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Factors in the biosynthesis of tryptophan and tyrosine by Lactobacillus arabinosus

Daniel Edward Atkinson
Iowa State College

NOTE TO USERS

FACTORS IN THE BIOSYNTHESIS OF TRYPTOPHAN AND TYROSINE BY *LACTOBACILLUS ARABINOSUS*

by

Daniel Edward Atkinson

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Signature was redacted for privacy.

Head of Major Department

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PART I

STUDIES ON THE CONVERSION OF ANTHRANILIC ACID TO TRYPTOPHAN BY *LACTOBACILLUS ARABINOSUS*

INTRODUCTION

The common use of *Lactobacillus arabinosus* as an assay organism for tryptophan depends on its complete inability to synthesize this amino acid. The ability of this species to perform the later steps in the synthesis (subsequent to anthranilic acid), however, makes it seem a promising organism for the study of those reactions. These later steps are isolated naturally and absolutely from those leading to anthranilic acid, obviating such ambiguities as those concerned with the effective level within the cell of intermediates which are both added to the medium and synthesized by the organism.

This thesis reports studies of some factors affecting the conversion of anthranilic acid to tryptophan and tests of the effects of some related compounds on that conversion.

HISTORICAL

Tryptophan is unusual in that microbiological studies concerning it antedate its chemical isolation and identification. One of the first clues to the existence and nature of tryptophan was the report of Nencki in 1874 that a pancreatic digest of flesh contained indole. Brieger (1879) found that pancreatic digestion of commercial blood albumin similarly gave rise to both indole and skatole. E. and H. Salkowski (1879) reported that these products arise also from putrefaction of proteins by randomly present bacteria (which they report were mainly Bacillus subtilis). Nencki (1880) found skatole and a trace of indole among the products of putrefaction of oxbrain. Salkowski (1884) reported the general production of both indole and skatole in putrefaction of flesh and of various proteins. Hewlett (1900, 1901) found indoleacetic acid[1] in cultures of diphtheria bacilli.

Considerable discussion ensued as to whether indole and skatole groupings per se existed in the protein

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1. The commonly used names indoleacetic acid, indolepropionic acid, etc., will be used in this thesis for 3-indolylacetic acid, β-(3-indolyl)propionic acid, etc.

molecule or were somehow formed during its breakdown. Simultaneously, intensive search for the substance in tryptic digests of proteins responsible for the protein-chrome or tryptophan reaction--the formation of a red compound with bromine--was under way. (The name tryptophan was first applied to this compound by Neumeister (1890)). Both of these questions were solved by Hopkins and Cole through their work on an apparently unrelated problem--the Adamkiewicz color reaction of proteins. These workers showed (1901a) that this reaction is due to glyoxylic acid rather than acetic acid as previously thought, and that it is not a furfural reaction and hence not evidence for the existence of carbohydrates in the protein molecule, as commonly supposed at the time. Continuing this line of work, they succeeded (1901b) in isolating a new substance from a tryptic digest of casein which gave an intense glyoxylic acid test, evolved indole and skatole on heating, and formed the red addition compound with bromine. Accordingly, the new amino acid was declared to be the long-sought tryptophan.

In a classic paper, Hopkins and Cole later (1903) reported that tryptophan is always liberated in protein putrefaction (as demonstrated by the halogen color test), passes through a maximum concentration, and then disappears, being presumably further broken down. It was also

observed that Escherichia coli (B. coli communis[1]) was able to convert tryptophan to indole, skatole, indoleacetic acid, and indolepropionic acid. At that time, it was believed that tryptophan, indoleacetic, and indolepropionic acids were skatole derivatives with the remainder of the side chains attached to the number 2 carbon atom of the ring. It is of interest that Nencki much earlier (1890) had predicted that a compound of this structure would be found to occur in proteins as the precursor of the indole and related products obtained on their putrefaction. However, Ellinger established by synthesis the correct formulas for indoleacetic acid (1904) and indolepropionic acid (1905), making the Nencki structure for tryptophan untenable. Final proof of the true structure of tryptophan came with its synthesis by Ellinger and Flamand (1907, 1908).

Microbiological Degradation of Tryptophan

The biological degradation of tryptophan received considerable attention during the following decade, and several products other than those found by Hopkins and Cole were reported. Oidium lactis was observed to con-

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1. In this thesis, Bergey's Manual of Determinative Bacteriology, 6th Ed., (1948) will be followed wherever possible. When the name used by the original author differs, it will be noted parenthetically at the time of first citation.

vert L-tryptophan[1] to levorotatory indolelactic acid (Ehrlich and Jacobsen 1911). A commercial pressed yeast converted tryptophan to β-indolylethanol (Ehrlich 1912), and an organism isolated from human feces decarboxylated the amino acid to yield tryptamine (Berthelot and Bertrand 1912). The first evidence for the breakdown of the indole nucleus was presented by Raistrick and Clark (1921) who reported that while Proteus vulgaris (B. proteus vulgaris) produces ammonia only from the amino group of tryptophan, Serratia marcescens (Bacterium prodigiosus) also slowly evolves ammonia from the indole nitrogen, and Pseudomonas fluorescens (B. fluorescens) and Pseudomonas aeruginosa (B. pyocyaneous) form ammonia from the ring nitrogen almost as rapidly as from the amino group.

Sasaki and Otsuka (1921) observed that Proteus (Proteusbakterien) produced levorotatory indolelactic acid from L-tryptophan. It was further shown (Sasaki and Kinose 1921) that the related but not naturally occurring amino acids α-naphthylalanine and furylalanine are similarly converted to α-hydroxy acids. These experiments constituted the first attempt to clarify the metabolism of tryptophan through the use of structurally related

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1. Amino acid isomer prefixes used in this thesis refer to configuration rather than rotation, and should be construed as small capitals in the official system for such designations (Crane 1947).

compounds.

Japanese workers also appear to have been the first to appreciate fully the stereochemical factors involved. Jackson (1927) had prepared levorotatory indolelactic acid by the method of Ehrlich and Jacobsen (1911) (fermentation of tryptophan by Oidium lactis) and found it ineffective as a tryptophan substitute for rats. A small amount of the material was presumably racemized by refluxing with barium hydroxide and was still inactive. The melting point was unchanged by this treatment. Ichihara and Iwakura (1931), however, found that complete racemization by autoclaving with barium hydroxide raised the melting point by nearly fifty degrees. The material reported by Jackson was thus obviously recovered (levorotatory) starting material. Authentic racemic indolelactic acid replaced tryptophan in the rat diet, while the inactivity of the levorotatory compound was confirmed. It was suggested that the dextrorotatory form might be the one corresponding structurally to naturally occurring tryptophan. Ichihara and Nakata (1936) pointed out that inversion of configuration had been shown to occur in the deamination of other amino acids by Oidium lactis. These workers resolved the racemic acid with quinine and showed that the dextrorotatory form was indeed twice as active as the racemic acid in replacing tryptophan in the rat

diet. Saito (1933) reported that the same specificity exists for E. coli (Colibakterien), racemic indolelactic acid being converted to indole by this organism, while levorotatory indolelactic acid gives no indole under the same conditions. Majima (1936) showed that indole production from the dextrorotatory acid is quantitative.

An attempt by Sasaki (1923) to isolate indolelactic acid from the action of Bacillus subtilis (Subtilisbakterien) on tryptophan yielded instead still another breakdown product, anthranilic acid. This important finding was confirmed by Kotake and Otani (1933).

Indole production was first studied quantitatively by Herzfeld and Klinger (1915), who also presented evidence for the use of both tryptophan and indole by several species of bacteria. Many intermediates have been tested in attempts to elucidate the mechanism of indole production. Frieber (1921) was unable to show indole production by E. coli in growth medium when supplied with skatole, indole-2-carboxylic acid, indole-3-carboxylic acid, indole-3-aldehyde, indoleacetic acid, indolepropionic acid, or β-(3-indolyl)ethylamine. Woods (1935b), using washed suspensions of E. coli (Bact. coli), failed to obtain indole from indole-3-aldehyde, indole-3-carboxylic acid, indoleacetic acid, indolepropionic acid, or indoleacrylic acid. Indolepyruvic

acid in the presence of ammonia gave a 10% yield of indole.

Washed suspensions of E. coli were shown to convert tryptophan to indole quantitatively, with the uptake of five atoms of oxygen for each molecule of tryptophan used (Woods 1935a). This corresponds to the complete oxidation of the side chain to carbon dioxide, water, and ammonia. It was also reported that the end product under anaerobic conditions is indolepropionic acid, and that D-tryptophan is attacked very slowly if at all.

Happold and Hoyle (1935) prepared killed suspensions of E. coli which retained most of the tryptophanase activity of the living cells. In agreement with observations on living cells, no indole was produced from indole-3-aldehyde or from indoleacetic, indoleacrylic, indolepyruvic, or indole-3-carboxylic acids.

Using such killed suspensions, Baker and Happold (1940) carried out systematic tests of many analogs and possible intermediates from which they concluded that the degradation of tryptophan requires at least a free carboxyl group, an unsubstituted amino group, and a beta-carbon atom susceptible to oxidative attack. An unsubstituted NH group in the indole nucleus was later added to this list (Baker, Happold, and Walker 1946). It was suggested (1940) that the side chain is probably

lost as a unit, with the alanine formed being subject to complete oxidation, thus accounting for the oxygen uptake observed by Woods.

From a review of previous work, Krebs, Hafez, and Eggleston (1942) concluded that enough possible intermediates had been tested to exclude initial attack on the side chain of tryptophan. They suggested that opening and reclosing of the heterocyclic ring occur, and showed that o-aminophenylethanol is converted to indole by E. coli. It was admitted, however, that the bacterium is able to oxidize ethanol to acetaldehyde, and that o-aminophenylacetaldehyde spontaneously goes to indole, so that the conversion of the alcohol to indole may have no relation to the tryptophan-indole reaction. In the light of present knowledge, this appears to be the case. Baker, Happold, and Walker (1946) reported that dimedon completely inhibits indole formation from o-aminophenylacetaldehyde, but has no effect on the bacterial tryptophan-indole conversion.

Microbiological Requirements for Tryptophan

The idea that tryptophan (or indeed any specific compounds) might be required for the growth of some strains of bacteria developed only very slowly. Braun and Cahn-Bronner (1921) emphasized the desirability in

studies of bacterial physiology of using chemically defined media containing no substances not required to support growth. In studying typhus and paratyphus bacilli, they found that some strains required tryptophan, and considered the heterocyclic component essential, since alanine, phenylalanine, or tyrosine could not be substituted. The same workers later (1922) showed that these tryptophan-requiring strains could not grow when supplied indole-2-carboxylic acid, indole-2,3-dicarboxylic acid, or indole plus alanine. They therefore concluded that tryptophan as such is required in the synthetic processes of these bacteria. Mueller et al. (1933) studied a strain of the diphtheria bacillus which required tryptophan.

Burrows (1932) reported that Clostridium botulinum in an acid-hydrolyzed casein medium required added tryptophan for growth. In a synthetic medium, however, tryptophan was found not to be essential (Burrows 1933), and a later paper (Burrows 1934) reported that tryptophan only stimulated growth in acid-hydrolyzed casein media.

Microbiological Synthesis of Tryptophan

Fildes, Gladstone, and Knight (1933) reported that Salmonella typhosa (B. typhosus) "ordinarily...will not grow without added tryptophan", but that it could be

"trained" to grow with ammonia as the sole source of nitrogen. Synthesis of tryptophan by a "trained" strain was demonstrated by the glyoxylic acid reaction. The tryptophan responses of various bacteria were then studied in acid-hydrolyzed casein media (Fildes and Knight 1933). About half of the species studied (notably pathogens) were found to require tryptophan, although several of these could be "trained" to grow in its absence. It was pointed out that since all bacterial protoplasm presumably contains tryptophan, those organisms not requiring an exogenous supply of the amino acid must be able to synthesize it. This elementary concept seems not to have been widespread at the time, although Logie many years before (1920) had expressed it somewhat differently, had demonstrated synthesis of tryptophan by E. coli (B. coli), and had shown indole utilization by this organism under certain conditions.

Burrows (1939a) studied the nutrition of eight strains of the typhoid bacillus, and found that for four of them tryptophan was the only amino acid which supported growth when supplied alone. It was claimed, however, that all of the strains were able to grow on a mixture of six amino acids not including tryptophan, so that the requirement was not specific. Tryptophan, at any rate, according to Burrows, determined neither the rate nor

the total extent of growth, but exhibited a "triggering" action. All of the strains were reported to synthesize tryptophan. A later paper (Burrows 1939b) presented extensive analytical data in support of the contention that even in cases where tryptophan was required for the initiation of growth it was then synthesized by the growing cultures. Although the data appear convincing, the amounts are very small, and much simpler and unequivocal proof of the hypothesis would have been obtained if the growth, once "triggered" off by tryptophan, could have been shown capable of serial transfer in tryptophanless medium. It is difficult to understand why this test was not made. At the present time the most likely explanation of Burrows' results would appear to be selection for tryptophan-producing mutants (see Part II of this thesis). The effect of "triggering" levels of tryptophan would then be the production of moderately large populations of normal (tryptophan-requiring) cells in which mutation could occur. The probability of occurrence of a given mutation is of course a function of population size.

Logie's (1920) report that E. coli could use indole was confirmed by Happold and Hoyle (1936), who suggested that it might be converted to tryptophan. Serious study of the biosynthesis of tryptophan began with the demon-

stration by Fildes (1940a) that indole could replace tryptophan for tryptophan-requiring strains of S. typhosa. Indoleacetic, indoleacrylic, indolepropionic, and indolepyruvic acids, indoleethylamine, indole-3-aldehyde, and skatole failed to replace tryptophan. Indole per se was therefore considered to be a precursor of tryptophan. Testing the new concept that structural analogs of intermediates might inhibit biosyntheses, Fildes (1941) showed that E. coli and "trained" S. typhosa were inhibited by indoleacrylic acid, and that the inhibition was counteracted by small amounts of tryptophan. Increasing the amount of indoleacrylic acid did not increase the amount of tryptophan required to permit growth, so it was concluded that synthesis, rather than utilization, of tryptophan was being inhibited. However, release of the inhibition by indole also failed to show a competitive ratio. This fact has not been satisfactorily explained.

"Trained" S. typhosa appeared to make indole from indoleacrylic acid, and the sequence indoleacrylic → tryptophan → indole was postulated (Fildes 1938). However, it was later shown (Fildes 1946) that the indole produced was often greater than could arise from the indoleacrylic acid supplied, and also that the rates of disappearance of indoleacrylic acid and production

of indole failed to coincide. These data suggested that indoleacrylic acid was causing the accumulation of indole by blocking its utilization rather than by conversion to indole, and were considered to prove that the organism can synthesize indole.

Because of Sasaki's discovery of anthranilic acid among the degradation products of tryptophan (cited above), Snell (1943) tested anthranilic acid for replacement of tryptophan for several species of lactobacilli. He found that both indole and anthranilic acid were capable of replacing tryptophan for Lactobacillus arabinosus and L. casei, but neither was utilized by L. pentosus, Streptococcus lactis R, or Leuconostoc mesenteroides P-60. By analogy with the then recently discovered sulfanilamide-p-aminobenzoic acid competition, Snell hoped to inhibit anthranilic utilization by addition of orthanilic acid, the sulfonic analog of anthranilic acid. This compound, however, neither inhibited nor was utilized. Salicylic acid and m- and p-aminobenzoic acids were not utilized. It was reported that anthranilic acid became toxic as the level approached one milligram per milliliter. Maximum growth was never observed when anthranilic acid replaced tryptophan. Snell assumed that anthranilic acid was a precursor of indole in tryptophan synthesis, but had no way of ruling

out definitely the possibility of the reverse order. Nothing was said of the efficiency of the conversion, but from data given in the paper it is possible to calculate that the mole-percent effectiveness of anthranilic acid relative to tryptophan ranged downward from 67% as the level of anthranilic acid increased. This paper is one of the most important dealing with tryptophan biosynthesis, and, despite intensive study, no new intermediates in the synthesis have since been found.

Tatum, Bonner, and Beadle (1944) found that one of their "tryptophanless" Neurospora mutants was able to utilize anthranilic acid as well as indole. Another used indole but not anthranilic acid, and in fact accumulated anthranilic acid in the medium. These observations established the sequence anthranilic acid → indole → tryptophan. Pimelic, salicylic, benzoic, and o-aminophenylacetic acids and aniline were inactive for both strains. o-Aminophenylethanol exhibited low activity for both strains, but it must be remembered (cf. reference above to Krebs et al. 1942) that the corresponding aldehyde yields indole spontaneously. Phenylglycine was inactive for both strains, but o-carboxyphenylglycine was active only for the strain able to use anthranilic acid. It was thought that this activity arose through conversion to anthranilic acid.

In further studies of the system, Tatum and Bonner (1943, 1944) employed a mutant strain using indole but not anthranilic acid in an attempt to find some intermediate coming after the genetic block but before indole in the synthesis. Skatole, indoleacetic acid, indolepropionic acid, l-kynurenine, tryptamine, indolelactic acid, and indolepyruvic acid all failed to promote growth. It was then suspected that the tryptophan molecule might arise by condensation rather than stepwise additions to the indole nucleus. Tests of this hypothesis showed that the addition of serine to the medium caused a rapid decrease in indole concentration. No such effect was exerted by alanine, β-alanine, pyruvic acid, glyceraldehyde, phosphoglyceric acid, glycolic acid, threonine, Cori ester, or sucrose. L-Serine was twice as effective as the racemic amino acid. The production of tryptophan was shown colorimetrically, by bioassay with a Neurospora mutant unable to use indole, and finally by actual isolation. Suspecting that the mechanism thus firmly established for the conversion of indole to tryptophan by Neurospora would be applicable to the same or the reverse reaction in other species, these workers reported, without adequate details, preliminary tests on E. coli indicating that the addition of serine slowed the production of indole from trypto-

phan. Under certain conditions in the presence of serine, it was in fact reported that indole decreased and tryptophan increased. Fildes (1946) was unable to repeat this last observation with "trained" S. typhosa, although he did find that serine had some effect in relieving inhibition caused by indoleacrylic acid, and hence felt that the indole-serine condensation probably occurred in this organism as well.

A cell-free Neurospora preparation capable of causing the indole-serine condensation was reported by Umbreit, Wood, and Gunsalus (1946). Pyridoxal phosphate was found to be a necessary cofactor for the activity. Schweigert (1947) accordingly tested the effect of pyridoxine, pyridoxal, and pyridoxamine on the conversion of indole to tryptophan by L. arabinosus, and found that any one of these compounds was required for the conversion. Pyridoxine was found to be much less active than the other forms. Schweigert (1947, Schweigert et al. 1946) reported better utilization of anthranilic acid and indole in the presence of serine than in its absence, thus suggesting that the indole-serine condensation observed in Neurospora might also occur in lactobacilli. Acetate, however, showed a more marked effect than serine, so that evidence for the direct participation of serine was not convincing.

A number of related compounds have been tested for tryptophan activity with L. arabinosus (Schweigert et al. 1946, Greene and Black 1943, Wooley and Sebrell 1945). Only indole and anthranilic acid have shown significant activity, ranging in Schweigert's work from 14% to 90% and from 19% to 50% respectively. Inactive compounds tested include indoleacetic acid, indolepropionic acid, indolebutyric acid, tryptamine hydrochloride, xanthurenic acid, kynurenic acid, indoleacrylic acid, aniline, skatole, p-aminobenzoic acid, orthanilic acid, and indole-3-aldehyde.

Fildes and Rydon (1947) found that the seven methylindoles and five methyltryptophans failed to show any tryptophan activity for S. typhosa. No inhibition was shown by indoles substituted on the pyrrole ring (1, 2, or 3), while all of those substituted on the benzenoid ring inhibited. The order of decreasing effectiveness was 4-, 6-, and 5-. The authors discussed this alternation effect in terms of its possible implications as to the electronic requirements for enzyme-substrate complex formation. It was suggested that the attachment to the enzyme involves the pyrrole ring, so that indoles with substituents in these positions fail entirely to affect the system. The inhibitions were released competitively by indole and non-competitively by tryptophan,

indicating that indole was a substrate (or precursor of a substrate) of the blocked reaction, while tryptophan was a product.

The situation with the methyl tryptophans was similar in that all of the derivatives having a methyl group attached to the benzene ring inhibited, while 2-methyltryptophan exerted no effect. The order of inhibitory effectiveness in this case was 4-, 5-, 6-, and 7-. (Substituted tryptophans were numbered to correspond with the methylindoles.) The inhibitions were competitively overcome by tryptophan, indicating that in this case utilization, rather than synthesis, of tryptophan was blocked. All of Fildes' growth and inhibition estimations are based on visual observations, so that too great value should perhaps not be placed on relative activities of the compounds.

2-Methyltryptophan had been earlier studied by Matsuoka and Nakao (1931), who reported that this derivative is more active than tryptophan itself in overcoming experimentally-induced anemia, but fails to substitute for tryptophan nutritionally. Rydon (1948) found that 4- and 5-methylanthranilic acids inhibit the growth of a strain of S. typhosa "trained" to synthesize tryptophan. The inhibition was overcome by anthranilic acid, indole, or tryptophan.

β-1-Naphthylacrylic acid (the naphthyl isoster of the indoleacrylic acid found inhibitory by Fildes) was found by Bloch and Erlenmeyer (1942) to inhibit E. coli (Bacterium coli). The inhibition was relieved, apparently in a competitive manner, by tryptophan. The naphthyl isoster of tryptophan itself was tested by Raoul, Chopin, and Ayrault (1947). When the growth (presumably the synthesis of tryptophan) of E. coli (Bacillus coli) was inhibited by indoleacrylic or naphthyl acrylic acids, the addition of the tryptophan isoster caused slight growth, although not comparable to that allowed by anthranilic acid. Inhibition by either acrylic acid was also overcome by indoleacetic acid, although this compound had no activity in uninhibited cultures (Raoult 1947).

Indoleacetic, indolepropionic, indolebutyric, naphthalene acetic, β-naphthoxyacetic, 2,4-dichlorophenoxyacetic, and 2,3,5-tri-iodobenzoic acids (compounds having hormonal activity for green plants) were all found inhibitory to several pathogens by Dubos (1946). Half-maximal growth occurred at concentrations varying from 0.01 to 0.001%, and the inhibition was reversed by ten times the concentration of tryptophan in each case. Handler and Kamin (1947), however, found that inhibitions of E. coli, L. arabinosus, L. casei, Strep-

tococcus faecalis, and Aerobacter aerogenes by indoleacetic acid were not overcome by either tryptophan or nicotinic acid. Mycobacterium tuberculosis (one of the pathogens used by Dubos) was not affected by indoleacetic acid in these tests. Since there was no correlation between requirement for exogenous tryptophan and tolerance for indoleacetic acid, and especially since tryptophan failed to reverse the inhibition, these workers doubted that the indoleacetic inhibition, where it exists, is related to the synthesis of tryptophan. The reported effectiveness of salicylic and p-aminosalicylic acids against human tubercule bacilli both in vivo and in vitro (Lehmann (1946a, 1946b) may relate to tryptophan synthesis or metabolism. It is of interest that anthranilic acid was among the related compounds reported to cause inhibition (1946a).

Attempting to repeat the preliminary E. coli experiments of Tatum and Bonner cited above, Dawes, Dawson, and Happold (1947) could find no evidence for conversion of indole to tryptophan on addition of serine. They confirmed the slowing of indole production from tryptophan by the addition of serine, but found that the effect was non-specific, since alanine caused a similar retardation. Rather than the mass-action relationship postulated by Tatum and Bonner, these workers

suggested that the phenomenon depended on the relative ease of oxidation of alanine, serine, and tryptophan. If the former two compounds, as seems likely, are more available as energy sources, the side chain of tryptophan will not be metabolized in their presence. This hypothesis could also explain the long-known ability of fermentable sugars to inhibit indole production by E. coli. (A similar idea was in fact advanced much earlier by Logie (1920) and by Frieber (1921), who suggested that the side chain of tryptophan serves as a carbon source, but is not required--and hence not metabolized--in the presence of glucose.) Logie (1920) and Happold and Hoyle (1936) had earlier shown that inhibition by glucose persists only as long as glucose remains in the medium as such. When all the glucose has been fermented, indole production begins at once.

Dawes, Dawson, and Happold (1947), using paper partition chromatography, were unable to demonstrate the presence of any products of the side chain in the normal system, presumably because of their too rapid oxidation. However, preferential inhibition by mepacrine, which reduced indole production by only one-half while nearly abolishing oxygen uptake, permitted the demonstration of alanine, which was therefore reported as an intermediate in tryptophan breakdown.

Considerable clarification of earlier data resulted from partial purification of the tryptophanase of E. coli by Wood, Gunsalus, and Umbreit (1947). Although pyridoxal phosphate is the prosthetic group of this enzyme as well as of that responsible for the indole-serine condensation in Neurospora, the reaction mediated is not the same. Products of tryptophan fission were found to be indole, pyruvic acid, and ammonia. The possibility that the pyruvic acid arose from a secondary reaction was ruled out by demonstration that the enzyme preparation caused no production of pyruvic acid from either serine or alanine. In the light of this finding, it seems likely that the suggestions of Logie, Frieber, and Dawes et al. were on the right track. Tryptophan can be used by E. coli as a source of pyruvic acid--i.e., of energy--and the characteristic indole reaction is merely a by-product. Presence of other, presumably more efficient, energy sources prevents the metabolism of tryptophan in this way. The alanine shown to arise from tryptophan breakdown (Dawes et al. 1947) may then be accounted for as a transamination product of pyruvic acid.

The existence of the indole-serine condensation in lactobacilli has been rather generally assumed, but, as has been seen, no satisfactory evidence for

this view has been obtained. A second possibility--reversal of the pathway of tryptophan breakdown shown by Wood et al. (1947)--must also be seriously considered. Demonstration of this reaction in a metabolizing cell with its plentiful supply of pyruvic acid would be difficult.

Tryptophan appears to be of unique importance in phage-bacteria systems. Anderson (1945a) reported that T_4 and T_6 phage attack E. coli very weakly in synthetic minimal medium, but strongly on the addition of low levels of tryptophan to the medium. Adsorption of the phage particles on the cells was the step affected by tryptophan. Anthranilic acid, indole, indole plus serine, and several other related compounds did not promote adsorption. It was later shown (Anderson 1945b) that B_z-3-methyltryptophan (5-methyltryptophan in Fildes' terminology) competitively inhibits tryptophan in E. coli, while having the same effect as tryptophan on the adsorption of the virus particles on the coli cells. Delbrück (1948) has shown that adsorption is inhibited by indole and skatole, while indoleacetic acid and ten other related compounds were without activity. A phage strain not requiring tryptophan was not affected by indole. Spizizen and Kenney (1949) report that salicylic acid inhibits multiplication of T_2 phage in E.

coli at one-tenth of the concentration required to inhibit bacterial growth. Further, inhibition of virus multiplication was "relieved completely by minute amounts" of anthranilic acid, indole, or tryptophan, while the bacterial inhibition was not relieved by these compounds. This strain is one of those not requiring tryptophan for adsorption on the host cells.

MATERIALS AND METHODS

Lactobacillus arabinosus 17-5 and mutant strains derived from it were used in the work reported in this thesis. The strain used had been maintained in the Chemistry Department at Iowa State College for several years, and had been originally obtained from the American Type Culture Collection (#8014). Toward the end of the investigation, a new culture was obtained from this source. Its ability to convert anthranilic acid to tryptophan and the quantitative effect of pyridoxine on this conversion were found identical with the strain under investigation within the limits of experimental error.

The stock culture was carried in stabs in peptone-yeast extract agar medium of the composition shown in Table 1. Stabs were stored in a refrigerator at about 2° after growth for approximately 24 hours at 36°.

Transfers were approximately monthly. Inocula were prepared by straight-needle transfer to broth medium of the same composition, except for the agar, as the stab cultures. After incubation for about 18 hours at 36°, the cells were centrifuged, the medium decanted, and the

Table 1. Composition of the Medium Used for Stock Cultures.

Constituent	Amount
Bacto-peptone	0.8 g
yeast extract	0.2 "
sodium acetate	0.5 "
glucose	1.0 "
agar	1.2 "
salt solution A*	0.5 ml.
salt solution B*	0.5 "
water	100 "

*See Table 2

cells resuspended in the original volume of sterile 0.9% sodium chloride solution. One further centrifugation and resuspension were made to wash the cells, and the suspension stored in the refrigerator until use. For the inoculation of ten milliliter cultures, this suspension was diluted to about 1/50 with sterile 0.9% sodium chloride solution. One drop of the diluted suspension was added to each tube from a pipette. When

smaller culture volumes were to be inoculated, proportionally greater dilution was made.

The basal medium was essentially that of Kuiken et al. (1943). Its composition is given in Table 2. The concentration of pyridoxine was generally increased, as will be specified in the text.

Amino acids used as standard substances were obtained from Merck and Company, Winthrop Chemical Company, Eastman Kodak Company, or Amino Acid Manufactures (UCLA). Those which were merely ingredients of the medium were obtained from these sources and also from Dow Chemical Company, National Aniline, and General Mills. Technical grade leucine, valine, methionine, phenylalanine, aspartic acid, and glutamic acid from the latter three sources were recrystallized one or more times from water before use.

Following common microbiological assay practice, the desired medium was made up at twice the final concentration and added to the culture tubes after the materials to be tested had been added, together with enough water to bring all tubes up to the desired final volume (usually ten millliliters.) Tubes were plugged with cotton and sterilized by autoclaving ten to fifteen minutes, depending on culture volume, at fifteen pounds pressure per square inch. After inoculation with the usual pre-

Table 2. Basal Medium.

(Composition of one liter of double-strength medium.)

Constituent	Amount
L-arginine.HCl*	400 mg.
DL-alanine	400 "
DL-aspartic acid	800 "
L-cystine	400 "
DL-glutamic acid.H_2O	800 "
L-histidine.HCl	400 "
DL-isoleucine	400 "
DL-leucine	400 "
L-lysine.HCl	400 "
DL-phenylalanine	400 "
L-proline	400 "
DL-serine	400 "
DL-tryptophan	400 "
DL-methionine	400 "
DL-tyrosine	400 "
DL-threonine	400 "
DL-valine	400 "
glucose	40 g.
sodium acetate	15 "
adenine sulfate	10 mg.
guanine.HCl	10 "
uracil	10 "
vitamin concentrate	10 ml.
salt solution A	10 "
salt solution B	10 "
water	1000 "

*See footnote 1, page 5.

Table 2. (Continued)

Constituent	Amount
Vitamin concentrate	
thiamin.HCl	2 mg.
pyridoxin.HCl	2 "
calcium pantothenate	2 "
riboflavin	4 "
nicotinic acid	8 "
folic acid	100 µg.
p-aminobenzoic acid	10 "
biotin	8 "
water	100 ml.
Salt solution A	
KH_2PO_4	25 g.
K_2HPO_4	25 "
water	250 ml.
Salt solution B	
$MgSO_4.7H_2O$	10.0 g.
NaCl	0.5 "
$FeSO_4.7H_2O$	0.5 "
$MnSO_4.4H_2O$	1.5 "
water	250 ml.

cautions as to sterility, the tubes were incubated at 36° to 37°. An air incubator equipped with a fan was used.

In tests in which metabolism was estimated by titration of the lactic acid produced, approximately 0.1 normal sodium hydroxide was used. Standardization of the base is of course unnecessary, since the only data required are relative responses among the cultures in the test. It is desirable that the base be of approximately constant normality from test to test, however, in order that any abnormalities with regard to either maximum growth or "blank growth" will be readily observed. Pyrex culture tubes 25 x 150 mm were used in these tests, and the titrations carried out directly in the tubes. Phenolphthalein was used as the indicator, since the equivalence point of the titration lies between pH 8.5 and 9.0 (depending on the extent of growth).

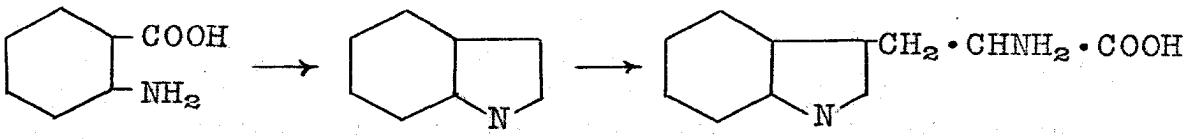
When periodic readings during the growth of the cultures were desired, cell multiplication was estimated by turbidity. A Coleman Universal Spectrophotometer Model 11 was used for these measurements. Readings were in terms of optical density, which is nearly proportional to cell concentration. Pyrex culture tubes 18 x 150 mm were used in these tests. Light of wavelength 575 millimicrons was used for all readings, since this wavelength

was found to give the maximum optical density for a given cell suspension in the medium used. The tubes used were individually matched by filling with a dilute copper sulfate solution and rotating in the tube carrier of the spectrophotometer until a point was found where transmission matched that of a standard tube. This position was then marked by scratching the tube to correspond to a mark on the tube carrier. It was found that about fifty percent of one commercial lot of tubes and about eighty percent of another could be satisfactorily matched in this manner.

EXPERIMENTAL

In preliminary studies of the conversion

anthranilic acid ⟶ indole ⟶ tryptophan,



the activities of anthranilic acid and indole were tested as functions of vitamin B_6 activity. The effects of pyridoxin and pyridoxamine additions to 10 milliliter cultures containing 10 micrograms of anthranilic acid per tube in lieu of tryptophan are shown in Table 3. The activity ranges and levels required for optimum utilization of anthranilic acid agree well with those

reported by Schweigert (1947).

By comparison with the tryptophan standard curve associated with this test, the tryptophan level corresponding to any titration value may be obtained for calculations of relative anthranilic acid activity. Such

Table 3. Effects of Pyridoxine and Pyridoxamine on Utilization of Anthranilic Acid by L. arab. 17-5.

(Each tube contained 10 micrograms of anthranilic acid and no tryptophan. Titration values are averages of duplicate 10 milliliter cultures. Incubated 3 days.)

Pyridoxine·HCl (γ/tube)	Titre (ml. 0.1 N NaOH)	Pyridoxamine·HCl (γ/tube)	Titre (ml. 0.1 N NaOH
0	1.2	0	1.3
1	1.6	0.005	2.7
2.5	4.1	0.01	3.8
5	5.8	0.025	7.0
10	7.4	0.05	8.9
25	8.9	0.1	9.9
50	9.7	0.5	10.3
100	10.1		

calculations for the tubes at the highest levels of pyridoxine and pyridoxamine gave anthranilic acid activities 82% and 85% that of L-tryptophan on a molar basis. Since these values are considerably higher than those previously reported from tests in synthetic media (Snell 1943, Greene and Black 1943, Schweigert et al. 1946,

Schweigert 1947), similar tests were made at varying levels of anthranilic acid. In repeated experiments, anthranilic acid activity relative to tryptophan on a molar basis has varied from 67% to 97% in the presence of 25 micrograms of pyridoxin per tube. Results of a typical experiment are shown in Table 4. It will be

Table 4. Effect of Pyridoxin on Conversion of Anthranilic Acid to Tryptophan by *L. arab. 17-5*.

(Values express tryptophan activity on a molar percentage basis. Incubated 60 hours.)

Anthranilic acid (γ/tube)	Pyridoxine·HCl 1 γ/tube	5 γ/tube	25 γ/tube
5	11	53	97
10	4	46	81
20	2	34	--

noted that the relative effectiveness of anthranilic acid decreases as its level is increased. In other words, the dosage-response curve of anthranilic acid is less steep than that for tryptophan when both are plotted on a molar basis.

The presence of tryptophan at very low levels was reported by Schweigert et al. (1946) to reduce

drastically the tryptophan activity of indole and anthranilic acid. Activities of both compounds were held below 10% by the addition of only 0.4 micrograms per milliliter of tryptophan to the otherwise complete synthetic medium in which the tests were made. This phenomenon appeared of interest in connection with the mechanism of the anthranilic acid-tryptophan conversion. Attempts to duplicate it, however, were unsuccessful. When tested at levels of 1 to 5 micrograms per 10 milliliter culture in the presence of tryptophan at 4 micrograms per tube, anthranilic acid activity ranged from 57 to 45% and indole activity from 66 to 57%, both expressed relative to L-tryptophan on a molar basis.

Anthranilic Acid Dosage-Response Relationships

Extension of the above tests to high levels of anthranilic acid led to an unexpected result. Snell (1943) reported that growth did not occur at high levels of anthranilic acid. The effect was readily confirmed here. Under the conditions of these experiments, with pyridoxin at 25 micrograms per tube and with titration at about 48 hours, a regular increase in growth occurred with increase of anthranilic acid level up to 100-250 micrograms per tube, followed by a sharp decline to 500 micrograms and essentially no growth at 1000 or 5000 micrograms per tube

(Figure 1a). Such a situation is not unusual, even among essential metabolites. Several examples involving amino acids are cited in Part II of this thesis. It was found, however, that the addition of either 4 or 8 micrograms of L-tryptophan per tube entirely prevented this effect. In the presence of these levels of tryptophan, maximum growth was obtained with anthranilic acid at 100, 500, 1000, or 5000 micrograms per tube. Since growth was much greater than could be accounted for on the basis of added tryptophan, it was obvious that anthranilic acid was being utilized. Failure of growth in the presence of high levels of anthranilic acid and absence of tryptophan thus appears to be caused by inhibition of some other essential cellular process and not to inability of the organisms to use anthranilic acid at these concentrations. The addition of very small amounts of tryptophan renders such levels of anthranilic acid available for conversion to tryptophan by abolishing the inhibitory effects in some manner. The data of Table 5 indicate that 5 micrograms per tube of either L-tryptophan or indole suffices to cause utilization of normally inhibitory levels of anthranilic acid, while 0.5 and 0.05 micrograms of tryptophan fail to do so.

The effects of more closely-spaced levels of tryptophan on growth of 10 milliliter cultures containing

1000 micrograms of anthranilic acid are shown in Fig. 1. It will be noted that a normal growth curve is obtained on the addition of 5 micrograms of L-tryptophan, and an essentially similar curve, though with the hint of a break at about 30 hours, resulted when 2 micrograms of

Table 5. Effects of Tryptophan and Indole on Utilization by L. arab. 17-5 of Anthranilic Acid Supplied at High Levels

(Tabulated values are averages of duplicate titrations with 0.1 N NaOH. Culture volumes 10 ml. Incubated 50 hours.)

Anthranilic acid γ/tube	L-Tryptophan				Indole
	0	0.05 γ/tube	0.5 γ/tube	5 γ/tube	5 γ/tube
100	9.4	10.4	10.9	10.8	11.6
250	2.6	2.5	2.9	10.7	11.5
500	2.7	2.3	2.4	10.4	11.3
1000	3.5	2.5	2.7	10.3	11.1

tryptophan were supplied. Curves for tryptophan at 1, 0.5, and 0.2 micrograms, on the other hand, reflect a definite tendency of the cultures to cease growth (presumably on attaining the limit of growth allowed by the tryptophan present, although unfortunately a tryptophan standard curve was not run with this experiment) and then to resume rapid growth after fifteen to twenty hours.

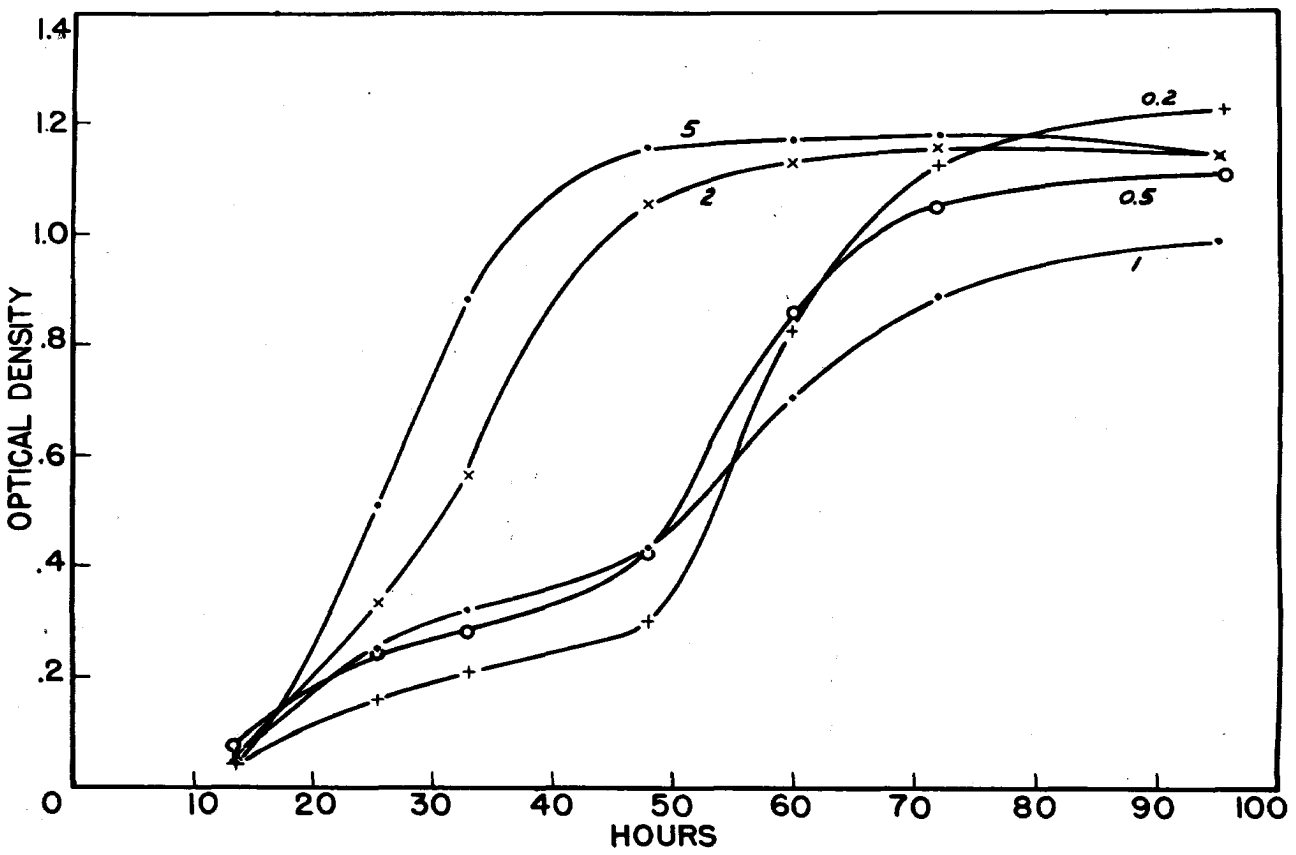


Figure 1. Effects of varying levels of tryptophan on growth of L. arabinosus 17-5 in 10 milliliter cultures containing 1000 micrograms of anthranilic acid and 25 micrograms of pyridoxine. Figures identifying curves indicate L-tryptophan level in micrograms per tube.

Some possible implications of such curves, and especially of the slower growth and lower terminal growth of the borderline cultures (those containing 1 microgram of tryptophan in this case) will be discussed later. For present purposes it need only be noted that the behavior of the cultures containing 0.2 and 0.5 micrograms of tryptophan strongly suggests the appearance of an adapted or mutant strain able to utilize anthranilic acid under the existing conditions. Visual observations had earlier shown that the effect of high levels of anthranilic acid is to delay, rather than to prevent, growth. It was therefore suspected that the two lowest tryptophan levels tested in the foregoing experiment had exerted no significant effect on the general course of growth, and that in these cases or in the total absence of tryptophan the growth which occurs after a time lag consists of cells differing genetically from those of the inoculum. To test this hypothesis, the growth of L. arabinosus in 10 milliliter cultures containing anthranilic acid at levels varying from 100 to 10,000 micrograms was followed turbidimetrically. The results are presented in Fig. 2. The curve obtained after 48 hours of incubation shows the sharp decline in growth on increasing the anthranilic acid level from 250 to 500 micrograms which had been noted in earlier experiments

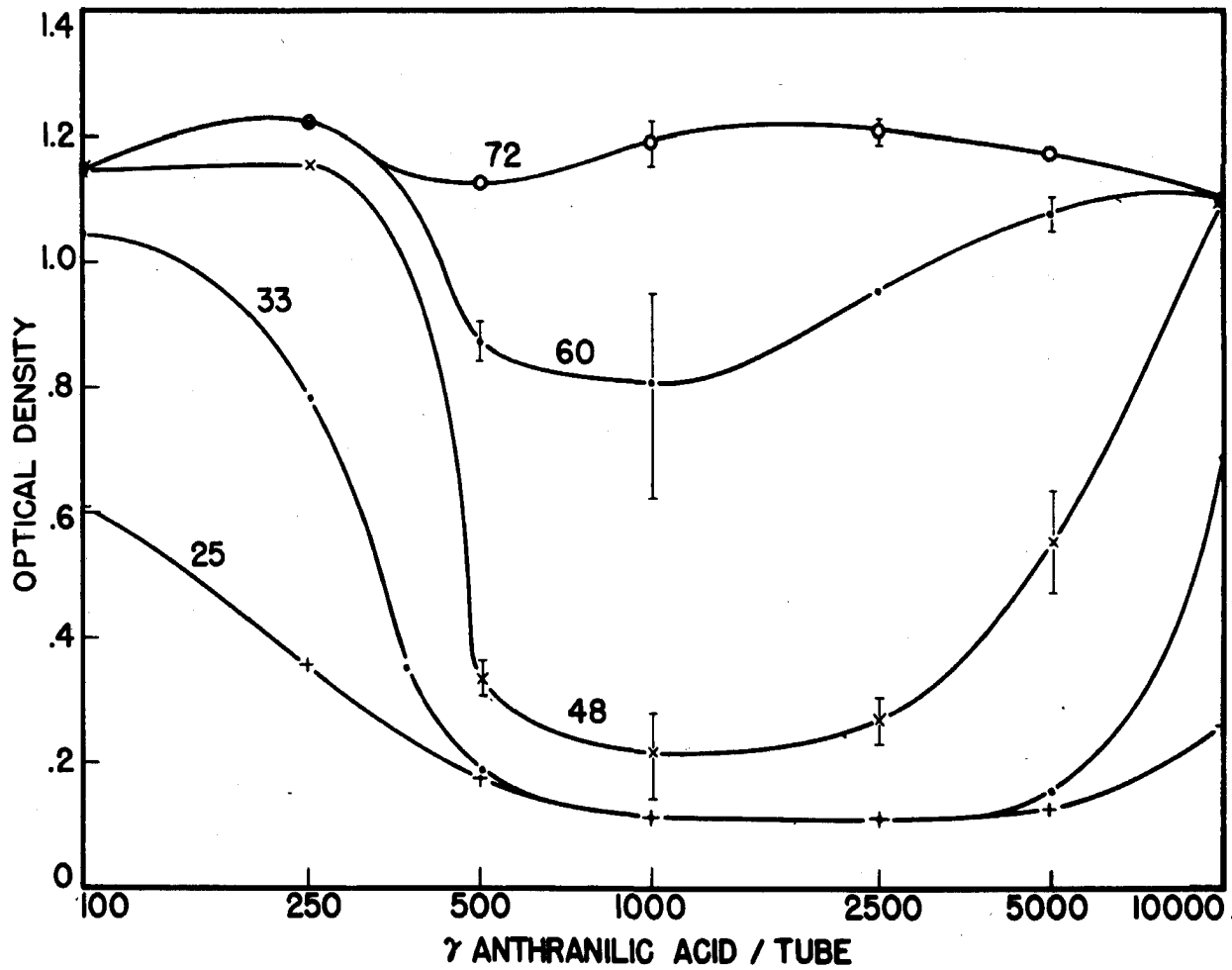


Figure 2. Anthranilic acid dosage-response curves for L. arabinosus 17-5 after different times of incubation. All cultures contain 25 micrograms of pyridoxin; culture volume 10 milliliters. Figures identifying curves indicate time of incubation in hours. Vertical lines show extent of difference between duplicates.

on titration at about 48 hours. However, this effect was clearly temporary. At levels of from 500 to 5000 micrograms, rapid growth had been initiated during the interval between 33 and 48 hours, and by 72 hours all cultures had reached essentially the same terminal growth level. The time factor is more clearly seen when growth is plotted against time, as is done for another experiment in Figure 3. The progressive decrease in both growth rate and extent of terminal growth with increase of anthranilic acid level to 500 micrograms is very similar to previously discussed responses to tryptophan additions of cultures containing 1000 micrograms of anthranilic acid (Figure 1) and to the curves obtained on increase of the molar ratio of a competitive inhibitor to its metabolite analogue (Part II of this thesis). This type of response is considered further evidence that above an optimum level in the neighborhood of 100 micrograms, at which enough tryptophan is formed for maximum growth, added anthranilic acid functions as an inhibitor of some other cell process.

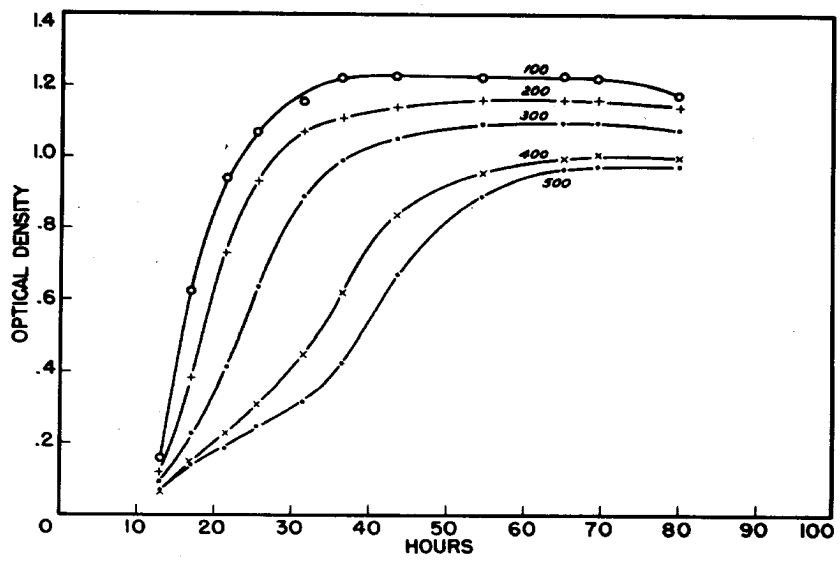
Growth in the retarded cultures, once it began, was as rapid as at optimum anthranilic acid levels, and reached essentially the same terminal levels. Such results are most easily explained on the hypothesis of selection of a mutant not inhibited by high levels of

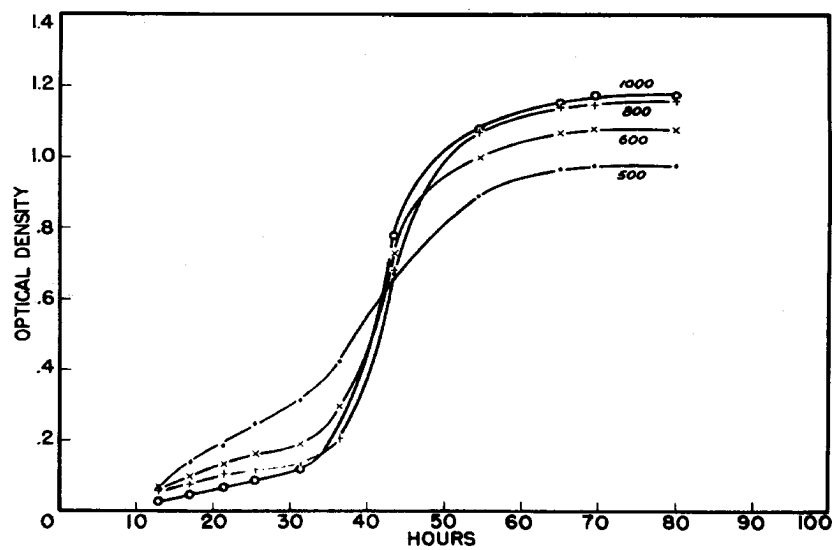
anthranilic acid. This possibility is strengthened by the fact that although no significant differences between duplicates occurred in cultures containing 500 micrograms or less of anthranilic acid, wide discrepancies were observed between duplicates at the three highest levels tested. This phenomenon was always seen at these levels in similar experiments, and is indicated in Figure 2 by the vertical lines signifying differences between duplicates greater than those attributable to reading error or tube variation (about 0.03 optical density units). Because of the very steep slope of the growth curves, differences of only a few hours in time of initiation of mutant growth will lead to very large differences between duplicates during the period of rapid growth. In several similar experiments, the only differences between such curves of adapted growth have been caused by differing times of initiation of growth rather than by varying rates of growth. The terminal growth levels reached by duplicate cultures have also generally agreed within experimental error. All of these observations are to be expected if the growth in the inhibited cultures consists of mutant cells not adversely affected by concentrations of anthranilic acid which are inhibitory to the parental type. Since averaging the values for cultures which initiate logarithmic growth

at different times distorts the curves (the slope of the average is less than that of either individual curve) only data from individual cultures should be plotted when duplicates disagree. In this thesis the arbitrary procedure will be followed of always plotting data from the first culture to initiate rapid growth. This has been done in Figure 3, where curves for the 600, 800, and 1000 microgram levels are for individual tubes.

After adaptive growth was complete, transfers were made from a culture containing 1000 micrograms of anthranilic acid to tubes containing 0, 25, 1000, and 4000 micrograms of anthranilic acid in 10 milliliters of medium. Growth at the higher levels was as rapid as that in the presence of 25 micrograms of the acid, with no initial retardation, and reached definitely higher final values. The altered strain was then tentatively considered a mutant and was designated L. arab. A.

After two transfers in peptone-yeast extract-glucose medium, the new strain was compared with the original type in the presence of 1000 micrograms of anthranilic acid and 25 micrograms of pyridoxine per tube. Culture volume in this experiment was 11 milliliters. Results are shown in Figure 4. The normal growth curve obtained with strain A demonstrates ability to utilize anthranilic acid freely under conditions in which it is inhibitory





Figures 3a (above) and 3b (below). Growth curves of L. arabinosus 17-5 cultures supplied varying levels of anthranilic acid. Figures identifying curves indicate micrograms of anthranilic acid per 10 milliliter culture. Cirves of levels up to and including 500 micrograms are from averages of duplicate cultures; those for the highest three levels are from single cultures (see text). The curve for 500 micrograms is included in both figures for comparison.

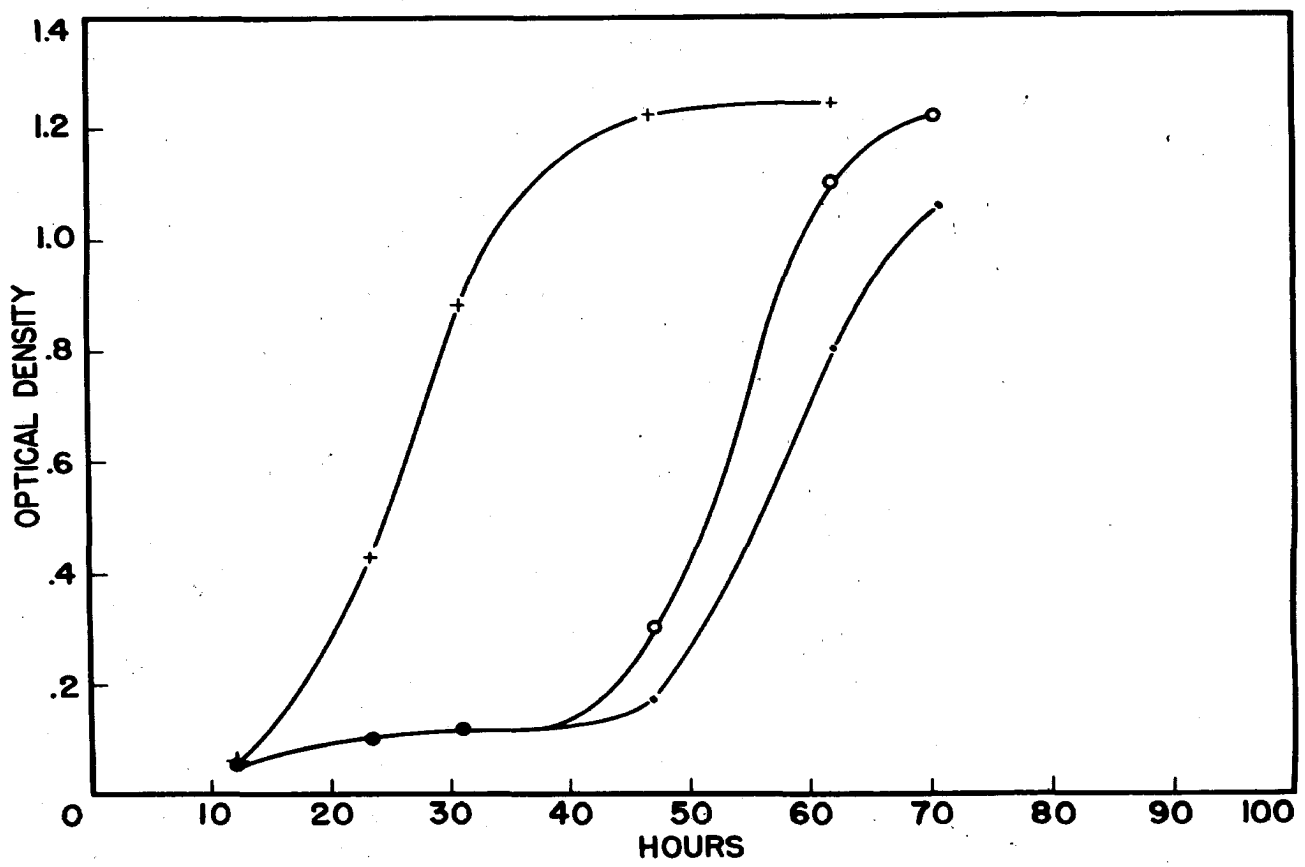


Figure 4. Growth of L. arabinosus 17-5 and L. arabinosus A cultures containing 1000 micrograms of anthranilic acid and 25 micrograms of pyridoxine per tube. Culture volume 11 milliliters. Crosses and dots represent readings of individual duplicate cultures of L. arabinosus 17-5; crosses indicate averages of duplicates of strain A.

to the original strain. The cultures of the parental type show the same delayed initiation of growth as previously noted, followed by a growth curve of normal shape and slope. These results are interpreted as strongly confirmatory evidence for the hypothesis that a genetic change is involved in the adaptation to ability to utilize anthranilic acid at high levels. Passage of strain A through successive transfers in the natural medium should have caused reversion to the original state if the adaptation were due to increased level of enzymes which the original strain is genetically capable of producing ("adaptive enzymes") or any similar non-heritable physiological change. Growth curves for the two tubes inoculated with the original strain are plotted separately to show that very large differences between duplicate tubes after a given period of incubation (e. g., 62 hours) may result from essentially identical growth behavior of the two cultures if growth is initiated in one only a few hours later than in the other. The close resemblance of these two curves to each other and to that for strain A is considered strong evidence that the same mutation which differentiates strain A from the parent strain had occurred and been selected in these tubes. In a large number of similar tubes in several different experiments, heavy growth in the presence of

initially unavailable levels of anthranilic acid has always appeared at from three to four days, indicating that the mutation concerned occurs with relatively high frequency.

Attempts to estimate mutation frequency by the method of Luria and Delbrück (1943), which involves plating into medium in which only the mutant will yield visible colonies, have been unsuccessful. In agar medium containing anthranilic acid at levels which are inhibitory in liquid medium, each normal cell gave enough growth to produce a colony barely visible to the naked eye when diluted suspensions were plated, or cloudiness of the agar when undiluted suspensions were used. Since from 10^5 to 10^9 cells were plated in these experiments, this amount of growth was sufficient to allow the production <u>on the plates</u> of several mutant cells. Small colonies of parental cells in which mutations occur during suitable periods of growth will develop into large mutant colonies which cannot be distinguished from those arising from mutant cells present in the inoculum. The latter colonies are the only ones which should be counted for use in the equations of Luria and Delbrück, however. The situation was further complicated by the fact that no large colonies formed during the first two days, when colonies from mutants present in the inoculum should

appear. The number of large colonies finally forming was nearly as great in plates receiving one milliliter of a 10^{-4} dilution of the washed culture as in those plated with a 10^{-2} dilution, while no large colonies arose from the plating of undiluted cultures. Radically different values for mutation frequency would thus be obtained if these data were used for estimation of mutation rate. Until such effects can be explained and prevented, neither determination of mutation frequency nor quantitative studies on relative growth rates of mutant and parent in different media can be valid.

Strain A does not differ from the parent strain in either rate or extent of its growth response to limiting levels of anthranilic acid, as shown in Table 6. The mutant strain gives a normal dosage-response curve, leveling off after reaching maximum growth at about 250 micrograms of anthranilic acid per tube. The parent strain gives identical responses up to slightly below this optimum level, followed by the aberrant behavior previously discussed.

The rapid growth which resulted from the addition of 10,000 micrograms of anthranilic acid (Figure 2) might conceivably result from earlier appearance of the mutant strain, from its more rapid growth, or from ability of the parent strain to grow under these

Table 6. Responses of L. arab. 17-5 and L. arab. A to Varying Levels of Anthranilic Acid.

(Tabulated values are optical densities x 100; averages of duplicate 10 milliliter cultures.)

Anthranilic acid (γ/tube)	Incubation time (hours)					
	20		45		75	
	17-5	A	17-5	A	17-5	A
0	6	6	8	7	6*	8
10	48	49	62	61	65	64
50	77	71	95	92	94	92
100	80	78	122	107	116	106
250	39	82	108	122	110	118
500	16	79	30	130	66	120
1000	10	78	48	130	107	122
2500	8	82	38	125	104	118

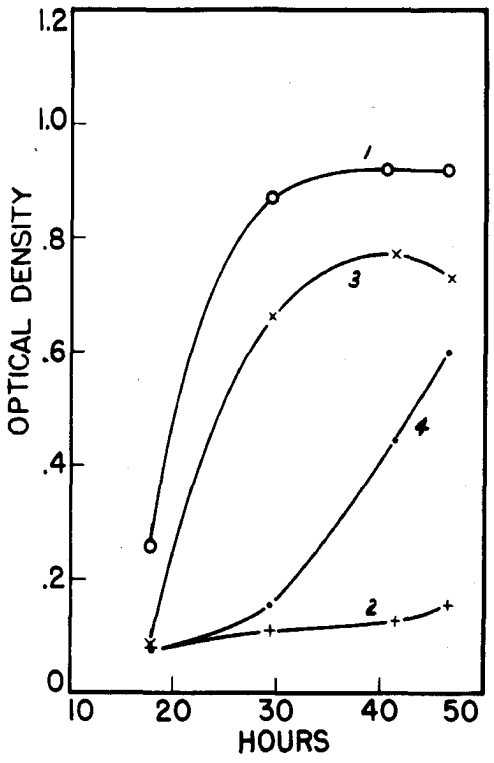
* one aberrant tube omitted.

conditions. The first alternative would require that the rate of mutation be affected by the level of metabolite, and was deemed highly unlikely. Figure 3 shows that growth rates were not responsible for the observed differences. It therefore seemed likely that further addition of anthranilic acid beyond the inhibitory levels had created conditions in which the parent strain could utilize the acid. This inference was strengthened by the agreement between duplicate tubes at the 10,000 microgram level.

In the experiment from which Figure 2 was constructed, the pH of the double-strength medium was adjusted to 6.8 before addition to the tubes containing the various levels of anthranilic acid. The final pH's of the individual tubes were unknown. Corresponding tubes were therefore made up for investigation of this point, and it was found that 1000 micrograms of anthranilic acid lowered the pH only to 6.7, while the addition of 10,000 micrograms resulted in a pH of 5.8. To test whether the observed growth response at the latter level might be related to the lower initial pH of the culture, the parent strain and strain A were inoculated into four media--three corresponding to the 50, 1000, and 10,000 microgram levels of the earlier test and the fourth containing 10,000 micrograms of anthranilic acid with

the pH readjusted to 6.9 after addition of the acid. Results obtained with the parent strain, shown in Figure 5a, were thought to indicate that lower pH was at least partially responsible for the rapid growth in the presence of 10,000 micrograms of anthranilic acid. No reason for the difference between the curve for the culture with pH adjusted after addition of 10,000 micrograms of anthranilic acid and that for the culture containing 1000 micrograms of the acid is suggested. In subsequent experiments, the former curve has generally diverged less widely from the latter, but has always lain above it. If pH were the sole factor responsible for the growth observed at 10,000 micrograms, these two curves should be identical. Evidently some other factors are involved. Responses of strain A to the same media are plotted in Figure 5b.

Repetition of the experiment from which Figure 2 was constructed (response of the original strain to different levels of anthranilic acid) resulted in essentially complete duplication of the earlier results except for a greater delay in growth initiation at the 5000 microgram level, which broadened the valley-like depression zone seen during the 48 to 60 hour period. Rapid growth was again observed in tubes containing 10,000 micrograms of the acid with pH not subsequently



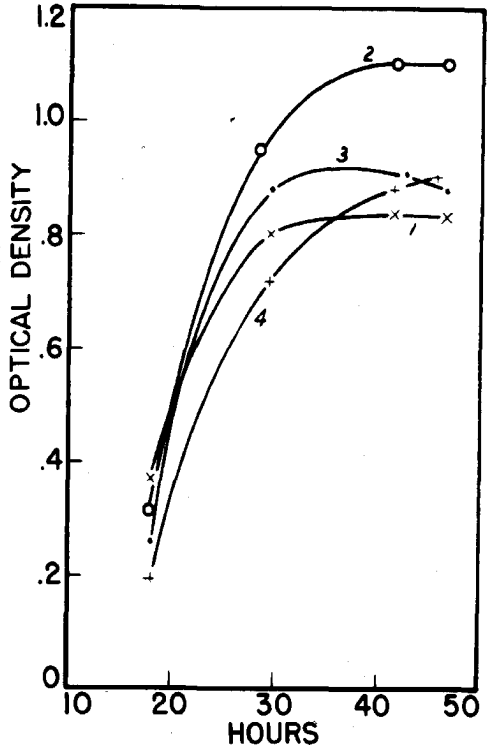


Figure 5a (left). Growth of L. arabinosus 17-5 in four media differing in anthranilic acid level and pH. Each culture contained 25 micrograms of pyridoxine and lacked tryptophan. Anthranilic acid levels in micrograms per tube and pH of the media were: 1) 50, pH 6.8; 2) 1000, pH 6.7; 3) 10,000, pH 5.8; 4) 10,000, pH 6.9.

Figure 5b (right). Growth of L. arabinosus A in four media differing in anthranilic acid level and pH. Media and conditions same as for Figure 5a.

adjusted. In this experiment, anthranilic acid was tested at levels ranging from 10 to 10,000 micrograms. Differences between duplicate tubes which were considered significant (greater than .03 optical density units) occurred at all levels in the delayed-growth zone (250 to 5000 micrograms) and at no other levels, again strongly suggesting the random appearance of a spontaneously occurring mutant.

After final growth had been reached in the experiment just described, transfers from tubes containing anthranilic acid at 50, 1000, and 10,000 micrograms were made to the four media previously described for comparison of strain A with the parent strain (see Figure 5). Transfers from the 50 and 10,000 microgram levels gave curves superposing almost perfectly on those of Figure 5a (except for slight differences in the curve for medium with pH adjusted after addition of 10,000 micrograms of the acid), while curves for the transfer from the 1000 microgram level were virtually identical with those of Figure 5b. These results could be interpreted as indicating ability of the parental strain to utilize anthranilic acid at moderate levels regardless of initial pH of the culture (at least up to neutrality), but at high levels only if the initial pH were well below 7 (since pH at the 10,000 microgram

level was not readjusted). It would follow that the disproportionate effect of small amounts of tryptophan or indole on availability of high levels of anthranilic acid might be due to lowering of culture pH by the limited growth (with consequent acid production) permitted by the tryptophan or indole.

If this hypothesis relating growth on high levels of anthranilic acid to the pH of the cultures were true, it should be possible to obtain maximum and rapid growth of the parent strain by allowing a small amount of "priming" growth to occur in the presence of a low level of anthranilic acid before the addition of initially inhibitory levels of the same substance. The results of such an experiment are shown in Figure 6. As inoculated, the cultures contained 50 micrograms of anthranilic acid. Curve A shows the growth resulting from this medium without further addition. The addition of 1000 micrograms of anthranilic acid ten hours after inoculation (curve marked 10) reduced growth nearly to the level occurring in control tubes containing 1000 micrograms of anthranilic acid at inoculation (curve B). At ten hours, when this addition was made, growth was barely visible as faint turbidity not detectable by the spectrophotometer used, and pH had not changed from the initial value of 6.5. By sixteen hours after inoculation,

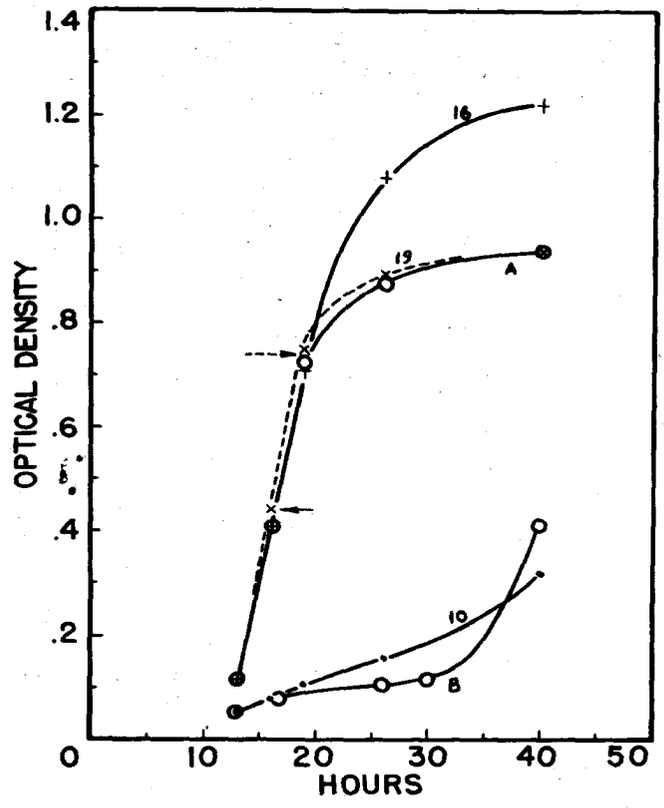


Figure 6. Effect of addition of 1000 micrograms of anthranilic acid during growth of L. arabinosus 17-5 cultures originally supplied 50 micrograms of the acid. Curves represent:

A) control--contained 50 micrograms of anthranilic acid when inoculated; no additions.

B) control--contained 1000 micrograms of anthranilic acid when inoculated; no additions.

10, 16, 19)--contained 50 micrograms of anthranilic acid when inoculated; 1000 micrograms added at 10, 16, and 19 hours respectively.

Solid and broken arrows indicate times of addition to cultures 16 and 19 respectively.

the optical density had reached 0.10 to 0.13, and pH was 5.7. Addition of the same amount of anthranilic acid at this time had no effect on the rate of growth, but caused it to continue on to a higher terminal level than that of cultures receiving no additional anthranilic acid. When the same addition was made at 19 hours, however, at which time optical density had reached 0.73 to 0.75 and pH was 4.0, no effect was observed (curve marked 19). These cultures did not differ significantly from those receiving no additional anthranilic acid. Apparently addition of this amount of anthranilic acid before a given amount of growth has occurred inhibits growth of the parental-type cells, and addition near the end of the logarithmic portion of the growth curve does not change the growth response, while there is an intermediate period during which added anthranilic acid is not inhibitory, but can be converted into tryptophan. These results are consistent with the hypothesis that inhibitory effect of anthranilic acid is related to pH (in the medium used growth is limited by acidity at pH 3.8), but are not critical evidence for that hypothesis, since many factors other than pH are of course changing during growth of the culture.

Attempts to determine directly the importance of initial pH by growth responses of the original strain

as a function of pH in medium containing anthranilic acid at 1000 micrograms and pyridoxine at 25 micrograms per tube have been inconclusive. Such media were made up with initial pH's ranging from 5.6 to 8.2. Decrease of pH on autoclaving is greater for initially basic media, and the pH's at the time of inoculation ranged only from 5.5 to 6.7. In two experiments, no appreciable growth occurred during the first thirty hours, with rapid growth being initiated in all tubes soon thereafter. This lag, together with the fact that duplicates generally differed significantly during the period of logarithmic growth, suggested that mutant cells were involved. This inference was confirmed by transfer from the tubes of one such experiment to liquid medium containing anthranilic acid at 50 and 1000 micrograms per tube. Typical strain A responses (see Figure 5b) were obtained in all cases, indicating that all cultures contained mutant cells at least in considerable number. Decreasing initial pH as far as 5.5, then, does not permit growth of parental-type cells in the presence of 1000 micrograms of anthranilic acid.

In both of the experiments just described, there was a tendency for cultures at initial pH's (at time of inoculation) of 5.8 to 6.2 to give somewhat more rapid adaptation than those at either higher or lower pH. To

test the significance of this observation, responses of the parental strain to levels of anthranilic acid ranging from 25 to 20,000 micrograms per tube were compared in media made up with solutions, including anthranilic acid, adjusted to 7.0 or 6.0 (6.5 and 5.8 at time of inoculation). Results are shown in Table 7. It is evident that initial pH exerts a marked effect only on cultures at the two highest levels of anthranilic acid tested. Thus, although the earlier observations as to the effect of pH on cultures containing 10,000 micrograms of anthranilic acid are confirmed, their extension to explain the peculiar shape of the anthranilic acid dosage-response curve is seen to be in error. Essentially the same shape is obtained at both of the pH values tested.

The effect of varying vitamin B_6 activity on the anthranilic acid high-level inhibition is shown in Table 8. Pyridoxamine was used in this experiment in order to avoid the very high levels which would be necessary were such a test run using pyridoxine. Inspection of the table indicated that the upper limit of the pyridoxamine activity range found for the low levels of anthranilic acid holds also for higher levels. Pyridoxamine at 0.1 microgram per tube was earlier shown (Table 3) to allow nearly maximum utilization of anthranilic

Table 7. Response of L. arabinosus 17-5 to varying levels of anthranilic acid at two initial pH values.

(Tabulated values are optical densities x 100; averages of duplicate 10 milliliter cultures.)

Anthranilic acid (γ/tube)	Incubation time (hours) 12	17	21	25	31	36	43	54	64
	pH 6.5 when inoculated								
25	18	76	88	94	100	100	100	102	101
50	16	80	106	114	119	123	122	124	124
100	15	73	108	121	129	128	128	128	128
250	11	42	82	102	114	122	124	125	125
500	6	15	23	31	46	68*	90	103	106
1000	4	8	9	12	15	30	85	114	120
2500	4	6	8	9	13	40*	91*	114*	120*
5000	5	8	9	10	16	34*	84*	112*	117*
10000	5	6	9	12	20	35	73	102	108
20000	4	8	14	25	59	85	102	108	110
	pH 5.8 when inoculated								
25	32	78	88	94	98	100	100	101	101
50	27	76	103	113	120	120	121	121	120
100	20	68	99	112	120	124	124	124	120
250	7	18	38	64	93	104	108	110	111
500	6	8	10	12	18*	36*	82*	108*	112
1000	2	3	4	6	10	33*	88*	112	118
2500	2	3	4	6	10	22*	63*	103*	112
5000	4	6	8	12	22	40	76	96	98
10000	3[a]	6	14	30	67	88	101	104	103
20000	4	14	40	66	88	98	104	104	106

* duplicates differ by more than 0.05 optical density units.

[a] values in this line are from a single tube because the duplicate was broken.

Table 8. Response of L. arabinosus 17-5 to varying levels of anthranilic acid and pyridoxamine.

(Tabulated values are optical densities x 100; averages of duplicate 10-milliliter cultures.)

Anthranilic acid (γ/tube)	Incubation time (hours) 12	17	21	25	31	36	43	54	64
	A. Pyridoxamine 0.01 γ/tube								
25	4	7	8	10	12	12	14	19	47*
50	6	12	16	20	22	24	27	30	38*
100	8	24	42	55	70	84	94	100	102
250	10	41	70	89	103	108	110	112	113
500	10	45	73	83	89	92	91	91	92
1000	10	46	63	68	74	74	74	76	78
2500	12	35	45	50	54	54	54	56	55
5000	4	6	6	8	10	11	14	30*	55*
10000	4	7	8	10	14	21	38	62	72
20000	4	9	13	18	34	52	68	78	80
	B. Pyridoxamine 0.1 γ/tube								
25	18	76	88	94	100	100	100	102	101
50	16	80	106	114	119	123	122	124	124
100	15	73	108	121	129	128	128	128	128
250	11	42	82	102	114	122	124	125	125
500	6	15	23	31	46	68*	90	103	106
1000	4	8	9	12	15	30	85	114	120
2500	4	6	8	9	13	40*	91*	114*	120*
5000	5	8	9	10	16	34*	84*	112*	117
10000	5	6	9	12	20	35	73	102	108
20000	4	8	14	25	59	85	102	108	110
	C. Pyridoxamine 0.5 γ/tube								
50	14	79	113	120	122	126	125	126	126
100	14	71	109	122	127	132	129	131	129
250	9	40	86	107	123	126	125	130	129
500	6	14	24	36	62	86	102	112	114
1000	4	8	10	12	14	32*	82*	117*	122
2500	4	6	7	8	12	24*	90*	121	125
5000	4	6	7	10	14	46*	100	122	127
10000	4	6	8	12	18	36	80	110	114
20000	8	11	16	24	56*	83	100	104	106

* duplicates differ by more than 0.05 optical density units.

acid supplied at 10 micrograms per tube; in the present experiment little or no significant difference could be seen between the responses to anthranilic acid in the presence of pyridoxamine at 0.1 and at 0.5 micrograms per tube. These responses also agreed well with those obtained in the presence of 25 micrograms of pyridoxine.

The cultures receiving pyridoxamine at 0.1 and 0.5 micrograms (series B and C) exhibited the typical pattern of inhibition in the range between about 500 and 5000 micrograms, followed by initiation of rapid growth, usually at slightly different times. Cultures receiving either higher or lower levels of anthranilic acid showed no such pronounced break in their growth curves and no significant differences between duplicates. (In the one case at the 20,000 microgram level for which a discrepancy between duplicates is indicated the difference was 0.06 optical density units, and may have arisen through reading error.) The cultures receiving only 0.01 micrograms of pyridoxamine, on the other hand, showed a markedly different response pattern. Tubes at the lower levels of anthranilic acid grew very weakly, in agreement with earlier tests at similar levels (e. g., see Table 3). At intermediate levels, however, steady and moderately rapid growth occurred,

and cultures in this series which contained 1000 or 5000 micrograms of anthranilic acid had reached essentially maximum growth by 30 hours, at which time similar cultures receiving more pyridoxamine had not yet initiated growth.

The differences between the different series of this experiment could be fairly well accounted for by the assumption that in the presence of only 0.01 microgram of pyridoxamine anthranilic acid is from one-fifth to one-tenth as active as with optimum levels of the vitamin, and that this differential in activity extends also to inhibitory action. Thus the growth in the series A tubes supplied 250 micrograms of anthranilic acid was comparable with that of series B or C in the presence of 25 or 50 micrograms, while marked inhibition up to 24 hours was seen in series A first at 5000 micrograms, but appeared in the other series at 500 micrograms. The absence of evidence for mutation (in the form of discrepancies between duplicates) in series A is difficult to explain. (The initiation of apparently mutant growth at the 25 and 50 microgram levels presumably does not concern the strain A mutation, and was not studied. These cultures reached terminal optical densities of about 0.80.)

Addition of p-aminobenzoic acid at levels of 10

and 100 micrograms per tube did not prevent inhibition of the parental strain by 1000 micrograms of anthranilic acid. Nicotinic acid also showed no effect when added at 50 micrograms per tube to cultures containing anthranilic acid at levels ranging from 50 to 5000 micrograms.

Tests of Possible Intermediates or Inhibitors

Several compounds related structurally to anthranilic acid, indole, or tryptophan have been tested for tryptophan activity or for ability to inhibit the conversion of anthranilic acid to tryptophan. It was felt that the discovery of compounds having either type of activity might throw some light on the steps involved in normal tryptophan biosynthesis.

The first compounds tested were a group of N-substituted anthranilic acid derivatives[1]. Substituents tested in this position were the acetyl, formyl, methyl, ethyl, and carboxymethyl ($-CH_2 \cdot COOH$) groups. The formyl and carboxymethyl derivatives appeared in early tests to be inhibitory when added at 5000 micrograms per tube to cultures containing 10 micrograms of anthranilic acid. As will be seen below, however, this effect can be attributed to conversion of the derivatives to anthranilic acid itself, which as previously shown does not support growth when present at high levels in neutral medium. None of the other compounds

1. These compounds were prepared by Dr. F. N. Minard.

showed any inhibitory activity.

For evaluation of relative tryptophan activities of the compounds, dosage-response experiments were run and response (titration value or optical density) plotted against the logarithm of dosage level. If a constant ratio of activities exists, such curves should be identical in shape, with their horizontal displacements depending on relative activities. Superposition of the curves bore out this expectation, agreement between their shapes being very good. A typical anthranilic acid dosage-response curve obtained after about two days' incubation (52 hours in this case) is shown in Figure 7a, and response to three of the N-substituted derivatives is shown in Figure 7b. The medium used for this test lacked tryptophan and contained pyridoxin at 25 micrograms per tube.

On superposition of the curve of anthranilic acid response on that of a derivative, the amount of derivative corresponding in tryptophan activity to any given level of anthranilic acid may be read directly from corresponding points on the two abscissas and the molar activity ratio calculated. Thus when the anthranilic acid and N-carboxymethyl anthranilic acid curves were matched, the 10-microgram point on the abscissa of the former lay on the 17.8-microgram point of the abscissa

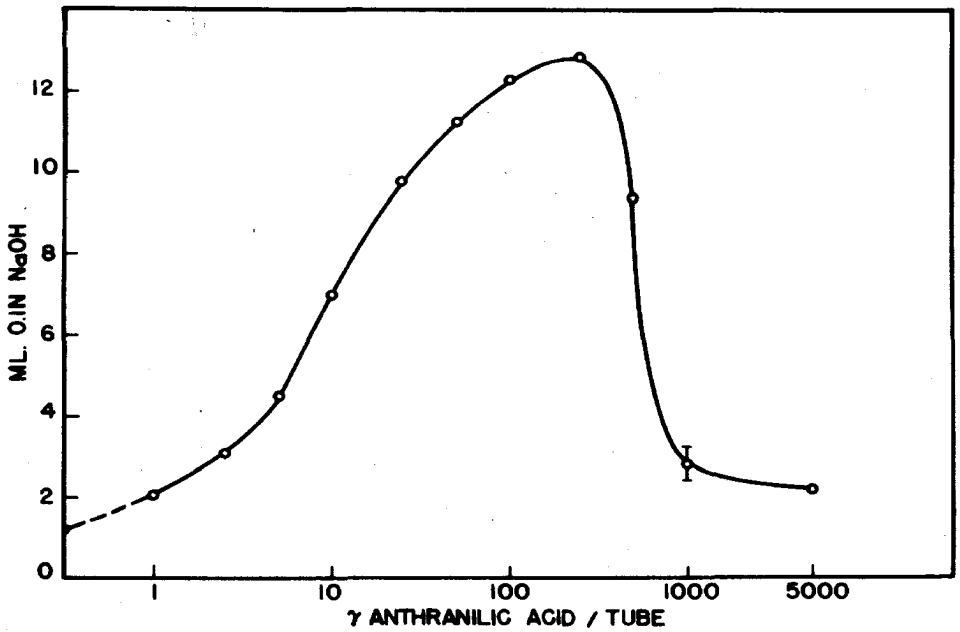


Figure 7a. Response of L. arabinosus 17-5 to varying levels of anthranilic acid. Pyridoxine 25 micrograms per culture; tryptophan omitted. Incubation time 52 hours.

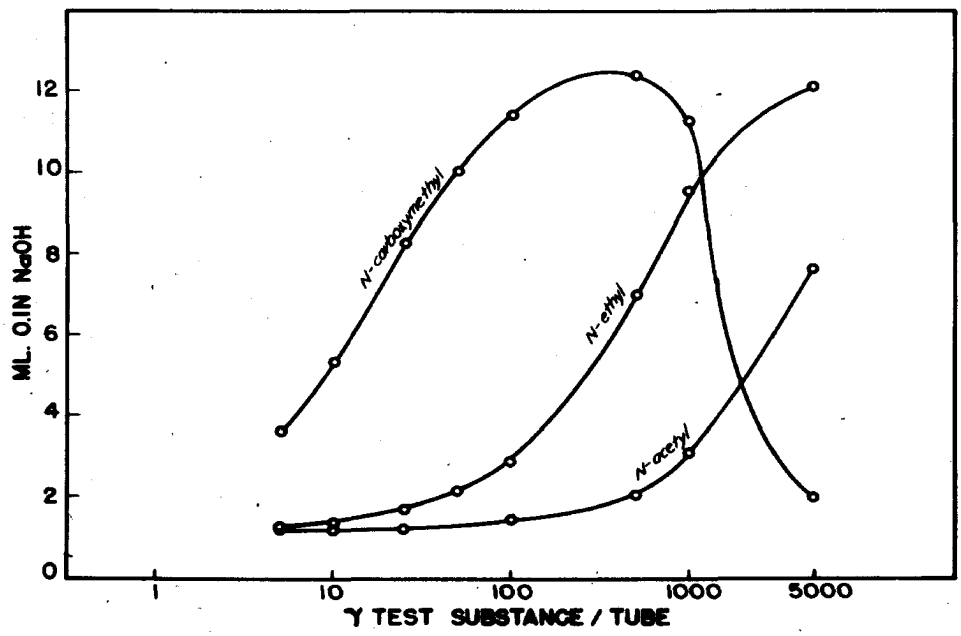


Figure 7b. Response of L. arabinosus 17-5 to varying levels of three N-substituted anthranilic acid derivatives. Conditions as for Figure 7a.

of the latter. From these figures, the activity of the carboxymethyl derivative was calculated as 80% that of anthranilic acid on a molar basis. Activities for N-substituted derivatives obtained in this way are presented in Table 9. The N-formyl derivative was sterilized by filtration and added after autoclaving for comparison with the usual procedure of addition before autoclaving. The increased activity resulting from the latter method is evidence for considerable hydrolysis of the compound in the autoclave.

Their relatively low activities appear to rule out all but one of the N-substituted anthranilic acids as possible intermediates in the conversion of anthranilic acid to tryptophan, while the close matching of their response curves with that for anthranilic acid suggests that such activity as they show is due to partial conversion to anthranilic acid itself. On the basis of the observed activities, only the carboxymethyl derivative was considered to merit further study. This compound was compared with anthranilic acid in an experiment in which both substances were added after autoclaving and growth was followed turbidimetrically. For each reading, relative molar activities were determined by curve matching as described above. The results, given in Table 10, are considered to confirm

Table 9. Growth-promoting Activities for L. arab. 17-5 of N-substituted Anthranilic Acid Derivatives in the Absence of Tryptophan.

(Activities are recorded as percentages of anthranilic acid activity on a molar basis. Incubated 52 hours.)

Substituent	Activity (%)
H (anthranilic acid)	100
carboxymethyl	80
ethyl	2
methyl	2
acetyl	4
formyl (added before autoclaving)	19
formyl (added after autoclaving)	6

the inference that the N-substituted derivative functions through conversion to anthranilic acid. This conversion is evidently slow enough that it determines the growth rate of the culture. Alternatively, anthranilic and

Table 10. Growth-promoting Activity for L. arab. 17-5 of N-carboxymethyl Anthranilic Acid as a Function of Incubation Time.

(Activities are recorded as percentages of anthranilic acid activity on a molar basis. Medium lacked tryptophan.)

Time of incubation (hours)	Activity (%)
18	18
24	25
37	49
87	106

N-carboxymethyl anthranilic acids might be converted to a common product, but in this case the rate of conversion of the latter compound would be relatively so slow as virtually to eliminate it from consideration as a participant in normal tryptophan synthesis.

As representative ring-substituted derivatives, 5-chloro- and 5-methylanthranilic acids were tested

for tryptophan activity or inhibition of tryptophan production[1]. The chloro derivative exhibited tryptophan activity of the order of one thousandth that of anthranilic acid, while the methyl compound lacked such activity altogether. Neither substance inhibited the utilization of anthranilic acid up to maximum weight ratios of 800 for the chloro derivative (4000 micrograms to 5 micrograms of anthranilic acid), and 2000 for the methyl derivative (10,000 micrograms to 5 micrograms of anthranilic acid).

Among compounds having other functional groups substituted for the carboxyl or amino groups of anthranilic acid, orthanilic acid (o-aminobenzenesulfonic acid) and salicylic acid (o-hydroxybenzoic acid) appear to be the only ones previously tested. Snell (1943) reported that these compounds neither replaced anthranilic acid nor inhibited its utilization. These findings were confirmed. The data for salicylic acid in Table 11 are typical of those interpreted as showing ability neither to replace nor to inhibit anthranilic acid. The slightly increased growth in the presence of 2 or 10 milligrams of salicylic acid per tube represents negligible ability to replace anthranilic acid (of the order of 0.1%). The decrease in growth

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1. These compounds were prepared by Robert Tichane.

Table 11. Effects of Some Structural Analogs of Anthranilic Acid on Growth of L. arabinosus in Presence of Anthranilic Acid or of Tryptophan.

(Tabulated values are averages of duplicate titrations with 0.1 N NaOH. Incubation time 53 hours.)

	Test cpd. level mg./tube	Control	o-Bromo-benzoic acid	o-Chloro-benzoic acid	o-Toluic acid	Salicylic acid
Anthranilic acid 5 γ/tube	2	2.9	2.9	2.9	2.9	3.8
	10		3.3	3.1	3.4	3.9
	50		4.0	3.7	3.5	2.0
L-tryptophan 100 γ/tube	2	8.7	9.1	8.8	8.1[a]	7.7
	10		7.5	7.7	8.0	7.2
	50		6.1	7.0	5.7	4.1

a. One widely aberrant value not averaged in.

caused by the addition of 50 milligrams of salicylic acid per tube, on the other hand, cannot be related to interference with tryptophan synthesis, since addition of an optimal level of tryptophan did not release the inhibition.

Other structural analogs of anthranilic acid tested included o-chlorobenzoic acid, o-bromobenzoic acid, and toluic acid (o-methylbenzoic acid). None of these compounds showed significant tryptophan-replacing activity, and all failed to inhibit conversion of anthranilic acid to tryptophan up to weight ratios of 10,000 (50,000 micrograms of test compound to 5 micrograms of anthranilic acid) as shown in Table 11. These compounds at low levels also failed to replace tryptophan, so that the data of Table 11 cannot be due to any high-level effects like those of anthranilic acid.

Skatole (3-methylindole) exhibited no significant tryptophan-replacing activity and no interference with the utilization of anthranilic acid or indole at weight ratios up to 200 (1000 micrograms to 5 micrograms of indole or anthranilic acid). At 5000 micrograms per tube skatole caused complete inhibition of growth. This growth inhibition, however, was not overcome by tryptophan even at 1000 micrograms, and so cannot be due to interference with tryptophan synthesis. Fildes

(1940a) had reported failure of skatole to replace indole in tryptophan synthesis by tryptophan-producing strains of S. typhosa.

Indoleacetic acid and cinnamic acid were also tested as possible inhibitors of a late step in tryptophan synthesis (in case an indole-serine condensation does not occur in Lactobacillus). Indoleacetic acid had no effect at ratios with respect to anthranilic acid or L-tryptophan of up to 2000 (10,000 micrograms of indoleacetic acid, 5 micrograms of anthranilic acid or L-tryptophan). Cinnamic acid inhibited in the presence of anthranilic acid, but at least as strongly in the presence of tryptophan, so that again tryptophan synthesis cannot have been the process interfered with.

Tests of Compounds which Might Condense with Indole

In attempts to test for participation of serine in the production of tryptophan by Lactobacillus, the effects of added serine on growth in the presence of tryptophan, indole, and anthranilic acid were compared. As would be predicted by the hypothesis of an indole-serine condensation like that operative in Neurospora, the addition of serine had little effect on tryptophan-containing cultures, but showed definite stimulation in those supplied indole or anthranilic acid. Threonine,

however, showed as marked an effect as serine, so that no support for the use of serine in tryptophan biosynthesis can be adduced from these experiments. Threonine variation was included in this study as a test of the possibility that this amino acid might condense with anthranilic acid to yield tryptophan directly. Both serine and threonine stimulated growth with either indole or anthranilic acid, however, so that no evidence for or against such an alternate pathway was obtained. There seemed to be some tendency, in fact, for threonine to be more effective in the presence of indole than when anthranilic acid was supplied.

Acetate level affected growth on indole or anthranilic acid more markedly than did either serine or threonine, and the efficiency of conversion of anthranilic acid to tryptophan in acetate-buffered medium devoid of both serine and threonine was about 50%. No separation was made of the direct effect of acetate from indirect effects due to differences in buffer range and capacity between the acetate-containing medium and the phosphate-buffered medium (2.5 g KH_2PO_4 per liter) with which it was compared. On consideration of the complexity of the media required for growth of lactic acid bacteria, and in view of the many effects that any added substance may have on other cell processes, it was felt that such

experiments were unpromising, and they were discontinued. Either acetate or threonine (but not both) is required even in the presence of tryptophan, for example, so that effects of addition of these compounds to media lacking tryptophan can not be related solely to its synthesis. The relatively high efficiency of the anthranilic acid-tryptophan conversion in the absence of both serine and threonine, noted above, is evidence that if either of these compounds is involved in tryptophan synthesis it is produced under the conditions of these experiments at such a rate that significant stimulation by exogenous material would be difficult to demonstrate. Finally, pyruvic acid and alanine, two compounds which must be most seriously considered as possible participants in tryptophan synthesis, will inevitably be present, since the former is the precursor of lactic acid in the normal fermentation of glucose and can produce alanine by transamination.

In the experiments just described, the conversion of precursors to tryptophan is inferred from the extent of growth occurring in the same culture in which the conversion is carried out. As discussed above, the results may be difficult to interpret because added test substances affect growth in ways unrelated to tryptophan synthesis. Such effects could be avoided

if non-growing cell suspensions could be caused to convert indole to tryptophan, which could then be determined quantitatively. Complete removal of indole without loss of tryptophan would allow determination of the latter substance by Lactobacillus assay without reintroduction of the type of ambiguities just discussed, since serine, alanine, and the other compounds to be tested are constituents of the assay medium. In the absence of anthranilic acid and indole, further additions of components of the medium have no effect on the accuracy of tryptophan assay. Ether extraction has been recommended for removal of indole from natural products to be assayed for tryptophan (Greene and Black 1943), and was found satisfactory under the conditions of the proposed experiments. In exploratory tests, 99% of the indole was removed from a solution containing 100 micrograms per milliliter by a single extraction with half a volume of ether, and no indole was detected after two such extractions. (The determinations were made by Lactobacillus assay, which in the absence of tryptophan is reliable for indole.) No tryptophan was lost in the extraction. A simple method for the detection of any tryptophan which might be formed by cell suspensions was thus available.

Attempts to obtain conversion of indole to tryptophan

by non-dividing cell suspensions were unsuccessful. Heavy suspensions were used (one-eighth the terminal density reached in complete medium, or about 10^8 cells per milliliter), and indole was present at the relatively high level of 100 micrograms per milliliter. Conditions of assay were such that a tryptophan yield of the order of 0.5% would have been readily detected. In the presence of pyridoxine and inorganic phosphate, additions of serine, alanine, or glucose resulted in no tryptophan production during one to five days incubation at 36° or room temperature. In some tests the cells used were harvested from cultures grown with indole replacing tryptophan, in an attempt to insure maximum activity of the enzyme system involved.

DISCUSSION

The results reported this thesis differ from those of Schweigert (1946; Schweigert *et al.* 1947) in regard to the efficiency of the anthranilic acid-tryptophan conversion and the effect of small amounts of tryptophan on this conversion. No particular explanation for these differences is suggested. It may be noted, however, that in systems involving as many variables as are concerned in these tests, such differences between laboratories are perhaps to be expected.

Quantitative differences, especially such as those between conversion efficiencies, need cause little concern. Except for the puzzling discrepancy noted above regarding low levels of tryptophan, all of the qualitative reports of other investigators which were checked in the course of this work have been confirmed. (Such confirmations relate to tryptophan activity, toxicity, or lack of effect of various compounds.)

Anthranilic Acid Dosage-Response Relationships

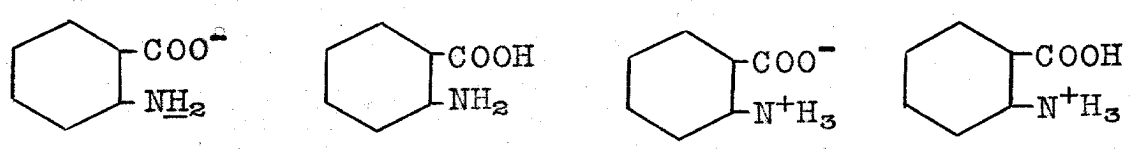
The cause of the peculiar anthranilic acid dosage-response curve of the parental type (L. arabinosus 17-5) has been by no means fully elucidated. The data obtained, however, warrant attempts to propose hypotheses which could be tested in future work.

For purposes of discussion, a review of pertinent experimental findings may be helpful. In the absence of tryptophan, anthranilic acid behaves as an essential metabolite because of the ability of the organism to convert this compound to tryptophan. When L. arabinosus 17-5 is supplied anthranilic acid at levels ranging from 0 to about 100 micrograms per 10 milliliters, growth increases regularly with anthranilic acid level and a plot of the resulting data yields a typical essential metabolite response curve of the type familiar in

microbiological assay. Further increase of anthranilic acid level to about 500 micrograms, however, results in a progressive decrease in both rate of growth and extent of final growth. In the range between 500 and 5000 micrograms, very little growth occurs for 30 to 40 hours, after which time rapid growth is invariably initiated. This growth appears to consist of cells differing genetically from the inoculum. At levels of 10,000 and 20,000 micrograms per culture moderately rapid growth of the original strain occurs. The behavior of the mutant selected at intermediate concentrations (strain A), on the other hand, is similar to that of the parental type up to 100 micrograms of anthranilic acid per culture, but levels off at about 250 micrograms. Further additions of anthranilic acid are without effect. The mutant in this case thus exhibits what may be termed normal behavior, while the parental type is aberrant. A curve qualitatively similar to that described for the parental strain has been reported by Shankman (1943) for the response of L. arabinosus to arginine. The first peak was reached at about 6 micrograms per culture, the minimum growth at about 30, and essentially full growth is again attained at 100 micrograms. No further study of the effect was reported.

In the experiment in which growth of parental-type cells in the presence of 10,000 micrograms of anthranilic acid was first observed (Figure 2), pH of the cultures at this level differed significantly from that of other cultures in the same test. When it was found that the rate of growth at this level was increased by lowering pH from 6.9 to 5.8 (Figure 5a), it seemed likely that pH variation might be of importance in determination of the response curve. As noted in the preceding section, the effects on the parental strain of anthranilic acid in the range 100 to 500 micrograms per tube are strikingly similar to those of a competitive inhibitor of a metabolite (Part II of this thesis). If such a mechanism is involved, increasing the concentration of anthranilic acid would of course increase its ratio to the metabolite, whether present in the medium, synthesized by the bacterial cells, or both.

Anthranilic acid contains two ionizable groups and so exists in four ionic species:



A B C D

The ionization constants for this compound at 35° are reported as 2.39×10^{-12} for the amino group and

1.25×10^{-5} for the carboxyl group (Lunden 1906). From these values it may be calculated that at pH 7 the acid exists over 99% as A. At pH 6, A forms about 92% of the total, with B amounting to 8%. At pH 5, the amount of A has decreased to 55%, with B making up the remainder. Forms C and D do not occur in significant amounts in this pH range. These changes in ratios might conceivably contribute to release of the inhibition on lowering culture pH to 6 or below. The growth of parental-type cells in cultures containing 10,000 or 20,000 micrograms of anthranilic acid would then be due to the lower pH resulting from addition of this amount of acid.

The hypothesis just developed would require that the effect of pH on lower concentrations of anthranilic acid be at least as marked as at 10,000 micrograms. Further, elimination of pH as a variable (for example, by adjusting the anthranilic acid solutions, as well as the medium, to the desired pH before addition to culture tubes) should cause the parental strain to respond no more favorably to anthranilic acid at 10,000 micrograms than at 1000. Figure 5a shows that the latter prediction was not fulfilled. Growth in medium 4, containing 10,000 micrograms of anthranilic acid at pH 6.9, was slow but definite, while in medium 2,

containing 1000 micrograms of anthranilic acid at pH 6.7, virtually no growth had occurred by 46 hours. Both predictions were tested in the experiment reported in Table 7, and both were found false. Variation of pH between 5.8 and 6.5 markedly affected growth rates in the presence of 20,000, 10,000, and probably 5000 micrograms of anthranilic acid, but exerted no comparable effect at lower levels. Holding of initial pH constant at either 5.8 or 6.5 failed to eliminate the differences between the 10,000 and lower levels. Attractive as the hypothesis which attributed these differences to pH had seemed in the light of earlier data, it had to be discarded. The effect of pH variations on growth rates at the highest levels tested may still relate to differential physiological effects of the ionic species of anthranilic acid.

Interference with an unknown metabolite still appears the most likely explanation of the high-level inhibition by anthranilic acid. Further addition of the metabolite involved should reverse the inhibition. The only compounds tested for such action, p-aminobenzoic acid and nicotinic acid, failed to cause reversal.

The absence of anthranilic acid inhibition in the presence of small amounts of tryptophan or indole, or in cultures "primed" by low levels of anthranilic acid,

was first explained also on the basis of lowered pH. This explanation is not tenable in the light of the discussion above. It seems likely that any factors permitting moderate growth will remove the anthranilic acid inhibition, probably through ability of the metabolizing cells to build up the substance required for counteraction of the anthranilic acid inhibition.

From the fact that low levels of indole or tryptophan allow growth in the presence of normally inhibitory levels of anthranilic acid, it would appear that the blocked step is involved in the conversion of anthranilic acid to tryptophan. This possibility enhances the interest of the inhibition both from the standpoint of its relation to tryptophan synthesis and because of the theoretical interest which would attach to such inhibition of a reaction by a precursor of one of the reactants.

The effect of reduced pyridoxamine level is perhaps the most difficult feature of the whole phenomenon to reconcile with any hypothesis which has been considered. It was known that reduction of B_6 activity level would reduce the efficiency of anthranilic acid in replacing tryptophan, as had been reported by Schweigert (1947) and in this thesis (Tables 3 and 4). It was expected, however, that the inhibitory activity

of anthranilic acid would not be affected, since this was thought probably due to some interfering action of the acid itself. The data of Table 8 clearly show that this expectation, too, was not borne out by experiment. As noted in the preceding section, the results are such as to suggest that reduction of pyridoxamine level from 0.1 to 0.01 micrograms per culture reduced anthranilic acid activity by about 4/5, both as tryptophan precursor and as inhibitor. Since pyridoxamine is considered to be involved in tryptophan synthesis only subsequent to indole production, this result, taken by itself, would suggest that some intermediate subsequent to indole is the true inhibitor. Decrease of B_6 activity would then decrease utilization of indole and production of the inhibitor, and hence reduce the apparent inhibitory effect of anthranilic acid. Such a situation is excluded, however, by the earlier finding that even low levels of indole remove the anthranilic acid inhibition.

The complexity of the interactions involved is illustrated by the fact that, if the experiments had been limited to anthranilic acid levels between 500 and 2500 micrograms per culture, the effect could have been spoken of as a pyridoxamine inhibition, since under those conditions much earlier growth occurs in cultures receiving 0.01 micrograms of the vitamin than in those

containing 0.1 or 0.5 micrograms.

The occurrence, selection, and probable importance of bacterial mutants will be more fully discussed in Part II of this thesis. The mutant type designated as L. arabinosus A is a good example of a mutant isolated and characterized solely on the basis of selective advantage over the parental strain in a given medium. Many similar cases will be cited later. As in a large number of the reported cases, the biochemical basis for the selective advantage is unknown. Three possibilities present themselves, however. First, the mutant might possess the ability to produce in large quantity the unknown metabolite which according to the hypothesis is antagonized by anthranilic acid, thus overcoming the inhibition. Second, the mutant might be able to utilize an alternate pathway for the synthesis of the product of the blocked reaction, thus by-passing the inhibition. Finally, the mutation might involve greater specificity on the part of the enzyme responsible for the inhibited reaction. Slight alteration in the active surface of an enzyme might prevent the adsorption of (and hence the interference by) a structural analogue of the normal substrate, while not affecting complex formation with the substrate itself. No means of distinguishing between these possibilities

is at hand, of course, as long as the identity of the inhibited step is unknown.

As noted in the preceding section, attempts to determine the rate of mutation to strain A failed because of inability to distinguish between the two types of cells on plates. Ryan and Schneider (1949a) reported very similar difficulties in connection with a histidineless mutant of E. coli. The difficulty was found to lie in the presence of minute amounts of histidine in the medium. This contamination allowed some growth of histidineless cells, with consequent possibility of mutation in the microcolonies thus formed. It follows that the number of large colonies will be related to the amount of growth which has occurred on the plate (i.e., to the probability of mutation within the microcolonies on the plate) rather than solely to the number of mutants which were actually plated. Amount of growth will depend in turn on the extent of contamination, and will be largely independent of the number of cells introduced onto the plate with the inoculum. This is the situation encountered when L. arabinosus 17-5 was plated into medium containing pyridoxine at 25 micrograms and anthranilic acid at 1000 micrograms per tube. From the results in liquid cultures, this agar medium should have inhibited the growth of

parental-type cells, allowing the determination of the number of strain A cells in the inoculum. However, each cell introduced grew sufficiently to produce a barely visible colony. Under such conditions, the number of large colonies formed obviously has little relation to the number of mutant cells initially present in the inoculum. This difficulty prevented not only the determination of mutation frequency, but also the estimation of number of mutant and parental cells in cultures supplied various levels of anthranilic acid. The alternate method using liquid cultures which was employed (see Figure 5) is capable of distinction only between cultures containing a large number of strain A cells and those containing few or none.

Tests of Possible Intermediates or Inhibitors

The significance of the results obtained in tests of compounds which might be suspected of participating in or affecting the conversion of anthranilic acid to tryptophan have been discussed in the preceding section. These results were such as to exclude all of the compounds tested from consideration as intermediates in the conversion, and no specific inhibitors of the process were found.

In these tests, and particularly in those of

N-substituted anthranilic acids, it was assumed as most likely that whatever the source of the number 2 carbon atom of indole, the nitrogen and number 3 carbon atoms were derived from anthranilic acid. It was hoped that the presence of different substituents on the nitrogen atom might facilitate or hinder the ring closure. It now appears, however, from a very recent paper by Nyc et al., (1949) that this assumption is probably in error. These workers used a tryptophanless Neurospora mutant able to use anthranilic acid, indole, or tryptophan (thus nutritionally similar to L. arabinosus in this respect). When carboxyl-marked anthranilic acid was supplied to this mutant, most of the isotopic carbon was recovered as carbon dioxide, and no appreciable amount was found in either tryptophan or nicotinic acid. Since the formation of tryptophan by condensation of indole and serine seems well established in Neurospora, this result strongly suggests that the carboxyl carbon of anthranilic acid is lost in the production of indole. The mechanism of biosynthesis of indole thus seems even more obscure than before.

Indole-Tryptophan Conversion

Failure to obtain clean-cut results from tests of compounds which might condense with indole in tryptophan

formation was probably related, as noted in the experimental section, to the complexity of the medium and the much greater complexity of the metabolic interconversions between these compounds. It seems unlikely that such a purely nutritional approach, without benefit of tracer techniques or either genetic or chemical blocks, could be made to yield conclusive data.

Several possible reasons for failure to obtain tryptophan production from indole by non-growing cells could be presented. Perhaps most important, the actual coenzyme involved both in production of tryptophan by Neurospora and in its splitting by E. coli is pyridoxal phosphate. Ability of actively metabolizing and growing L. arabinosus cells to produce this coenzyme from pyridoxine and inorganic phosphate does not necessarily imply the same ability on the part of the nondividing cell suspensions; indeed the avoidance of similar interconversions was one reason for use of such suspensions. These experiments were of a preliminary nature, and by no means exclude the possibility that through variation of conditions or by supplying pyridoxal phosphate or other possible cofactors, as well as other possible reactants, such suspensions might be made to produce tryptophan. In such a case, more critical tests of suspected intermediates and participants could be

made than in growing cultures.

If the history of the tryptophanase reaction of E. coli points any lesson in this regard, however, it is that isolation of the responsible enzyme systems allows such reactions to be studied with somewhat more confidence. After twenty-five years of intensive research on the reaction in suspensions and growing cultures (and forty-five years after its discovery) the primary products of the reaction seem finally to have been demonstrated by study of a partially purified enzyme preparation (Wood, Gunsalus, and Umbreit 1947). A similar approach should permit unambiguous tests of compounds which are possible participants in the reverse reaction in Lactobacillus.

SUMMARY

The Lactobacillus arabinosus 17-5 response curve for anthranilic acid in the absence of tryptophan is characterized by regular increase in growth to about 100 micrograms per 10 milliliter culture, steady decrease to about 500 micrograms, nearly complete inhibition from 1000 to 5000 micrograms, and fair growth above 10,000 micrograms. The inhibition is prevented by low concentrations of indole or tryptophan or by allowing initiation of growth at lower levels of

anthranilic acid. Although added at normally inhibitory levels, anthranilic acid is then converted to tryptophan and heavy growth ensues.

The inhibition is believed to involve interference with some essential cellular process, possibly a step in the conversion of anthranilic acid to tryptophan.

A mutant strain (designated L. arabinosus A) overgrows inhibited cultures in two to three days. This mutant apparently differs from L. arabinosus 17-5 only in its insensitivity to anthranilic acid at levels inhibitory to the latter strain.

Five N-substituted anthranilic acid derivatives were tested for inhibition of or participation in tryptophan synthesis. No inhibition was observed, and such tryptophan activity as exists is apparently due to conversion to anthranilic acid. Similar tests of 5-methyl- and 5-chloroanthranilic acids and of o-chloro-, o-bromo-, and o-methylbenzoic acids revealed neither type of activity.

Attempts to determine the course of the indole-tryptophan conversion were unsuccessful.

PART II

TWO MUTATIONS AND A STRUCTURAL ANALOG WHICH AFFECT SYNTHESIS AND METABOLISM OF ARYLALANINES IN *LACTOBACILLUS ARABINOSUS*

INTRODUCTION

Metabolic pathways are frequently studied by methods which aim at the prevention of one specific reaction in otherwise normal organisms. Various enzyme poisons have classically been used to produce such metabolic blocks. The activities of these poisons in general depend on properties of the enzymes (*e.g.*, presence of sulfhydryl groups), and often are not sharply specific.

Two types of more specific metabolic blocks have recently been used in biochemical studies. One of these is genetic, and depends on the selection of mutants, either naturally-occurring or induced, which differ from the wild type by gain or loss of a single metabolic step. The other type of block is chemical, and depends on competition for available enzyme between a metabolite and a structural analog. Both methods may allow identification of intermediates and their assignment to positions preceding or following the blocked step.

This thesis reports applications of both methods to the question of the interrelationships in *Lactobacillus arabinosus* between the three naturally-occurring aryl-

alanines tyrosine, phenylalanine, and tryptophan. Both methods have been previously used in studies of synthesis or metabolism of these amino acids in other organisms, as cited in the following section. (Applications of the genetic block method in studies on tryptophan biosynthesis were discussed in Part I of this thesis.)

HISTORICAL

Probably no recent controversy in the fields of microbiology and biochemistry has been as extensive or as longlived as that regarding the origin of bacterial variants. The question seems at the present time to be as well answered as any other scientific problem of equal complexity; nevertheless the controversy continues in the journals of half a dozen related fields. The explanation for this state of affairs may be found, at least in part, in the varied backgrounds of the many investigators working with bacteria. The science of bacteriology was for many years dominated to a considerable extent by workers whose approach was largely clinical. Dubos (1945, p. 339) writes that

> the first period of the bacteriological era is chacterized by the discovery of a number of phenomena and techniques...which often do not exhibit any obvious relation to the contemporary biological sciences. By the beginning of the twentieth century, bacteriology was following an independent course, almost uninfluenced by

the doctrines and methods of classical biology. It is not surprising, then, that the extensive early literature on the subject of variation and "dissociation" of bacteria contains almost no references to the possibility of considering such phenomena from the standpoint of genetics.

In recent years many of the variations most intensively studied have been physiological or biochemical in nature. Many investigators with predominately chemical training have become interested in such phenomena as the gain or loss of ability to synthesize or to utilize a given compound, or the acquisition of tolerance to drugs. Many of these workers, of course, lack a knowledge of current genetic concepts.

In view of the diverse directions from which bacterial phenomena are being approached, it is not surprising that an amount of evidence much greater than was required to lay the ghost of Lamarckism in regard to higher organisms has not convinced the many workers who still adhere to a belief in the inheritance of acquired characters by bacteria. In the case of higher plants and animals, there were in general only geneticists to be convinced; in the case of bacteria, books and monographs on variations and their transmission are written also by biochemists, physical chemists,

and clinical workers. Because the point of view and even the nature of some of the experimental work reported in this thesis depend on the assumption that the occurrence and transmission of heritable variations in bacteria are in essence the same as in higher plants and animals, some of the relevant scientific literature will be briefly reviewed.

Origin of Bacterial Variants

As early as 1901, when the mutation theory was in its crude infancy, Beijerinck (cited by Braun 1944) suggested that the occurrence of visibly variant colonies of bacteria might be due to mutation. Jordan (1915) pointed out that the large populations and short generation times of bacteria effect a speeding up of selective processes, so that apparent environmental induction of a modification might very well be actually selection of the most adapted type. Cole and Wright (1916) supported the thesis that inheritance in bacteria is probably essentially the same as in other organisms--a point of view which they noted "appears to be novel, or at least unappreciated by most bacteriologists."

The first important experimental test of this concept was made by Lewis (1934). Working with so-called

mutabile strains of coliform bacteria, Lewis showed that a very low proportion of the cells of normal populations possessed the ability to utilize sugars or alcohols which the majority of cells were unable to metabolize. When the cultures were placed in the presence of these substrates, selection of these pre-existing variants, rather than induction of variation, led to the production of an altered strain. Although this paper pointed out in most convincing fashion the implications of this finding and the errors of earlier workers which had led to other conclusions, it seems to have received little attention.

Parr (1938) reported that 29 of over 5000 strains of coliform bacteria tested contained cells able to utilize citrate as an energy source.

Gratia (1921) interpreted his experiments on the lysis of E. coli by phage as indicating that normal bacterial populations contain individuals differing in resistance to phage. Gratia's evidence was inconclusive, but his conclusions were confirmed in most convincing fashion by Burnet (1929).

It is probably in regard to the origin of strains possessing increased tolerance to drugs or having decreased nutritional requirements that the concept of inheritance of acquired characters is most firmly rooted. Knight (1936), in a monograph on bacterial

nutrition, admitted the theoretical possibility that decreased nutritional requirements might arise through spontaneous mutation. He objected, however, that a high rate of mutation would be required, and dismissed the mutation hypothesis by accusing those favoring it of hindering attempts to explain adaptation by hiding behind the word "spontaneous". Both of these arguments are still being used, but both depend on lack of understanding of the experimental conditions or of genetic terminology. The populations of bacteria ordinarily dealt with in single culture tubes are larger than the human population of the earth, and the generation time may be measured in minutes. Even the inocula customarily used contain millions of cells. Mutation rates do not need to be high in order to account for the observed phenomena. In fact, the estimated mutation rates, as will be seen below, are of the same order of magnitude as some estimates for higher organisms. Knight's second argument stems from a misconception of the meaning of the word "spontaneous" as applied to mutations. Rather than implying that there is no cause, the term indicates that the cause is unknown. The only implication as to the origin of the changes, to the present writer at least, is that they occur randomly and that they are not <u>directed</u> by any factor of the external environment.

The literature dealing with the development of strains resistant to sulfonamides will serve to illustrate the points of view encountered in the literature regarding the nature of such variations. Early reports of sulfa-resistant strains (MacLean, Rogers, and Fleming 1939, MacLeod and Daddi 1939) indicate that the authors considered the change to be acquired in response to the environment, but include no explicit statements on this point. Strauss, Dingle, and Finland (1941) state without evidence that "the development of fastness is dependent upon contact between the drug and the organisms." Schmidt and Sesler (1943) concluded from the stepwise manner in which resistant strains were developed that "sulfonamide-resistant organisms are formed through some action of these drugs on the sensitive organisms." When the development of such resistance was finally studied by appropriate techniques, which will be described below, it was found (Oakberg and Luria 1947) that at least five different random mutations are involved. Additive effects account for the stepwise increase in culture tolerance.

Hinshelwood has recently written extensively on bacterial kinetics, with particular reference to drug adaptation. He has insisted repeatedly (e.g., 1946, p. 268) that such adaptations are "automatic responses to changes

in relative reaction velocity which lead to new enzyme balances." He declares (1946, p. 202) that "one can only deny the active participation of the training agent at the cost of assuming an almost unbelievable liveliness in the spontaneous variability," and adds that "phenomena like the transformations of coliform organisms into streptococci are not observed." It is argued that considerations of entropy and free energy requirements show "abnormal division" (mutation) to be unlikely. The same point of view and similar arguments are repeated in a very recent paper (Hinshelwood 1949).

It may be appropriate to point out that the assignment of probabilities to mechanisms necessarily depends on analogy with other better-known processes. Such arguments can be no more valid than the analogies on which they are based. By analogy with certain non-living systems, adaptation by selection of random mutants may seem unlikely; by analogy with heritable variations in other organisms, this mechanism seems extremely likely. The latter analogy seems much the more reliable. A bacterial culture is a large population of individual organisms under extreme selective pressure, and changes in its population characteristics resemble only superficially the simple systems for which the concepts of thermodynamics have at present any experimental meaning.

Fortunately, the matter need not be settled by recourse to analogy. A reliable technique for experimental choice between the hypotheses of random mutation and induced variation was supplied by Luria and Delbrück (1943). If the adaptation is caused by test conditions (presence of a drug, absence of an essential metabolite, etc.), their effects on similar cultures should be similar. If the adaptation arises through occurrence of random mutations, similar cultures before exposure to the altered conditions should contain random numbers of mutant cells already capable of growth under these test conditions. The number of such mutants in any given culture will depend on the time at which mutations happened to occur during the growth of that culture, early mutants having more progeny than those occurring toward the end of the growth period. It follows that if adaptation is caused by the conditions of test, the variance between similar cultures will be no greater than that among samples from a single culture. On the other hand, if adaptation is due to random mutations the former variance will greatly exceed the latter. If the mutant cells can be enumerated separately by plating under conditions such that each mutant cell develops into a colony while growth of the parental type does not occur, the data required for the statistical test are available. Luria and Delbrück (1943)

applied this criterion to the production of phage-resistant E. coli strains and found that beyond any possible doubt the resistance arose through random mutation. For samples from a homogeneous population, the variance should approximate the mean, and this condition was met for samples from a single culture. For samples from different cultures, however, the variance invariably greatly exceeded the mean, often by a factor of 100 or more, even though the cultures were identical in preparation and treatment.

Two methods were supplied by Luria and Delbrück for estimating mutation rates. Either method can give only rough approximations because of the mathematical and biological assumptions which had to be made in their derivations. By making it possible to estimate the order of magnitude of the rate of any bacterial mutation which can be distinguished by plating, however, these formulas have been largely instrumental in rescuing studies of bacterial variation from the empirical morass into which they had fallen and placing them on a reasonably quantitative footing in harmony with accepted biological generalizations.

The demonstration that bacterial mutants could be obtained by irradiation (e.g., Haberman and Ellsworth 1940, Haberman 1941, Gowen 1941) greatly strengthened the parallel between the genetics of bacteria and other forms of life. Biochemical mutants of E. coli were obtained by the

use of X-rays (Roepke, Libby, and Small 1944) or ultraviolet light (Gray and Tatum 1944). Irradiation has now become a standard laboratory method for obtaining random variants in bacteria, as it has long been for other organisms.

The criterion for random mutation proposed by Luria and Delbrück has been very widely applied. Demerec (1945) showed that the development of penicillin-resistant strains of Staphylococcus aureus involved the summation of numerous random variations, each of which gives only a small increase in tolerance. Demerec and Fano (1945) confirmed the finding of Luria and Delbrück that resistance of E. coli to virus is genetic, and showed further that mutation to resistance to one strain of virus does not affect the rate of mutation to immunity to another. Resistance to different strains are thus due to independent factors in a classical genetic sense.

Three species of bacteria were found by Severens and Tanner (1945) to adapt readily to high concentrations of inorganic salts. The adaptations were shown to be due to the presence in normal cultures, before exposure to the salts, of random numbers of cells capable of growing in salt concentrations inhibitory to the culture as a whole.

Random mutations conferring increased resistance to the lethal effects of X-rays or ultraviolet irradiation

were demonstrated by Witkin (1946). The frequency of occurrence of such mutations is increased by irradiation. This fact, however, cannot be taken as evidence for directed mutation, since these types of radiations indiscriminately increase other mutation rates as well.

Clinical experience has shown that a high degree of streptomycin resistance is readily acquired by most pathogens. Klein and Kimmelman (1946) showed that this ease of adaptation is accounted for by the fact that, at least in the Shigellae, resistance to high levels of the drug is conferred by a single mutation. Growth in even low concentrations of the antibiotic thus selects the mutant able to grow also at much higher levels. The same single-step acquisition of streptomycin resistance was later shown for many common bacteria, both pathogenic and non-pathogenic (Klein 1947). In contrast, no individuals highly resistant to penicillin were found in normal cultures of any of the species tested, in agreement with Demerec and Fano's previously cited finding that resistance in this case is developed stepwise by the accumulation of many mutations. The adaptations of Influenzae (Alexander and Leidy 1947, 1948), meningococci (Miller and Bohnhoff 1947a,b), and mycobacteria (Yegian and Vanderlinde 1948) to streptomycin resistance depend on random mutation.

A more detailed analysis with E. coli showed that at least three different mutations are concerned with streptomycin resistance in E. coli (Newcombe and Hawirko 1949). One of these conferred slight resistance, another produced the high degree of resistance previously shown due to a single mutation, and the third causes not only tolerance to high levels of the antibiotic but also a requirement for this compound. The data indicated complete independence of the different mutations.

At least five different mutations were shown by Oakberg and Luria (1948) to be involved in the acquisition of resistance to sulfonamides by Staphylococcus aureus. One or possibly two of these involve increased production of p-aminobenzoic acid, the metabolite involved in the competitive inhibition.

Publications on bacterial variation and genetics up to late 1946 have been reviewed by Braun (1947) and by Luria (1947). The latter author states that "in all thoroughly analyzed cases, we see that bacterial variation, including apparent hereditary adaptation, is the result of sudden spontaneous mutation." The only results which could be construed as contradictory to this statement are those concerning the serological type transformations in the pneumococci (reviewed by McCarty 1946) and the similar effects reported for E. coli by Boivin (1947).

In these cases, however, material which may actually constitute part of the physical basis of inheritance is transferred from one cell to another of the same species. The results are of the greatest interest, but they most certainly furnish no evidence for environmental direction of mutation.

Fildes, who has long been a protagonist of "training" in bacteria (his work on the "training" of S. typhosa to dispense with exogenous tryptophan is cited in Part I of this thesis), has recently re-examined the situation (Fildes and Whitaker 1948). He now finds that although normal cultures of S. typhosa require tryptophan, they contain individuals not requiring this amino acid, and that "training" is in reality the selection of these mutant cells. The absence of the nutrient is not involved in the actual production of the variant cells, but only in their becoming dominant in the population. This paper is important in that it brings the views of a very productive research group into harmony with established biological concepts. Garzo Curcho (1948) independently demonstrated that "training" of S. typhosa to produce tryptophan involved the selection of mutant cells, and estimated the rate of occurrence of the mutation.

The mutation rates which have been reported for single phenotypic characters in bacteria (these may often

amount to summations of different mutations having similar expressions) usually fall in the range of 10^{-5} to 10^{-11} mutations per cell per generation. A large proportion of them are of the order of 10^{-8}. Such values effectively dispose of objections based on the belief that an improbably high degree of instability must be assumed if mutations are to account for bacterial variation and adaptation. Such reproducibility is of course incomparably beyond that of *in vitro* chemical reactions, and might be compared to a highly complicated synthesis in which one molecule of a side product is formed for each hundred million molecules of the primary product.

Interactions between Structural Analogs

Fruitful speculations on the mode of action of compounds having adverse effects on bacterial growth date at least from the time of Ehrlich, whose postulations of lock-and-key relationships and of cellular "receptors" underlie many of the current concepts of enzymology, immunology, and chemotherapy. It is only in the last decade, however, that something approaching an understanding of one type of mechanism which may account for bacteriostatic effects has been achieved.

Woods (1940) discovered that yeast cells produce something which reduces the effect of sulfanilamide on hemo-

lytic streptococci. The behavior of the active agent suggested that it might be related to sulfanilamide, and when p-aminobenzoic acid was shown to possess the same activity in marked degree the yeast factor was presumptively identified with the latter substance. The data were interpreted as indicating that p-aminobenzoic acid plays some essential part in the cellular metabolism which is interfered with by the sulfonic analog. The degree of inhibition appeared to depend on the ratio of sulfanilamide to p-aminobenzoic acid rather than on the absolute amount of either compound. This was taken as evidence that the metabolism of p-aminobenzoic acid, rather than its synthesis, was being interfered with, since in the latter case the administration of the required amount of the metabolite should have completely reversed the action of any amount of sulfanilamide.

From Woods' data, Fildes (1940b) suggested that any structural analog of an essential metabolite might exhibit the property of competing with the metabolite for a specific enzyme. This was proposed as the mode of action of sulfanilamide. In order for the interference to be sufficiently severe to affect growth of the organism, Fildes suggested that the analog must be closely enough related to the metabolite to allow adsorption by the enzyme, but sufficiently unrelated to be devoid of essential

metabolite activity in itself.

Woods' findings were extended and confirmed by the demonstration by Selbie (1940) that p-aminobenzoic acid prevented the normal effect of sulfanilamide on streptococci in mice as well as *in vitro*, and by Snell's report (1940) that the *in vitro* activity of peroxidase on p-aminobenzoic acid is reduced by the sulfonic analog. A mathematical analysis of growth rate data was considered by Wyss (1941) to offer conclusive proof for the hypothesis of competitive interaction between p-aminobenzoic acid and sulfanilamide.

With the first demonstrated case of such interaction thus firmly established, Fildes' hypothesis stimulated further research by many workers, and such inhibitory analogs, natural or synthetic, soon took an important place in both chemotherapy and cellular biochemistry. The early work was reviewed by McIlwain (1942), while later research has been comprehensively surveyed by Welch (1945), Roblin (1946), and Woolley (1947). In this thesis only some of the interactions involving amino acids will be considered.

Amino Acids Other than Substituted Alanines

Many interrelationships and toxicities of amino acids were reported before the advent of the concept of interfering analogs. For example, Gladstone (1939) reported that

under certain conditions valine and leucine each counteracted the toxic effect exerted by the other on Bacillus anthracis when present singly. Both valine and leucine were required for prevention of inhibition by either isoleucine or norleucine. Interactions were also demonstrated between valine and threonine, valine and α-aminobutyric acid, and threonine and serine.

The first analogs of amino acids prepared for test as inhibitors were a group of α-aminosulfonic acids (McIlwain 1941). These compounds were inhibitory to several strains of bacteria, most markedly to those requiring amino acids in the medium. In some cases the inhibitions were reversed by aminocarboxylic acids, but not always necessarily those of corresponding structure. The author noted that "the reversing action is, however, specific in that among simple nutrients it is practically confined to α-aminocarboxylic acids."

Harris and Kohn (1941) found that ethionine, the ethyl analog of methionine, inhibited E. coli. The inhibition was reversed by methionine. Methionine also reversed inhibitions caused by norleucine and norvaline. This last observation holds also for Proteus (Porter and Meyers 1945). An aspartic acid inhibition of Lactobacillus casei under some conditions was found by Feeney and Strong (1942) to be reversed by asparagine, glutamic acid, or

glutamine. These workers were the first to attempt an explanation of such amino acid interrelations in terms of biosynthetic pathways. They suggested that glutamine is required by the cell and is produced from glutamic acid by transamination from asparagine. Interference by aspartic acid with the NH_2 donation would account for the observed effects.

Phenylalanine, norleucine, and norvaline were reported to inhibit the activity of isoleucine and valine for a mutant strain of Neurospora thought to require both of the latter two amino acids although differing from the wild type by a single gene (Bonner, Tatum, and Beadle 1943). It was later shown that the apparent double requirement was in itself the result of a competitive interaction between the keto-acids corresponding to isoleucine and valine, and that the mutant really lacked only the ability to make isoleucine (Bonner 1946).

A strain of Pasteurella pestis "weaned" to grow in simplified medium was inhibited by leucine (Doudoroff 1943). The inhibition was overcome by isoleucine and valine, but by neither alone. Hutchings and Peterson (1943) reported rather complex inhibitions and reversals among amino acids for Lactobacillus casei. Glycine, serine, threonine, and β-alanine were found inhibitory for some lactic acid bacteria by Snell and Guirard (1943). Inhi-

bition by any of these amino acids was overcome by alanine or pyridoxine, but by no other amino acid or vitamin, and the mechanism of inhibition was thought to be interference with production of pyridoxine from alanine.

Doermann (1944) reported that arginine inhibited the utilization of lysine by a lysineless mutant of Neurospora. Arginine was without effect on the wild-type strain which could synthesize lysine. It was suggested that such a result might be explained on the assumption that normal protein synthesis does not involve amino acids as such. (Such a suggestion has been frequently made on other grounds: e.g., Linderstrøm-Lang 1939, Kalckar 1941). In such a case, the action of arginine might be interference with the conversion of lysine to the active compound. In the parental type this step would not occur, so that arginine would not inhibit.

Threonine was shown by Meinke and Holland (1948) to inhibit the utilization of serine by several lactic acid bacteria, and the possible effects of such interactions on the accuracy of microbiological assay were discussed.

Methoxinine, the oxygen analog of methionine, inhibited the growth of E. coli and Staphylococcus aureus (Roblin et al. 1945). The inhibition was overcome by methionine and to a lesser extent by choline (an alter-

nate methyl donor) or by p-aminobenzoic acid (which seems to function in the biosynthesis of methionine).

The inhibition of tryptophan synthesis by indoleacrylic acid (Fildes 1941, 1946), β-1-naphthylacrylic acid (Bloch and Erlenmeyer 1942), several methylindoles (Fildes and Rydon 1947), and two methylanthranilic acids (Rydon 1948), as well as the inhibition of tryptophan utilization by some methyltryptophans (Fildes and Rydon 1948), have been discussed in the first part of this thesis.

Inhibition of bacterial growth is shown by certain D amino acids. This type of inhibition appears to differ from most of those discussed above, but may be responsible for some of the inhibitions which have been reported for DL forms. When first reported, the inhibitory effect of D-leucine on L. arabinosus was thought probably related to the metabolism of L-leucine or other amino acids (Fox, Fling, and Bollenback 1944). Very high levels of the D form were used (20 milligrams per milliliter), and the tests were run in the presence of levels of the natural isomer considerably in excess of that required for maximum growth. It was later found that D-valine exerts a similar effect, while D-alanine failed to do so (Fling and Fox 1945). The natural isomers of these three amino acids at the same high levels did not reduce

growth of the cultures. Similar results, except that D-alanine showed inhibitory activity, were obtained when E. coli was tested in nutrient broth (Kobayashi, Fling, and Fox 1948). Preliminary experiments indicate that, for the inhibition of E. coli by D-leucine at least, the inhibition is due to some interaction between D-leucine and some other component of the complex medium (either in the medium or within the cell) since the same levels of D-leucine are without effect in a glucose-salts minimal medium (Atkinson and Kobayashi 1947). It was also shown that simple competition between optical isomers is apparently not involved, since the level of L-leucine seemed not to affect the extent of inhibition.

From the first enunciation of the concept of metabolite-analog inhibitions, inferences concerning the course of metabolism have been drawn from the occurrence of such inhibitions and the nature of the substances effective in overcoming them. Shive and coworkers have recently proposed the term inhibition analysis for the use of analog inhibitions in studies of biosynthetic pathways. Such inhibitions are considered chemical blocks of specific metabolic steps, and are analogous to the genetic blocks which have been used so effectively in study of the biochemistry of Neurospora. The postulate underlying Shive's work is that inhibition of a specific biosynthetic step

will be reversed competitively by a reactant (or precursor of a reactant), but non-competitively by a product of the inhibited reaction, as proposed originally by Woods (1940) and Fildes (1940b). The important contributions of Shive and his colleagues lie in their demonstration of the utility of chemical blocks in studies of metabolic pathways and in the results obtained from their extensive application of the method.

As has been seen from several papers cited above, inhibitions by amino acids or amino acid analogs are frequently reversed by other amino acids having little or no structural analogy to the inhibitor. This situation has often been considered evidence for the non-specific nature of such inhibitions. In such cases, however, the actual mechanism of inhibition may still be interference with one specific reaction, as pointed out by Harding and Shive (1948). These workers list the following types of substances which may be expected to overcome competitive inhibitions:

a) the analogous metabolite,

b) the product of the inhibited reaction,

c) precursors of the metabolite,

d) substances having sparing action on the product (these will usually be substances normally formed by further metabolism of the product, but could also include substances which yield the same product by an alternate reaction),

e) substances increasing the effective concentration of the enzyme responsible for the blocked step.

The known and suspected facility of conversions between amino acids makes it likely that many amino acids will fall into classes c and d--that is, they may be converted to some extent to the metabolite, the product, or substances normally formed from the product. Compounds exerting type c activity may be recognized by their decreased activity on addition of higher levels of the metabolite. No unequivocal means of separating type d and type e activity is available, but it is assumed that type e will be encountered fairly seldom.

By application of the lines of reasoning discussed in the last paragraph, Harding and Shive (1948) were able to reconcile some rather complex data involving apparently non-specific reversals with the hypothesis that norleucine competitively inhibits the use of methionine in some step which is common to the biosyntheses of leucine, isoleucine, and valine. With the earlier demonstration that p-aminobenzoic acid is involved in the synthesis of methionine (Shive and Roberts 1946) and the finding of competitive interference between the keto-acids corresponding to isoleucine and valine (Bonner 1946) the hypothesis could also be extended to explain puzzling interactions between p-aminobenzoic acid, methionine, leu-

cine, norleucine, isoleucine, and valine reported, among others, by Harris and Kohn (1941), Porter and Meyers (1945), Bonner, Tatum, and Beadle (1943), and Doudoroff (1943) and later by Brickson et al. (1949). This point has been discussed at some length because of the importance of the concept that apparent non-specificity of an inhibition may be due to conditions of the experiment or to unsuspected interconversions performed by the organism, so that in such cases the primary effect may still be interference with one specific metabolic reaction.

Hydroxyaspartic acid inhibits E. coli, and the inhibition is reversed competitively by aspartic acid (Shive and Macow 1946). From the effects of other compounds on this inhibition it was concluded that aspartic acid is normally converted to β-alanine, which is used in the synthesis by the cells of pantothenic acid. The reaction blocked by hydroxyaspartic acid is involved in this conversion.

Arylalanines

Research on the β-arylalanines--phenylalanine, tyrosine, and tryptophan--will be treated separately, both because of its closer relation to the subject matter of this thesis and because these amino acids are involved in a larger number of clearly demonstrated competitive inhi-

bitions than are any others.

The first analog of phenylalanine tested for inhibitory activity was β-2-thienylalanine. du Vigneaud et al. (1945) showed that this compound inhibited the growth of Saccharomyces cerevisiae, and that the inhibition was reversed by phenylalanine. Tyrosine was without effect. The same group of workers (Dittmer et al. 1946) later showed that the thienyl compound was inhibitory also for E. coli, Strep. faecalis, and L. arabinosus. In all cases the inhibition was reversed by phenylalanine, but there were marked differences between E. coli and S. cerevisiae in regard to the effect of other amino acids. For the former organism, tyrosine was reportedly more effective on a molar basis than phenylalanine, and the activity of tryptophan approached that of phenylalanine. For yeast, on the other hand, no other amino acids tested were over 13% as active as phenylalanine in reversing the thienylalanine inhibition, and the only three exhibiting even that order of activity were leucine, isoleucine, and tryptophan. Because ratio studies were not made, it is impossible to deduce whether tyrosine was a product of an inhibited reaction in the case of the E. coli inhibition. The human tubercle bacillus is also inhibited by thienylalanine, and the inhibition is overcome by phenylalanine (Drea 1948).

Ferger and du Vigneaud (1948) showed that the inhibitory action of the thienyl compound resides in the L isomer. The D isomer was without effect on S. cerevisiae or E. coli, and on Lactobacillus delbruckii LD5, which requires phenylalanine and can use only the L isomer. It is of interest that the same workers later (1949) found that the rat, which can utilize either optical form of phenylalanine (Rose and Womack 1946), is inhibited by D-, L-, or DL-thienylalanine. Added DL-phenylalanine counteracted the inhibition of all three forms, while tyrosine failed to do so.

It was reported by Clark and Dittmer (1948) that β-2-furylalanine exhibited the same sort of action as the thienyl analog. The inhibition was reversed most effectively by phenylalanine, and the pattern of reversal by other amino acids was similar to that observed with thienylalanine. Thus, leucine, tryptophan, and isoleucine were most effective (none over one-third the activity of phenylalanine) in reversing the inhibition of S. cerevisiae, while tyrosine and tryptophan were most active with E. coli. Both β-1-naphthylalanine and β-2-naphthylalanine failed to inhibit S. cerevisiae, E. coli, L. casei, L. arabinosus, and two strains of Neurospora (Dittmer, Herz, and Cristol 1948).

A series of phenylalanine derivatives and analogs

have been prepared by Elliott, Fuller, and Harington (1948) for testing against pathogenic bacteria. Against hemolytic streptococci in broth of unspecified composition, β-(ω-amino-p-tolyl)alanine, β-4-(6-methoxyquinolyl)alanine, and β-4-pyridylalanine were inhibitory at moderately high concentrations. Under the same conditions, p-aminophenylalanine, ω-amino-p-tolylalanine, p-dimethylaminophenylalanine, and β-4-quinolylalanine showed no significant activity. No tests for reversal were made.

Shive and coworkers have applied their technique of inhibition analysis to the metabolism of the arylalanines, mostly in E. coli. Beerstecher and Shive (1946) found that inhibitions of E. coli by either phenylserine or thienylalanine were competitively reversed by phenylalanine. Since tryptophan was about one-tenth as effective as phenylalanine in reversing either inhibition, with the relative effectiveness decreasing at higher levels, it was concluded that tryptophan functioned in the synthesis of phenylalanine. At much higher levels, however, tryptophan competitively interfered with phenylalanine metabolism (Beerstecher and Shive 1947c).

The inhibition by thienylalanine was later shown (Beerstecher and Shive 1947b) to be prevented completely by low levels of tyrosine. The non-competitive nature of the reversal showed that tyrosine was the product of the

inhibited reaction, thus establishing the conversion of phenylalanine to tyrosine in E. coli. In the case of the inhibition by phenylserine, however, tyrosine was without effect. These results clearly demonstrated that the two analogs interfere with different metabolic steps, both of which require phenylalanine. The conversion of phenylalanine to tyrosine appears to be irreversible, since added tyrosine does not increase the effective phenylalanine level, as shown by its lack of effect on the phenylserine inhibition.

By serial transfers in medium containing phenylalanine, Beerstecher and Shive (1947b) selected a "sensitized" strain which was strongly inhibited by low levels of tyrosine. A small amount of exogenous phenylalanine prevented the inhibition, which was thus shown to be due to interference with the synthesis of phenylalanine. The biochemical difference between the "sensitized" and normal strains was not further investigated, and the effect of lower levels of tyrosine was not determined.

In summary, these workers have presented evidence that tryptophan is involved in the synthesis of phenylalanine, which is converted to tyrosine. Tryptophan at high levels can interfere with the metabolism of phenylalanine, while, at least for one variant strain, tyrosine can interfere with the synthesis of phenylalanine.

Mitchell and Niemann (1947) tested several halogenated phenylalanines and tyrosines on wild type Neurospora crassa. In the presence of 0.03 milligrams per milliliter of DL-phenylalanine, 0.04 milligrams per milliliter of 3-fluoro-DL-phenylalanine reduced the growth rate by 50%. The period over which growth occurred was not specified. Strong inhibition was also produced by all three optical forms of 3-fluorotyrosine. The order of decreasing activity was L, DL, and D, but differences were not great. It was stated without presentation of data that these inhibitions were specific and competitive in nature. 2-Chloro-, 3-chloro-, 4-chloro-, 3-bromo-, and 3-iodo-DL-phenylalanine and 3,5-difluoro-DL-phenylalanine were not inhibitory at ratios up to 150.

Melvin (1947) found that 4-fluoro-DL-phenylalanine strongly inhibited the growth of Lactobacillus arabinosus in nutrient broth. Tolerance to the fluoro derivative was readily developed.

MATERIALS AND METHODS

The materials and procedures used in the investigations to be reported were the same as those described in Part I of this thesis.

EXPERIMENTAL

Nutritional Mutants

In a test of p-fluorophenylalanine and p-chlorophenylalanine for ability to replace tyrosine, barely transferrable growth occurred in the first cultures. On transfer into the same medium, heavy growth occurred in several tubes after about 100 hours. Because of the very long lag period and the fact that growth was not obtained in all of the identical cultures, it was suspected that mutations had occurred in those cultures which showed heavy growth. In order to test whether the mutation was to ability to replace tyrosine by the halo derivatives or to dispense with exogenous tyrosine altogether, transfers were made to the same media and to medium containing neither tyrosine nor either halo derivative. Growth was prompt and heavy in all cases. The altered strain was thus shown to be able to grow in the absence of an external supply of tyrosine. After eight serial transfers in medium lacking tyrosine, the presumptive mutant was designated L. arabinosus Q. It has since been carried in peptone-yeast extract agar stabs and has shown no tendency to revert to the parental type. The occurrence of such a mutant had earlier been observed in this laboratory (Bollenback and Fox 1945), and the population kinetics of its selection are being studied by James (1949).

In later tests of the nutritional responses of this mutant, growth was observed in several tubes in the absence of both tyrosine and phenylalanine. After three further transfers in medium lacking both of these amino acids, the progeny of one such culture was designated L. arabinosus H.[1] Since phenylalanine is known to be converted to tyrosine in the rat (Moss and Schoenheimer 1940) and in E. coli (Beerstecher and Shive 1947), it was thought likely that strain Q had acquired the ability to perform this oxidation. The parental strain requires tyrosine and phenylalanine in approximately equal amounts, so that if the mutant is producing tyrosine from phenylalanine, its requirement for the latter amino acid should be at least doubled. The data of Table 12, however, show the responses of the two strains to be nearly identical. On low levels of phenylalanine and especially after prolonged incubation, the mutant strain gave significantly higher growth than the parental type when supplied with the same amount of phenylalanine. This could be interpreted as due to slight conversion of tyrosine to phenylalanine by the mutant. At any rate, the difference is in the opposite direction from that to be expected if strain Q obtained its tyrosine from the oxidation of phenylalanine. At high levels of phenylalanine, definitely higher terminal growth was reached by

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1. James (1949) has isolated a similar mutant.

Table 12. Growth Responses of L. arabinosus 17-5 and L. arabinosus Q to Varying Levels of Phenylalanine.

(Tabulated values are optical densities x 100; averages of duplicate 10 milliliter cultures. Medium lacks phenylalanine and tyrosine except as shown in table.)

DL-phenylalanine (micromoles per tube)	Incubation time (hours)				
	14	20	31	44	87
	A. L. arab. Q.		Medium lacks		tyrosine.
0	1	2	5	6	26
0.04	8	12	18	22	40
0.08	13	22	32	40	58
0.2	19	36	53	60	66
0.4	22	49	70	77	82
0.8	21	54	76	84	88
2.0	24	62	78	85	92
	B. L. arab. Q.		Medium contains		tyrosine.
0	2	2	3	4	44
0.04	8	8	8	10	39
0.08	12	14	16	18	23
0.2	24	31	38	42	50
0.4	36	53	64	68	75
0.8	40	73	87	91	94
2.0	43	89	108	114	114
	C. L. arab. 17-5.		Medium contains		tyrosine.
0	0	0	0	0	2
0.04	6	6	8	8	9
0.08	12	14	16	17	18
0.2	21	32	35	38	44
0.4	34	49	58	62	68
0.8	38	70	82	88	91
2.0	42	86	104	112	110

either the mutant or the parental strain in the presence of tyrosine than by the mutant in the absence of this amino acid. The readings for 44 hours represent normal terminal growth.

The appearance of the strain H mutation is seen in Table 12 at 87 hours in the cultures of L. arabinosus Q containing low levels of phenylalanine. The occurrence of phenylalanine-independent growth in many such tubes of strain Q, but in none of the corresponding cultures of the parental strain, has been repeated in other tests, and appears to have some significance. Apparent mutations from the parental strain to phenylalanine independence have, however, occurred (Table 21, ratio 0), but they have not been isolated.

The responses of strain Q and the original strain to tyrosine are compared in Table 13. It will be seen that growth of the mutant is essentially independent of tyrosine level, although there is a tendency for cultures containing the higher levels of this substance to grow more rapidly, but to a somewhat lower terminal level.

In order to test the possibility that either of the altered strains might have arisen through contamination, rather than from the original strain by mutation, the responses of all three strains to tryptophan were compared. The results, which are presented in Figure 8, appeared to

Table 13. Growth Responses of *L. arabinosus* Q and *L. arabinosus* 17-5 to Varying Levels of Tyrosine.

(Tabulated values are optical densities x 100; averages of duplicate 10 milliliter cultures.)

L-tyrosine (micromoles per tube)	Incubation time (hours)		
	20	32	46
	L. arabinosus Q		
0	66	97	102
0.02	64	93	100
0.04	65	94	101
0.1	63	94	103
0.2	59	86	97
0.4	74	88	92
1.0	73	90	89
	L. arabinosus 17-5		
0	4	8	9
0.02	16	26	27
0.04	24	35	40
0.1	42	55	58
0.2	56	68	70
0.4	68	93	96
1.0	68	98	96

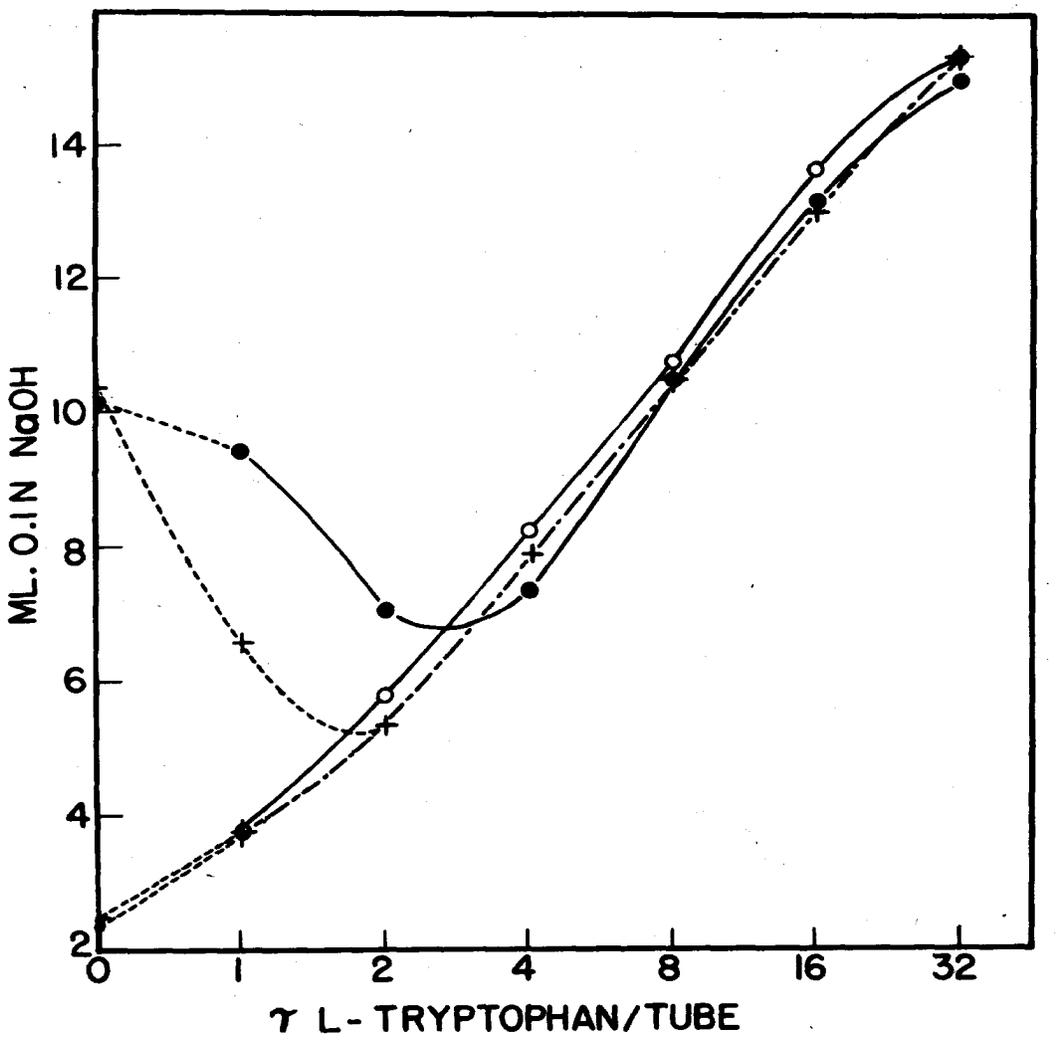

Figure 8. Responses of three L. arabinosus strains to graded levels of L-tryptophan. Strain 17-5, open circles; strain Q, crosses; strain H, solid circles. Duplicates for strain Q are shown separately below 2 micrograms.

demonstrate conclusively that the altered strains arose from the L. arabinosus inoculum, since such close agreement of response curves between this organism and any contaminant would be highly unlikely. The behavior of the two mutant strains in the presence of low levels of tryptophan was highly interesting. One of each pair of tubes of strain Q at the lowest level of tryptophan and in the control exhibited growth which can be reasonably accounted for only on the hypothesis that a strain producing tryptophan has appeared. In the case of strain H, both tubes of the control and of the two lowest tryptophan levels showed the same type of behavior. These results seemed to indicate that the ease of selecting tryptophan-producing mutants from these strains already exceeding the parent strain in their synthetic capacities was considerably greater than from the parental strain itself. In a large number of similar tests and standard curves for tryptophan assay, similar growth in the absence of tryptophan in cultures inoculated with the parental strain has occurred only once. Such a mutant strain has been reported by Wright and Skeggs (1945).

The effect of phenylalanine and tyrosine on growth of strain H is shown in Table 14. The addition of tyrosine, either alone or in addition to phenylalanine, was without effect. Phenylalanine, however, markedly stimulated the

early rate of growth. No differences remained at 44 hours or later. When strain H was inoculated into media lacking tyrosine and containing phenylalanine at levels varying from 0 to 2 micromoles per 10 milliliter culture, the

Table 14. Effect of Phenylalanine and Tyrosine on the Growth of L. arabinosus H.

(Tabulated values are optical densities x 100; averages of duplicate 10 milliliter tubes.)

DL-phenylalanine (micromoles per tube)	L-tyrosine (micromoles per tube)	Incubation time (hours)			
		14	20	31	44
0	0	10	45	79	94
0	0.2	10	38	79	92
0.2	0	20	52	82	94
0.2	0.2	24	48	76	85
2.0	2.0	28	68	86	92

results were similar to those of Table 13. That is, increasing levels of phenylalanine gave increasing rates of early growth, but no differences remained at 40 hours.

The responses of the three strains to tryptophan in media differing with respect to phenylalanine and tyrosine are shown in Table 15. These data confirm and extend the

Table 15. Growth Responses of L. arab. H, L. arab. Q, and L. arab. 17-5 to Varying Levels of Tryptophan.

(Tabulated values are optical densities x 100; averages of duplicate 10 milliliter cultures except that duplicate tubes at the first two levels are tabulated separately. Media lack tryptophan, tyrosine, and phenylalanine except as noted in the table.)

L-tryptophan (γ/tube)	Incubation time (hours)					
	48	71	77	85	90	98
	A. L. arab. Q. Medium contains phenylalanine and tyrosine.					
0	4	4	5	20	44	70
	5	6	6	21	44	69
1	13	14	18	38	51	63
	13	13	14	15	14	14
2	19	20	21	22	22	22
4	32	34	--[a]	--	--	36
8	58	58	--	--	--	56
16	78	76	--	--	--	74
32	106	106	--	--	--	104
	B. L. arab. Q. Medium contains phenylalanine					
0	3	4	12	46	61	74
	6	6	8	33	49	67
1	14	14	15	15	15	15
	13	14	14	14	14	14
2	18	18	19	20	20	20
4	33	34	--	--	--	33
8	58	58	--	--	--	56
16	79	76	--	--	--	74
32	102	103	--	--	--	100

Table 15. Continued.

L-tryptophan (γ/tube)	Incubation time (hours) 46	79	85	93	98	116
	C. L. arab. H. Medium contains phenylalanine and tyrosine.					
0	3	4	4	4	5	4
	3	8	37	72	83	87
1	11	13	13	14	14	13
	11	13	14	14	14	13
2	16	19	19	20	20	20
4	31	34	--[a]	--	--	34
8	56	54	--	--	--	53
16	78	78	--	--	--	75
32	105	105	--	--	--	102
	D. L. arab. H. Medium contains phenylalanine					
0	3	5	22	63	80	90
	3	4	4	4	4	3
1	12	14	15	15	15	14
	12	13	14	18	31	55
2	18	19	20	20	21	21
4	29	30	--	--	--	32
8	54	54	52	--	--	51
16	73	71	--	--	--	69
32	103	104	--	--	--	100
	E. L. arab. H.					
0	6	7	7	7	6	7
	4	6	4	4	4	5
1	7	9	16	35	45	56
	10	11	11	12	12	13
2	16	18	18	18	18	19
4	26	30	--	--	--	31
8	50	51	--	--	--	52
16	76	75	--	--	--	74
32	94	96	--	--	--	95

Table 15. Continued.

L-tryptophan (γ/tube)	Incubation time (hours)					
	48	71	77	85	90	98
	F. L. arab. 17-5. Medium contains phenylalanine and tyrosine.					
0	3	3	3	3	3	3
	5	5	5	5	5	5
1	13	13	15	15	14	14
	17	18	18	19	19	18
2	19	20	21	21	21	21
4	31	34	--[a]	--	--	36
8	60	60	--	--	--	58
16	77	77	--	--	--	74
32	104	104	--	--	--	101

a. Readings not taken because cultures appeared to have reached maximum growth.

results plotted in Figure 8. Except for somewhat lower growth of strain H at the highest level of tryptophan in medium lacking both phenylalanine and tyrosine, growth rate and extent of final growth are nearly identical except in cultures in which tryptophan-independent strains appeared. Duplicate tubes are tabulated separately for the control and the lowest tryptophan level in order to show the pattern of adaptation to growth in the absence of tryptophan. Two features of the adaptation seem noteworthy: the long lag period before its occurrence and the fact that it occurs only when tryptophan is absent or present at very low levels. Growth is quite severely limited by tryptophan deficiency at levels up to 8 micrograms of the amino acid per culture, but appreciable growth of the tryptophan-independent mutant does not occur at tryptophan levels higher than 1 microgram. This feature will be discussed in the following section.

In the experiment summarized in Table 15, nine cultures adapted to growth which was apparently independent of tryptophan. Transfers from these nine tubes were made to medium lacking tryptophan. By 17 hours all cultures showed heavy growth, while controls from stock cultures of Q and H were very faintly turbid. On transfer in peptone-yeast extract slants, however, these strains have

reverted to behavior characteristic of the strains from which they were derived (Q and H).

Since plating results have been erratic, attempts were made to determine relative probabilities of adaptation to tryptophan production among the three strains by transfers into the three media listed for strain H in Table 15. Twenty-five transfers of each strain were made into two milliliter volumes of each medium. Only 12 of these 225 cultures adapted to tryptophan production, however. These adapted cultures were divided evenly among strains Q and H. This percentage of adapting cultures was very much lower than in the previous experiment (Table 15). The same inoculum was used in both tests (1 drop of a 1/50 dilution of washed peptone-yeast extract culture). Five times as many cells per milliliter were thus introduced into the cultures of the latter experiment, where culture volume was only 2 milliliters.

Enough growth to produce faint turbidity always occurs in media deficient in a single amino acid, and the long lag times observed make it seem probable that the mutants are produced in the tubes during this growth rather than being introduced into the tubes in the inoculum. It was thought that smaller inocula might allow the occurrence of more growth and thereby increase the probability of mutation. A test was therefore set up in which the usual inoculum

was used straight, diluted to 10^{-2}, and diluted to 10^{-4}. Medium lacking only tryptophan was used and the three dilutions of inoculum were introduced into five tubes containing 2 milliliters. Concurrent plate counts showed that 1.8×10^5, 1.8×10^3, and 18 cells were introduced by the use of the different inocula. Since the series in which 1.8×10^{-5} cells were inoculated into 10 milliliters of medium was as close a duplication as possible of the technique used in the experiment from which Table 15 was constructed, it was expected that at least several of these tubes would adapt. In the whole experiment only one tube showed heavy growth, however, and this was a small tube receiving inoculum of the intermediate dilution. The reason for these discrepancies is not known.

In the absence of phenylalanine and tyrosine (Table 15, series E), strain H grows to an optical density of 0.95 when supplied 0.16 micromoles (32 micrograms) of L-tryptophan. This growth is comparable with that reached by the parental strain (optical density 1.01) when supplied the same quantity of tryptophan in addition to abundant phenylalanine and tyrosine. To attain comparable growth, the parental strain requires 0.8 micromoles of DL-phenylalanine when only this amino acid is limiting (Table 12, series C). These results appear to exclude tryptophan from participation in phenylalanine synthesis by strain H. Even if the

0.16 micromole of tryptophan were not required as such for growth, it could hardly function as precursor for the much larger amount of phenylalanine which is necessarily being synthesized by the mutant strain. Since phenylalanine is not used in tyrosine production by strain Q (Table 12), neither step of the sequence

tryptophan ⟶ phenylalanine ⟶ tyrosine,

which has been reported for E. coli (Beerstecher and Shive 1946, 1947b), is operative in L. arabinosus H.

Competitive Inhibition by p-Fluorophenylalanine

The experiments of Melvin, cited in the historical section, on the effects of p-fluoro-DL-phenylalanine[1] on L. arabinosus 17-5 were extended to synthetic medium. A preliminary test in which results were estimated visually indicated that the growth inhibition was due to competitive interaction with phenylalanine, and that tyrosine had no effect on this interference. A similar experiment in which the fluoro derivative was substituted for phenylalanine or tyrosine in the medium showed that there was no tendency for the analog to replace either of the natural amino acids nutritionally. In this latter test, identical results were obtained with the original strain and with Melvin's strain adapted to growth in nutrient broth with

1. The lots of this compound used were prepared by Sam Melvin and Kenneth Hartz.

the addition of 5 milligrams per milliliter of the fluoro compound.

The specificity of the interference of fluorophenylalanine with phenylalanine metabolism suggested the possibility that, if the effect is primarily steric, p-chlorophenylalanine might similarly interfere with tyrosine, since the larger chlorine atom more nearly resembles the hydroxyl group in size. This compound[1], however, was found completely inactive against both phenylalanine and tyrosine at molar ratios up to 80, and also failed to replace either of these amino acids nutritionally.

The preliminary experiment on inhibition by the fluoro derivative indicated a 50% molar inhibition ratio of the order of 1. It follows from the postulates of Shive and colleagues that ratios below this value should give inhibitions of lesser extent. In order to test this prediction, the fluoro derivative was added at molar ratios of 1 or less with regard to phenylalanine. The results, shown in Table 16, clearly show that the expectation was not realized. In the presence of low levels of phenylalanine, sub-inhibitory levels of the fluoro analog definitely stimulated growth. When enough phenylalanine was present to allow nearly maximum growth the analog was without effect at these ratios.

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1. The p-chlorophenylalanine used in these tests was prepared by Kenneth Hartz.

The data of Table 16 represent terminal growth. The same response pattern was already evident in readings taken at 14 hours.

The effects of p-fluorophenylalanine on strain H in the

Table 16. Effect on Growth of L. arabinosus 17-5 of p-Fluorophenylalanine at Concentrations Equal to or Less that That of Phenylalanine.

(Tabulated values are averages of duplicate 10 milliliter cultures. Incubated 87 hours.)

DL-phenylalanine (micromoles per tube)	DL-p-fluorophenylalanine (micromoles per tube)	DL-p-fluorophenylalanine (ratio to phenylalanine)	Optical densities x 100 test cultures	Optical densities x 100 controls lacking p-fluorophenylalanine
0.04	0.04	1	16	9
0.08	0.04	0.5	24	18
0.2	0.04	0.2	51	44
0.2	0.1	0.5	53	44
0.4	0.1	0.25	78	68
0.8	0.1	0.125	94	91

presence or absence of phenylalanine and tyrosine are shown in Table 17. The results are those to be expected from the data obtained with the parental strain. In the absence of exogenous phenylalanine, quite low levels of the fluoro

Table 17. Effects of Phenylalanine and Tyrosine on the Inhibition by p-Fluorophenylalanine of *L. arab. H.*

(Tabulated values are optical densities x 100; duplicate 10 milliliter cultures.)

DL-phenylalanine (micromoles per tube)	L-tyrosine (micromoles per tube)	DL-p-fluorophenylalanine (micromoles per tube)	Incubation time (hours) 20	44	60	87	112	142
0	0	0	41	94	102	97	--	--
			49	94	101	97	--	--
0	0	0.1	0	2	3	69	88	86
			0	2	3	67	83	84
0	0	0.4	0	1	1	3	5	68
			0	0	0	1	0	63
0	0	1.0	1	2	2	2	0	1
			0	0	0	0	0	0
0.8	0	0.4	61	84	87	88	86	83
			58	83	86	86	85	83
0.8	0	1.0	48	75	79	82	81	81
			54	80	83	84	81	79
0	0.8	0.4	0	0	12	59	66	67
			0	0	2	45	49	48
0	0.8	1.0	0	0	0	0	13	78
			0	0	0	2	53	82

analog were inhibitory. The addition of phenylalanine at levels giving an exogenous analog/metabolite ratio of the order of 1 reversed the inhibition, while the addition of tyrosine was without effect. The most interesting aspects of these results were the increased lag time resulting from increase of the inhibitory compound, and the random times of initiation of growth in these tubes. As has been previously discussed, such results suggest the occurrence of a mutation permitting growth under conditions inhibitory to the parental type.

The results of a similar experiment with strain Q are presented in Table 18. As in Table 17, it is clearly seen that tyrosine had little or no effect on the inhibitory action of the fluoro analog. This result indicates lack of conversion of tyrosine to phenylalanine. A prolonged time lag followed by rapid growth was again observed in those cultures containing the analog at levels allowing no initial growth. When present at half the concentration of phenylalanine (0.2 micromoles per culture), the fluoro analog exhibited an amazing degree of growth stimulation. This effect was particularly marked during the early growth of the cultures, and was already evident in readings taken at 14 hours, which are not included in the table. In all cases but one where apparent mutation allowed delayed growth in the presence of the analog, the terminal growth

Table 18. Effect of Tyrosine on the Inhibition by p-Fluorophenylalanine of L. arab. Q.

(Tabulated values are optical densities x 100; duplicate 10 milliliter cultures. DL-Phenylalanine at 0.4 micromoles per tube.)

L-tyrosine (micromoles per tube)	DL-p-fluorophenylalanine (micromoles per tube)	Incubation time (hours) 20	31	44	60	87	112
0	0	12	18	23	30	40	44
		12	17	22	29	39	43
0	0.2	40	54	63	65	66	65
		40	58	62	64	65	65
0	1.0	1	4	11	46	54	55
		0	3	6	47	55	55
0	3.0	0	0	2	27	35	36
		0	0	21	45	51	52
2.0	0.2	46	58	63	65	65	64
		48	58	62	64	64	63
2.0	1.0	0	5	49	64	69	69
		1	5	49	63	69	69
2.0	3.0	0	0	36	71	76	76
		0	2	53	68	72	73

reached was markedly higher than that allowed by the same amount of phenylalanine in the absence of analog. One hypothesis which might be suggested by these data is that in this case the development of tolerance to the analog involves the acquiring of ability to produce phenylalanine. Such a mutation might both reverse the inhibition and remove the ceiling on growth set for the original strain by the limitation of exogenous phenylalanine.

The apparent occurrence and selection, in the experiments just described, of mutants unaffected by p-fluorophenylalanine suggested that the time course of growth in inhibited cultures should be followed. Such experiments were set up with the parental strain and strain H. Some of the results obtained from one such experiment with the parental strain are presented in Figure 9. In this series, the level of phenylalanine was constant at 2 micromoles per culture and the molar ratio of p-fluorophenylalanine to phenylalanine was varied between 0.5 and 40. Readings were taken each three hours from eight to thirty-five hours, and at intervals not exceeding eleven hours as long as growth continued. Five sets of readings were selected for Figure 9. The general pattern of the curves plotted in this figure has been obtained in several similar experiments. The minimum at a ratio of 2 has, in fact, often been more marked than is shown in this figure. The short horizontal

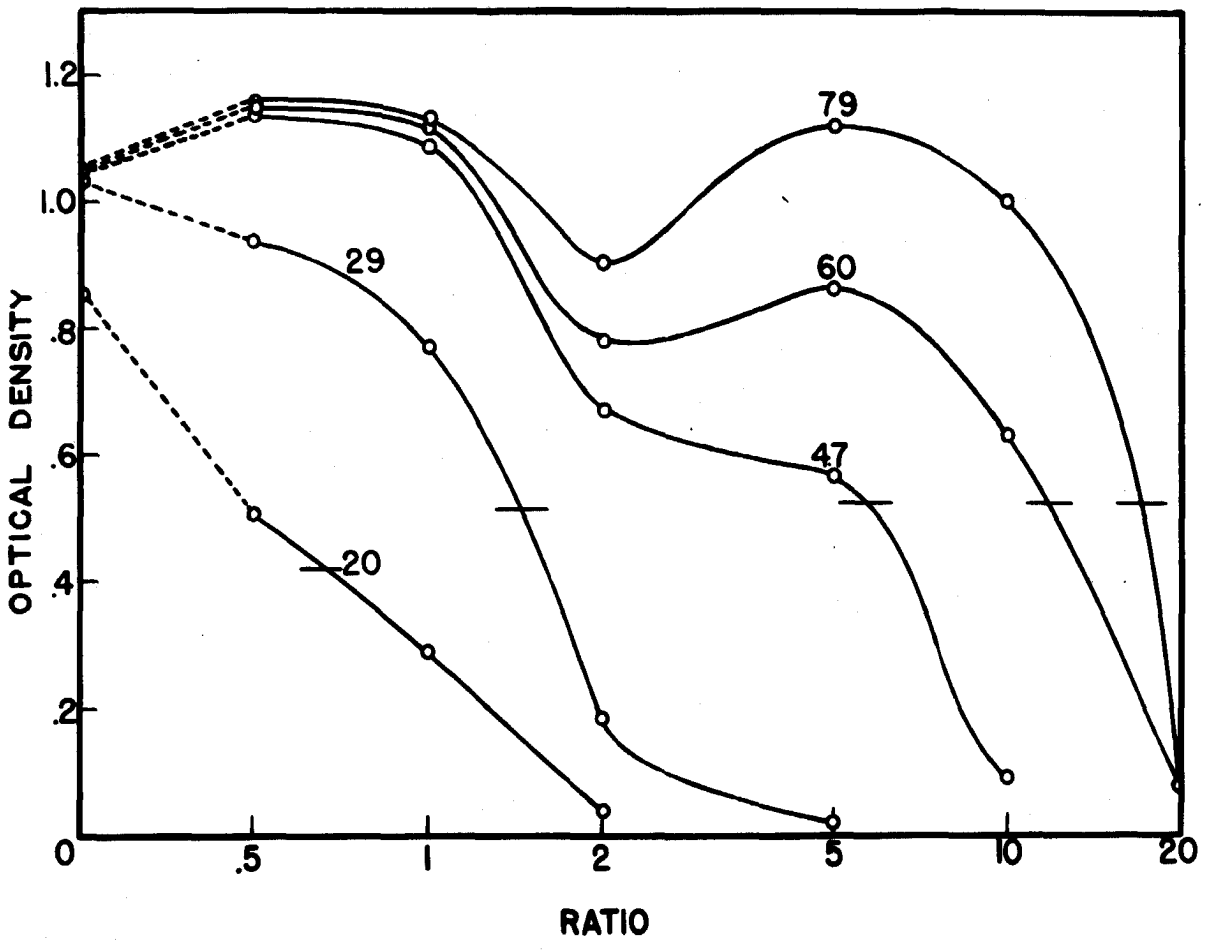

Figure 9. Response of L. arabinosus 17-5 to varying molar ratios of p-fluorophenylalanine to phenylalanine. Culture volume 10 milliliters; 2 micromoles of DL-phenylalanine per culture. Figures identifying curves indicate time of incubation in hours. Horizontal lines represent 50% of control growth.

marks on the curves indicate 50% of the growth obtained up to that time by cultures containing the same amount of phenylalanine but none of the analog. It will be noted that the ratio allowing half of the normal or uninhibited growth increases with time of incubation. Table 19 gives the values for the 50% inhibition ratio determined graphically in this manner and also a range for the antibacterial index as defined by McIlwain and used by Shive. This index is the smallest molar ratio causing essentially complete inhibition.

The significance of the data of Table 19 will be discussed in the following section. It can be readily seen from study of this table or of Figure 9, however, that the effect primarily responsible for the progressive increase in both the 50% inhibition ratio and the antibacterial index is the initiation of growth in cultures which were at first apparently completely inhibited.

The time relations are seen more clearly when growths of the cultures are plotted against time, as is done in Figure 10. The curves are obtained in each case by plotting readings of the first tube of each duplicate pair in which growth was initiated. This figure shows that increase of the ratio of analog to metabolite gives a progressive decrease in growth rate and (except for the stimulation previously noted for low ratios) a concurrent de-

Table 19. Inhibition of L. arab. 17-5 by p-Fluorophenylalanine; Antibacterial Index (Ratio Giving Complete Inhibition) and 50% Inhibition Ratio as Functions of Time of Incubation.

(Ratios refer to moles of fluoro analog per mole of phenylalanine. Antibacterial index range given as between the highest ratio allowing growth and the lowest ratio giving complete inhibition. Fifty per cent inhibition ratio determined as described in text. Culture volume 10 milliliters; 2 micromoles of phenylalanine per culture.)

Incubation time (hours)	Antibacterial index lies in range	50% Inhibition index
17	1- 2	0.5
20	1- 2	0.7
23	1- 2	1.0
26	2- 5	1.3
29	2- 5	1.4
32	2- 5	1.6
35	2- 5	1.8
42	5-10	2.2
47	10-20	4.6
52	10-20	12
60	10-20	16
79	10-20	17

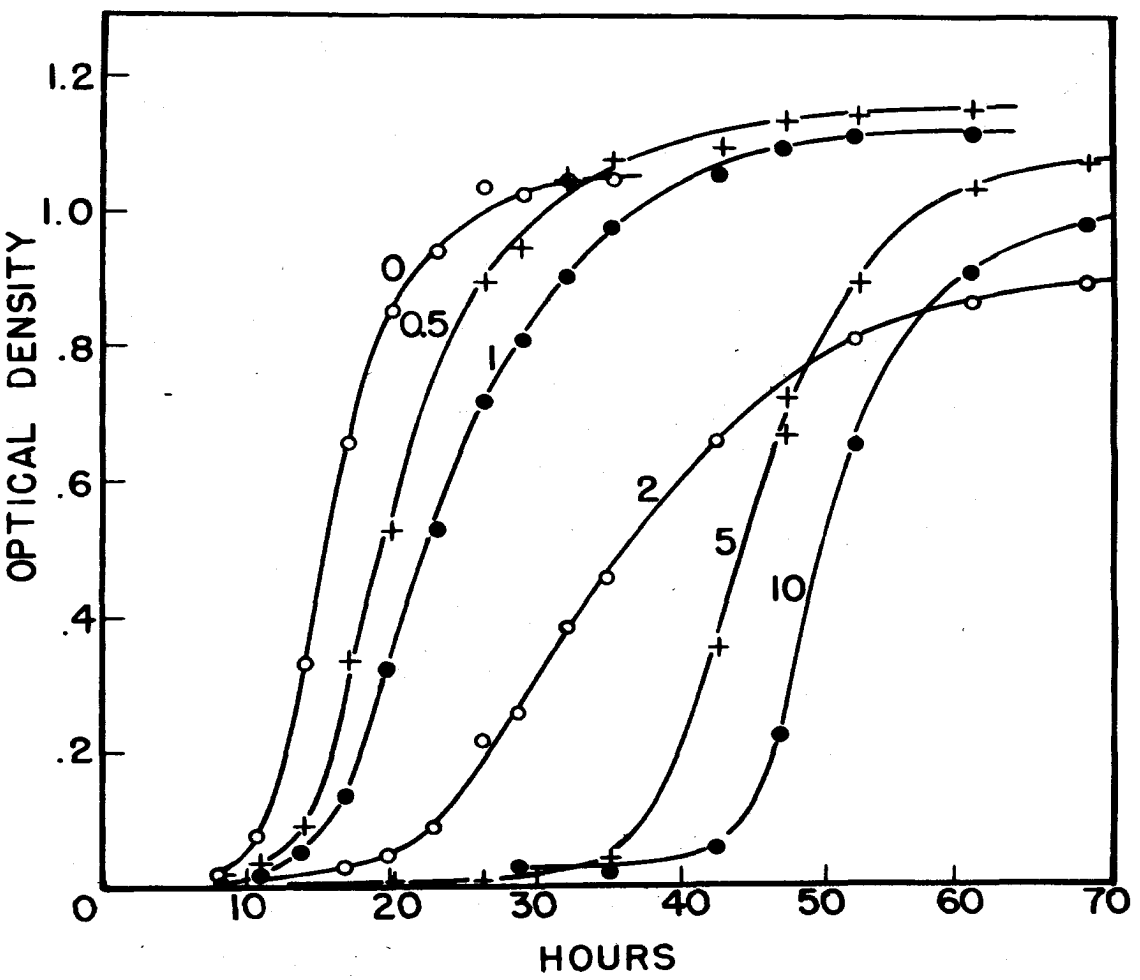

Figure 10. Growth of L. arabinosus 17-5 in media containing 2 micromoles of DL-phenylalanine and varying levels of DL-p-fluorophenylalanine. Figures identifying curves indicate molar ratio of the analog to phenylalanine.

crease in the terminal growth level. A critical ratio exists (in this case at about 2) beyond which no measurable growth of the parental strain occurs. In the presence of inhibitor at levels above the critical ratio, a prolonged time lag is usually followed by the appearance of mutant growth insensitive to the inhibitor, at least in the concentration present. The time lag may be even longer than shown by the graph; one culture at a ratio of 20 initiated growth at about 100 hours. The rate of the delayed growth is seen to be essentially equal to that of the uninhibited parental strain, and the terminal growth level is usually markedly higher than for cultures at or near the critical level. The dip in the curves of Figure 9 at a ratio of 2 is of course another aspect of the same effect.

The effects of the fluoro analog on the parental strain in the presence of lower levels of phenylalanine are shown in Tables 20 and 21. Table 20 presents results obtained when the inhibitor was added to cultures containing 0.5 micromoles of phenylalanine. It will be seen that the results are practically identical with those of Figure 10. Growth is slowed appreciably when the inhibitor/metabolite ratio is 0.8, and markedly decreased in both rate and extent when this ratio has a value of 2. When the amount of the analog is increased beyond this point, growth occurs only after a pronounced time lag and is rapid. The length

Table 20. Time-Course of Inhibition of L. arab. 17-5 by p-Fluorophenylalanine

(Tabulated values are optical densities x 100. Duplicate 10 milliliter cultures; 0.5 micromoles of phenylalanine per culture. Ratios refer to moles of p-fluorophenylalanine per mole of phenylalanine.)

Ratio	Incubation time (hours)													
	14	17	20	23	26	29	32	35	42	47	52	60	68	79
0	28	52	55	59	62	63	65	66	--[a]	--	--	--	--	--
	32	50	53	58	60	61	62	64	--	--	--	--	--	--
0.8	9	29	50	60	64	66	69	69	--	--	--	--	--	--
	8	29	49	59	63	65	67	68	--	--	--	--	--	--
2.0	2	4	8	15	25	33	40	44	49	51	53	--	--	--
	3	5	9	19	27	34	40	44	48	51	53	--	--	--
4.0	--[a]	--	--	--	--	--	3	7	52	58	61	--	--	--
	--	--	--	--	--	--	2	7	56	60	62	--	--	--
8.0	--	--	--	--	--	--	--	2	3	11	39	58	--	--
	--	--	--	--	--	--	--	3	14	48	61	65	--	--
20	--	--	--	--	--	--	--	--	--	--	2	3	3	2
	--	--	--	--	--	--	--	--	--	--	3	6	40	61

a. For the sake of clarity, readings before initiation of growth and after its cessation are omitted from the table.

Figure 21. Time-Course of Inhibition of L. arab. 17-5 by p-Fluorophenylalanine.

(Tabulated values are optical densities x 100. Duplicate 10 milliliter cultures; 0.1 micromoles of phenylalanine per culture. Ratios refer to moles of p-fluorophenylalanine per mole of phenylalanine.)

Ratio	Incubation time (hours)												
	14	17	20	23	26	29	35	42	47	52	60	68	79
0	13	16	18	19	23	23	23	28	39	57	77	88	93
	13	16	17	19	20	20	21	27	41	57	77	86	90
1.0	11	19	23	26	27	27	29	30	30	31	33	--[a]	--
	12	19	22	25	27	27	29	30	30	31	33	--	--
2.0	5	16	21	25	27	29	30	31	31	33	--	--	--
	9	18	23	27	30	31	30	32	32	34	--	--	--
4.0	--[a]	4	10	18	22	23	28	32	33	36	37	39	--
	--	4	10	17	23	23	28	31	33	35	38	39	--
10	--	--	--	--	--	--	3	12	23	28	33	36	--
	--	--	--	--	--	--	3	11	21	26	31	34	--
20	--	--	--	--	--	--	--	3	2	3	14	22	28
	--	--	--	--	--	--	--	3	5	16	27	34	37

a. For the sake of clarity, readings before initiation of growth and after its cessation are omitted from the table.

of the lag tends to vary with the analog/metabolite ratio. No growth was observed at a ratio of 40.

When the level of the metabolite is quite low, the same essential pattern is seen with some modifications. Table 21 shows the results of addition of p-fluorophenylalanine to cultures containing phenylalanine at 0.1 micromole. The apparent occurrence in the control tubes of mutation to production of phenylalanine tends to obscure any conclusions which might be drawn regarding terminal growth levels. It seems that the constant readings obtained between 26 and 35 hours represent the greatest growth of the parental strain possible in the presence of this amount of phenylalanine, and the later growth might be due to cells able to produce this amino acid. If this is true, such mutants may have also appeared and contributed to the behavior of other cultures in this series, but their effects would be impossible to isolate.

Aside from the abnormal growth curve of the controls which is discussed above, the only significant difference between the data of Table 21 and those presented in Table 20 and Figure 10 is that in the former the critical ratio above which the parental strain is unable to grow seems to lie in the vicinity of 4, rather than 2. Growth again failed to occur in cultures having an analog/metabolite ratio of 40.

The effect of p-fluorophenylalanine on the growth of strain H in the absence of tyrosine and phenylalanine, both of which it is able to synthesize, is shown in Table 22. Very low levels of the analog are seen to be capable of causing severe inhibition. The lowest level tested, 0.1 micromoles per culture (1.8 micrograms per milliliter), gave the type of results which have been interpreted as evidence for practically complete inhibition of growth of the inoculum. No measurable growth occurred for over 36 hours, after which the two duplicates differed widely in behavior. Doubling the concentration of the inhibitor had no effect on the lag period, but caused a sharp reduction in the terminal level. This effect seems not to be explainable in terms of other results. Further increase of the analog concentration, however, caused the expected increase in length of the lag period. Cultures containing 2 micromoles or more of the analog showed no growth whatever.

The addition of a small amount of phenylalanine to cultures of strain H very markedly affected the action of the fluoro analog, as is seen in Table 23. The first five lines of this table correspond exactly to Table 22 except that the cultures from which Table 23 was constructed contain 0.1 micromole of phenylalanine per tube (1.6 micrograms per milliliter). This level of exogenous

Table 22. Time-Course of Inhibition of L. arab. H. by p-Fluorophenylalanine in the Absence of Exogenous Phenylalanine or Tyrosine.

(Tabulated values are optical densities x 100. Duplicate 10 milliliter cultures.)

DL-p-fluorophenylalanine (micromoles per tube)	Incubation time (hours)											
	14	20	26	35	42	47	52	60	79	94	103	119
0	12	56	81	95	98	100	--a	--	--	--	--	--
	--b	--	--	--	--	--	--	--	--	--	--	--
0.1	--a	--	--	3	11	15	18	22	47	63	66	--
	--	--	--	2	30	60	86	105	110	--	--	--
0.2	--	--	1	3	16	27	34	37	39	--	--	--
	--	--	4	4	14	25	32	34	37	--	--	--
0.4	--	--	--	0	0	0	0	0	0	25	53	62
	--	--	--	1	6	10	14	15	36	65	72	79
1.0	--	--	--	--	--	--	--	--	1	0	1	1
	--	--	--	--	--	--	--	--	2	6	46	61

a. For the sake of clarity, readings before initiation of growth and after its cessation are omitted from the table.

b. The second tube of this pair was accidentally broken.

Table 23. Time-Course of Inhibition of L. arab. H. by p-Fluorophenylalanine in the Presence of Phenylalanine.

(Tabulated values are optical densities x 100. Duplicate 10 milliliter cultures; 0.1 micromoles of phenylalanine per culture. Ratios refer to moles of p-fluorophenylalanine per mole of phenylalanine.)

Ratio	Incubation time (hours)											
	14	17	20	23	29	35	42	47	52	60	68	79
0	19	45	62	75	90	96	98	100	--a	--	--	--
	33	53	68	78	90	96	97	100	--	--	--	--
1	9	24	36	50	64	75	81	83	85	--	--	--
	15	27	41	52	64	74	80	81	83	--	--	--
2	7	16	25	30	36	36	39	38	40	--	--	--
	7	16	25	31	35	36	39	40	40	--	--	--
4	3	6	9	13	18	19	25	28	29	32	37	40
	2	4	8	12	16	19	25	28	31	34	37	39
10	--a	--	--	--	--	4	11	23	31	39	41	45
	--	--	--	--	--	1	15	26	33	35	38	38
20	--	--	--	--	--	3	27	38	44	48	52	53
	--	--	--	--	--	1	3	12	26	38	44	47
40	--	--	--	--	--	--	--	--	2	18	34	42
	--	--	--	--	--	--	--	--	0	0	0	0

a. For the sake of clarity, readings before initiation of growth and after its cessation are omitted from the table.

metabolite was sufficient to alter the whole aspect of response to the inhibitor. The behavior shown in Table 23 is very similar to that of the parental strain (17-5) as seen in Table 21. The series from which these two tables were constructed differed only in the inoculum.

When phenylalanine was supplied at higher levels, the response of strain H to fluorophenylalanine resembles even more closely that of the parental strain, as is shown in Table 24. Comparison of the data of this table with the response of strain 17-5 to the same conditions (Figure 10) shows that in both cases rate of growth decreases somewhat when the analog/metabolite ratio is 1. For both strains at this phenylalanine concentration, the critical ratio, at which growth rate and terminal growth are most strongly depressed and beyond which measurable growth occurs only after a definite lag, lies in the vicinity of 2.

Attempts to determine the rate of mutation to fluorophenylalanine tolerance by plating have been unsuccessful because of the growth of barely visible colonies in very great numbers (apparently from every cell plated) in media characterized by analog-metabolite ratios which completely prevent the appearance of visible growth in liquid medium. This result is the same as obtained in attempts to estimate rates of mutation to the other variant types discussed in this thesis, and like them may relate to the presence

Table 24. Time-Course of Inhibition of L. arab. H by p-Fluorophenylalanine in the Presence of Phenylalanine.

(Tabulated values are optical densities x 100. Duplicate 10 milliliter cultures; 2 micromoles of phenylalanine per culture. Ratios refer to moles of p-fluorophenylalanine per mole of phenylalanine.)

Ratio	Incubation time (Hours)											
	14	17	20	23	26	29	32	35	42	47	52	60
0	32	61	78	90	95	98	100	101	104	--a	--	--
	40	60	76	86	92	96	98	100	102	--	--	--
0.5	30	57	77	86	93	100	103	105	106	--	--	--
	--b	--b	75	83	88	98	100	103	104	--	--	--
1	21	49	68	82	87	100	101	105	105	108	--	--
	24	49	67	81	85	97	99	103	104	104	--	--
2	11	28	47	59	62	74	78	83	84	86	--	--
	9	26	44	53	56	66	70	75	77	78	--	--
5	--a	--	--	--	--	--	--	4	23	54	75	90
	--	--	--	--	--	--	--	4	17	46	65	84

a. For the sake of clarity, readings before initiation of growth and after its cessation are omitted from the table.

b. These readings were missed.

of undesired amino acids as trace contaminants of the medium. Because of slower diffusion in agar media and especially because the cells are held together in colonies, local pH and local concentrations of metabolites, inhibitor, and products of metabolism will vary more widely in the solid medium than in liquid cultures. Some of these factors may be involved in the unexpected behavior on plates.

DISCUSSION

Nutritional Mutants

It seems reasonable to suspect that morphological characters in higher organisms, in those cases where they have been shown to depend on single genes, are ultimately due to single metabolic steps. Means for the identification of the biochemical reaction related to a given visible character are in general not available, however. Such relationships are more directly attacked experimentally in the case of factors where fewer intervening stages between gene and observed character are likely to exist, as with genetic differences involving altered cellular biochemistry. Here the end product of the affected metabolic pathway is usually known directly, so that the investigator is at the outset much closer to the problem than if he dealt with morphological characters. The obvious advantages of microorganisms for studies of cel-

lular economy have led to the recent spectacular increase in the amount of genetic work relating to them.

Up to the present, Neurospora has been doubtless the microorganism most profitably employed in biochemical genetic studies. The occurrence of sexual processes and the possibility of isolation of the products of individual meioses make this organism peculiarily valuable. For many purposes, however, bacteria are more convenient, and they are at present receiving increasing attention in this regard.

There is little evidence at present that the mechanisms of distribution of genetic factors in cell divisions of bacteria is exactly the same as in other cells. It has often been suggested that the "genes" of bacteria may exist individually in the protoplasm, without being organized into structures corresponding to chromosomes. Recent data indicating linkage among characters in E. coli (Lederberg 1947), however, strongly imply some sort of linear arrangement of the hereditary units.

Regardless of the exact manner of their transmission, present evidence definitely indicates the presence of discrete units, comparable to genes, in the bacterial cell which determine its heredity. As has been seen, these units mutate, both spontaneously and under the influence of such non-specific stimuli as high-energy radiations,

just as do the genes of other organisms. This type of control of the metabolism of the cell intrudes genetic considerations into any studies of bacterial physiology or biochemistry. In fact, it may be said with much reason that no real distinctions exist here between genetics, biochemistry, and physiology.

Most studies of biochemical genetics have dealt with Neurospora or E. coli, both of which are able to synthesize all required metabolites if supplied an energy source, mineral salts, and, in the case of Neurospora, biotin. Comparisons have been made between the original or wild type and mutants having synthetic deficiencies which are expressed experimentally as nutritional requirements. Because the mutants are derived from the parental strains (usually by selection after irradiation) and in the case of Neurospora can be shown to differ from it by a single gene, it may be assumed that all of the necessary enzymes for the affected synthesis are present except those controlled by the altered gene. If the postulated one-to-one relationship of gene to enzyme is valid, only a single enzyme has been effectively lost by the altered strain.

In the work reported here, the approach was in a sense the reverse of that applicable to Neurospora or E. coli. Lactobacillus arabinosus is characterized by many nutrition-

al requirements, but the development of synthetic media supporting rapid and heavy growth for indefinite serial transfers has made it available for biochemical studies. The variant cells which have been selected apparently differ from the parental type only in the added ability to synthesize one metabolite. The statistical criterion of Luria and Delbrück has not been applied to these variants because of technical difficulties described in the preceding section. As pointed out in the experimental portions of both parts of the thesis, the criteria for genetic change which have been used are random times of initiation of growth and retention of the changed nutritional requirements after serial culture in complete medium. These criteria are not rigorous, but in the framework of the present knowledge of bacterial variation, they appear conclusive.

Since these mutations confer increased synthetic ability, it is easy, by supplying a medium lacking the metabolite involved, to select those arising spontaneously in a normal population. The elaborate and ingenious techniques required for isolation of loss mutations are thus not needed.

It is commonly assumed that organisms having complex nutritional requirements have arisen by accumulation of loss mutations from a remote autotrophic ancestral strain.

The L. arabinosus mutants able to synthesize tyrosine or both tyrosine and phenylalanine may then be supposed to have regained an ancestral ability, possibly through restoration of a "damaged" non-functional gene which has been carried along automatically despite its lack of utility to the organism. Such a supposition seems much more likely than the alternative possibility of de novo appearance in a single step of a unique pathway for these syntheses. The demonstration in the preceding section that L. arabinosus H does not utilize the sequence

tryptophan ⟶ phenylalanine ⟶ tyrosine

in its synthesis of the latter two amino acids is therefore considered to indicate, not only that some alternative pathway is possible, but that it probably was used by some ancestor of L. arabinosus 17-5, and hence may be of general occurrence.

The demonstration of the conversion of phenylalanine to tyrosine by E. coli (Beerstecher and Shive 1947b) appears conclusive. The conclusion of the same workers (1946) that tryptophan functions in phenylalanine synthesis in this species seems less well substantiated. The evidence offered was that tryptophan was about 10% as effective as phenylalanine in reversing inhibitions caused by phenylserine or thienylalanine. It would seem dangerous to draw conclusions from such a low degree of activity. The same

data might result, for example, if phenylalanine is a precursor of tryptophan, or if both amino acids can be used interchangeably in some other process. In either case, the drain on the phenylalanine supply by a non-inhibited pathway would be obviated by an exogenous supply of tryptophan. The relative importance of this drain, and hence the effect of tryptophan, would decrease with increase in the concentration of phenylalanine. This effect was observed. It is therefore believed that, while the data indicate different pathways of tyrosine synthesis in E. coli and the L. arabinosus mutants, the apparent difference with regard to phenylalanine synthesis might well be due to overextension of data by Beerstecher and Shive.

It has often been suggested (e.g., Luria 1947) that the loss of a single step in a synthetic pathway will tend to lead to the loss of other steps in the same path, since they are no longer of benefit to the organism. This process is considered especially likely if the end product of the blocked synthesis normally occurs more frequently in the surroundings of the species than do intermediates coming after the metabolic block. Under such conditions,

> mutations producing blocks in other steps in the same reaction chain can then be accumulated without adverse selection, and the process may lead to loss of the whole series of reactions involved in the synthesis of the end product. (Luria 1947).

Strains of bacteria unable to synthesize a given

metabolite often give rise to mutant strains capable of the synthesis, however, and it seems necessary to assume that a single mutation is responsible. The mutant strains of L. arabinosus reported in this thesis are examples of this phenomenon, as are the tryptophan producing strains of S. typhosa selected by Fildes (Fildes, Gladstone, and Knight 1933, Fildes and Whitaker 1948) and by Garzo Curcho (1948), and the tryptophan producing L. arabinosus strain of Wright and Skeggs (1945). Many other examples could be cited. In such cases it seems reasonable to suppose that despite loss of one step in the synthesis, all of the other enzymes have been retained in functional form, so that a single mutation, by removing the block, restores the whole synthesis. The ability of L. arabinosus 17-5 to produce tryptophan from anthranilic acid furnishes more direct evidence that the loss of one step in a synthesis need not lead to loss of others.

Consideration of the probable order of magnitude of the rates of the mutations which are postulated to accumulate and cause loss of unused enzymes shows why this accumulation probably occurs much less often than is commonly supposed. If an average rate of 10^{-8} mutations per cell per generation causes loss of a given enzyme, and if no selective advantage or disadvantage results, it is

easily calculated that at the end of 1000 generations there will be only about 1 mutant cell for every 10^5 cells of the parental type, even assuming the complete absence of back mutation. With the same assumptions, after 10^8 generations there will still be nearly 40% of the original type present.

In the case of L. arabinosus, for example, it seems certain that tryptophan will be more generally available than anthranilic acid, so that loss of steps in the conversion of anthranilic acid to tryptophan would be expected from the line of reasoning suggested by Luria. If, however, once in several million generations the species happens into surroundings where anthranilic acid is available but tryptophan is limiting, the mutant cells unable to perform the conversion, which had been increasing in the population for these millions of generations, will be diluted to insignificance in a very few hours.

As the above figures show, selective advantage, rather than rate of mutation, will determine the composition of bacterial populations. This is true even for the cases in which it has been reported that neither strain possesses any selective advantage over the other, since there is no means of measuring selective advantages so small that they approach usual mutation rates. It has been suggested (e.g., Lwoff 1946) that the fact that parasites generally require

a number of metabolites preformed in their environment proves that there is a selective advantage associated with the loss of synthetic abilities. A cell which dispensed with an unnecessary synthesis, for example, might for that reason possess a greater supply of metabolic energy for other cellular processes and thus slightly outgrow cells still performing that synthesis. This argument may not have the generality which it has sometimes been ascribed (see Luria 1947), but even if it is generally valid, it does not predict the loss of other steps in a blocked synthesis. The first block in a given synthetic chain stops the entire synthesis and confers any selective advantage which may acrue to that stoppage; later losses should lead to no added advantage. It seems probable, then, that the retention of many steps in blocked pathways, which is indicated in three cases by the data of this thesis, is of common occurrence.

Competitive Inhibitors

The discovery by Woods (1940) that the inhibition caused by sulfanilamide was related more closely to the sulfanilamide/p-aminobenzoic acid ratio than to the level of either substance has been cited in the historical section, as has some of the work of Shive and colleagues on inhibitors as chemical blocks of metabolic pathways. The

latter group of workers have also attempted to place Woods' empirical findings on a theoretical foundation, and have presented a mathematical derivation based on mass-action considerations (Shive and Macow 1946) which purports to predict such a ratio effect. The relationship derived for any defined degree of inhibition is

$$(1). \qquad \frac{[I]}{[S]} = \frac{K_1 [EI]}{K_s [ES]}$$

where brackets denote concentration terms, I represents the inhibitor, S the substrate, EI the enzyme-inhibitor complex, K_1 the dissociation constant of this complex, ES the enzyme-substrate complex, and K_s the dissociation constant of this complex. The right-hand member of this equation is supposed to be constant for any defined inhibition, since under defined conditions of time and temperature of incubation, composition of medium, etc., growth is a function of the concentration of the product P of the inhibited system. Synthesis of P is represented by the usual formulation

$$(2). \qquad E + S \rightleftharpoons ES \rightarrow E + P \ ,$$

and it follows that the rate of production of P is proportional to $[ES]$. Any defined degree of inhibition (defined level of growth) is then supposed to define the production of the limiting metabolite P and, since the time of

incubation is constant, define $[ES]$. On the assumption of enzyme saturation, the total enzyme concentration $[E_t]$ is equal to the sum of the complexes,

(3). $$[ES] + [EI] = [E_t].$$

With the further assumption that $[E_t]$ remains constant during growth, it follows from (3) that $[EI]$ is also constant, since the other two terms of this equation have been assumed not to vary. All of the terms of the right-hand side of equation (1) have then been shown or assumed to be constant, so that the lefthand ratio $[I]/[S]$ must be constant for any defined inhibition.

There seem to be two weaknesses in the derivation sketched above. In the first place, no evidence for constant enzyme concentration exists. It has been pointed out in a later paper (Harding and Shive 1948) that substances altering the effective enzyme concentration will alter the $[I]/[S]$ ratio required for any given degree of inhibition. It may be added that if variation in substrate level causes variation in total enzyme concentration, as seems generally to be the case (Spiegelman 1945), no basis remains from the equations for prediction of constant degree of inhibition from a given inhibitor-substrate ratio.

More important, the total growth depends on the <u>total</u> amount of product P formed in the culture during the period

of growth. The concentration terms, while not all specifically defined, can have meaning only if referred to concentrations within the cell. Since the amount of protoplasm is constantly changing during the growth of the culture, the deduction of constant [ES] must be re-examined. This deduction was based on the production of a defined amount of product P in the whole culture in a given time of incubation, and so could logically follow only if [ES] is defined for this purpose on the basis of concentration in the culture, rather than in the cell. It could even then refer only to an effective average level of ES during the growth period. The total amounts of protoplasm, enzyme, and both enzyme complexes are constantly increasing during growth, so that the situation is not as simple as pictured by Shive and Macow. It seems necessary at the outset to define all concentration terms exclusively in relation to concentrations within the cell. Rate of production of P must then be expressed by the differential equation

$$(4). \qquad \frac{d[P]}{dt} = K_1 [ES] = K_2 v,$$

where v is the instantaneous volume of protoplasm in the culture . It follows that equation (1), rigorously derived, really predicts that any given ratio of inhibitor

to metabolite should produce a constant logarithmic growth rate, rather than a constant growth in any specified time. Only in the special case where no lag follows inoculation and the experiment is concluded before any cultures have begun to diverge from logarithmic growth will this growth rate be proportional to total growth. Only under these conditions are the conclusions of Shive and Macow valid. Errors due to changes in total enzyme concentration have not, of course, been eliminated by this re-derivation.

It must be noted that in their own work Shive and colleagues have made growth measurements usually at 14 to 18 hours, so that deviation from logarithmic growth of the controls could not have begun many hours previously. Reference to Figure 10 makes clear, however, the difficulties which are involved in estimation of inhibition ratios from readings taken at any one time. The uninhibited control had already deviated significantly from exponential growth at 20 hours, by which time the culture at a ratio of 2 had not yet attained sufficient growth to allow accurate estimation by the spectrophotometer used. The progressive increases in antibacterial index and in 50% inhibition ratio from 17 to 35 hours, which are shown in Table 19, are due to the tendency of the slower-growing inhibited cultures to catch up with the control as the latter slows down on approaching maximum growth. (Later increases are related

to the appearance of mutant cells, as will be discussed below.) The most accurate estimations of 50% inhibition ratios will thus be those taken about 16 to 20 hours with L. arabinosus and considerably earlier for faster-growing species like E. coli. (The size of inoculum will of course be important in determining the duration of exponential growth.) It may be noted that the statement of Shive and Macow (1946) that the antibacterial index will vary with time should apply only after the controls have ceased exponential growth.

It is not believed that the above considerations cast doubt on the conclusions which Shive and colleagues have derived from their applications of inhibition analysis to problems of biosynthesis. By keeping time of incubation short and by attaching no significance to less than threefold variations in the antibacterial index, these workers have avoided the errors which might otherwise have been involved. It is felt, however, that the above discussion and data demonstrate the desirability of following growth as a function of time in studies of inhibitors. It is also believed that redefinition of inhibition ratios in terms of growth rates would eliminate much of their dependence on such factors as size and physiological condition of inoculum or slight variations in temperature or in composition of the medium.

The 50% inhibition ratio has often been favored over the ratio causing complete inhibition because the response curve is fairly steep in the region of 50% growth, while it may approach zero more or less asymptotically. The 50% inhibition ratio can hence be determined graphically with precision, while the antibacterial index (ratio causing complete inhibition) depends on the definition taken for complete inhibition. The situation is shown graphically in Figure 9. It is evident that if readings were taken at only one time, as is customary in such studies, considerable confidence would probably be placed in the graphically determined value of the 50% inhibition ratio. This was the point of view held by the author when these investigations were begun. Taking readings periodically during the growth of the cultures, however, dispels this illusion of precision. Consideration of the several curves of Figure 9 or of the values for the inhibition ratio given in Table 19, which were obtained from such curves, shows that either index, at least as determined by <u>extent of growth</u> rather than <u>rate of growth</u> data, can be only an order of magnitude value. Choice between the two seems largely a matter of individual preference.

As noted in the preceding section, it follows from the postulates of analog interference with formation of enzyme-substrate complexes that as the level of the inhi-

bitor is diminished its effect should likewise decrease regularly until growth equal to the controls is reached. At still lower ratios there should be no effect. This is not the pattern obtained in the presence of low ratios of p-fluorophenylalanine, however. The stimulatory effects of low levels of the analog shown in Figure 10 and Tables 16, 18, and 21 has been observed in all similar tests. This stimulation occurs only when the level of phenylalanine, as well as the analog/metabolite ratio, is low.

Such a pattern of response to the analog may be explained by the hypothesis that two essential metabolic processes requiring phenylalanine are involved, in one of which the fluoro analog can substitute, while it interferes with the other. When the level of phenylalanine is limiting and the analog/metabolite ratio is below that required for significant inhibition, the analog should show phenylalanine-sparing activity by virtue of its ability to replace the natural amino acid in one reaction. At higher ratios, of course, the inhibited reaction becomes the growth-limiting factor and any sparing effect on other processes is obliterated. This explanation is naturally only tentative. It seems reasonable, however, especially in view of the report that 3-fluoro-p-aminobenzoic acid competed with p-amino-

benzoic acid, while the 2-fluoro derivative replaced the metabolite (Wyss, Rubin, and Strandskov 1943).

The inability of tyrosine to reverse the inhibitory effect of p-fluorophenylalanine indicates that tyrosine is not converted to phenylalanine, at least in significant yield, by either the parental type or strain H. Taken with the demonstration discussed above that phenylalanine does not participate in synthesis of tyrosine by strain Q, and that tryptophan is not used in phenylalanine synthesis by strain H, this result appears to demonstrate a surprising lack of interrelationship in the syntheses and metabolic functions of these three related amino acids in L. arabinosus 17-5 and the mutant strains derived from it.

Mutation to p-Fluorophenylalanine Tolerance

In one of the earliest papers on competitive structural analogs, McIlwain (1940) reported three types of inhibitions. Type I inhibition reduced the growth rate and the final level of growth but did not cause an initial time lag. Type II inhibition was characterized by an initial time lag which increased in length with increase in inhibitor level. Growth, when it occurred, depended on the level of the metabolite present. Type III inhibition was similar to II, except that when growth occurred it was independent of metabolite level.

McIlwain's results were based on visual observations, which necessarily leave much to be desired, especially when growth rates are compared. However, his Type I results are in excellent agreement with those reported in this thesis for the fluorophenylalanine inhibition at levels below the critical ratio, and his Type II pattern corresponds to that seen above the critical ratio. McIlwain reported that pyridine-3-sulfonamide interfered with nicotinic acid metabolism in Staphylococcus in such a manner as to give the Type I pattern, while pyridine-3-sulfonic acid showed Type II activity. In view of their correspondence to the results reported in this thesis, it may be suggested that the real distinction between these compounds lies in degree of activity, rather than in any qualitative difference in mode of action. If the free sulfonic acid is a more potent inhibitor, and was tested solely in the range above the critical ratio, the results McIlwain called Type II would be expected. The amide is probably less active, and, being tested below its critical ratio, gave the classical competitive inhibition pattern which McIlwain referred to as Type I. Type III inhibition would result when the mutants gain resistance to the analog by increased ability to synthesize the metabolite (nicotinic acid in this case).

From the point of view taken in this thesis, McIlwain's

types could then be reworded as follows: Type I--below the critical level, so mutants are not selected; Type II--mutations occur which confer tolerance to the analog without causing synthesis of the metabolite; Type III--mutations confer tolerance by allowing metabolite synthesis (or by permitting the analog to replace the metabolite nutritionally). Types I and II, and probably III, have been reported in this thesis for the competitive interference by p-fluorophenylalanine with phenylalanine metabolism. It seems likely that all three could be found for most inhibitions. Thus Ivanovics (1942) divided immunity to sulfanilamide into two classes: that due to greater production of p-aminobenzoic acid, and that caused by stronger action of this compound in reversing the inhibition. The latter class might perhaps be better described as reduced ability of sulfonamide to interfere in p-aminobenzoic acid metabolism. In a paper previously cited, Oakberg and Luria (1947) reported that of five mutations reducing the effect of sulfonamides on Staphylococcus aureus, one or possibly two cause increased p-aminobenzoic acid production. The cause of tolerance in the other cases is not known, but they clearly fall into McIlwain's Type II or Ivanovic's second class. Most of the adaptations reported in this thesis are also of this type.

Since mutations are currently thought to lead in many cases to altered enzymes, results of the type under discussion seem most logically explained on the basis of randomly occurring gene-controlled steric variations in enzyme active surfaces. Any such variations which happen to retain the ability to adsorb the substrate and mediate the appropriate reaction, while having a decreased affinity for the analog, will of course confer resistance to the analog. In the presence of the analog, such mutants will be at a selective advantage and will soon dominate the population.

It seems likely that a mechanism of the type postulated above has been operative in the evolutionary development of enzyme specificities. Many pairs or groups of substances of very similar configuration are involved in metabolism. Although some mutual antagonisms can be demonstrated at ratios well outside those normally encountered by the organism (some such cases involving amino acids have been cited, e.g., Beerstecher and Shive 1947c), in general the enzymes seem not to be affected by even very closely related natural compounds. It is logical to assume that this high degree of specificity has been developed by selection, among random alterations of the active surfaces of enzymes, of those which caused increased specificity for the substrates. Naturally-occurring sub-

stances must therefore be added at high ratios before significant interference results.

The situation is different when synthetic analogs which do not occur in nature are added to the medium. In the case reported in this thesis, for example, p-fluorophenylalanine is evidently adsorbed by the enzyme approximately as readily as is phenylalanine, since the analog, when present at the same molar concentration as the natural amino acid, reduces growth rate by about 50%. (If the relation derived by Shive and Macow (1946), which is discussed above, is taken as a first approximation, it follows that for 50% inhibition $K_i = K_s$, which indicates equal affinities of the enzyme for substrate and inhibitor.) Among the random variants constantly occurring at very low rates, some will confer increased specificity for phenylalanine as compared to the fluoro derivative. Under natural conditions these alterations are of no selective advantage, but in the presence of p-fluorophenylalanine at levels beyond the critical ratio, they confer 100% advantage. It is to be expected that growth of these mutant strains in the presence of high ratios of p-fluorophenylalanine to phenylalanine would result in the accumulation of further random variations increasing the sharpness of enzymic distinction between the analogs, and that practically any desired degree of speci-

ficity could be developed in this way.

Tolerance to some metabolic inhibitors develops only as fast as is required; that is, cultures are resistant to the drug at the level to which they have been exposed, but not at higher levels until after further "training". Such behavior has been used by workers cited in the historical section, notably Hinshelwood, and by many others not cited, as evidence for direct action of inhibitors on enzymes or on enzyme balances in such a manner as to confer resistance.

It seems more likely that such adaptation patterns are due to the type of selective enzyme alteration proposed above. The enzyme concerned might of course be one able to inactivate the inhibiting compound without destroying related metabolites, in which case specificity and high turnover number would still presumably be selected for.

Although it has been shown that such stepwise adaptations can be due to accumulation of mutations (e.g., penicillin resistance, Demerec 1945a,b), there seems to have been no discussion in the literature of altered enzyme specificity as a probable mechanism of action of these mutations. The gradual alteration of enzyme surfaces proposed here will occur only in the presence of the inhibitor, and is superficially similar to the

automatic enzyme alteration under the influence of drugs proposed by Hinshelwood. In essence, however, the two mechanisms are diametrically opposed, since the one involves selection of random mutants and the other postulates direct action by the inhibitor on the makeup and inheritance of the cell.

Interactions between Bacterial Strains

A similar pattern of interaction between parental and altered strains has been observed for all of the mutations studied in both parts of this thesis. Inoculation of the parental strain into the complete medium as normally used leads of course to prompt and heavy growth. Under conditions highly unfavorable to the parental strain the appropriate mutant is sharply selected. (For the mutants studied, these conditions are lack of tyrosine, lack of phenylalanine, lack of tryptophan, or presence of p-fluorophenylalanine or of anthranilic acid at inhibitory levels.) A time lag ensues because the mutant strain must increase from the very few cells present in the inoculum or even from a single mutant cell formed after inoculation, but growth, once initiated, usually equals the normal growth of the parental strain in both rate and extent.

Under conditions intermediate between those allowing normal growth of the parental strain and those causing clean selection of the mutant, however, a strikingly different response is observed. Growth begins with little or no delay, but is abnormally low in both rate and terminal level. When the parental strain is inoculated into a graded series of media covering the range between the usual medium and that strongly favoring a given mutant, therefore, terminal growth measurements will show heavy

growth at the two extremes, with an intermediate zone of growth depression. For the p-fluorophenylalanine-phenylalanine system, this depressed zone occurs at an analog/metabolite ratio of 2, as shown in the terminal growth data of Figure 9 (curve for 79 hours) and in the growth rate data of Figure 10. In the selection of a tryptophan-producing mutant from strains H and Q, minimum growth occurs in the neighborhood of 2 to 4 micrograms of L-tryptophan per 10 milliliters (Figure 8). Table 12, series B, shows that when the mutation to phenylalanine production (strain H) is being selected from strain Q a similar minimum in terminal growth occurs at about 0.08 micromoles of DL-phenylalanine. In the selection of the tyrosine producing strain Q from L. arabinosus 17-5, a similar minimum is seen at 0.04 micromoles of L-tyrosine.

In the case of the nutritional mutants, growth of the parental strain occurs to a limited but turbidimetrically measurable extent in the intermediate media before the appearance of mutant cells in significant numbers. Growth curves are therefore complex, and include the early growth and leveling off characteristic of the parental strain under the given conditions, followed by growth of the mutant strain. Interactions involving the mutant characterized by increased tolerance to fluorophenylalanine do not exhibit this complicating factor of early growth of the

parental type at a normal rate but to a limited extent. They therefore show the interaction in a more clear-cut and isolated manner.

The pattern of adaptation in the presence of varying p-fluorophenylalanine/phenylalanine ratios, shown in Figure 10, typifies the inhibition type of interactions.

The term "critical ratio" has been used in this thesis to designate the analog/metabolite ratio (2 in the case of the p-fluorophenylalanine/phenylalanine competition) which gives the maximum depression in rate of growth and terminal growth level, and beyond which visible growth occurs only after a time lag. This term is used empirically to describe the experimental observations and is not intended to imply that growth of the parental strain is absolutely stopped when the inhibitor is present at levels exceeding the critical ratio. It seems more likely that growth occurs, but is limited to such an extent that it is not detected turbidimetrically with the spectrophotometer used. The tendency for the lag time to depend on the analog/metabolite ratio could most easily be explained on this basis. In cultures containing the analog at levels above the critical ratio, the probable time of appearance of the mutant type will depend on the population size. If growth is occurring in these cultures, the cultures in which the growth rate is least retarded, and hence the population at any

given time is largest, should initiate mutant growth earliest, except for statistical fluctuations. The observations supported such a statistical explanation, since an increased ratio led in general to a longer lag time, but the differences between duplicates were occasionally greater than between levels. The earlier of the duplicates at a ratio of 10, for example, might initiate growth slightly before the later duplicate at a ratio of 5.

A preliminary experiment in which viable cell counts in cultures beyond the critical level were taken periodically by plating into complete medium supported the above interpretation. Extremely slow growth (before the initiation of mutant growth) occurred in a culture characterized by an analog/metabolite ratio of 8. When the ratio was 4, growth was slightly more rapid, and was somewhat faster yet at a ratio of 2. Cultures at the latter ratio, as in previous experiments, gave the lowest terminal growth.

Perhaps the clearest picture of interaction obtained is that involving the mutation to tolerance to high levels of anthranilic acid reported in Part I of this thesis (Figure 3). Increasing the level of anthranilic acid from 100 to 500 micrograms per 10 milliliter culture caused a progressive decrease in both initial rate of growth and terminal growth level. Further increase in the anthranilic acid concentration led to further decrease in initial

growth level; the rate and extent of the later mutant growth, however, increased. The pronounced change at about 32 hours in growth rates of the cultures containing 400 and 500 micrograms of anthranilic acid indicates that mutant cells played an important role in these cultures. Final growth level, however, was limited by interaction with the parental cells. The effect of parental cells on the growth of mutants is even more clearly seen at anthranilic acid levels above 500 micrograms. In the case of the anthranilic acid inhibition, growth in cultures beyond the critical level (500 micrograms) although extremely slow, occurs at a turbidimetrically measurable rate. It is evident from the curves of Figure 3b that the greater the growth of the parental strain before the mutant becomes dominant the less the terminal growth level attained by the mutant.

The depressed growth zones do not represent conditions unfavorable to growth of the mutants, as is shown when the corresponding mutant strain, rather than the parental type, is inoculated into such graded media. No region of depressed growth occurs in this case. Such experiments using appropriately graded media have been performed with strain Q (Table 13), strain H (not reported in the experimental section), and the anthranilic acid tolerant strain A (Table 6). Although such a test has

not been made with the strain resistant to p-fluorophenylalanine, it seems evident from the data at hand that growth of this strain is not retarded by any intermediate analog/metabolite ratios.

The presence of mutant cells in the depressed cultures cannot be doubted, since the populations of parental-type cells are much larger than those occurring at higher ratios from which mutant cultures invariably arise. The presence of mutants is sometimes evident from the growth curves themselves, as discussed above for Figure 3. It seems impossible to escape the conclusion that the slow but earlier growth of the parental strain in some manner limits growth of the mutants under the intermediate conditions. At higher levels of inhibitor (lower levels of metabolite in the case of the nutritional mutants) less growth of the parental type occurs, and mutant growth is less affected. Although delayed in initiation, the growth of mutants in such media therefore approaches the parental type in both rate and terminal level.

A very similar response pattern has been reported by Ryan and Schneider (1948) for the interaction of histidine-producing mutants with the parental population of histidine-requiring strain of *E. coli*. Perhaps because of the simpler medium required, these workers have been able to enumerate mutant and parental cells separately by plating.

(Difficulties similar to those reported in this thesis were overcome by Ryan and Schneider (1949a) through the purification of constituents of the medium.) Such separate enumeration allowed unequivocal demonstration that below the depressed growth zone the final population consists largely of mutant cells, and that above this zone mutant cells are essentially absent. A sharp decrease in the mutant population, expressed as percentage of the total population, occurs at the histidine level resulting in the greatest depression of final growth. It was shown further that the degree of depression of mutant growth depended on the number of parental-type cells present. The situation thus clearly demonstrated for the E. coli mutant is the same as that deduced from the results reported in this thesis for the mutant L. arabinosus strains. It has been impossible to test the hypothesis directly in this latter case because of plating difficulties, as discussed previously.

In later papers of the series (kindly made available by Dr. Ryan in advance of publication), Ryan and Schneider have shown that although gross growth response is similar in either case, the mechanism of the interaction depends on the presence or absence of oxygen. It was shown (1949b) that depression of mutant growth under anaerobic conditions involves a combined effect of pH and an inhib-

itor produced by the parental strain. The nature of the inhibiting substance is not known. Under aerobic conditions, the limitation of mutant growth was shown to be due to consumption of glucose by the parental strain, even after its growth had stopped because of histidine limitation (1949c). Multiplication of mutants proceeded until stopped by glucose exhaustion, and was resumed upon further addition of this sugar. It was possible, by determination of relative rates of glucose utilization by growing and "resting" cells and of the normal generation time, to predict the growth level which should result from inoculation of various mixtures of the strains into media differing in histidine and glucose contents (1949d). The predictions agreed very well with experimental results.

Lactobacillus arabinosus lacks a functional terminal oxidase system, so that this organism is indifferent to the presence or absence of oxygen. It appeared unlikely that glucose utilization by non-growing cells could be responsible for the observed interactions involving this species, since it had been observed that when L. arabinosus is used for amino acid assay lactic acid production stops with cessation of growth. This belief was supported by a preliminary experiment in which the addition of glucose to depressed cultures characterized by a p-fluorophenylalanine/

phenylalanine ratio of 2 failed to permit renewed growth.

No further experiments on the mechanism of the interaction were made. The mode of suppression of growth of L. arabinosus mutants by the parental type under conditions where the latter strain grows weakly is therefore unknown.

Wright and Skeggs (1945) reported that a tryptophan-producing mutant strain of L. arabinosus exhibited growth depression at intermediate levels of tryptophan. Such behavior on the part of the mutant strain itself is different from the results reported in this thesis for other L. arabinosus mutants, and from results obtained with E. coli by Ryan and Schneider. It has been found in work related to this thesis that tryptophan-producing strains of L. arabinosus appear to revert to tryptophan requirement on serial transfer in complete medium, suggesting that this mutation may confer serious competitive disadvantage in the presence of tryptophan. This behavior may be related to the results obtained by Wright and Skeggs.

Ryan and Schneider (1949d) suggest that interactions of the type discussed above may be of common occurrence. It is felt that the demonstration of five similar cases involving a different organism strongly supports this suggestion.

SUMMARY

Two L. arabinosus mutants, one able to synthesize tyrosine and the other synthesizing both tyrosine and phenylalanine, have been isolated. (Both mutations had been observed previously.) The relation of such mutations to the hypothesis that unused biochemical abilities are normally lost is discussed.

Neither step of the sequence

tryptophan ⟶ phenylalanine ⟶ tyrosine,

which has been reported for E. coli, is utilized by the L. arabinosus mutants.

The time-course of the inhibition of L. arabinosus by p-fluorophenylalanine has been followed. The inhibition is reversed competitively by phenylalanine with a 50% inhibition ratio (20 hours) of about 0.7. Tyrosine is without effect on the inhibition, demonstrating that this amino acid is not converted to phenylalanine in appreciable yield.

Increasing the molar ratio of fluorophenylalanine to phenylalanine causes a decrease in rate and extent of growth until a critical ratio (about 2) is reached. At levels of analog above this critical ratio, visible growth occurs only after a marked time lag, and appears to consist of a mutant strain resistant to the inhibitor. The

lag period tends to vary with the level of inhibitor.

Increased specificity for phenylalanine of the blocked enzyme is proposed as the most likely mechanism of the mutation. The implications of such a mechanism for the development of drug resistance and of enzyme specificity in general are discussed.

The three mutants studied, as well as one reported in Part I of this thesis, are suppressed in some manner by the parental strain under conditions allowing only moderate growth of the latter strain. This suppression is denoted by the occurrence of a depression in total growth under conditions intermediate between those allowing normal growth of the parental strain and those causing clean selection of the mutant. The mechanism of this suppression is not known.

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