate, from $10^6$ organisms exposed to penicillin). These colonies consisted practically exclusively (90 to 100\%) of a variety of strains unable to grow on minimal medium.
The introduction of the extra stage of cultivation was based on the hypothesis that the previous failures were caused by the syntrophic effect of metabolites released by the relatively dense populations of bacteria exposed to penicillin following irradiation. This hypothesis was supported by experiments on mixtures of the washed tryptophane-less mutant and the stock culture; these showed that the mutant became increasingly susceptible to penicillin in minimal medium when the density of stock bacteria, either normal or sterilized by irradiation, exceeded $10^5$ cells per milliliter. Since in the early experiments the suspensions used as sources of mutants were irradiated until the proportion of viable survivors was $10^{-4}$ to $10^{-5}$, it was necessary, in order to expose appreciable numbers of viable bacteria to penicillin, that the total population density be very high. The extra stage of cultivation eliminated the harmful excess of metabolically active but non-viable products of irradiation.

Following this adaptation of the method, it was found that the basis on which the extra cultivation had been introduced was not correct after all, even though the procedure was successful. Once the irradiated bacteria had passed through the intermediate cultivation, a certain number of mutants could be obtained even at very high population densities. It was therefore impossible to account for the requirement of intermediate cultivation simply on the basis of the syntrophic effect. We believe the main factor is rather the following. Mutations of the desired type are produced by eliminating the function of a gene which normally produces molecules of a given species of enzyme; these enzyme molecules in turn control a step in the biosynthesis of a given metabolite. But when the gene has undergone a mutation following irradiation, even though the product of the gene can no longer be formed, the pre-existing gene products (enzyme molecules) continue to function and to synthesize their metabolites. The cell is therefore able to grow to a certain extent on minimal medium, and is consequently sterilized by penicillin before the products of the mutated gene are exhausted. During the process of intermediate cultivation, however, the original gene products are so diluted out or exhausted that the mutants are unable to grow on minimal medium, and hence are insensitive to penicillin. To describe this "phenotypic lag" more briefly one might say that the new genotype finds phenotypic expression as a biochemically deficient mutant only after it has undergone sufficient growth (possibly requiring several generations) to lose the physiologic character of the old genotype. The syntrophic effect appears also to play a definite but secondary rôle in determining survival from penicillin. Experiments are in progress to analyze the syntrophic effect and the phenotypic lag quantitatively.

By this technique a variety of mutants of E. coli ("Waksman" strain, ATCC #9637) have been obtained with individual requirements for all the
naturally occurring amino acids except alanine, aspartic acid and hydroxyproline; for several multiple sets of amino acids; for the naturally occurring purines or their nucleosides or nucleotides; for the naturally occurring pyrimidines or their nucleosides or nucleotides; for the vitamins biotin, niacin, \( p \)-amino benzoic acid, thiamin, pyridoxin and panthothenic acid; and for unidentified factors in yeast extract. Mutants requiring vitamins have been isolated with less efficiency than the others; we have evidence that the syntrophic effect is much more striking with at least some of the vitamins, which act as nutrilites in much smaller traces, than with amino acids or nucleic acid components, which form the bulk of the protoplasm.

The experimental details underlying this paper will be published later. A brief protocol of a typical experiment follows, in which only amino acid mutants were sought.

Experimental.—A 24-hour turbid culture of \( E. \text{coli} \) ("Waksman" strain, ATCC #9637) in minimal medium \(^{19}\) was irradiated for 2 minutes in a quartz flask with constant shaking at a distance of 39 inches from an ultraviolet mercury lamp (General Electric "Sterilamp"). This procedure reduced the viable count from \( 10^8 \) per milliliter to \( 10^7 \) per milliliter. (In most experiments the irradiation was more extensive.) One milliliter of the suspension was added to 2 ml. of minimal medium supplemented with 0.2\% tryptic hydrolyzate of casein (Sheffield "N-Z-Case"), and the culture was incubated for 24 hours at 37°C., at which time the viable count had reached \( 1.5 \times 10^9 \) per milliliter. The bacteria were centrifuged, washed once with water and resuspended in 3 ml. of water. Serial tenfold dilutions of the suspension were prepared in water, and 0.1 ml. of each dilution added to 3 ml. of minimal medium containing 300 units of crystalline penicillin per milliliter. Following incubation at 37°C. for 24 and 48 hours, 0.1 ml. from each tube was plated in 10 ml. of minimal agar and in the same medium supplemented with 0.2\% casein hydrolyzate. (It was determined that this dilution of the inoculum in agar was sufficient to remove the antibacterial effect of the penicillin.) Because of the frequently delayed appearance of colonies following exposure to penicillin, the plates were incubated for 48 rather than 24 hours. Table 1 shows the much larger numbers of colonies appearing in the enriched medium. Ten colonies chosen at random from one of the plates of enriched medium were all found to be biochemically deficient mutants, unable to grow on minimal agar. The mutant strains were spot-tested \(^{22}\) for their response to all of the naturally occurring amino acids. Because of the multiplication during the intermediate cultivation between irradiation and selection by penicillin, a number of replicate colonies with the same requirement, presumably a clone derived from a single mutation, are generally found in the same plate. In a typical experiment 5 to 10 distinct types are found on a single plate.
Discussion.—E. coli was the organism chosen because of its hardiness, its ability to grow on the simplest medium, its completely dispersed growth in liquid medium, and the large amount of information already available on its biochemical mutants. Though it requires much higher concentrations of penicillin than those species which are considered fully sensitive to the drug, it fortunately shares with these species the requirement of bacterial growth for bactericidal action. With more sensitive species the separation of mutants from non-mutants by penicillin might well be even more quantitative.

It may be emphasized that those mutants which have survived penicillin under these special conditions are not penicillin resistant in the ordinary sense. In the presence of their growth requirements they display no resistance to the bactericidal action of penicillin. It would not be expected that biochemical deficiencies should be associated with resistance to penicillin; on the contrary, it has been pointed out that the development of a high degree of penicillin resistance by Staphylococcus aureus is accompanied by loss of most of the growth requirements of this normally fastidious organism.\(^{23}\)

<table>
<thead>
<tr>
<th>INOCULUM IN 3-ML. TUBE</th>
<th>AFT. 24 HRS. IN PENICILLIN</th>
<th>AFT. 48 HRS. IN PENICILLIN</th>
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<tr>
<td></td>
<td>MINIMAL MEDIUM</td>
<td>ENRICHED MEDIUM</td>
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<td>(10^6) bacteria</td>
<td>ca. 400</td>
<td>12</td>
</tr>
<tr>
<td>(10^7)</td>
<td>36</td>
<td>152</td>
</tr>
<tr>
<td>(108)</td>
<td>7</td>
<td>79 (10/10 mut.)</td>
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So far as is known to the author, penicillin is the only antibacterial agent which has been shown to require growth for its bactericidal action, but few others have been tested as carefully. Streptomycin\(^9\) and sulfonamides have other modes of action. For microbial species which are completely resistant to penicillin it might be worth while to search for at least partial selection of mutants by other antimicrobial agents. It is well known that a variety of disinfectants sterilize cells from the logarithmic phase of growth more rapidly than cells from the stationary phase.

It was incidentally anticipated, in undertaking this investigation, that light might be thrown on the mode of action of penicillin, since a selective survival of certain classes of mutants, but not of others, would have furnished an indication of the site of action of the drug. As it turns out, the apparently universal survival of all types of non-multiplying mutants makes this procedure particularly valuable as a tool for selecting mutants, but valueless for narrowing down the search for the point of metabolic attack of penicillin. One can conclude only that sterilization by this com-
pound requires extensive protoplasmic synthesis—an integrated process which apparently is interrupted at all points (including the point or points of attack of penicillin) soon after a deficiency appears at any one point. It may be pointed out, however, that actual cell division is not required. Considerable sterilization by penicillin occurs during the lag phase, before the number of cells has increased in a parallel culture without penicillin.\textsuperscript{9} 

This method as developed so far does not appear to yield quantitative survival of mutants, and hence cannot be used for accurate determination of mutation frequencies. The total fraction of mutants obtained with casein hydrolyzate as a supplement is approximately $10^{-3}$ of the viable cells exposed to penicillin, whereas a frequency of approximately $10^{-2}$ was obtained when the same irradiated suspension was tested by another method\textsuperscript{2} which permits survival of all the members of a small population. The virtue of the penicillin method lies chiefly in the use it permits of huge populations. It is possible by this method to isolate certain specific types of mutants conveniently. For example, when it turned out that all of the cystine-requiring mutants on hand responded to sulfide, an experiment was performed to isolate mutants blocked at another level. Cystine was added in the intermediate cultivation, and sulfide in the penicillin tube to eliminate the sulfide mutants. Subsequent plating yielded several kinds of cystine mutants which did not respond to sulfide.

The relatively high frequency of several per cent of detectable biochemical deficiencies in irradiated bacteria has been interpreted by Burkholder and Giles\textsuperscript{16} as implying a haploid, uninucleate state of the bacterial cell. Otherwise, it was suggested, the heterozygous sets of genes in the same cell would lead to mixed colonies, in which the unmaturated strain would regularly have prevented detection of mutants by the methods in use, which involved formation of a colony from each cell immediately following irradiation. Although this argument was advanced in connection with \textit{Bacillus subtilis} spores, it would apply equally, if valid, to other bacteria, which have similar mutation frequencies following ultra-violet irradiation. Actually, this interpretation is in conflict with recent cytological evidence that bacilli, including \textit{E. coli}, are multinucleate.\textsuperscript{24} Although the residence in these nuclear bodies of the genes for biosyntheses has not been proved, it seems very probable. The argument of Burkholder and Giles, in any case, is open to question if we recall that the irradiated suspensions do not contain 1% mutants and 99% non-mutants; rather, in a typical case, with $10^{-4}$ viable survivors, the proportions would be $10^{-6}$ mutants, $99 \times 10^{-6}$ non-mutants, and 0.9999 non-viable cells which may be regarded in a general way as lethal mutants. It is obvious that under these circumstances a viable mutant set of genes in a multinucleate cell would almost invariably be accompanied by a lethal set which could not give rise to a mixed colony, and presumably would not interfere with the propagation of the
viable set of genes. This consideration is also applicable to the suggestion\textsuperscript{25, 26} that polyploidy might be a cause of the delayed appearance of phage-resistant bacterial mutants.

Delayed phenotypic expression appears to be much the most likely, though not the only possible, explanation of the failure to isolate mutants by exposure to penicillin immediately after irradiation; for it seems not only plausible, but practically inevitable, that enzymes should persist in the cell for some time after radiation has altered the corresponding gene. It is not permissible to refer to this process as a “cytoplasmic lag,” such as has been demonstrated with Paramecium,\textsuperscript{27} for we have no certain knowledge of the location in bacteria of either the genes or their products. It would be desirable, however, for the biochemical analysis of genetic processes, to have a collective term for the products of gene action, defined on a functional rather than a morphological basis. \textit{Genome} has long been used to denote the sum total of the autoreproductive units (genes) in a cell. We here propose the term \textit{phenome}\textsuperscript{28} for the sum total of extragenic, non-autoreproductive portions of the cell, whether cytoplasmic or nuclear. The phenome would be the material basis of the phenotype, just as the genome is the material basis of the genotype. What has been called a delay in phenotypic expression would, then, depend on the transformation of the phenome to correspond to the new genome. Since the word phenotype is purely formal and has somewhat special implications as ordinarily used in genetics, it is suggested that the process under discussion be referred to more concretely as a \textit{phenomic lag}.

A delay in the appearance of mutants induced by irradiation has also been described by Demerec for phage resistance.\textsuperscript{25, 26} Newcombe\textsuperscript{29} has recently demonstrated indirectly a similar delay among spontaneously appearing phage-resistant mutants, and favors the view that in both induced and spontaneous phage resistance the delay is one of phenotypic expression of the mutation. Biochemically deficient mutants are a simpler case in the sense that we have some conception of the biochemical nature of the change, whereas the mechanism underlying phage resistance is more obscure. The similar delay in phenotypic expression of both types of mutation therefore supports the notion that in phage resistance there is also an alteration of the composition of the phenome, presumably primarily involving one or more enzymes controlled by a single gene.

\textbf{Summary.}—A method is described for the isolation of biochemically deficient mutants of bacteria. This procedure is based on the fact that penicillin sterilizes only growing bacteria; mutants selectively survive exposure to penicillin in a minimal medium which is inadequate for their nutrition. By this technique a large variety of mutants of \textit{E. coli} have been obtained with requirements for amino acids, nucleic acid components, and vitamins.

Mutants induced by ultra-violet irradiation do not survive exposure
to penicillin in minimal medium unless they are first permitted a period of growth, during which the new genotype achieves physiologic expression as a deficient phenotype. The term phenome is proposed to denote the total extragenic material of the cell, and the term phenomic lag to describe the delay in phenotypic expression.

Syntrophism also contributes to the susceptibility of mutants to penicillin in minimal medium.

The excellent technical assistance of Mrs. Harlean Cort is gratefully acknowledged.

* The use of penicillin to isolate biochemically deficient mutants was also developed independently by J. Lederberg and N. Zinder. Communications by these investigators and by the author are being published in the December 1948 issue of the Journal of the American Chemical Society.

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15 Knight, B. C. J. G., Vit. & Hor., 3, 105 (1945).
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19 The minimal medium had the following composition: Na$_2$HPO$_4$$\cdot$7H$_2$O 6 g.; KCl 4 g.; K$_2$SO$_4$ 1 g.; NH$_4$ lactate 2 g.; MgSO$_4$$\cdot$7H$_2$O 0.025 g.; glucose (autoclaved separately) 2 g.; H$_2$O ad 1000 ml.; pH adjusted to 7.6. This medium contains considerably higher concentrations of K and Mg than certain other media which have been recommended for E. coli, including Medium “F” which is widely used in bacteriophage work. It was found that these high concentrations are necessary for the initiation of growth by small inocula (10 to $10^4$ cells) and for the efficient sterilization of non-mutants by penicillin, although the extra K and Mg do not affect the final density of growth attained with large inocula. A small amount of sodium citrate (0.01%) was also found to substitute for the high concentrations of K-and Mg, and was sometimes added to the minimal medium since it augmented the bactericidal rate of penicillin.
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RIBOFLAVIN-SENSITIZED PHOTOOXIDATION OF INDOLE-ACETIC ACID AND RELATED COMPOUNDS

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Indoleacetic acid (IAA) is known to exert profound effects upon growth and morphogenesis in higher plants (cf., the review by Went and Thimann17). Since its natural occurrence in plants has been directly demonstrated by chemical isolation (Haagen-Smit, et al.,7, 8) its status as a plant hormone seems well established. Obviously, any information concerning the genesis and disappearance of this hormone is of fundamental importance to plant physiology.

During a series of experiments on the physiology of light action in etiolated peas (Galston and Hand5), we noticed that the addition of small amounts of riboflavin (Rbf) to the growth medium resulted in a marked growth inhibition if the tissue were exposed to light. No such inhibition occurred in the absence of light. Since this inhibition could be partially reversed by the addition of relatively large quantities of IAA to the medium, it appeared possible that riboflavin in some way caused the photo-inactivation of indoleacetic acid. This interpretation was completely confirmed by in vitro experiments, as described below.

Methods.—The test solutions were put into Erlenmeyer flasks of such capacity that a layer 1–2 cm. deep was formed. Duplicate series were prepared, one being mixed and stored in a dark room, the other being exposed to about 200 foot-candles of light from “Daylight” fluorescent bulbs. Such light as was necessary in the “darkroom” was supplied by a 7 1/2-watt ruby-red bulb. In the determination of IAA, a 1-cc. aliquot of the reaction mixture was removed to a test tube, mixed with 4 cc. of Salkowski reagent (see Tang and Bonner14), and allowed to stand for 30 minutes. The intensity of the resultant pink color was then measured in a Klett-Summerson photoelectric colorimeter, using a green filter, and the concentration of IAA in the aliquot determined by reference to a previously prepared standard curve.