Reprinted from

METHODS IN
Medical Research

Volume 3

RALPH W. GERARD, *Editor-in-Chief*

GENETICS OF MICRO-ORGANISMS, *S. E. Luria, Editor*

ASSAY OF NEUROHUMORS, *J. H. Gaddum, Editor*

SELECTED PSYCHOMOTOR MEASUREMENT METHODS,
Walter R. Miles, Editor

METHODS FOR STUDY OF PEPTIDE STRUCTURE, *Choh Hao Li, Editor*

THE YEAR BOOK PUBLISHERS, INC.

200 EAST ILLINOIS STREET, CHICAGO

Lederberg

Isolation and Characterization of Biochemical Mutants of Bacteria

J. LEDERBERG, *University of Wisconsin*

ALTHOUGH MOST mutations may be ultimately referable to biochemical changes, the term "biochemical mutant" generally refers to a mutant detected by its effects on the nutrition or on a specifically recognizable enzymatic process of the organism (1).

The use of mutagens to increase the proportion of mutants is discussed by Witkin (pp. 23 ff.). The methods presented here aim at separating mutant cells from the nonmutants which usually outnumber them. The techniques have been applied especially to *Escherichia coli,* among bacteria, but have also been used with other organisms.

Media.—The recognition of a nutritional mutant depends on a comparison of its growth on "minimal" and "complete" medium. These terms are relative to the purposes of the investigation. A minimal medium consists only of components essential for the

TABLE 1.—MEDIA FOR *Escherichia coli**

A. MINIMAL MEDIUM† (B. D. Davis)	
Glucose	1
K_2HPO_4	7
KH_2PO_4	2
Na_3 citrate$\cdot 5H_2O$	0.5
$MgSO_4\cdot 7H_2O$	0.1
$(NH_4)_2SO_4$	1

B. COMPLETE MEDIUM	
Casein digest (N Z Case)	10
Yeast extract	5
K_2HPO_4	3
KH_2PO_4	1
Glucose	5

C. MINIMAL EMB MEDIUM ("EMS") (20)	
Sugar	10
Sodium succinate	5
NaCl	1
$MgSO_4$	1
K_2HPO_4	2
$(NH_4)_2SO_4$	5
Eosin Y	0.4
Methylene blue	0.065

D. COMPLETE EMB MEDIUM	
Sugar	10
Casein digest (N Z Case)	8
Yeast extract	1
NaCl	5
K_2HPO_4	2
Eosin Y	0.4
Methylene blue	0.065

*Agar is always used at a final concentration of 1.5% if required. All concentrations are g/l of distilled water. The media may be prepared by adding the materials and autoclaving together. However, minimal medium A is better prepared by sterilizing the sugar (and agar) in a separate aliquot and mixing with the sterilized solution of the other constituents just before pouring agar plates.

†Trace elements have been dispensed with, being present in adequate amounts in other chemicals of ordinary chemical purity.

growth of the wild-type strain; a complete medium contains a variety of supplements covering the range of interest of growth factors for which mutants are to be sought. Yeast extract, peptones and similar organic preparations are commonly used as complex supplements which are expected to contain adequate amounts of such growth factors. When specific mutants are being looked for, however, it is important to verify that the "complete" medium is sufficiently enriched in the particular factor and that it does not contain antagonists which might interfere with the expected responses (23).

Media suitable for *E. coli* are tabulated in Table 1. They are certainly not the only formulae that will prove satisfactory; for specific purposes it will always be necessary to formulate the appropriate substitutes.

RANDOM ISOLATION OF NUTRITIONAL MUTANTS (2, 8)

PROCEDURE

Cells grown from an irradiated inoculum in a complete medium are diluted to 100–500/sample and poured into or spread on the surface of complete agar plates. After 24 hr of incubation, colonies are picked at random with a fine platinum needle. It is convenient to cool the needle in a small tube containing 1 ml of synthetic medium, touch the colony lightly, immerse it in the 1 ml tube, and then spot the needle on a complete agar plate. One can also use a 2d tube containing complete medium for maintaining the isolate. After 24 hr the tubes or spots on complete agar corresponding to clear minimal tubes (no growth) are transferred to slants for later verification as mutants. Where a spot shows no growth, there may not have been successful inoculation into the minimal medium, and the test is discarded.

Evaluation.—Tatum (35) recovered about 1% characterizable mutants from *E. coli* treated with x-rays or nitrogen mustard. Roepke and Mercer (29) obtained yields of 1–2% from cells plated immediately after x-ray treatment. They noted that storage of treated cells in electrolyte-containing medium resulted in a markedly lower yield. In *Bacillus subtilis,* Burkholder and Giles (4) obtained 3–6% yields of mutants, a high yield which may be related to the resistance of their material to lethal effects of radiation. Substantially the same procedure has been used for Neurospora, where Beadle and Tatum (2) report the isolation of 380 mutants from 68,000 tested ascospores, or about ½%. However, the genetic procedure used in Neurospora is only 50%

efficient because the ascospores are the progeny of a cross of treated with untreated nuclei.

DELAYED ENRICHMENT (LAYER PLATING) (21)

Much of the work needed to obtain a collection of mutants is spent on separating them from their nonmutant neighbors, which often predominate 100:1. The most direct but least efficient procedure is random isolation followed by individual test on minimal medium. By delayed enrichment of minimal agar plates, several hundred colonies can be screened at once.

PROCEDURE

Plates are prepared with 15–20 ml of minimal agar medium and allowed to solidify (bottom layer). The bacterial suspension is diluted to contain 200–400 cells/sample; each sample is incorporated in a few ml of minimal agar and poured over a plate (seeded layer). When this has hardened, 5–10 ml of minimal agar is poured on top (cover layer). Thus all the colonies will develop in the agar and will not be disturbed by subsequent manipulation. The seeded plate is incubated for 24 hr or longer to permit the development of prototroph cells (i.e., cells as nutritionally competent as the wild type) into uniform large colonies. The mutant cells will have developed very slightly, if at all, and will be invisible to the naked eye. They are brought to view by pouring a few ml of complete agar over the layer plates (supplement layer). The growth factors diffuse down through the agar and cause growth of the mutants, which are picked up as small or as new colonies after 6–12 hr of further incubation. With some practice and use of a fine needle, the tiny mutant colonies can be picked to complete medium for verification.

Modifications.—Other methods of delayed enrichment suggest themselves. The supplement need not be poured on top, but may be injected below the bottom layer with a hypodermic needle through a penicillin cup, or placed in a penicillin cup itself, or may be poured in a trench cut out of the minimal agar either with a sterile spatula or by pulling up a strip of cloth sterilized with the plates. These and other modifications, e.g., transfer of the entire agar layer to a plate of complete agar, may be particularly useful in dealing with aerophilic bacteria or molds which will not develop readily as deep colonies.

MARGINAL OR LIMITING ENRICHMENT (6)

A marginal or limiting concentration of growth factor can usually be found at which a mutant will form colonies character-

istically smaller than prototrophs in a supplemented minimal agar. This principle is especially useful in counting a particular mutant (whose quantitative responses are already known) in the presence of a preponderance of prototrophs or of other mutants (31, 6).

The greatest drawback of this procedure is the uncertainty of the proper concentrations of supplements to use. One can use a graded series of concentrations in the hope of finding the correct level empirically, though uneconomically.

In principle, a more rational pre-estimate can be made by determining the content of a given factor in hydrolysates of wild-type bacteria grown on minimal medium. On the assumption that the wild-type content is not very different from the needs of the organism, the amount of supplement which will limit growth of a mutant can be calculated from this content. This assumption also implies that, in general, a level of bacterial hydrolysates which suffices for any 1 growth factor requirement will also be suitably rich in other factors. Conversely, a concentration of hydrolysate that is marginal for 1 growth factor should be approximately marginal for other substances, known and unknown. Therefore, a concentration of bacterial hydrolysate that suitably limits the growth of any mutant already isolated should be appropriate for the isolation of new mutants.

Since the assumptions made here, that biosyntheses are not markedly over- or underproductive and that losses of growth factors in preparing hydrolysates are uniform, hold only very approximately for many factors, these generalizations are far from precise. However, the technique has been successfully employed for the isolation of mutants of *E. coli* and of *Azotobacter agilis* (12) both by using graded concentrations (6) and by using bacterial hydrolysates (19).

Comment by Bernard D. Davis

A possible advantage of limiting enrichment is that it does not require the survival of isolated nongrowing cells, a consideration that may be important with organisms less hardy than *E. coli*.

ISOLATION OF MUTANTS BY PENICILLIN (4a, 5, 22)

Considerable improvement can be achieved with penicillin. Its application depends on the fact that the bactericidal action of penicillin is only exerted on cells in a medium in which they could grow in the absence of the antibiotic. In a synthetic medium in which biochemical mutants are unable to grow, a differential between the survival of mutants and prototrophs after treatment

with penicillin permits of considerable concentration of mutants. With Salmonella and *E. coli* K-12, the logarithmic ratio is of the order of 2; i.e., for each 10-fold reduction in viable mutants, the prototrophs are diminished 100-fold. 10^{-6} is the largest degree of killing that leaves a convenient number of survivors; this will often result in 1000-fold augmentation of the ratio of mutants to prototrophs in the population treated in synthetic medium.

PROCEDURE

A suspension of bacteria (grown in a complete medium from an irradiated inoculum to a density ca. 2×10^9 cells/ml) is washed *twice* with sterile saline and diluted to 20 times the original volume in minimal medium. A freshly prepared solution of penicillin is added to yield a final concentration of 300 units/ml. The tubes are incubated at 37 C on a shaker for 4–24 hr. Samples of 0.1, 0.01 and 0.001 ml are spread on complete medium plates. After 24 hr incubation, isolated colonies are picked at random and tested for growth on minimal medium. In successful runs, half the colonies may show no growth on the 1st test, but ⅓ or more of these supposed mutants may subsequently behave as prototrophs after subculture on complete medium. No satisfactory explanation has been found for this transitory behavior, which parallels that of the "mutants" described by Peacocke and Hinshelwood (27) and of chilled cultures of *Achromobacter fischeri* (26).

Evaluation.—The penicillin method is undoubtedly the most efficient but the most erratic. There appears to be considerable difference among strains of *E. coli* and Salmonella in sensitivity to penicillin. Adjustments in the time of treatment and concentration of penicillin must be made empirically. For isolation of the widest range of mutants, especially those with vitamin requirements, it may be desirable to use lower initial cell concentrations (5). However, *E. coli* mutants requiring thiamine, nicotinamide and pantothenate have been isolated with the described procedure.

Freshly irradiated cells should not be subjected directly to the penicillin selection. In our laboratory, cells grown several hours or overnight in complete broth from irradiated inocula were used uniformly on theoretical grounds, to permit of nuclear segregation (8, 19). Davis (5), however, ascribed the beneficent effects of preincubation to the exhaustion of biosynthetic mechanisms which might persist for a time and permit growth after the occurrence of genetic changes that suppress syntheses. Probably both effects play a role. At any rate, the caution that treated popula-

tions be grown for some time after irradiation before applying selective methods for isolating mutants has been empirically justified.

If specific classes of mutants are desired, the synthetic medium can be supplemented with the irrelevant growth factors, so that unwanted mutants are killed along with prototrophs.

The mutants obtained by the penicillin methods can be subjected to a 2d treatment to produce double mutants (the penicillin medium should, of course, now contain the growth factors needed by the original mutant). This is possible because, in general, the mutant survivors of this technique are not resistant to penicillin.

The 300 units/ml concentration of penicillin is required because of the low penicillin sensitivity of organisms such as Salmonella or Escherichia. Lower concentrations may be sufficient for other organisms.

Comment by Bernard D. Davis

In the penicillin method the chief source of sterilization of mutants appears to be the syntrophic effect of traces of growth factors excreted by the wild-type organisms. In some cases the mutants show no sterilization by penicillin until the wild-type population has reached densities exceeding 10^6 cells/ml. Since large inocula increase the number of mutants present but decrease the efficiency of survival, we expose a variety of population densities to penicillin for long periods, then plate out in the largest amount compatible with elimination of the penicillin effect by dilution. We usually inoculate in 3 ml of minimal medium containing penicillin (300 units/ml), 10^{-1}, 10^{-2} and 10^{-3} ml of a turbid suspension of washed cells, previously irradiated and grown overnight. We incubate for 4–24 hours (without shaking), then plate 0.1 ml from each tube in minimal and in enriched medium. Pour plates seem preferable to surface inoculation because of the danger of sterilization of mutant cells when exposed to nutrilites in the presence of a high, though transient, local concentration of penicillin. Colonies are usually picked only from those enriched plates which show a significantly higher colony count than the corresponding minimal plates, although mutants are frequently obtained even when no such differential is evident.

A modification of the method, which substantially improves its efficiency (6a), consists in preincubating the washed organisms for 4–6 hours in a medium lacking nitrogen before placing them into penicillin. Preincubation helps exhaust stored metabolites. The minimal medium is then completed by addition of ammonium sulfate, and penicillin is added. The preincubation particularly improves the recovery of certain vitamin-requiring mutants.

Since crude penicillin may be contaminated by growth factors, it is desirable to use only crystalline material. Penicillin solutions are stable in the refrigerator for only a few days, but may be kept for at least a month in

the freezing compartment of a refrigerator. A salt-ice mixture may be necessary initially to induce freezing.

CHARACTERIZATION OF BIOCHEMICAL MUTANTS (2)

Two approaches are available for determining the requirements of nutritional mutants: single-additions and single-omissions. By the 1st method, possible growth factors are added singly and in mixtures to determine minimal requirements for growth of the mutant. By the 2d approach, a comprehensive mixture of growth factors that will support growth of the mutant is prepared, and single factors or groups of factors are then omitted to determine those whose removal prevents growth of the mutant. The single-additions method fails when the mutant requires multiple supplements; the single-omissions when 1 growth factor can replace another (e.g., glutamic acid and proline, or methionine and cystine). For routine purposes, single additions are more economical and convenient, especially if certain complex requirements, which have been discovered empirically, are kept in mind (*vide infra*).

PROCEDURE

Most mutants require 1 of the following substances: (*a*) amino acids, (*b*) water-soluble vitamins, (*c*) purine bases and similar nucleic acid components, or (*d*) further components, known or unknown, of complex supplements such as malt or yeast extract. In tracking down the specific requirement of a mutant, it is convenient 1st to classify it in 1 of 4 categories by inoculating the mutant into minimal media supplemented as follows: (1) no addition; (2) 0.1% acid-hydrolyzed casein (vitamin-free) plus 0.5 μg/ml tryptophane; (3) a mixture of water-soluble vitamins (see Table 2); (4) 0.1% alkali-hydrolyzed yeast nucleic acid, and (5) 0.3%

TABLE 2.—RECOMMENDED LIST OF WATER-SOLUBLE VITAMINS WITH CONCENTRATIONS* SUGGESTED FOR *Escherichia coli*

1.	Thiamine	0.001	
2.	Riboflavine	0.5	
3.	p-aminobenzoic acid	0.1	(0.001)
4.	Nicotinic acid	0.1	
5.	Pantothenic acid	0.1	
6.	Pyridoxine	0.1	
7.	Pteroylglutamic acid	0.01	
8.	Choline	2	
9.	Inositol†	1	
10.	Biotin	0.0001	(0.001)
11.	"Vitamin K" (methyl-naphthaquinone)	1	
12.	"Vitamin B_{12}" (Cobione)	10^{-6}	(0.001)

*In mg/l. Concentrations given are believed to be in considerable excess. Values given in parentheses were found adequate for *E. coli* mutants in our laboratory.

†*Inositol is believed to be absent from cells of E. coli.*

yeast extract. According to personal preferences, one may conduct these growth tests either in tubes of liquid medium or on agar plates. Inocula are most conveniently taken by suspending a loopful of growth from an agar slant and diluting so that the suspension is only barely turbid. A drop or loopful of such a suspension is then added to the liquid medium tubes or brushed on the surface of the supplemented minimal agar plates. Usually 24 hr is a suitable time for incubation, but this will depend on the organism. Since almost all of the supplements may be more or less contaminated with traces of vitamins, a response to the vitamin mixture takes precedence over the others. After a specific vitamin requirement has been established, it is left to the investigator's judgment whether to regard additional responses to other compounds as due to simple contamination of the chemicals or to more subtle replacement mechanisms.

Once the major group response is established, it remains to determine which compound or compounds within the group are active. If an amino acid requirement is indicated by growth on hydrolyzed casein, it is preferable not to proceed with tests on 28 individual amino acids, but instead to categorize the mutant within an amino acid subgroup. The amino acids may be subdivided arbitrarily, if desired, but Table 3 represents an attempt to

TABLE 3.—LIST OF AMINO ACIDS WITH RECOMMENDED GROUPINGS*

1. Lysine, arginine, methionine, cystine
2. Leucine, isoleucine, valine
3. Phenylalanine, tyrosine, tryptophane
4. Histidine, threonine, glutamic acid, proline, aspartic acid
5. Alanine, glycine, serine, hydroxyproline
6. (Optional miscellaneous): nor-valine,† nor-leucine,† alpha-amino-butyric acid, cysteine

*It is suggested that all amino acids be administered at a concentration of 10 mg/l, except cystine (50 mg) and threonine (20 mg). When available, the L- (natural) isomers are preferable, but the DL- compounds are generally suitable for routine purposes (however, see 6b).

†Probably not naturally occurring amino acids.

group them to facilitate the characterization of mutants that may have complex or alternative requirements. Once the amino acid subgroup is settled, there is a much smaller number of single-addition or single-omission tests to be made within the group. In *E. coli* K-12, L-valine by itself is inhibitory and must be accompanied by isoleucine in growth tests.* Also, in this strain (and others), methionine plus lysine is a fairly common complex requirement. Methionine or cystine, proline or glutamic acid, and glycine or serine exemplify alternative requirements that have

*DL-serine inhibition of *E. coli* was found to be due to the D-isomer (6b).

frequently been found and for which there is an obvious biochemical basis. The distinction of complex requirements from double deficiencies, occasionally produced by radiation, has been discussed by Davis (6a).

Table 4 lists the substances which may be tested in the nucleic acid "breakdowns."

TABLE 4.—Components of (Yeast) Nucleic Acid and Related Extractives*

Purines†
xanthine, hypoxanthine, adenine, guanine

Pyrimidines†
uracil, thymine, *cytosine*

Nucleosides†
[ribose] uridine, cytidine, xanthosine, inosine, adenosine, guanosine
[desoxyribose] *thymidine*

Nucleotides†
adenosine-3-phosphoric acid (yeast adenylic acid)
adenosine-5-phosphoric acid (muscle adenylic acid)
xanthylic acid, guanylic acid, uridylic acid, cytidylic acid

*Italicized compounds may be difficult to obtain from commercial sources.
†Suggested concentration, 10 mg/l.

Previous experience in biochemical genetics (cf. 3) has shown that many mutants can be characterized more precisely than in terms of the end-metabolite if the stage at which the synthesis is blocked can be found. Table 5 gives some *ad hoc* suggestions for tests on particular kinds of mutants which may help to distinguish them further.

TABLE 5.—Suggestions for Further Tests after Primary Characterization of Nutritional Mutants

If Mutant Responds to:	Also Test (or Retest):
Arginine	Ornithine, citrulline
Tryptophane	Indole + serine, anthranilic acid, kynurenine, nicotinic acid
Cystine or methionine	Sulfite, sulfide, thiosulfate, thioglycollate, homocystine, cystathionine
Proline	Glutamic acid, ornithine, alpha-ketoglutaric acid
Tyrosine	Phenylalanine
Thiamine	"Vitamin-thiazole;" "Vitamin-pyrimidine," methionine, thioformamide, Buta-3-one-1-ol (acetopropanol)
Pteroylglutamic acid	p-aminobenzoic acid
Nicotinamide	Nicotinic acid, hydroxyanthranilic acid, and compounds listed with tryptophane
Pantothenic acid	Pantoyl lactone, beta-alanine, aspartic acid
Pyridoxine	Pyridoxal, pyridoxamine, thiamine
Choline	Ethanolamine, N-methylaminoethanol, N-dimethylaminoethanol
Biotin	Desthiobiotin, pimelic acid
Isoleucine	Alpha-aminobutyric acid
Vitamin B_{12} or PABA	Methionine

Comment by Bernard D. Davis

Attention has naturally been concentrated mainly on mutants with absolute requirements. Mutants of 2 other classes are, however, quite common: those with relative requirements, which grow slowly on minimal medium and rapidly with the proper supplement, and "slow-growers," which cannot be hastened by any available supplements. As might be expected, such mutants are isolated less frequently by the penicillin method than by the methods in which small colonies are selected. Mutants of these types are also frequently found among the reversions, spontaneous or ultraviolet-induced, from strains with absolute requirements.

Interpretation of negative results.—Frequently, mutants are found which respond to yeast extract or even to hydrolyzed casein but which cannot readily be classified further. Before such a mutant is labeled as requiring a new or unknown growth factor, the following possibilities should be considered.

1. A requirement for a balanced amino acid combination. E.g., certain Neurospora mutants requiring arginine are very sensitive to inhibition by lysine; another mutant requires isoleucine and valine in a very precise ratio (3). A single-omission test series usually will give the necessary clues as to the compounds required. It may be necessary to test a number of amino acids in varying proportions before optimal growth responses are obtained.

2. A requirement for fat-soluble factors. Sterols, unsaturated fatty acids and carotenoids are essential for certain organisms and should be considered as possible requirements for bacterial mutants, although such requirements have not been encountered. Such compounds can be incorporated into aqueous media with the aid of non-ionic detergents such as Tween 80 (concentration 0.1%).

3. Water-soluble factors not previously listed. Some of these—glutamine, glutathione and ascorbic acid—are very heat-labile and should be filter-sterilized before addition to autoclaved medium. Other compounds include ethyl alcohol, organic acids (acetic, malic, fumaric, succinic, lactic, pyruvic), strepogenin, pyridoxal phosphate, putrescine, hemin, hexose phosphates, cocarboxylase (thiamine pyrophosphate) and cozymase (diphosphopyridine nucleotide). It is impossible to offer an exhaustive list of potential growth factors.

4. The carbon, sulfur or nitrogen sources offered may be unsuitable for the mutant; e.g., some *E. coli* mutants cannot grow on glucose (7). Such a mutant would probably grow in yeast extract or in casein hydrolysate media, but in few with single synthetic supplements in small quantities. The solution is either to determine whether the carbon source offered is attacked in a

complete medium or to supply an alternative carbon source (e.g., asparagine). In Neurospora, mutants have been described which can assimilate ammonia but not nitrate, but ammonia is the usual nitrogen source in most media for bacteria. *E. coli* mutants have been described which cannot reduce either sulfate or sulfite, but which respond to sulfide (14). Owing to the preparative methods, many amino acids may be contaminated with sufficient sulfide to give confusing responses, but such mutants are usually first picked up as requiring cystine. Sulfide should be tried in any instance of a broad response to many amino acids. H_2S is sufficiently volatile to contaminate media autoclaved together with sulfide-supplemented media. Difco agar, unless repeatedly rinsed with water, is also contaminated with materials that allow growth of sulfide-requiring mutants.

5. Physical conditions. A supplemented medium might favor growth of a mutant by virtue of physical characteristics, such as pH, rH, ionic strength or osmotic pressure rather than its chemical constituents. These factors must be considered in analyzing mutants which do not give well defined responses to specific chemicals. Also, some mutants may have unique temperature responses which cause confusion if this parameter is not controlled. The usual pattern (25, 10) is biochemical deficiency (for known or unknown substances) at a higher temperature and competence at a lower. Since it is impossible to predict the characteristics of mutants so far not isolated, the choice of temperatures at which to conduct these tests is arbitrary. We have had fortunate results with 40 and 30 C as the differential temperatures in studies on *E. coli* and Salmonella mutants.

Modifications.—Choice of procedures depends largely on personal preferences, because the methods have not been documented precisely. The details of nutritional characterization tests—inoculum size, volume of medium, temperature and time of incubation, use of liquid vs. solid medium, etc.—must all be left to individual judgment.

The *auxanographic method,* devised by Beijerinck, has certain advantages (16). Here, washed cells of the mutant are heavily (about 10^6–10^8 cells/ml) and uniformly seeded in a thin top layer of minimal agar, over a prepoured minimal agar plate. After the agar has hardened and dried for 2 hr, the different supplements are dropped at different points on the agar surface. The supplements may be used either in solution or in the form of a few crystals. Sterile technique is unnecessary, but gross contamination should be avoided. A growth response is indicated by turbidity in a circular or annular zone where the growth factors have diffused.

Since this zone is usually less than 2 cm in diameter, there is usually sufficient room for a complete characterization of a mutant on a single plate. The plates can be read 6–10 hr after supplementation, and unsupplemented areas of the same plate can be used for single additions after the major grouping has been established. The advantages of this procedure are (*a*) erratic growth responses due to reversion are eliminated, since reverse mutations in the agar give rise to single colonies; (*b*) the diffusion gradient exposes the cells to a wide range of concentrations; (*c*) complex requirements can be detected as turbidity at the common boundary of 2 zones (to take full advantage of this, the single supplements should be strategically placed in relation to each other), and (*d*) the supplements need not be sterilized. When small inocula, of the order of 1000 cells /plate, are used with a single supplement /plate, this method is the most reliable to study the requirements of unstable, reversible mutants.

Comment by Bernard D. Davis

Although agar has been suspected of contamination with interfering amounts of vitamins, we have found only negligible impurities. Unwashed Difco agar provides no growth stimulation for mutants of *E. coli* requiring pantothenate and thiamine and permits only microscopic to barely visible growth of streaks of mutants requiring pyridoxine, PABA, biotin, niacin and β-alanine. This slight background growth can be largely eliminated by washing the agar.

REVERSE MUTATION (30)

Many or most nutritional mutants have the potentiality of "adapting" so that they can grow in minimal medium in the absence of the substance which they originally required. Although this process superficially suggests a direct action of the environment in educating the mutant bacteria to dispense with their erstwhile requirements, a more thorough analysis indicates that the adaptation is probably the result of spontaneous mutations toward nutritional competence, the reverted mutants being strongly selected for by the minimal medium (31, 32). Limited investigation in Neurospora and *E. coli* has shown that these mutations are usually back-mutations restoring the wild-type condition genetically as well as physiologically; some apparent reversions, however, are the result of "modifier" or "suppressor" mutations, involving further gene mutations which merely mimic the wild type (11).

The possibility of reversion must be considered in all nutritional and metabolic studies on micro-organisms, be they artificial mutants or microbes that carry nutritional deficiencies, probably

originated by mutation, when isolated from nature. This interference is best countered by carrying mutant stocks on adequate medium, so that random reversions will not be selected for; by frequent reisolations, to get rid of accumulated reversions, and, above all, by verifying all critical or doubtful responses by reinoculation into minimal medium to insure the persistence of the nutritional requirements.

SYNTROPHISM (16)

Even when biochemical and genetic information is not available, mutants with similar nutritional requirements can often be distinguished by syntrophism (mutual feeding). As has been demonstrated most clearly in Neurospora work, genetic blocks in the biosynthesis of an end-product may result in the accumulation and excretion of the intermediate whose further utilization is blocked. The excreted intermediate may be capable of stimulating the growth of another mutant which is unable to make the same end-product but which is blocked at an earlier step in the reaction chain. The ability of a mixture of mutants to grow on minimal medium is a definite indication of physiologic differences between them and, by inference, of genetic nonidentity.

PROCEDURE

The most sensitive test for syntrophic interaction is probably a quantitative comparison of the turbidity of separate and mixed cultures which are supplied with concentrations of the growth factor requirement which limit the individual mutants to about 10% of optimal. Syntrophism can also be demonstrated by streaking the 2 cultures across or near each other on a minimal agar plate.

Evaluation.—This test is roughly comparable to the heterocaryon test for "allelism" in Neurospora (p. 61). A syntrophic reaction indicates physiologic difference, and probably, though not necessarily, nonallelism. Failure to obtain effective syntrophism does not prove physiologic or genetic similarity. Lampen *et al.* (14) have successfully applied this method to the classification of a group of cystine- and methionine-requiring mutants of *E. coli*.

Comment by Bernard D. Davis

The increased growth of a mixture of mutants, compared with their separate growth, is usually predominantly or exclusively an increase in the growth of 1 component at the expense of an intermediate accumulated by the other component. If the term "syntrophism" is used to mean the feeding of 1 strain by another, mutual syntrophism is a special case, occurring, for

example, with certain tyrosine- and phenylalanine-requiring mutants. We have found parallel streaking to be the most convenient and useful test for syntrophism, since it tells at a glance which strain is being fed (6a). A possibly more sensitive test for syntrophism is the satellite phenomenon observed in a pour plate containing 10–100 feeder cells surrounded by a large number of cells of the strain to be fed (10^4–10^5).

FERMENTATION MUTANTS

Fermentation mutants have proved useful in genetic and chemical work in *E. coli* (17, 20, 7). They are readily obtained and classified and are usually distinctive and independent in behavior. Their most useful attribute is their ability to be classified by colony appearance on a plate without further test. This permits of their application to population, segregation and other studies, where counts of the relative proportions of 2 genetic types must be made.

The essential for finding and using fermentation mutants is an indicator medium which will permit of colony classification. Eosin-methylene blue agar (see Table 1, p. 5) is the most reliable and informative indicator medium tried. On this medium, colonies or even sectors of colonies can be scored for fermentative capacity, even on a crowded plate. Colonies which ferment the sugar take up a dense purplish black coloration, often with a green sheen, while nonfermenters remain of a white or pink color, later sometimes turning a transparent blue, which indicates alkali production. Slow or late fermenters may assume varying shades of purple, all against the dark but transparent orange-purple background. Particularly when the colonies are crowded, the contrast does not favor the finding of nonfermenters, but close examination will usually permit their discovery.

PROCEDURE

The most convenient method of obtaining fermentation mutants has consisted in spreading a drop (about 0.05 ml) of a fully grown culture of *E. coli* in any complete broth over the surface of each of a number of EMB plates with appropriate sugars. Each plate is then exposed to ultraviolet light long enough to reduce the colony survival to 200–400. With a Hanovia high pressure lamp, 125 w at 15 cm distance, this requires about 7 sec. After 18–24 hr the plates are examined for mutant colonies and sectors. With lactose, maltose, galactose, etc., yields of 0.02–0.10% of nonfermenting mutants are found after this heavy dose of radiation. The mutants often occur as sectors, indicating that a mutated

nucleus may segregate from an unmutated nucleus in the formation of the colony, as suggested earlier. The mutants are tested and purified by streaking them out on EMB agar. After they are purified, they should be tested for their fermentation reactions on other sugars, since different patterns of effects may occur (7).

Modifications.—For large scale platings, triphenyltetrazolium chloride may be a useful indicator in place of EMB (18). The indicator is added (after being sterilized separately) to a 0.05% concentration in nutrient agar-sugar medium. The acid produced by fermenters inhibits the intracellular reduction of the indicator to a colored, water-insoluble formazan, which gives a brilliant red color to the nonfermenters. Since the background is neutral, the contrast here favors the mutants. Unfortunately, although most runs with tetrazolium have given excellent results, occasionally all colonies on a batch of plates, fermenters or not, take up enough pigment to obscure the results. For further study and verification, it is advisable to transfer suspected fermentation mutants to EMB agar.

APPLICATIONS OF BIOCHEMICAL MUTANTS

The most generally interesting use of biochemical mutants is probably in the study of biosyntheses of growth factors. Most work of this type has been done with mutants of fungi (3) and with naturally occurring, exacting organisms such as lactobacilli, but bacterial mutants have begun to assume a prominent role in this type of work. We may cite studies on the synthesis and utilization of proline (33), tyrosine (34), purines (9), sulfur amino acids (14), p-aminobenzoic acid (13) and isoleucine (cf. 3). In addition, mutants have been used in the analysis of such diverse processes as light production in luminous bacteria (24), nitrogen fixation (37) and synthesis of starch from maltose in *E. coli* (7). There is every reason to believe that nutritional mutants could compete favorably with the lactobacilli in the search for new vitamins, especially since most workers who have produced bacterial mutants have accumulated cultures that respond to yeast extract but not to any known pure substance. However, this expectation remains to be realized.

Along the same lines, mutants should be suitable for microbiologic assays of various vitamins, amino acids and nucleic acid derivatives. However, this avenue has been pre-empted by the painstaking work already done with lactic acid bacteria and with Neurospora mutants. Except for substances for which suitable assay methods are not yet available, the reward does not seem

to justify the tedious investigations needed to perfect this potential application.

Aside from these more biochemical uses, biochemical mutants are indispensable in genetic studies on bacteria. In the first place, they provide excellent "markers" for studies in natural selection and experimental evolution, i.e., in population dynamics. Fermentative mutants are especially useful here, as they can be enumerated by inspection on indicator media.

Second, nutritional markers have proved especially useful for the genetic analysis of the sexual cycle that has been discovered in *E. coli,* strain K-12 (36). The necessity for genetic methods to investigate the question of sex in bacteria is clearly shown by the ambiguity and inconclusiveness of the morass of previous cytologic work. In principle, any sufficiently stable genetic markers can be used to look for the recombination of characters, which is the function and the proof of sexual reproduction; nutritional requirements have the special advantage that they allow for the efficient selection of minute numbers of recombination types amidst large populations of the parents.

The selection for recombinants can be accomplished by using, as test parents, complementary nutritional mutants, such that each is competent to synthesize the requirements of the other, e. g., a biotin-methionineless and a threonine-leucineless pair. Double mutants of this sort must be used to obviate ambiguity of results caused by reverse mutation. If genetic recombination occurs between these strains in a mixed culture, some of the resulting recombinants should be prototrophic. The prototrophs can, of course, be readily selected by plating washed suspensions of the mixed culture into minimal agar medium.

The recovery of prototrophs (at the rate of about 1 for each million mutant cells inoculated) from mixed cultures, and not from the parents kept separately, was presumptive evidence for recombination in strain K-12 (36). Much more pertinent evidence was provided, however, with the help of other markers such as phage resistance and sugar fermentations, which are not directly affected by selection for prototrophs. If the prototrophs are the result of recombination, unselected markers should likewise be reassorted among the prototrophs, and this was found to be the case.

The segregation behavior of unselected markers has led to the inference of a linear linkage system, or chromosome, in this bacterial strain. The typical life cycle resembles that of the ascomycetes, with a transient diplophase and a vegetative haplophase (21). Mutant stocks have been found, however, in which the diplo-

phase may be prolonged, thus paralleling a transition from the Zygosaccharomyces to the Saccharomyces type of life cycle. However, the diplophases currently available show abnormalities in their segregation behavior whose basis is not yet clearly understood, but which preclude a facile application to genetic problems (20).

REFERENCES

1. Beadle, G. W.: Biochemical genetics, Chem. Rev. 37:15, August, 1945.
2. Beadle, G. W., and Tatum, E. L.: Neurospora: II. Methods of producing and detecting mutations concerned with nutritional requirements, Am. J. Bot. 32:678, December, 1945.
3. Bonner, D. M.: Biochemical mutations in Neurospora, Cold Spring Harbor Symp. 11:14, 1946.
4. Burkholder, P. R., and Giles, N. H., Jr.: Induced biochemical mutations in *Bacillus subtilis,* Am. J. Bot. 34:345, June, 1947.

4a. Davis, B. D.: Isolation of biochemically deficient mutants of bacteria by penicillin, J. Am. Chem. Soc. 70:4267, December, 1948.

5. Davis, B. D.: Isolation of biochemically deficient mutants of bacteria by means of penicillin, Proc. Nat. Acad. Sc. 35:1, January, 1949.
6. Davis, B. D.: Isolation of biochemically deficient mutants of bacteria by limited enrichment, Arch. Biochem. 20:166, January, 1949.

6a. Davis, B. D.: Studies on nutritionally deficient bacterial mutants isolated by means of penicillin, Experientia 6:41, February, 1950.

6b. Davis, B. D., and Maas, W. K.: Inhibition of *E. coli* by D-serine and the production of serine-resistant mutants, J. Am. Chem. Soc. 71:1865, May, 1949.

7. Doudoroff, M., *et al.*: Direct utilization of maltose by *Escherichia coli,* J. Biol. Chem. 179:921, May, 1949.
8. Gray, C. H., and Tatum, E. L.: X-ray induced growth factor requirements in bacteria, Proc. Nat. Acad. Sc. 30:414, December, 1944.
9. Guthrie, R.: Studies of a purine-requiring mutant strain of *Escherichia coli,* J. Bact. 57:39, January, 1949.
10. Horowitz, N. H.: The one gene-one enzyme hypothesis, Genetics 33:612, November, 1948.
11. Houlahan, M. B., and Mitchell, H. K.: A suppression in Neurospora and its use as evidence for allelism, Proc. Nat. Acad. Sc. 33:223, August, 1947.
12. Karlsson, J. L., and Barker, H. A.: Induced biochemical mutants of *Azotobacter agilis,* J. Bact. 56:671, November, 1948.
13. Lampen, J. O.; Roepke, R. R., and Jones, M. J.: The replacement of p-aminobenzoic acid in the growth of a mutant strain of *Escherichia coli,* J. Biol. Chem. 164:789, July, 1946.
14. Lampen, J. O.; Roepke, R. R., and Jones, M. J.: Studies on the sulfur metabolism of *Escherichia coli*: III. Mutant strains of *Escherichia coli* unable to utilize sulfate for their complete sulfur requirements, Arch. Biochem. 13:55, April, 1947.
15. Lederberg, E. M.: The mutability of several Lac$^-$ mutants of *Escherichia coli,* Genetics 33:617, November, 1948.
16. Lederberg, J.: Studies in bacterial genetics, J. Bact. 52:503, October, 1946.

17. Lederberg, J.: Gene recombination and linked segregations in *Escherichia coli*, Genetics 32:505, September, 1947.

18. Lederberg, J.: Detection of fermentative variants with tetrazolium, J. Bact. 56:387, November, 1948.

19. Lederberg, J.: Problems in microbial genetics, Heredity 2:145, September, 1948.

20. Lederberg, J.: Aberrant heterozygotes in *Escherichia coli*. Proc. Nat. Acad. Sc. 35:178, April, 1949.

21. Lederberg, J., and Tatum, E. L.: Detection of biochemical mutants of microorganisms, J. Biol. Chem. 165:381, September, 1946.

22. Lederberg, J., and Zinder, N.: Concentration of biochemical mutants of bacteria with penicillin, J. Am. Chem. Soc. 70:4267, December, 1948.

23. Lein, J.; Mitchell, H. K., and Houlahan, M. B.: Methods for selection of biochemical mutants of Neurospora, Proc. Nat. Acad. Sc. 34:435, September, 1948.

24. McElroy, W. D., and Farghaly, A.-H.: Biochemical mutants affecting the growth and light production in luminous bacteria, Arch. Biochem. 17:379, April, 1948.

25. McElroy, W. D., and Mitchell, H. K.: Enzyme studies on a temperature sensitive mutant of Neurospora, Federation Proc. 3:376, September, 1946.

26. Miller, H.; Farghaly, A.-H., and McElroy, W. D.: Factors influencing the recovery of biochemical mutants in luminous bacteria, J. Bact. 57:595, June, 1949.

27. Peacocke, A. R., and Hinshelwood, C. N.: The changes induced in *Bact. lactis aerogenes* by irradiation with ultraviolet light, Proc. Roy. Soc. London, s. B 135:454, December, 1948.

28. Pontecorvo, G.: Auxanographic techniques in biochemical genetics, J. Gen. Microbiol., 3:122, January, 1949.

29. Roepke, R. R., and Mercer, F. E.: Lethal and sublethal effects of x-rays on *Escherichia coli* as related to the yield of biochemical mutants, J. Bact. 54:731, December, 1947.

30. Ryan, F. J.: Back-mutation and adaptation of nutritional mutants, Cold Spring Harbor Symp. 11:215, 1946.

31. Ryan, F. J.: On the stability of nutritional mutants of bacteria, Proc. Nat. Acad. Sc. 34: 425, September, 1948.

32. Ryan, F. J., and Schneider, L. K.: Mutations during the growth of biochemical mutants of *Escherichia coli*, Genetics 34:72, January, 1949.

33. Simmonds, S., and Fruton, J. S.: The utilization of proline derivatives by mutant strains of *Escherichia coli*, J. Biol. Chem. 174:705, May, 1948.

34. Simmonds, S.; Tatum, E. L., and Fruton, J. S.: The utilization of phenylalanine and tyrosine derivatives by mutant strains of *Escherichia coli*, J. Biol. Chem. 169:91, June, 1947.

35. Tatum, E. L.: X-ray induced mutant strains of *Escherichia coli*, Proc. Nat. Acad. Sc. 31:215, August, 1945.

36. Tatum, E. L., and Lederberg, J.: Gene recombination in the bacterium *Escherichia coli*, J. Bact. 53:673, June, 1947.

37. Wyss, O., and Wyss, M. B.: Mutants of Azotobacter which do not fix nitrogen, Proc. Soc. Am. Bact., 49th Meeting, May, 1949, p. 12.