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Studies on Nutritionally Deficient Bacterial Mutants Isolated by Means of Penicillin[1]

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Studies on the mode of action of chemotherapeutic agents have not shown any direct effect on the known catabolic processes of bacteria. There is good reason, by exclusion, to suppose that they act by means of interfering with anabolic reactions, about which much less is known. In the last decade, BEADLE and his school have made striking contributions to this area in biochemistry, as well as to genetics, by the use of nutritionally deficient ("biochemical") mutants of the mold, Neurospora—mutants which have lost one or another specific enzymic activity[3]. More recently, bacteria have been found to yield mutants of the same sort[4]. These microbial mutants offer a promising approach to the major theoretical problems of chemotherapy: the mechanism of chemotherapeutic action and the biochemical alterations responsible for drug-resistance in microorganisms. Furthermore, the phenomenon of drug-resistance presents a direct problem in microbial genetics. Finally, just as the nutritional requirements of various wild-type microorganisms have been of tremendous value in isolating the known vitamins, so the extension of this approach to the induction of artificial nutritional requirements offers promise as a method for detecting metabolites peculiar to microorganisms—metabolites of special promise as models for the construction of inhibitory analogues. For these several reasons this laboratory, interested in chemotherapy, has felt warranted in undertaking an excursion into microbial genetics.

It is a simple matter to isolate, from huge microbial populations, rare mutants that can survive or proliferate in a medium which suppresses the parent strain. Examples include mutations to drug-resistance, to bacteriophage-resistance, or to decreased nutritional requirements. Comparable techniques have not been available for the nutritionally deficient mutants, though these are in some respects more interesting. In the early investigations, deficient mutants were isolated by testing of spores or colonies selected at random—a tedious process, since the total frequency of recognizable mutants, even after optimal irradiation, rarely exceeds 1 or 2 per cent. Improved techniques of isolation have recently been introduced for Neurospora[1] and other molds[2], and for bacteria[3]. In the case of the bacteria, the techniques depended upon delayed or limited enrichment of the medium, with consequent production of small colonies of mutants which could be distinguished from the larger colonies of the parent strain. Since the colonies must not be too crowded, these techniques were limited to a few hundred colonies per Petri dish.

The penicillin method

For the isolation of rare mutants, a method permitting selection from much larger populations would be desirable. The possibility of such a method suggested itself on the basis of the report that penicillin has the remarkable property of sterilizing ("killing") bacteria only under conditions which permit growth[4]. We have confirmed and extended this conclusion. In a minimal medium containing glucose, lactate, ammonia, and sulfate as sole sources of carbon, nitrogen, and sulfur, various amino acid-requiring mutants of *E. coli* are completely resistant to the bactericidal action of penicillin. The addition of the required amino acid, however, renders the mutant as sensitive to penicillin as its parent wild-type strain. In applying this method to the isolation of new mutants, a suspension of *E. coli* was irradiated with ultraviolet light, further cultivated in an enriched medium, washed, and then incubated, in inocula of suitable size, with penicillin in minimal medium for 24 hours. Up to 100 p. c. of the large number of survivors, recovered by plating in agar media supplemented with casein hydrolysate or yeast extract, were found to be nutritionally deficient mutants. The penicillin method has been independently developed in

[1] Based on material delivered in a symposium on *The Relation of Genetics to Biochemistry* at the annual meeting of the American Chemical Society in San Francisco, Cal., March 30, 1949.

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this laboratory[1] and by LEDERBERG and ZINDER[2]. After isolation, the growth requirement of a mutant can be simply determined by distributing tiny drops of solutions of nutrilites on the surface of a heavily seeded pour plate[3] (BEIJERINCK's "auxanography").

Since the publication of this method, a modification has been introduced which substantially improves its efficiency. Instead of inoculating the washed organisms directly into minimal medium containing penicillin, the inocula are placed in a medium lacking nitrogen. After 4 to 6 hours of incubation, during which dissimilation of glucose promotes the exhaustion of stored metabolites, the minimal medium is completed by addition of ammonium sulfate. Penicillin is added, and the procedure is continued in the usual way. The period of dissimilation particularly improves the recovery of certain vitamin-requiring mutants.

Although the penicillin method provides a marked improvement in the efficiency of isolating mutants, it nevertheless has serious limitations. The population density cannot be indefinitely large, as is possible with the drug-resistant mutants, for at population densities above 10^5 to 10^7 cells per ml, the non-mutants release enough of various metabolites ("syntrophism"; cf.[4]) to permit certain mutants to grow slightly and hence be sterilized by penicillin. In addition, even at low population densities the recovery of mutants is not quantitative. Finally, the use of this method for estimating mutation rates is limited by the requirement of a stage of intermediate cultivation between the irradiation and the exposure to penicillin, in order to permit phenotypic expression of the induced mutation. During this cultivation, the distribution of the population will undoubtedly be distorted.

Delayed phenotypic expression

The requirement of a certain amount of growth for the phenotypic expression of an induced mutation is itself an interesting point, and has also been reported for phage-resistance[5]. The explanation, at least with the nutritionally deficient mutants, appears to be that the cell must undergo some growth, possibly several generations, before the premutational products of the mutated gene (enzymes; possible intermediates between genes and enzymes) are exhausted. Only then will the pattern of enzymes in the cell correspond to the new pattern of genes. For this particular mechanism of delayed phenotypic expression we have proposed the term "phenomic delay"[6], the "phenome" being defined as the total non-self-reproducing part of the cell, under the control of the self-reproducing genes. The occurrence of the same phenomenon in mutations in the reverse direction will be discussed later.

Other possible explanations of the requirement of intermediate cultivation in the penicillin method include segregation of mutant and non-mutant nuclei from a multinucleate cell, and a syntrophic effect of the non-viable irradiated bacteria, which would promote sterilization of mutants by penicillin. Evidence will be published elsewhere that neither of these mechanisms furnishes an adequate explanation, while the phenomic delay accounts for all the available facts. A similar conclusion was reached by Newcombe in a thorough analysis of delayed phenotypic expression of phage resistance[1].

Biochemical advantages of bacteria

Bacteria are less easily studied genetically than molds such as Neurospora, which can be made to multiply sexually or asexually at will. While genetic recombination in bacteria has recently been demonstrated by TATUM and LEDERBERG[2], it apparently occurs in only a few strains and a tiny proportion of the population. For biochemical investigation, however, bacteria appear to have several advantages. Not only can a variety of mutants be isolated relatively quickly, but it is possible to demonstrate very simply, by the syntrophic interaction of adjacent streaks on solid media, the instances in which one mutant accumulates a metabolic intermediate which is utilized as a nutrilite by another mutant. Metabolite accumulations, when present, are extremely useful in analysing biosynthetic pathways[3].

A further advantage of bacteria is the uniformly dispersed growth of certain species in liquid media, which permits simple and precise quantitative experiments, using colony counts for low population densities and turbidimetry for high densities. On solid media, the production of uniform colonies has made possible a variety of experiments involving prolonged cultivation, without risk of confusion from back-mutants, which are readily distinguished from the rest of the population. In addition, slight variations in colony size and syntrophism have made it possible to recognize unexpected phenomena which might easily have gone unnoticed in a mycelial mat. Finally, in relation to chemotherapy, the metabolism of bacteria is of particular interest. Although some of the problems to be described here are still under investigation, it seems desirable to illustrate at this time the types of phenomena that can be revealed by these primitive techniques, especially by the test for syntrophism. This effect has been long

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known in microbiology as the satellite phenomenon; systematically employed, it has been our vade-mecum.

Mutants obtained

Mutants of *E. coli* ("Waksman" strain[1], Amer. Type Culture Collection 9637) have been obtained with requirements for all the naturally occurring amino acids except alanine, aspartic acid, and hydroxyproline. A number of mutants respond to either serine or glycine; thus far, none of our strains has been specific for either of these interconvertible amino acids. The sulfur-deficient mutants, which are a very common class, respond to cystine or less rapidly to methionine, and are blocked in the reduction of sulfate to sulfite or of sulfite to thiosulfate or sulfide. In addition, there are mutants, unresponsive to thiosulfate or sulfide, with specific requirements for cystine and others for methionine. Besides specific proline-requiring mutants, there are others which respond to either proline or glutamic acid or α-ketoglutaric acid (but not ornithine).

Many of these types of mutants have already been isolated from *E. coli* by earlier techniques[2]. In addition, we have isolated strains with more complex requirements which should throw light on certain metabolic relationships. Alternative requirements exist for lysine or threonine, and, in another mutant, for α-amino butyric acid or isoleucine (or, curiously, D-threonine but not L-threonine). One peculiar mutant responds either to methionine or to thiamine: to methionine in the concentrations of several micrograms per ml usual for amino acid mutants; to thiamine or its pyrimidine in the concentrations, one thousandth as great, required by other thiamine mutants. Another mutant similarly requires, under special conditions, either methionine or vitamin B-12. Finally, there are several mutants with a multiple requirement apparently due to a single genetic block: isoleucine plus valine; phenylalanine plus tyrosine; phenylalanine plus tyrosine plus tryptophan; and these three aromatic amino acids plus *p*-amino benzoic acid. No peptide-requiring mutants have been obtained; although much of our isolation work has been done with tryptic casein hydrolysate, which contains many peptides, all the mutants isolated from this enrichment have grown on a mixture of known amino acids. One serine or glycine mutant and one methionine mutant, like several reported Neurospora mutants, are temperature sensitive, with an absolute requirement at 37°C, and none at 25°C.

With yeast extract or hydrolysed yeast nucleic acid, mutants have been obtained with requirements for purines or pyrimidines. The purine mutants, however, have all responded to adenine or guanine or hypoxanthine or their ribosides or nucleotides, while several have also responded to xanthine; the pyrimidine mutant has responded to cytosine or thymine or uracil or their ribosides or nucleotides. Because of this non-specificity, this group has not been further studied.

Among the vitamins, mutants have been obtained with individual requirements for thiamine, nicotinamide or nicotinic acid, pyridoxin or its amine or aldehyde, *p*-amino benzoic acid (PABA), pantothenic acid, and biotin. In spite of a number of attempts, none have been obtained with requirements for riboflavin, inositol, choline, or hemin. The reason for these failures is not apparent. One mutant requiring an unknown factor in yeast extract has been obtained twice.

Microbiological assay

We have not engaged in extensive studies on the use of these mutants for microbiological assay. Since the medium employed is so simple, mutants might be expected to have some advantage over the wild-type species with complex requirements which are generally used. In addition, turbidimetric assay of bacterial growth is more convenient than the measurement of dry weight of pellicle which is required with Neurospora mutants[1]. The advantage of the mutants would be largely lost, however, if a heavily enriched medium were required in order to prevent other substances present in the material under assay from altering the quantitative response to the required nutrilite. Since secondary effects of other substances have been observed in many instances with wild-type organisms, it would be necessary with each mutant to determine the conditions for assay. Furthermore, the instability of most of the mutants requires caution. With several mutants of ordinary stability (phenylalanine, tyrosine) we have been able to obtain satisfactory growth curves, without significant appearance of reversions, at 24 hours, but by 48 hours irregular increases in turbidity due to reversions had appeared.

Syntrophism

The syntrophic technique is illustrated with three arginine-requiring mutants which are shown in Fig. 1 to be blocked at different stages in the well-known Krebs-Henseleit scheme: ornithine→citrulline→arginine. Sets of mutants of both *Neurospora* and *E. coli*, blocked at these stages, had previously been reported[2], having been recognized by their response to these precursors of arginine, In the case of growth factors whose precursors are unknown, however, and which

[1] The initiation of this work with the "Waksman" strain has been accidental. For any new program, it would undoubtedly be preferable to use the K-12 strain of *E. coli*, with which genetic recombination can be studied[3].

[2] E. L. TATUM, Cold Spring Harbor Symp. Quant. Biol. *11*, 278 (1946). – B. D. DAVIS, Arch. Biochem. *20*, 166 (1949).

[3] E. L. TATUM, and J. LEDERBERG, J. Bact. *53*, 673 (1947).

[1] F. J. RYAN, Feder. Proc. *5*, 366 (1946).

[2] A. M. SRB, and N. H. HOROWITZ, J. Biol. Chem. *154*, 129 (1944). – R. R. ROEPKE, quoted by E. L. TATUM, Cold Spring Harbor Symp. Quant. Biol. *11*, 278 (1946).

are therefore the most interesting, this technique of substituting precursors cannot be used, except by guesswork, to recognize differences in the site of genetic blocks. For this reason, the alternative technique of syntrophism is particularly useful. This

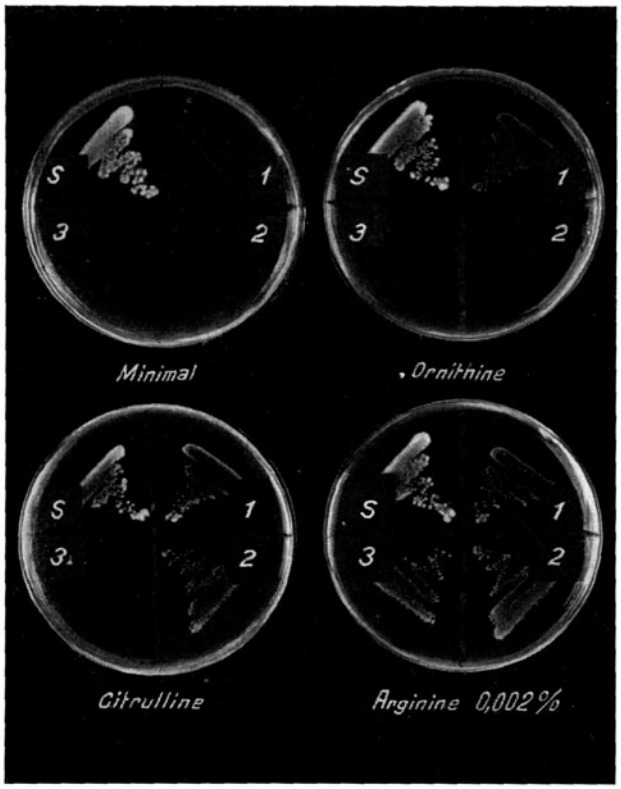


Fig. 1. – Growth of mutants and wild-type (*S* stock) on minimal medium enriched with ornithine, citrulline, or arginine. It is seen that one mutant responds to any of the three related compounds, another to either citrulline or arginine, the third to arginine only.

technique is shown in Fig. 2, in which the mutant specifically requiring arginine is seen to excrete a factor, presumably citrulline, which stimulates the growth of the two mutants blocked earlier; while the citrulline mutant in turn feeds the ornithine-requiring mutant. The gradient of observed growth reflects the gradient of diffusion through the agar.

Similar relationships, involving unknown precursors, have been observed among proline and among histidine-requiring mutants and among certain mutants requiring aromatic amino acids. Syntrophic accumulation of precursors appears to be quite widespread with *E. coli* and has been observed among sets of mutants (e. g. arginine) which fail to show accumulation in Neurospora. Its absence between two mutants with a common requirement, however, does not prove that they are blocked at the same enzymic site, since precursors may fail to accumulate because of instability, diversion along an alternative path, or the inability of the cell to build up a concentration adequate for excretion.

Causes of syntrophism

Since syntrophism leads to recognition of the accumulation of a precursor whose subsequent isolation and identification will contribute to the analysis of a biosynthetic chain, it becomes important to be aware of other possible sources of this phenomenon. Several have so far been observed.

(1) *Accumulation of the precursor of a genetically blocked reaction.* This mechanism is the one discussed above.

(2) *Excretion of metabolites by wild-type E. coli.* It is known that bacteria not only remove nutrilites from the medium, but contribute metabolic products to it. In order to find whether these include growth factors for any available mutants, the wild-type strain was streaked on minimal medium adjacent to mutants with single requirements for each of the factors listed above. After 48 hours, three of the mutants were so heavily fed as to produce maximal growth. These were the strains requiring biotin, PABA, and pantothenic acid. In addition, the nicotinamide-less mutant was moderately fed. The other available vitamin-requiring mutants (thiamine and pyridoxin), as well as a purine, a pyrimidine, and all the amino acid-requiring mutants, showed that the amounts of their required factors excreted by wild-type were negligible, supporting at most only microscopic growth after 2 to 5 days. It is concluded that this strain of *E. coli* is economical in its synthesis of all the growth factors that could be tested except four vitamins. It is of interest to note, however, that the amounts of the other factors excreted, at

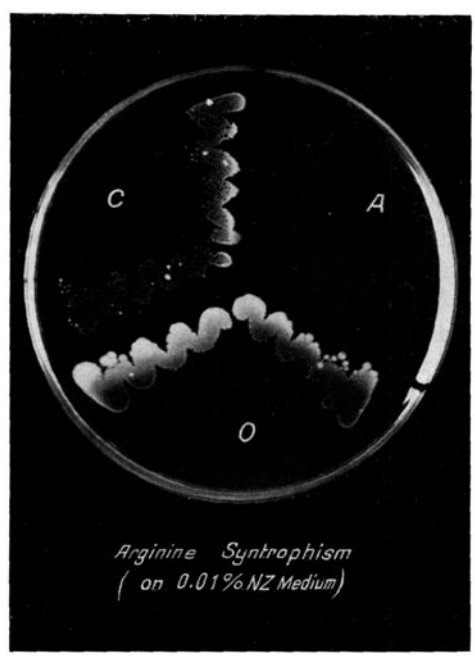


Fig. 2. – Syntrophism among arginine-requiring mutants of Fig. 1. Mutant *O* responds to ornithine or citrulline or arginine; *C* responds to citrulline or arginine; *A* responds to arginine only. 48 hours of growth (37° C) on medium enriched with very small amount of casein hydrolysate ("NZ Case").

least in the presence of penicillin, are sufficient to limit the permissible population density in the penicillin method of mutant isolation; this procedure is apparently much more sensitive to traces of syntrophism than is the technique of adjacent streaks.

(3) *Conversion of a precursor.* It has been observed that wild-type, growing on an excess of the keto acid precursor of isoleucine, will feed a mutant that responds to isoleucine but not to its keto acid. The same conversion and excretion is carried out by a mutant, blocked earlier, which can use this precursor as well as isoleucine itself. It has also been observed with wild-type acting on certain other precursors. In some reactions, however, such as the conversion of ornithine or citrulline to arginine, it has not been possible with an excess of either precursor to stimulate the excretion of arginine by wild-type or by a mutant. It is evident that the organism possesses more than one type of mechanism for determining the rate of synthesis of an amino acid; in the case of isoleucine, the capacity for amination of the keto acid exceeds the requirement, and hence cannot be the rate-governing mechanism.

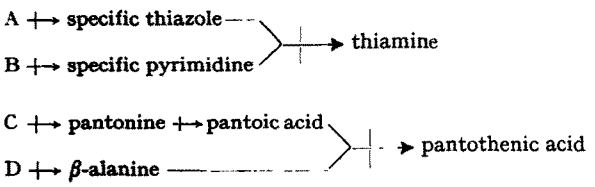


Fig. 3. – Synthesis of thiamine and pantothenic acid. Blocked arrows represent sites of genetic blocks.

(4) *Excretion of a lone conjugant.* Thiamine is composed of two moieties, a substituted thiazole and a substituted pyrimidine (Fig. 3). Thiamine-less mutants were obtained which respond respectively to the pyrimidine, to the thiazole, and to neither (implying a deficiency in the conjugation of the two); a fourth type, whose site of genetic deficiency is not readily interpreted, responds to the specific thiazole plus pyrimidine, as well as to thiamine itself, but not to either moiety alone. Tests for syntrophism showed that the thiazole-less mutant feeds the pyrimidine-less mutant, but not vice versa. Mutants with similar blocks and accumulations have been observed with Neurospora[1].

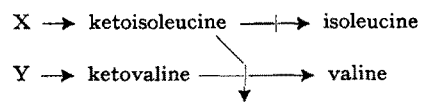
Another vitamin, pantothenic acid, also consists of two components, pantoic acid and β-alanine (Fig. 3). W. K. MAAS in this laboratory has isolated various pantothenic acid-less mutants which respond respectively to pantoic acid or pantonine; to β-alanine; to pantothenic acid only; and to a mixture of pantoic acid and β-alanine. The pantoic-less mutant feeds the β-alanine-less mutant, but not vice versa.

In both these instances of syntrophism, the cause of the accumulation of the intermediate is not absence of the enzyme concerned with its further conversion, but inability of that enzyme to perform the conversion in the absence of the second conjugant.

(5) *Release of physiological brake on a synthetic process.* One of the most interesting observations to turn up is mutual syntrophism between certain tyrosine-less and phenylalanine-less mutants. Paper chromatography confirmed the inference that the block in phenylalanine synthesis resulted in excretion of tyrosine itself, rather than a precursor, while a block in tyrosine synthesis caused excretion of phenylalanine. Feeding of a tyrosine-less mutant by culture filtrates of a phenylalanine-less mutant has also been observed with another strain of *E. coli*[1].

From the presence of mutants with a double requirement for these two compounds, as well as others with triple and quadruple requirements for these plus other aromatic compounds, all resulting from single mutations, it had been inferred that phenylalanine and tyrosine arose from a common precursor (Fig. 4). A possible explanation for the mutual syntrophism therefore appeared to be the diversion of this precursor in one rather than the normal two directions, with resultant excretion of the excess of phenylalanine or tyrosine. Further study, however, showed this explanation to be inadequate, as the amount of phenylalanine (or tyrosine) excreted was much larger than the amount of tyrosine (or phenylalanine) consumed. Since the requirements of mutants of *E. coli* for these two compounds are of the same order of magnitude, simple diversion could not account for the large production.

An alternative explanation has been developed, stimulated largely by the interesting work of BONNER[2]. He showed that a single block in isoleucine synthesis, between the α-keto and the amino acid, accounted for the double requirement of a Neurospora mutant for isoleucine and valine. Apparently the accumulated isoleucine precursor competes as a structural analogue with the corresponding compound in the valine-chain, causing a requirement for this amino acid as well:



Recently ADELBERG[3] has found that the compound accumulated by this mutant is not ketoisoleucine, but is rather the α-β-dihydroxy acid. The principle of internal inhibition by a normal metabolite, however remains unchanged.

This evidence of inhibition of a normal reaction by increased concentration of a normal metabolite has seemed to us to point to the possibility of a general mechanism of integration of various parallel sequences

[1] E. L. TATUM and T. T. BELL, Amer. J. Bot. *33*, 15 (1946).

[1] S. SIMMONDS and J. S. FRUTON, personal communication.
[2] D. BONNER, J. Biol. Chem. *166*, 545 (1946).
[3] E. A. ADELBERG, personal communication.

of biosynthesis. If the accumulation of normal metabolite A in excessive concentration completely blocks a certain enzymic reaction in the production of compound B, then the normal concentration of metabolite A might exert a governing effect on that reaction, while a complete absence of metabolite A, resulting from a genetic block, might permit excessive synthesis of compound B, out of proportion to the remainder of the metabolites being synthesized, with resulting excretion of compound B.

To apply this concept to the present problem, we would postulate a tyrosine precursor which interferes with phenylalanine synthesis, and vice versa. The excretion of phenylalanine would therefore be due to a block early enough in the synthetic chain of tyrosine to cause absence of that tyrosine precursor which normally governs the rate of phenylalanine synthesis, and the same consideration would apply to the phenylalanine mutant which secretes tyrosine. On the other hand, there is one tyrosine mutant which fails to feed phenylalanine; its block would occur *after* the governing intermediate (*E* in Fig. 4). This scheme is strongly supported by the fact that the excretion of phenylalanine by the tyrosine mutant is prevented in the presence of an excessive concentration of tyrosine; the same is true of the phenylalanine mutant whose excretion of tyrosine is inhibited by an excess of phenylalanine or phenylpyruvic acid, which can be substituted for phenylalanine as a growth factor for this mutant. Presumably the excess of the amino acid causes reversal of the normal processes of synthesis, restoring from without the governing compound whose synthesis is genetically blocked.

This scheme for explaining the output of phenylalanine and tyrosine is on speculative grounds, since the intermediates in the synthesis of these amino acids (except for phenylpyruvic acid) are as yet unknown. The concept of normal physiological interaction among separate biosynthetic paths, however, is further strengthened by returning to the better documented isoleucine-plus-valine case. We have isolated a mutant of this type with *E. coli* whose mechanism appears to be identical with that of the similar Neurospora mutant. In addition, another *E. coli* mutant has been isolated which responds equally well to isoleucine, its α-keto acid, or α-amino butyric acid. The double-requiring mutant, which cannot use ketoisoleucine, is known to accumulate an inhibitor of valine synthesis; the single-requiring mutant, which can use ketoisoleucine, and which is fed by the double, must be blocked earlier; it would therefore follow, from the reasoning outlined above, that the single-requiring mutant should not form the isoleucine intermediate that governs valine synthesis, and hence might be expected to excrete valine. On testing, this mutant was indeed found to feed a valine mutant heavily, and paper chromatography on the culture filtrate showed a dense spot corresponding to valine. While in this case, as in those of tyrosine and phenylalanine, neither microbiological assay nor paper chromatography alone furnishes complete proof that the compound excreted is the amino acid rather than a related compound, it seems unlikely that the non-specificity of these two techniques would overlap to give a false answer when used together.

In several instances, therefore, interference with synthesis of one amino acid leads to excessive synthesis of another. This evidence encourages us to feel that the mutants lend themselves not only to determining the normal steps in biosynthesis, but also to unravelling the mechanisms of integration by which the normal cell determines that its metabolites should be synthesized in proper proportions and not wasted. Recent experiments have shown that this economy of the organism is upset in diverse ways by a single mutation. A tyrosine mutant, for example, not only excretes phenylalanine, but also, to a smaller degree, feeds the mutants requiring tryptophan, lysine, valine, and leucine. This type of study has just begun, but it is already clear that the interrelations between various biosynthetic paths are complex (Fig. 4).

Precursors of aromatic compounds

The mechanism of biosynthesis of aromatic compounds has long been a subject of speculation. The availability of mutants with multiple aromatic requirements (Fig. 4) offers an opportunity to obtain definite information on this matter. A wide variety of aromatic compounds, with single and multiple sub-

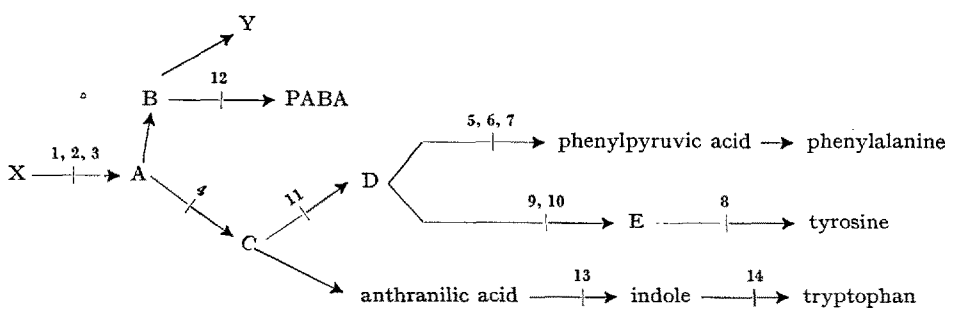
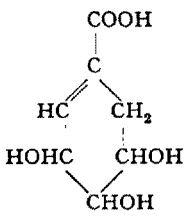


Fig. 4.–Scheme of aromatic synthesis.

stitutions of carboxyl, amino, and phenolic groups, were tested for their capacity to substitute for the multiple requirements of the aromatic-less mutants. None of these was effective. Similarly cyclohexane carboxylic acid, cyclohexanol, and inositol were not utilized. Shikimic acid, however (suggested and furnished by R. STANIER) was used by aromatic-less mutants with quadruple, but not with triple or double requirements.



shikimic acid

It therefore appears that the precursor of the benzene ring is at least partly saturated. These mutants do not use quinic acid, in which the double bond of shikimic acid is hydrated.

It will be noted that Fig. 4 contains an unknown "end-product", presumably aromatic, labelled Y. The evidence for the existence of this postulated compound is as follows. (a) The aromatic-less mutants with quadruple requirements require more PABA than does the PABA mutant, and their growth is accelerated by high concentrations of PABA, suggesting that the synthesis of PABA may be reversed, to yield a precursor common to PABA and an unknown compound. (b) Growth of the aromatic-less mutants on large amounts of the four aromatic compounds is slow; similarly, growth on shikimic acid alone is slow. But shikimic acid plus the three amino acids yields much faster growth, practically as fast as wild-type. These results suggest that shikimic acid may occupy position B, rather than A, serving rapidly as a precursor of Y plus PABA (for which PABA alone serves only slowly), but serving only slowly, by reversal of the normal process, as precursor for the amino acids.

The scheme of Fig. 4 must be considered quite tentative. Certain phenomena are difficult to explain, such as the fact that mutants with a double aromatic requirement (tyrosine plus phenylalanine) heavily feed the quadruples, while the triple neither feeds the quadruples nor is fed by the doubles. One must therefore consider more seriously the possibility, always theoretically present, that some of these multiple requirements may depend on internal inhibition, as with isoleucine and valine, rather than on block at an early stage of synthesis.

Partial back-mutants

Almost all the mutants are detectably unstable; that is to say, spontaneous reversions to nutritional independence (prototrophs) occur with a high enough frequency (10^{-7}–10^{-8}) to give rise after several days to a few large colonies of back-mutants in a streak on a medium which is sufficiently enriched to permit limited growth (cf. Fig. 2). For this reason practically all of our experiments, even on quantitative response, are carried out on solid media; in tubes of liquid media the greater precision of measurement by turbidimetry is accompanied by greater difficulty in distinguishing growth of back-mutants from that of parent mutants. Maintaining transfer cultures of mutants in liquid media, however, has caused no difficulty provided selection of back-mutants is avoided by the presence of an excess of the nutrient requirement.

Our study of back-mutants had a casual origin which is pointed out here since it illustrates the possibility, based on the uniform colonial growth of bacteria, of encountering interesting phenomena by simple observations on plates. Two presumptive back-mutant colonies from a PABA-less mutant were isolated and streaked on minimal medium, along with wild-type, to verify their nutritional independence. One of these grew as rapidly as wild-type; the other was by chance observed at an early time (18 hrs.) to form slightly smaller colonies than wild-type. Further study showed that the slightly slow prototroph grew as rapidly as wild-type in a medium supplemented with PABA; it apparently had recovered the capacity to synthesize PABA, but not rapidly enough to permit optimal growth.

Since PABA is one of the factors excreted by wild-type in large amounts, this hypothesis was easily tested. The two PABA back-mutants were compared with wild-type for their output of PABA on minimal medium, by pouring plates of minimal medium containing few cells of the prototroph and many PABA-requiring cells. (This technique is more sensitive to slight differences in syntrophism than is the technique of adjacent streaks.) As expected, the wild-type colonies were surrounded by an extensive halo of satellites, while the slow prototroph had none. This fact confirmed the earlier conclusion that its growth rate was limited by its rate of synthesis of PABA; it had none to spare. Unexpectedly, however, the other back-mutant, previously indistinguishable from wild-type, was surrounded by a smaller halo of satellites than was wild-type. Apparently its recovered capacity to synthesize PABA was not as great as that of wild-type.

Following this a dozen different back-mutant strains, spontaneous and ultraviolet induced, were isolated from a PABA mutant; among these 6 different rates of growth in the absence of PABA were recognized. In addition, all were more susceptible than wild-type to sulfonamide inhibition, which is not surprising since all synthesized PABA at a lower rate than wild-type. Some of the slower strains had their growth rate

restored to optimal by the addition of PABA; others did not. Similar studies were carried out with mutants requiring 6 amino acids, purines, biotin, and pantothenic acid. In all cases but one amino acid, a variety of degrees of back-mutation were observed; in only a fraction of the strains were slow growth rates restored to optimal by addition of the parent's growth requirement.

In the absence of the required genetic techniques it has not been possible to demonstrate whether these various degrees of restoration of a nutritional deficiency represent quantitative alleles of the same gene, or mutations in other genes which modify the deficiency. In the cases where the back-mutant is slow either with or without the growth factor, an allelic change seems much less likely than does a mutation of another gene which imposes a rate-limiting alteration of metabolism at the same time that it restores the deficiency. In any event, it is clear that back-mutants, which have lent themselves to quantitative genetic studies, represent a genetically very heterogeneous class. Indeed, it is not evident that any of the back-mutants are truly identical with wild-type. Perhaps they should be understood as "backward" mutants rather than true back-mutants or reversions, having mutated in the direction, but not necessarily to the precise position, of wild-type. One study of back-mutants of Neurospora[1] has failed to reveal such frequent and varied partial restorations of a deficiency. Incidentally, in this connection, it should be noted that mutations from wild-type to partial deficiencies of various factors are frequent, with Neurospora as well as with bacteria; they are not often mentioned in the literature since they have not seemed to lend themselves to genetic or biochemical analysis as well as mutants with absolute requirements.

Reversion of multiple requirements

Another genetic question, more essential for biochemical investigation, is whether certain multiple requirements arise from a single mutation and hence presumably from a change in a single enzyme. While we have not tried to solve this problem directly by recombination techniques, it has been possible to circumvent it by use of back-mutations[2]. In several instances it has been easy to obtain spontaneous prototrophs, with no growth requirements, from mutants with multiple requirements (e. g. quadruple and double aromatic), and has not been possible to obtain split reversions, with loss of only part of the requirements. From these results it is inferred that a single mutation has occurred and then reverted. In another instance, requiring histidine and the three aromatic amino acids, it has been possible, in appropriate media (limited histidine, excess of the other requirements) to lose the histidine requirement alone, but it has not been possible to lose all four requirements in one step. It is therefore concluded that this mutant had been altered in two separate steps. Non-allelic reversions from mutant to apparent wild-type in higher organisms have been described in which a mutation of a second gene at a different locus (modifier gene) restores the normal condition. In the absence of a test for allelism it cannot be decided whether our reverse mutations involve a change of the same gene or of another one. It is conceivable that a mutation of a modifier gene might suppress two independent growth requirements. But whatever the genetic mechanism of the reversions may be, one would expect with a double mutant to recover the single back-mutants as well as the prototrophs. Complete failure to isolate the single reversions, together with isolation of the total reversions, is therefore considered excellent evidence for a single mutation producing a multiple requirement. On the other hand, it should be pointed out that the recovery of split reversions is not conclusive evidence for the presence of independent mutations, unless it is accompanied by failure to isolate any total reversions. With the isoleucine-plus-valine mutant, for example, one back-mutant was obtained with a relative requirement for valine (slow growth without it), but no requirement for isoleucine. Since many completely prototrophic back-mutants were also obtained from this strain, a probable mechanism for the split reversion would be incomplete restoration of the isoleucine enzyme, leaving enough residual accumulation of the intermediate to inhibit valine synthesis.

This stress on the importance of negative as well as positive results is warranted only because of the great efficiency, essentially 100%, with which reversions to nutritional independence can be isolated from huge bacterial populations.

Delayed phenotypic expression of back-mutation

The number of spontaneous back-mutant (prototroph) colonies observed on a minimal medium plate is proportional to the size of the inoculated population. In a medium with limited enrichment, in contrast, the inoculum grows until the population reaches a size which is limited by the amount of enrichment; the mutants observed are therefore largely "plate-mutants" (i.e., those arising during generations occurring on the plate), and their number is, as a first approximation, a function of the enrichment rather than the inoculum size. Following ultraviolet irradiation, however, an entirely different situation was encountered.

Ultraviolet irradiation increases the frequency of back-mutants among the survivors by as much as many

[1] N. H. GILES, JR., and E. Z. LEDERBERG, Amer. J. Bot. 35, 150 (1948).

[2] R. R. ROEPKE, quoted by E. L. TATUM, Cold Spring Harbor Symp. Quant. Biol. *11*, 278 (1946).

thousandfold. Under these circumstances, the number of plate-mutants in a heavily inoculated plate with limited enrichment should be only a negligible fraction of the number of mutants inoculated. The number of ultraviolet induced prototroph colonies observed would therefore be expected to be relatively independent of the enrichment. To our surprise, with certain mutants the dependence was found to be extreme. With a washed inoculum of a mutant, almost no ultraviolet-induced prototrophs could be detected unless the medium was enriched with a trace of the factor required by the parent strain. To look at one instance: an inoculum of 10^8 cells of a tryptophan-requiring mutant yielded 1 to 3 visible back-mutant colonies after three days on minimal agar, and 1 to 6 colonies on agar slightly enriched with 0·01 to 0·5 γ/ml of tryptophan. The suspension was irradiated until about 2 and 50% of the cells survived, and the same volumes were inoculated. On minimal agar, 0 to 20 colonies developed; on slightly enriched agar, 200 to 600! Of these, 25 were picked at random, ranging from the large to the microscopic. All were prototrophs, growing on minimal agar, though many of the smallest grew very slowly. Qualitatively similar observations have been made with several amino acid, purine, and vitamin requiring mutants.

It appears that a certain amount of growth by the parent strains is necessary to permit the ultraviolet-induced back-mutants to get started. It is known, however, that small inocula of various bacteria often fail to initiate growth in a medium which is adequate for larger inocula; the small inocula are presumably unable to accumulate enough CO_2 or other essential metabolites. To rule out the possibility that the growth of the parent strain was promoting the appearance of back-mutant colonies by some such non-specific mechanism, variously enriched plates were inoculated with an irradiated strain, an unirradiated strain with a different requirement, and a mixture of the two. Prototrophs appeared in large numbers only from the irradiated strain, and when the medium was supplemented with its growth factor rather than that of the companion unirradiated strain.

We are therefore brought back to the consideration of the phenomic lag, introduced into this work at the outset by the initial failures of the penicillin method. It now appears that a lag occurs not only in exhausting premutational enzymes, but also in building up new enzymes which the mutated cell is capable of constructing; to build up the new enzyme the cell requires a complete set of building blocks, including the product of the previously deficient enzyme. In other words, to start the cycle, the pump must first be primed. This phenomenon stands in contrast to the behavior of certain adaptive fermentative enzymes, which are reported to be formed by bacteria or yeasts in a nitrogen-free medium, in which no net growth can take place.

Use of mutants in studying mechanisms of bacterial inhibition

D-Serine exerts a marked inhibitory effect on the growth of our wild-type strain of *E. coli;* many amino acids are able to antagonize the inhibition, but aspartic acid, in contrast, enhances the effect, although by itself it has no influence on growth[1]. Werner Maas, further studying this problem in this laboratory, found that the inhibition is overcome by extremely low concentrations of pantothenic acid, and by somewhat higher concentrations of β-alanine. The antagonistic action was apparently non-competitive with pantothenate and competitive with β-alanine. The results suggested that β-alanine is the substrate and pantothenate the product of the inhibited reaction. This type of inhibition analysis has been widely used to determine whether a given antagonist serves as substrate, precursor, or product of the inhibited reaction. For several reasons, however, which are discussed elsewhere[2], it seems difficult to draw rigorous conclusions as to the site of the inhibition, especially in those cases where only a narrow range of concentration of the inhibitor is possible before other reactions become affected. With appropriate mutants, on the other hand, it is possible to dissect out the system under investigation and produce more direct evidence of the site of inhibition. In addition, mutants show whether an apparent product of the inhibited reaction, acting non-competitively on wild-type, has done so in "physiological" concentrations.

Mutants blocked at various stages in the synthesis of pantothenic acid were therefore isolated (Fig. 3). A mutant blocked in the synthesis of β-alanine showed a competitive relation between β-alanine and D-serine; a mutant blocked in the synthesis of pantoic acid showed inhibition by D-serine when grown on pantoic acid, but none when grown on pantothenic acid; and a mutant blocked in the formation of pantothenic acid grew in proportion to the amount of pantothenic acid present, regardless of the presence or absence of D-serine. It has therefore been demonstrated, more conclusively than would be possible with wild-type alone, that D-serine (or a product of it) interferes with the conversion of β-alanine to pantothenic acid[2]. Similarly, salicylic acid, which is known to be antagonized by pantothenic acid[3], has been found[4] to interfere with the synthesis rather than the utilization of pantoic acid. Furthermore, several of the amino acids which antagonize D-serine inhibition of wild-type were tested against D-serine inhibition of a mutant unable to synthesize β-alanine. No antagonism of the inhibition

[1] B. D. Davis and W. K. Maas, J. Amer. Chem. Soc. *71*, 1865 (1949).
[2] W. K. Maas and B. D. Davis, to be published.
[3] G. Ivanovics, Acta Med. Szeged *12*, 1 (1944).
[4] W. K. Maas, unpublished observations.

was observed. This result indicates that these amino acids antagonize D-serine only indirectly, acting via the final common path of β-alanine synthesis. Finally, serine-resistant and salicylate-resistant mutants have been isolated and are under investigation. We feel that the pitfalls in interpreting the mode of action of antibacterial agents can sometimes be avoided by the combination of biochemical studies with the use of appropriate mutants.

In connection with the studies on pantothenic acid, certain pantothenate-requiring mutants have been observed to respond to either pantoic acid or to pantonine (the α-amino analogue of the α-hydroxy acid, pantoic acid); others respond to pantoic acid only (Fig. 3). It may be inferred that pantonine is either a normal precursor of pantoic acid, or is convertible to such a precursor.

A number of the problems described above are as yet only partly solved; yet so closely intertwined are the various metabolic sequences that each step toward a solution has revealed unexpected new problems. Herein lies much of the fascination of this field. It has been the main purpose of the present paper to illustrate the ease with which a variety of problems can be revealed and solved, up to a point, by simple methods: the isolation of desired bacterial mutants by the penicillin method, followed by direct observation of their behavior, especially with respect to syntrophism and back-mutation, on solid media. Some of the studies described in this paper will be published elsewhere in more detail.

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Zusammenfassung

In dieser Arbeit wird eine Methode beschrieben, die es gestattet, in größerer Zahl Stoffwechsel-Minusmutanten von Bakterien zu isolieren. Sie beruht auf der bemerkenswerten Eigenschaft des Penicillins, ausschließlich wachsende und sich vermehrende Bakterien abzutöten. Wird demzufolge eine große Bakterienkultur, die verschiedene, durch vorangegangene Ultraviolettbestrahlung herbeigeführte Mutanten enthält, in einem eben genügenden Nährmedium der Wirkung von Penicillin ausgesetzt, so wird die Mehrzahl der Kultur, die aus den normalen Bakterien des Stammes besteht, abgetötet, während die Minusmutanten, die nicht zu wachsen vermögen, selektiv überleben.

Mit Hilfe dieser Technik wurden Mutanten von *E. coli* mit individuell verschiedenen Ansprüchen auf nahezu sämtliche Aminosäuren, Vitamine, Purine und Pyrimidine isoliert. Einzelne Mutanten mit alternativen oder multiplen Stoffwechselbedürfnissen erlaubten Einblicke in den Gang von Biosynthesen. So wachsen eine Anzahl von «ringfreien» Mutanten, die gleichzeitig Tyrosin, Phenylalanin, Tryptophan und Paraaminobenzoesäure benötigen, auch in alleiniger Gegenwart von Shikimisäure, einem teilweise dehydrierten Derivat der Cyclohexancarbonsäure. Dies läßt vermuten, daß im Organismus aromatische Verbindungen aus wenigstens teilweise gesättigten Ringverbindungen entstehen können. Ferner ist bekannt, daß der Gang einer Biosynthese durch die Isolierung von Zwischenprodukten analysiert werden kann, die von gewissen Mutanten angestapelt werden. Derartige Ansammlungen lassen sich technisch mit Hilfe von Bakterien leicht nachweisen, wenn verschiedene Mutanten, die sich gegenseitig ernähren, nebeneinander ausgestrichen werden. Nicht nur werden so Stoffwechselvorstufen (precursors) angestapelt; in gewissen Fällen führt das Unvermögen, eine bestimmte Aminosäure (z. B. Tyrosin, Phenylalanin, Isoleucin) zu synthetisieren, zu Anhäufungen anderer Aminosäuren. Diese Beobachtung leitet zur Annahme, daß normale Zwischenstufen einer Verbindung einen regulierenden Einfluß auf die Synthese anderer Stoffe ausüben.

Ferner lassen sich diese Mutanten gut verwenden, um die Wirkungsweise bestimmter antibakterieller Wirkstoffe endgültig festzulegen. Es konnte nachgewiesen werden, daß die Umwandlung von β-Alanin in Pantothensäure durch D-Serin verhindert wird, während Salicylsäure auf die Bildung der Pantoinsäure störend einwirkt.

Durch Ultraviolettbestrahlung hervorgerufene Mutanten, sowohl Stoffwechsel-Minusvarianten als Rückmutierungen zur Norm, brauchen eine bestimmte Zeit, bis sie phänotypisch erkennbar sind, was darauf schließen läßt, daß sich die Fermentzusammensetzung erst nach einer Einstellungsphase der neuen Genzusammensetzung angleicht (phenomic lag).